Endophytic Fungi from Four Indonesian Medicinal Plants and Their Inhibitory Effect on Plant Pathogenic *Fusarium oxysporum*

Nampiah Sukarno1, Rohani Cinta Badia Ginting1,2, Utut Widyastuti1,2,3, Latifah Kosim Darusman1,4,5, Shigehiko Kanaya6, Irmanida Batubara4,5, I Nyoman Pugeg Aryantha2,8, Mashuri Waite9

1Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Darmaga Campus, Bogor, Indonesia
2Indonesian Soil Research Institute, Bogor, Indonesia
3Research Center for Bioresources and Biotechnology, IPB University, Darmaga Campus, Bogor, Indonesia
4Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Darmaga Campus, Bogor, Indonesia
5Tropical Biopharmaca Research Center, IPB University, Bogor, Indonesia
6Graduate School of Information Science, Nara Institute of Science and Technology, Takayama, Ikoma, Nara, Japan
7School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung, Indonesia
8Research Center for Biosciences and Biotechnology, Institut Teknologi Bandung, Bandung, Indonesia
9Gill Ewa Lands, Honolulu, Hawaii, USA

ABSTRACT

The medicinal plants *Centella asiatica*, *Curcuma xanthorrhiza*, *Guazuma ulmifolia*, and *Hydrocotyle verticillata* are widely used in Indonesian traditional medicine, but little is known about their associated endophytic fungi. This research aimed to study the diversity of endophytic fungi derived from functional parts of these plants and to evaluate their potential as antifungal agents against the plant pathogenic fungus *Fusarium oxysporum*. A total of 17 isolates of endophytic fungi were obtained: nine from leaves of *G. ulmifolia*, three each from leaves of *C. asiatica* and *H. verticillata*, and two from rhizomes of *C. xanthorrhiza*. The genus *Colletotrichum* was found in all plants studied, but each plant was associated with different species. *Colletotrichum aeschynomenes* was associated with *C. xanthorrhiza*, *C. siamense* was associated with *C. asiatica*, and *C. tropicale* was associated with *G. ulmifolia* and *H. verticillata*. The species *Curvularia affinis*, *Diaporthe tectonae*, *Lasiodiplodia mahajangana*, *Parengyodontium album*, *Talaromyces trachyspermus*, and *Speiropsis pedatospora* were found only in *G. ulmifolia*; while *Didymella coffeae-arabicae* and *Muyocopron laterale* were found only in *H. verticillata*. The endophytic fungi showed inhibition activity against *F. oxysporum* with inhibition values of 6.0-78.9%. *T. trachyspermus JBd10* and *C. affinis JBd14* gave the highest inhibition activity.

ARTICLE INFO

Article history:
Received October 15, 2020
Received in revised form February 15, 2021
Accepted March 1, 2021

KEYWORDS:
Antagonistic activity, Biodiversity, Endophytic fungi, ITS rDNA, Phylogenetic analysis

1. Introduction

The medicinal plants *Centella asiatica* L. (asiatic pennywort), *Curcuma xanthorrhiza* Roxb. (java turmeric), *Guazuma ulmifolia* Lamk. (bay cedar), and *Hydrocotyle verticillata* Thunb. (whorled pennywort) are well known for their usage and medicinal properties. The raw material for medicines can be obtained from different parts of a medicinal plant based on their various active ingredients. The dried sample can be obtained from a particular part of the medicinal plant such as the leaf, stem, rhizome, or root that contains a high concentration of the active compound, or from the whole plant. In traditional medicines, *Curcuma* dried sample is derived from rhizomes, while *Centella*, *Guazuma*, and *Hydrocotyle* dried samples are derived from leaves.

Endophytes are microorganisms that live within plant tissues for at least part of their life cycle without causing apparent disease. Fungi and bacteria are the most common microbes living as endophytes, but the most commonly isolated are fungi (Hardoim et al. 2015). There are relatively few reports on the endophytic fungi associated with medicinal plants. Among them, Hammerschmidt et al. (2015) isolated *Xylaria* sp. from healthy leaves of plants collected on the island of Timor, Indonesia, and the fungus produced a new compound (resacetophenone). Septiana et al. (2017) successfully isolated eleven
endophytic fungi from the turmeric plant, some of which showed antibacterial and anti-histamine activities. Endophytic fungi reported for *C. asiatica* included *Colletotrichum higginsianum*, *Guignardia mangiferae*, and *Glomerella cingulata* (Rakotoniriana et al. 2008). *Penicillium* sp. derived from turmeric leaves produced alkaloids, phenols, flavonoids, tannin, glycosides, and cellulase enzyme (Devi et al. 2012). Endophytic fungi associated with *G. ulmifolia* were *Mucor albus* (Strobel et al. 2007), *Pestalotiopsis* sp. (Russell et al. 2011), and *Nigrograna mackinnonii*, which produced limonene compound (Shaw et al. 2007), *Muscodor albus* (Strobel et al. 2007), *Pestalotiopsis* sp. derived from turmeric leaves, while *Fusarium oxysporum* is a plant pathogenic fungus listed in Indonesian remedies, the medicinally used organs of *C. asiatica, C. xanthorrhiza, G. ulmifolia*, and *H. verticillata* are the rhizomes. Samples were then put in clean plastic bags, transported to the laboratory, and processed within 24 hours of collection. Each sample was washed thoroughly with running tap water and followed up by rinsing with sterilized reverse osmosis water three times and pooling them to make a composite sample.

2.2. Isolation of Endophytic Fungi

All preparations and isolation processes were carried out in a biosafety cabinet. Leaf samples were cut into small pieces of 2 x 2 cm² size. The rhizomes of *C. xanthorrhiza* were peeled and then cut into 2 x 2 x 2 cm³ pieces. Surface sterilization was conducted by immersing the sample in 70% ethanol for 1 min, soaking in 0.5% hypochlorite solution for 5 min, and 70% ethanol for 1 min, and finally washing with sterilized distilled water six times. Then the samples were blotted on sterile Whatman filter paper for 12 hours. Four pieces from each cutting sample were randomly chosen and cultured on potato dextrose agar (PDA, dicro) plates containing rose bengal (30 mg L⁻¹) and chloramphenicol (0.5 g L⁻¹). Media plates were sealed and incubated at 28°C over 21 days, during which time they were checked daily for hyphal growth (Hallmann et al. 2007). The hyphal tips arising from the colonies having different characteristics were picked and transferred onto new PDA plates without being supplemented with either rose bengal or chloramphenicol. Each fungal isolate was purified to obtain a single colony.

2.3. Identification of Endophytic Fungal Isolates

The pure isolates having different characteristics were identified by a combination of morphological characteristics (Barnett and Hunter 1998) and molecular analyses. DNA extraction was prepared according to the CTAB-based extraction method (Sambrook and Russell 2000). The fungi were cultured in potato dextrose broth (PDB, dicro) and incubated in a shaker at 120 rpm 28°C for seven days. Mycelia were harvested through sterilized filter paper by vacuum filtration. The mycelia were frozen in liquid nitrogen and ground in a sterile mortar. About 0.5 g of mycelia powder was mixed with warm extraction buffer (600 μl PVP and 1.2 μl...
CTAB) in a 1.5-ml Eppendorf tube. It was inverted and incubated at 65°C for 30 min. The tube was then incubated on ice for 5 min and then 600 μl of a mixed solution of chloroform: alcohol (24:1) was added. It was then inverted and centrifuged for 10 min at 10°C, 25,000×g. The aqueous phase was removed carefully to a new tube, then added with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The tube was inverted and centrifuged again for 5 min at 4°C, 25,000×g. The supernatant was mixed with an equal volume of 2M NaOAc pH 5.2 and 2x volume of cold EtOH in a new tube, and incubated for 30 min at 20°C, and then centrifuged at 25,000×g, at 4°C for 30 min. DNA pellets were collected and washed with 500 μl 70% cold ethanol and then centrifuged for 5 min at 4°C, 25,000×g. DNA pellets were dried briefly using a vacuum, resuspended in 20 μl of sterilized double-distilled water, added with 0.2x volume of RNAse, and incubated for 10 min at 37°C. The DNA was then incubated for 10 min at 70°C to inactivate the RNAse. Fungal DNA was then stored in a freezer until used.

The DNA was then subjected to PCR amplification using the universal primers pair of ITS1 (forward) (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (reverse) (5′-TCC GCT TAT TGA TAT GC-3′) (White et al. 1990). The amplified fragments consisted of the internal transcribed spacer (ITS) regions of the extracted DNA, including the 5.8S rDNA. The PCR reaction was performed in a 60 μl reaction mixture which consisted of 42.6 μl sterilized ddH2O, 6 μl buffer (10x), 1.2 μl 2 mM dNTP, 1.5 μl 10 pmol of each forward and reverse primer, 1.2 μl 5 U Taq DNA polymerase, and 6 μl DNA template. The PCR amplification reaction was carried out under the following conditions: initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation, annealing, and extension at 72°C for 1 min, 94°C for 30 seconds, and 52°C for 30 seconds, respectively. This process was followed by a final re-extension step of 72°C for 5 min and finally stored at 25°C for 10 min using a Gene Amp 9700 thermal cycler (Applied Biosystems, USA).

The PCR products were purified and sequenced by First Base (Malaysia) using the same primers. The sequence was analyzed using the BioEdit Ver.7 (Hall 1999) and aligned using Clustal W (Thompson et al. 1994). The sequence similarity was determined by using available DNA fungal sequences at MycoBank (https://www.mycobank.org) and GenBank (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analyses were conducted using Maximum Likelihood methods in MEGA6 (Tamura et al. 2013). The reference GeneBank accession was used to construct the phylogenetic tree of *Aspergillus* based on Samson et al. (2014), *Colletotrichum* based on Cannon et al. (2012), *Curvularia* based on Marin-Felix et al. (2020), *Diaporthe* based on Dissanayake et al. (2017), *Didymella* based on Scarpari et al. (2020) and Chen et al. (2017), *Neocosmospora* based on Sandoval-Denis and Crous (2018), *Lasiodiplodia* based on Abdollahzadeh et al. (2010), *Parenygodontium* based on Tsang et al. (2016), and *Talaromyces* based on Adhikari et al. (2015). Sequences used for phylogenetic analysis are shown in Table 1. The maximum likelihood tree was constructed using the best DNA model (Nei and Kumar 2000). The genera *Aspergillus*, *Colletotrichum*, *Curvularia*, *Neocosmospora*, and *Lasiodiplodia* used the K2+G model; *Diaporthe* used the K2+G+I model; *Parenygodontium* used the T92+G model; and the genera *Didymella* and *Talaromyces* used the T92+G+I (G Gamma distributed, I evolutionarily invariable, K2 Kimura 2-parameter, K2 Kimura 2-parameter, T92 Tamura 3-parameter). Phylogenetic analyses of the genera *Muyocron* and *Speiropsis* were not done due to the limited availability of DNA sequences in the database. Gaps and missing data were treated as complete deletions. Initial trees for ML were made by the NJ/BioNJ algorithm and the branch swap filter was set very strong. Support for specific nodes on the ML tree was estimated by bootstrapping 1,000 replications. The nucleotide sequences generated in this study were deposited in GenBank (https://www.ncbi.nlm.nih.gov/).

### 2.4. In Vitro Antagonistic Bioassay

The antagonistic activities of the endophytic fungi isolates were evaluated against the plant pathogenic fungus *F. oxysporum* (IPBCC.88.0.12 or CBS 254.52) using the antagonist assays method of Morton and Stoube (1955) by using a dual culture technique in *vitro* assay on PDA. First, the endophytic fungi isolates and pathogenic fungus *F. oxysporum* were grown on separate PDA plates for seven days at 28°C. A 5 mm diameter mycelial plug of endophytic fungus was placed 1 cm away from the periphery at one end of a Petri dish containing PDA and incubated at room temperature for 4 days. A 5 mm in diameter culture plug of *F. oxysporum* was placed in the same Petri dish at a distance of 1 cm from the edge, at the opposite side to the endophytic fungus. In the control treatment, an agar disc (5 mm in diameter) was placed 1 cm away from the periphery.
Table 1. DNA sequence accession numbers of the isolates included in this study

| Species                      | Isolates          | ITS GenBank accession numbers | Sources                      |
|------------------------------|-------------------|-------------------------------|------------------------------|
| Aspergillus aculeatus        | NRRL 5094<sup>T</sup> | EF661221                      | Soil                         |
| A. avenaceus                 | CBS 109.46<sup>NT</sup> | AF104446                      | Pisum sativum seed           |
| A. calidoustus               | CBS 121601<sup>T</sup> | HE616558                      | Bronchoalveolar lavage specimen |
| A. clavatus                  | NRRL 1<sup>T</sup>  | EF669942                      | Soil                         |
| A. fischeri                  | NRRL 181<sup>NT</sup> | EF669936                      | Canned apples                |
| A. flavipes                  | NRRL 302<sup>LT</sup> | EF669591                      | Soil                         |
| A. flavus                    | NRRL 1957<sup>NT</sup> | AF027863                      | Moldy cellophane             |
| A. fumigatus                 | NRRL 163<sup>T</sup> | EF669931                      | Chicken lung                 |
| A. fumiculosus               | NRRL 4744<sup>T</sup> | EF661223                      | Soil                         |
| A. glaucus                   | NRRL 116<sup>NT</sup> | EF652052                      | House lumber                 |
| A. montevidensis             | NRRL 108<sup>NT</sup> | EF652077                      | Tympanic membrane            |
| A. nidulans                  | NRRL 187<sup>NT</sup> | EF652427                      | Soil                         |
| A. ochraceus                 | NRRL 398<sup>NT</sup> | EF661419                      | Unknown                      |
| A. penicillioides            | NRRL 4548<sup>NT</sup> | EF652036                      | Human skin                   |
| A. pseudoterreus             | NRRL 4017<sup>HT</sup> | NR_137472                     | Soil                         |
| Talaromyces flavus           | IPBCC 11.758      | SUB9428972                    | Rhizomes of Curcuma xanthorrhiza |
| A. sparsus                   | NRRL 1933<sup>NT</sup> | EF66181                       | Soil                         |
| A. terreus                   | NRRL 255<sup>T</sup>  | EF669586                      | Soil                         |
| A. togoensis                 | CBS 272.89<sup>T</sup> | AJ874113                      | Seed                         |
| A. versicolor                | NRRL 238<sup>NT</sup> | EF652442                      | Unknown                      |
| C. acerbum                   | CBS 128530<sup>HT</sup> | JQ948459                      | Bitter rot of Malus domestica fruit |
| C. aeschynomenes             | ICMP 17673<sup>HT</sup> | JX010176                      | Aeschynomene virginica       |
| C. anthrisci                 | CBS 125334<sup>T</sup> | GU227845                      | Anthriscus sylvestris        |
| C. boninense                 | CBS 123755<sup>HT</sup> | AB051400                      | Crinum asiaticum var. sinicum |
| C. clivia                    | CBS 125375<sup>HT</sup> | JK519223                      | Clivia miniata               |
| C. curcumae                  | IMI 288937<sup>PT</sup> | GU227893                      | Curcuma longa                |
| C. dracaenophilum            | CBS 118195<sup>HT</sup> | JK519222                      | Dracaena sanderana           |
| C. fructi                    | CBS 346.37<sup>HT</sup> | GU227844                      | Malus sylvestris             |
| C. jasminigenum              | MFLUCC 10-0273<sup>HT</sup> | HM131513                      | Jasminum sambac              |
| C. kahawae                   | ICMP 17816<sup>HT</sup> | JX010231                      | Coffea arabica               |
| C. lindemuthianum            | CBS 144.31<sup>LT</sup> | JQ005779                      | Phaseolus vulgaris           |
| C. lineola                   | CBS 125337<sup>T</sup> | GU227829                      | Apiaceae, dead stem          |
| C. orbiculare                | CBS 514.97<sup>HT</sup> | JQ005778                      | Cucumis sativus              |
| C. pseudoacutatum            | CBS 436.77<sup>HT</sup> | JQ948480                      | Pinus radiata                |
| C. pyricola                  | CBS 128531<sup>T</sup> | JQ948445                      | Pyrus communis, fruit rot    |
| C. rhombiforme               | CBS 129953<sup>T</sup> | JQ948457                      | Olea europaea                 |
| C. siamense                  | ICMP 18578<sup>T</sup>  | FJ972613                      | Coffea arabica               |
| C. truncatum                 | IPBCC 13.1092      | SUB9446440                    | Leaves of Centella asiatica  |
| C. toterosa                   | CBS 128544<sup>HT</sup> | JQ005164                      | Solanum melongena            |
| C. tropicalis                | ICMP 18653<sup>HT</sup> | GU994331                      | Theobroma cacao              |
| C. truncatum                 | IPBCC 11.752       | SUB9446618                    | Leaves of Hydrocotyle verticillata |
| C. yunnanense                | CBS 132135<sup>HT</sup> | EF369490                      | Buxus sp.                    |
| Monilochaetes infuscans      | CBS 869.96         | GU180626                      | Ipomoea batatas              |
### Table 1. Continued

| Species | Isolates | ITS GenBank accession numbers | Sources |
|---------|----------|------------------------------|---------|
| **Curvularia** | | | |
| affinis | CBS 154.34<sup>T</sup> | KJ909780 | Unknown |
| C. asiatica | IPBCC 13.1088 | SUB9445050 | Leaves of Guazuma ulmifolia |
| C. beerburrumensis | MFLUCC 10-0711<sup>T</sup> | JX256424 | Panicum sp. |
| C. cactivora | BRIP 12942<sup>T</sup> | MH414894 | Eragrostis bahiensis |
| C. cactivora | CBS 580.74 | MN688803 | Member of Cactaceae |
| C. chiangmaiensis | CPC 28829<sup>T</sup> | MF490814 | Zea mays |
| C. coicis | CBS 192.29<sup>T</sup> | JN192373 | Coix lacryma |
| C. crassiseptata | CBS 503.90<sup>T</sup> | LT631310 | Plant material |
| C. cymbopogonis | CBS 419.78<sup>T</sup> | HG778985 | Yucca sp. |
| C. dactyloctenii | BRIP 12846<sup>T</sup> | KJ415545 | Dactyloctenium radulans |
| C. ellisi | CBS 193.62<sup>T</sup> | JN192375 | Air |
| C. eragrosticola | BRIP 12538<sup>HT</sup> | MH414899 | Eragrostis pilosa |
| C. gladioli | CBS 210.79 | HG778987 | Gladialos sp. |
| C. heteropogonis | CBS 284.91<sup>T</sup> | JN192379 | Heteropogon contortus |
| C. intermedia | CBS 334.64 | HG778991 | Avena versicolor |
| C. isaehemi | CBS 630.82<sup>T</sup> | JX256428 | Ischaemum indicum |
| C. microspore | GUCC 6272<sup>T</sup> | MF319088 | Hippaestrum striatum leaf spot |
| C. mosaddeghi | IRAN 3131<sup>CT</sup> | MG846737 | Syzygium cumini leaf spot |
| C. neergaardii | BRIP 12919<sup>HT</sup> | KJ415550 | Oryza sativa |
| C. nodosa | CPC 28800<sup>T</sup> | MF490816 | Digitaria ciliaris |
| C. noludosa | CBS 160.58 | JN192383 | Eleusine indica |
| C. oryzae | CBS 169.53<sup>HT</sup> | KP400650 | Oryza sativa |
| C. ovariicola | CBS 470.90<sup>T</sup> | MN688809 | Ergrostris interrupta |
| C. pallescens | CBS 156.35<sup>T</sup> | KJ922380 | Air |
| C. palmonicola | MFLUCC 14-0404<sup>T</sup> | MF621582 | Acoelorrhaphe wrightii |
| C. papendorfii | CBS 308.67<sup>T</sup> | KJ909774 | Acacia karroo |
| C. paterae | CBS 198.87<sup>T</sup> | MN688810 | Triticum durum seed |
| C. perotidis | CBS 350.90<sup>T</sup> | JN192385 | Perotis rara |
| C. petersonii | BRIP 14642<sup>T</sup> | MH414905 | Dactyloctenium aegyptium |
| C. portulacae | BRIP 14541<sup>HT</sup> | KJ415553 | Portulaca oleracea |
| C. pseudointermedia | CBS 553.89<sup>T</sup> | MN688819 | Cultivated pasture soil |
| C. soli | CBS 222.96<sup>HT</sup> | KY905679 | Soil |
| C. sorghina | BRIP 15900<sup>HT</sup> | KJ415558 | Sorghum bicolor |
| C. sporobolica | BRIP 23040<sup>HT</sup> | MH414908 | Sporobolus australasicus |
| C. thailandica | MFLUCC 15-0747<sup>HT</sup> | MH275057 | Dead leaf of Pandanus sp. |
| C. trifolii | CBS 173.55 | HG779023 | Trifolium repens |
| C. tuberculare | CBS 146.63<sup>HT</sup> | JX256433 | Zea mays |
| C. xishuangbannaensis | MFLUCC 17-2271<sup>T</sup> | MH275058 | Dead leaf of Pandanus sp. |
| Bipolaris maydis | CBS 136.29<sup>T</sup> | KJ909769 | Zea mays |
| B. sorokiniana | CBS 110.14 | JN192381 | Hordeum sp. |

**DNA sequence accession numbers for phylogenetic analysis of Diaporthe**

| Species | Isolates | ITS GenBank accession numbers | Sources |
|---------|----------|------------------------------|---------|
| ambigua | CBS 114015<sup>T</sup> | KJ343010 | Pyrus communis |
| aquatica | IFRDCC 3051<sup>T</sup> | JQ797437 | Aquatic habitat |
| brasiliensis | CBS 133183<sup>T</sup> | KC343042 | Aspidosperma tomentosum |
| caatingensis | CBS 141542<sup>HT</sup> | KY085927 | Tacinga inamoena |
| citrassana | ZJUD 30<sup>T</sup> | JQ54645 | Citrus unshiu |
| compacta | CGMCC 3.17536<sup>T</sup> | KP267854 | Camellia sinensis |
| ganjae | CBS 180.91<sup>T</sup> | KC343112 | Cannabis sativa |
| goyleri | BRIP 55657a<sup>HT</sup> | KJ197290 | Helianthus annuus |
| longispora | CBS 194.36<sup>T</sup> | KC343135 | Ribes sp. |
| malorum | CAA734<sup>HT</sup> | KY435638 | Malus domestica |
| mayteni | CBS 133185<sup>HT</sup> | KC343199 | Maytenus acuminata |
| neoaonikayaporum | MFLUCC 14-1136<sup>T</sup> | KJ712449 | Tectona grandis |
| oxe | CBS 133186<sup>T</sup> | KC343164 | Maytenus ilicifolia |
| paranensis | CBS 133184<sup>T</sup> | KC343171 | Maytenus ilicifolia |
| passiflorica | CBS 141329<sup>T</sup> | KX228292 | Passiflora foetida |
| raonikayaporum | CBS 133182<sup>T</sup> | KC343188 | Spondias mombin |
| Species | Isolates | ITS GenBank accession numbers | Sources |
|---------|----------|-----------------------------|---------|
| D. sclerotioides | CBS 296.67T | KC343193 | Cucumis sativus |
| D. siamensis | MFLUCC 10-0573T | JQ619879 | Dasymaschalon sp. |
| D. tectonae | MFLUCC 12-0777HT | KU712430 | Tectona grandis |
| D. tulliensis | BRIP 62248HT | KR936130 | Theobroma cacao fruit |
| D. corylina | CBS 121124 | KC343004 | Corylus sp. |
| Didymella acetosellae | CBS 179.97 | GU237793 | Rumex hydrolapathum |
| D. aeria | LC 7441T | KY742051 | Air |
| D. arachidicola | CBS 333.75T | GU237833 | Arachis hypogea |
| D. chloroguttulata | LC 7435 | KY742057 | Air |
| D. coffeae-arabicae | CBS 123380HT | FJ426993 | Coffea arabicaes |
| D. dactylidis | CBS 124513HT | GU237766 | Dactylis glomerata |
| D. exigua | CBS 183.55NT | GU237794 | Rumex arifolius |
| D. glomerata | CBS 528.66HT | FJ427013 | Chrysanthemum sp. |
| D. longicolla | CBS 124514HT | GU237767 | Opuntia sp. |
| D. molleriana | CBS 229.79 | GU237802 | Digitalis purpurea |
| D. molleriana | CBS 109179 | GU237744 | Digitalis sp. |
| D. ocimicola | LC 8137 | KY7420782 | Ocimum sp. |
| D. pinodes | CBS 525.77HT | GU237883 | Pisum sativum |
| D. protuberans | CBS 381.96 | GU237853 | Lycium halfiolium |
| Didymella pteridis | CBS 379.96 | KT389504 | Pteris sp. |
| D. rhei | CBS 109177 | GU237743 | Rheum hypogonictum |
| D. ruminicola | CBS 683.79HT | KT389503 | Rumex obtusifolius |
| D. sancta | CBS 281.83HT | FJ427063 | Allianthus altissima |
| D. senecionica | CBS 160.78 | GU237787 | Senecio jacobae |
| D. suiyangensis | CBS 109177 | GU237802 | Digitalis purpurea |
| Phoma herbarum | CBS 615.75T | KT389536 | Rosa multiflora cv. cathayensis |
| Didymella pteridis | CBS 379.96 | KT389504 | Pteris sp. |
| D. rhei | CBS 109177 | GU237743 | Rheum hypogonictum |
| D. ruminicola | CBS 683.79HT | KT389503 | Rumex obtusifolius |
| D. sancta | CBS 281.83HT | FJ427063 | Allianthus altissima |
| D. senecionica | CBS 160.78 | GU237787 | Senecio jacobae |
| D. suiyangensis | CBS 109177 | GU237802 | Digitalis purpurea |
| Phoma herbarum | CBS 615.75T | KT389536 | Rosa multiflora cv. cathayensis |

**DNA sequence accession numbers for phylogenetic analysis of Lastiodiplodia**

| Species | Isolates | ITS GenBank accession numbers | Sources |
|---------|----------|-----------------------------|---------|
| Lastiodiplodia brasiliensis | CMM 4015HT | NR_147338 | Mangifera indica |
| L. laelio-cattleyae | CBS 167.28T | MH854963 | Laelio cattleya |
| L. citricola | IRAN1521T | GU945353 | Citrus sp. |
| L. crassispora | CBS 118741T | DQ103550 | Santalum album |
| L. gilanensis | IRAN 1523HT | GU945351 | Unknown |
| L. jatrophiolica | CMM 3610HT | NR_147348 | Jatropha curcas |
| L. magnoliae | CBS 122515HT | EU144050 | Adansonia gibbsos |
| L. mahajangana | CBS 122065 | EU144051 | Adansonia gibbsos |
| L. missouriana | CBS 128311HT | NR_145222 | Viris sp. |
| L. gonubiensis | CBS 115812T | DQ458892 | Syzigium cordatum |
| L. magnoliae | CBS 116355 | AY639594 | Syzigium cordatum |
| L. mahajangana | CBS 124925HT | MH863425 | Terminalia catappa |
| L. parva | CBS 494.78T | EF622084 | Cassava-field soil |
| L. pseudotheobromae | CBS 116459T | EF622077 | Gymelina arborea |
| L. theobromae | CBS 164.96T | AY640255 | Fruit on coral reef coast |
| L. venezuelensis | WAC12539HT | DQ103547 | Acacia mangium |
| L. viticola | CBS 128313HT | MH864855 | Wedge-shape canker of grapevine cv. Vigneoles |
| Diplodia mutila | CBS 112553 | AY259093 | Vitis vinifera |
| D. seriatia | CBS 112555HT | AY259094 | Vitis vinifera |
Table 1. Continued

| Species | Isolates | ITS GenBank accession numbers | Sources |
|---------|----------|------------------------------|---------|
| DNA sequence accession numbers for phylogenetic analysis of *Neocosmospora* | | | |
| *Fusarium brasiliense* | NRRL 31757 | EF408514 | Glycine max |
| *F. solani* f. sp. *batatas* | NRRL 22400 | AF178407 | Ipomoea batatas |
| *F. solani* f. *psii* | NRRL 22278 | DQ094309 | Pisum sativum |
| *F. solani* f. sp. *xanthoxyli* | NRRL 22277 | AF178401 | Xanthoxylum sp. |
| *F. sriatum* | NRRL 22101 | AF178398 | Cotton cloth |
| *Neocosmospora catenata* | NRRL 54993 | KC808256 | Zebra shark multiple tissues |
| *N. croci* | CBS 142423 | LT746264 | Citrus sinensis |
| *N. cyanescens* | CBS 518.82 | EU329684 | Human foot |
| *N. falciformis* | CBS 475.67 | MH859035 | Human bronchoalveolar lavage fluid |
| *N. gamsii* | NRRL 32323 | DQ094420 | Human bronchoalveolar lavage fluid |
| *N. illudens* | NRRL 22090 | AF178393 | Beilschmiedia tawa |
| *N. lichenicola* | NRRL 28030 | DQ094355 | Human |
| *N. macrospora* | CBS 142424 | LT746266 | Citrus sinensis |
| | IPBCC 11.756 | SUB9433318 | Leaves of Centella asiatica |
| *N. mahasenii* | CBS 119594 | JF433045 | Dead branch of live tree |
| *N. petrolihila* | NRRL 32315 | DQ094412 | Human groin ulcer |
| *N. plagianthi* | NRRL 22632 | AF178417 | Hoheria glabrata |
| *N. pseud沁orme* | CBS 125729 | KC691584 | Unknown dead tree |
| | IPBCC 11.748 | SUB9431884 | Leaves of Guazuma ulmifolia |
| *N. solani* | CBS 140079 | KT313633 | Solanum tuberosum |
| *N. suttoniana* | CBS 143214 | DQ094617 | Human wound |
| *N. vasinflecta* | CBS 130182 | EF453092 | Human |
| *Gejeayessia cicatricum* | CBS 125552 | HJ728145 | Buxus sempervirens |
| *G. atrofusca* | NRRL 22316 | AF178423 | Staphylea trifolia |
| DNA sequence accession numbers for phylogenetic analysis of *Parengyodontium* | | | |
| *Akanthomyces arachnophilus* | BCC17655 | GQ249995 | Unknown |
| *A. novoguineensis* | BCC22910 | GQ250003 | Insecta |
| *Beauveria amorpha* | ARSEF 2641T | NR_111601 | Hymenoptera: Formicidae |
| *B. caledonica* | ARSEF 2567T | HQ880817 | Soil |
| *B. vermiconia* | ARSEF 2922T | HQ880822 | Soil |
| *Cordyceps ninchukispora* | BCC1422 | FJ765278 | Insecta |
| *C. pruinose* | ARSEF 5413 | JNO49826 | laguides fasciata (Lepidoptera) |
| *C. takaomontana* | BCC 1409 | EU807995 | Pupa |
| *Engyodontium parvisporum* | IHEM 22910 | LC092896 | Indoor contamination |
| *E. rectidentatum* | CBS 641.74 | LC092895 | Buried keratinous substance |
| *I. amoenerosea* | CBS 107.73 | AY624168 | Coleopteran pupa |
| *I. tenuipes* | CBS 153.83 | NR_111170 | Adoxophyes privatana |
| *I. cicae* | BCC 2574 | AY624175 | Cicada nymph |
| *L.荩putolus* | ARSEF 5135 | NR_119512 | Lepidopteran pupa |
| *Leccanillia acerosum* | CBS 418.81T | NR_11268 | Crinipellis perniciosa |
| *L. antillanum* | CBS 350.85T | NR_11097 | Agaric |
| *L. aphanocladii* | CBS 376.77 | AJ292431 | Agaricus bitorquis |
| *L. attenuatum* | CBS 170.76T | EF79164 | Caterpillar of Carpocapsa pomonella |
| *L. dimorphum* | CBS 363.86T | NR_11101 | Agaricus bisporus |
| *L. flavidum* | CBS 342.80T | NR_111266 | Decaying needle of Abies alba |
| *L. fungicola var. aleophilus* | CBS 357.80T | NR_111064 | Agaricus bisporus |
| *L. fungicola var. fungicola* | CBS 992.69T | NR_119653 | Agaricus bisporus |
| *L. indonesiacum* | BTCC-F36T | AB378516 | Araneae |
| *L. kalimantanense* | BTCC-F23T | NR_112100 | Coleoptera in suspended soil |
| *L. longisporm* | IMI 012167T | NR_11095 | Icerya purchasi (coccidae) |
| *L. nodulorum* | IMI 338014R | EF513012 | Insect coccidae |
| *L. primulinum* | JCM 18425T | NR_119418 | Soil |
| *L. saksenae* | CBS 532.81T | JNO49846 | Forest soil |
| *L. tenuipes* | CBS 658.80 | LC092897 | Spider |
| *L. wallacei* | CBS 101237T | NR_111267 | Lepidoptera |
| *Parengyodontium album* | CBS 504.83ET | LC092880 | Human brain abscess |
| | IPBCC 11.755 | SUB9446667 | Leaves of Guazuma ulmifolia |
instead of endophytic fungus, while a 5 mm diameter culture plug of pathogenic fungi *F. oxysporum* was placed 1 cm away from the edge of the same Petri dish at the opposite side from the agar disc. All the plates were incubated at room temperature for seven days. The antagonistic activity was checked after incubation by measuring the growth radius of *F. oxysporum* on days 4 and 7 after inoculation. The magnitude of the inhibitory activity was calculated with the formula: $PI = \left(100 \times \frac{R1 - R2}{R1}\right)$, where $PI$ is percentage inhibition of radial growth, $R1$ is the growth radius of *F. oxysporum* colony in the control plate, and $R2$ is radial growth of *F. oxysporum* in dual culture with the endophytic fungus. All of the endophytic fungi obtained in this study were tested, and each assay was repeated five times. Statistical analysis was done using the MSTAT program (University of Wisconsin-Madison), and mean values were analyzed by DMRT ($p<0.05$).

### 3. Results

#### 3.1. Diversity of Endophytic Fungi
A total of 17 isolates of endophytic fungi having different colony characteristics were obtained from the four medicinal plants. Nine isolates of endophytic fungi were obtained from *G. ulmifolia* leaves, three isolates were obtained each from the leaves of *C. asiatica* and *H. verticillata*, and two isolates from the rhizomes of *C. xanthorrhiza*. Based on spore morphological characteristics, twelve of the fungal isolates could be classified into six genera, while the other five isolates were mycelia sterilia without spores. Leaves of *C. asiatica* were inhabited by *Aspergillus*, *Colletotrichum*, and *Fusarium*. Rhizomes of *C. xanthorrhiza* were inhabited by *Aspergillus* and *Colletotrichum*. Leaves of *G. ulmifolia* were occupied by *Aspergillus*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Phomopsis*, *Talaromyces*, and three mycelia.
sterilia. Leaves of *H. verticillata* were occupied by *Colletotrichum* and two mycelia sterilia (Table 2).

Identification to species level with sequence analysis of ITS1-5.8S-ITS2 rDNA gave 16 good E value results out of 17 isolates (94.1%), with the one remaining JBd11 isolate having poor quality DNA (Table 3). The JBd11 isolate was identified as *Speiropsis pedatospora* but with a low E value (6e-141). Based

### Table 2. The morphological characteristics of the endophytic fungi associated with the medicinal plants

| Medicinal plants | Endophytic fungi/IPBCC collection number | Mycelium, conidiophore, and spore characteristics |
|------------------|----------------------------------------|--------------------------------------------------|
| *C. asiatica*    | Aspergillus sp.1 PLd3 (IPBCC 11.760)   | Mycelium septate, conidiophores upright, simple, terminating in a globose bearing phialides at the apex, conidia 1 celled, globose, in dry basipetal chains, size 2.9-4.5 x 3.6-4.9 μm |
|                  | *Colletotrichum* sp.1 PLd6 (IPBCC 13.1092) | Mycelium septate, conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong without appendages, size 17.5-23.2 x 5.9-6.5 μm |
|                  | *Fusarium* sp.1 PLd1 (IPBCC 11.756)     | Mycelium septate, conidiophores hyaline, slender and simple cell bearing phialides, conidia hyaline, two kinds, macroconidia several-celled, slightly curved with canoe-shaped, size 43.4-62.4 x 6.5-9.5 μm, microconidia 1-2 celled, ovoid, orblong or slightly curved, size 10.3-16.6 x 3.4-4.6 μm |
| *C. xanthorrhiza* | *Aspergillus* sp.2 TLr5 (IPBCC 11.758)  | Mycelium septate, conidiophores upright, simple, terminating in a globose bearing phialides at the apex, conidia 1 celled, globose, in dry basipetal chains, size 5.3-6.4 x 4.1-5.5 μm |
|                  | *Colletotrichum* sp.2 TLr2 (IPBCC 11.757) | Mycelium septate, conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong without appendages, size 5.3-11.4 x 4.1-5.5 μm |
| *G. ulmifolia*   | *Aspergillus* sp.3 JBd3 (IPBCC 11.749)  | Mycelium septate, conidiophores upright, simple, conidia 1 celled, globose, in dry basipetal chains, size 5.3-6.4 x 4.1-5.5 μm |
|                  | *Colletotrichum* sp.3 JBd1 (IPBCC 11.747) | Mycelium septate, conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong without appendages, size 20.8-29.3 x 6.1-8.3 μm |
|                  | *Curvularia* sp. JBd14 (IPBCC 13.1088) | Mycelium septate, conidiophores brown, simple, bearing spores apically or on new sympodial growing points, conidia dark, end cells lighter, 3-5 celled, one of the central cells enlarged, size 37.4-65.3 x 15.1-18.2 μm |
|                  | *Fusarium* sp.2 JBd2 (IPBCC 11.748)     | Mycelium septate, conidiophores hyaline, slender and simple cell bearing phialides, conidia hyaline, two kinds, macroconidia several-celled, slightly curved with canoe-shaped, size 34.0-59.9 x 4.6-6.8 μm, microconidia 1 celled, ovoid, oblong or slightly curved, size 9.2-18.3 x 3.6-5.8 μm |
|                  | Mycelia sterilia 1 JBd7 (IPBCC 11.751)  | Mycelium septate, no conidia observed |
|                  | Mycelia sterilia 2 JBd11 (IPBCC 11.754) | Mycelium septate, no conidia observed |
|                  | Mycelia sterilia 3 JBd13 (IPBCC 11.755) | Mycelium septate, no conidia observed |
|                  | *Phomopsis* sp.1 JBd4 (IPBCC 11.750)    | Mycelium septate, conidiophores simple, conidia hyaline, 1 celled, size 10.8-15.2 x 4.1-6.7 μm |
|                  | *Talaromyces* sp. JBd10 (IPBCC 11.753)  | Mycelium septate, conidiophores arising from the mycelium with verticilate bearing phialide, conidia hyaline, 1 celled, mostly ellipsoidal, size 7.3-8.6 x 6.9-8.3 μm |
| *H. verticillata* | *Colletotrichum* sp.4 PBd3 (IPBCC 11.752) | Mycelium septate, conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong without appendages, size 3.5-6.2 x 8.9-12.5 μm |
|                  | Mycelia sterilia 4 PBd2 (IPBCC 13.10895) | Mycelium septate, no conidia observed |
|                  | Mycelia sterilia 5 PBd6 (IPBCC 13.1097) | Mycelium septate, no conidia observed |
Table 3. The molecular identification and GenBank accession number of the endophytic fungi associated with the medicinal plants

| Fungal identity/GenBank accession number* | Host                        | Fungal code/IPBCC collection number | References of GenBank accession number used | Maximum score | Similarity (%) | Query coverage | E value |
|------------------------------------------|-----------------------------|-------------------------------------|-------------------------------------------|---------------|----------------|----------------|---------|
| Aspergillus pseudoterreus (SUB 9428972)  | Curcuma xanthorrhiza         | Tlr5 (IPBCC 11.758)                | NR_137472.1                               | 1.002         | 98.76          | 83             | 0.0     |
| Aspergillus versicolor (SUB 9427202)     | Guazuma ulmifolia           | Jbd3 (IPBCC 11.749)                | NR_131277.1                               | 944           | 95.94          | 89             | 0.0     |
| Aspergillus versicolor (SUB 9403371)     | Centella asiatica           | Pld3 (IPBCC 11.760)                | NR_131277.1                               | 1.005         | 98.60          | 99             | 0.0     |
| Colletotrichum aeschynomenes (SUB 94327477) | C. xanthorrhiza            | Tlr2 (IPBCC 11.757)                | NR_120133.1                               | 1.022         | 98.95          | 89             | 0.0     |
| Colletotrichum siamense (SUB 9446440)    | C. asiatica                 | Pld6 (IPBCC 13.1092)               | JX010171.1                                | 979           | 97.89          | 97             | 0.0     |
| Colletotrichum tropicale (SUB 9433263)   | G. ulmifolia                | Jbd1 (IPBCC 11.747)                | MH863435.1                                 | 990           | 99.63          | 98             | 0.0     |
| Colletotrichum tropicale (SUB 9446618)   | Hydrocotyle verticillata    | Pbd3 (IPBCC 11.752)                | MH863435.1                                 | 1.027         | 99.13          | 99             | 0.0     |
| Curvularia affinis (SUB 9445050)         | G. ulmifolia                | Jbd14 (IPBCC 13.1088)              | HG778981.1                                 | 1.020         | 99.12          | 96             | 0.0     |
| Diaporthe tectonae (SUB 9431627)         | G. ulmifolia                | Jbd4 (IPBCC 11.750)                | NR_147590.1                                | 968           | 99.07          | 92             | 0.0     |
| Didymella coffeae-arabicae (SUB 94031855) | H. verticillata             | Pbd2 (IPBCC 13.1095)               | MH863293.1                                 | 915           | 97.74          | 97             | 0.0     |
| Lasiodiplodia mahajangana (SUB 9431869)  | G. ulmifolia                | Jbd7 (IPBCC 11.751)                | MH_863425.1                                | 955           | 99.06          | 97             | 0.0     |
| Muyocopron laterale (SUB9857667)          | H. verticillata             | Pbd6 (IPBCC 13.1097)               | NR_164055.1                                | 1.136         | 99.84          | 99             | 0.0     |
| Neocosmospora macrospora (SUB 9433318)   | C. asiatica                 | Pld1 (IPBCC 11.756)                | NR_163291.1                                | 848           | 99.57          | 88             | 0.0     |
| Neocosmospora pseudosiforme (SUB 9431884) | G. ulmifolia                | Jbd2 (IPBCC 11.748)                | MH863652.1                                 | 992           | 99.63          | 98             | 0.0     |
| Parengyodontium album (SUB 9446667)      | G. ulmifolia                | Jbd13 (IPBCC 11.755)               | LC092881.1                                 | 974           | 100.00         | 96             | 0.0     |
| Speiropsis pedatospora (SUB9856920)      | G. ulmifolia                | Jbd11 (IPBCC 11.754)               | MH857901.1                                 | 508           | 85.52          | 74             | 6e-141  |
| Talaromyces trachyspermus (SUB 9431993)   | G. ulmifolia                | Jbd10 (IPBCC 11.753)               | MH859701.1                                 | 850           | 99.36          | 99             | 0.0     |

*All fungal ITS rDNA sequences were submitted to GenBank (NCBI) to obtain the accession number.
on BLAST analysis, the similarity of the isolated fungi to the closest species available in MycoBank and GenBank varied from 85.52% to 100.00%, of which 13 isolates had a similarity value >98%. All of the isolates belong to Phylum Ascomycota in 3 classes, 8 orders, 11 families, and 15 identified species. The species are Aspergillus pseudoterreus, A. versicolor, Colletotrichum aeschynomenes, C. siamense, C. tropicale, Curvularia affinis, Diaporthe tectonae, Didymella coffeae-arabicae, Neocosmospora pseudensiforme, Lasiodiplodia mahajangana, Muyocopron laterale, Neocosmospora macrospora, Parengyodontium album, Speiropsis pedatospora, and Talaromyces trachyspermus (Table 3).

Fungal identification based on morphological characteristics uses microscopic observation of asexual spores (conidia). However, some isolates could not be differentiated based on morphological identification due to a lack of conidia. Mycelia sterilia 1 JBd7 and mycelia sterilia 3 JBd13 obtained from leaves of G. ulmifolia were identified as Lasiodiplodia mahajangana with 99.06% similarity and Parengyodontium album with 100.00% similarity. Similarly, mycelia sterilia 4 PBd2 derived from leaves of H. verticillata were successfully identified as Didymella coffeae-arabicae and mycelia sterilia 5 PBd6 was identified as Muyocopron laterale with 97.74% and 99.84% similarity to the sequences available in MycoBank, respectively (Table 2 and 3).

The JBd14 isolate was identified as Curvularia affinis with 99.12% similarity, and it was supported by phylogenetic analysis with 82% bootstrap support (Figure 1a). Based on sequence similarity comparison of the ITS region and phylogenetic analysis, PBd2 and JBd7 isolates were identified as Didymella coffeae-arabicae and Lasiodiplodia mahajangana with 97.74% and 99.06% similarity, respectively. Phylogenetic analysis showed that these isolates were in the same clade as those species with >50% bootstrap support (Figure 1b and c). These three species of fungi belong to the Dothideomycetes class (https://www.mycobank.org).

The TLr5 isolate has sequence similarities of 98.76% with the species of A. pseudoterreus, and the isolate is in the same clade as this species with 98% bootstrap support (Figure 2a). The PLd3 isolate had relatively higher sequence similarities (98.6%) with the species A. versicolor than with any other sequences, while the JBd3 isolate had sequence similarities at 95.94%. In the phylogenetic tree, the two isolates were in one clade with A. versicolor (Figure 2a). Based on sequence similarity comparison of the ITS region and phylogenetic analysis, the JBd10 isolate is identified as T. trachyspermus with 99.36% similarity, and the isolate is in the same clade as this species with 100% bootstrap support (Figure 2b). These two species of fungi belong to the Eurotiomycetes class (https://www.mycobank.org).

JBd1 and TLr2 isolates were identified as C. tropicale with a relatively high homology value of >98%, while PLd6 and PBd3 were identified as C. siamense and C. aescynomenes, respectively. Further analysis by phylogenetic tree showed that these isolates formed a sister clade with 88% bootstrap support (Figure 3a). The JBd4 isolate was closer to D. tectonae with 99.07% similarity. The result was supported by phylogenetic analysis with 78% bootstrap support (Figure 3c). The PLd1 isolate is closer to N. macrospora species with a relatively higher similarity value of >99%, and phylogenetic analysis shows that the isolate belongs to the N. macrospora CBS 142424 clade with 97% bootstrap support (Figure 3c). Based on sequence similarity comparison of the ITS region and phylogenetic analysis, the JBd13 isolate was identified as P. album and phylogenetic analysis showed that this isolate was in the P. album CBS 504.83 clade with 99% bootstrap support (Figure 3d). All four endophytic fungal genera belong to the Sordariomycetes class (https://www.mycobank.org).

3.2. Antifungal Activity of Endophytic Fungi

All isolated endophytic fungi showed inhibition activity against F. oxysporum. The percentage of inhibition varied from 6.0 to 78.9%, and the differences are statistically significant. The inhibition values of endophytic fungi derived from leaves of C. asiatica, G. ulmifolia, H. verticillata, and rhizomes of C. xanthorhiza against F. oxysporum ranged 28.3-40.2%, 6.0-78.9%, 22.9-36.1%, and 41.0-44.8%, respectively. The endophytic fungi T. trachyspermus and Curvularia affinis derived from leaves of G. ulmifolia showed the highest biocontrol activities against F. oxysporum with values of 78.9% and 60% inhibition, respectively (Table 4).
Figure 1. Maximum Likelihood phylogenetic analysis of ITS rDNA sequences of the Dothideomycetes endophytic fungi isolated from Guazuma ulmifolia and Hydrocotyle verticillata belonging to the genera (a) Curvularia, (b) Didymella, and (c) Lasiodiplodia. A phylogenetic tree was constructed using the MEGA ver. 6 program. Sequences obtained in the study are shown in bold. The marks ET, HT, IT, NT, T, and ST indicate epitype, holotype, isotype, neotype, type, and syntype strain, respectively. Numerical values (>50) on branches are the bootstrap values as a percentage of bootstrap replication from a 1,000 replicate analysis.
Figure 2. Maximum Likelihood phylogenetic analysis of ITS rDNA sequences of the Eurotiomycetes endophytic fungi isolated from Centella asiatica, Curcuma xanthorrhiza, and Guazuma ulmifolia that belonging to the genera (a) Aspergillus and (b) Talaromyces. A phylogenetic tree was constructed using the MEGA ver. 6 program. Sequences obtained in the study are shown in bold. The marks HT, LT, NT, and T indicate holotype, lectotype, neotype, and type strain, respectively. Numerical values (>=50) on branches are the bootstrap values as a percentage of bootstrap replication from a 1,000 replicate analysis.
Neocosmospora suttoniana CBS 143214^HT (DQ094617)
Neocosmospora lichenicola NRRL 28030 (DQ094355)
Neocosmospora falciformis CBS 475.67^T (MH859035)
Neocosmospora pseudensiforme CBS 125729^IT (KC691584)
Neocosmospora pseudensiforme Jbd2 (SUB9431884)
Neocosmospora solani CBS140079^IT (KT313633)
  Fusarium solani f. sp. xanthoxyli NRRL 22277 (AF178401)
  Fusarium solani f. sp. batatas NRRL 22400 (AF178407)
  Neocosmospora plagianthi NRRL 22632 (AF178417)
  Neocosmospora illudens NRRL 22090 (AF178393)
Neocosmospora vasinfecta CBS 130182 (EF453092)
  Fusarium solani f. pisi NRRL 22278 (DQ094309)
  Neocosmospora gamsii NRRL 32323^HT (DQ094420)
Neocosmospora croci CBS 142423^HT (LT746264)
  Fusarium sriatum NRRL 22101 (AF178398)
  Neocosmospora catenata NRRL 54993^HT (KC808256)
    Neocosmospora petroliphila NRRL 32315 (DQ094412)
      Neocosmospora cyanescens CBS 518.82T (EU329684)
    Neocosmospora macrospora CBS 142424^IT (LT746266)
      Neocosmospora macrospora PLd1 (SUB9433318)
    Fusarium brasiliense NRRL 31757 (EF408514)
Neocosmospora mahasenii CBS 119594^HT (JF433045)
  Fusarium atrofusca NRRL 22316 (AF178423)
    Geejayessia atrofusca NRRL 22278 (DQ094309)
    Geejayessia cicatricum CBS 125552 (HQ728145)
Figure 3. Maximum Likelihood phylogenetic analysis of ITS rDNA sequences of the Sordariomycetes endophytic fungi isolated from Centella asiatica, Curcuma xanthorrhiza, Guazuma ulmifolia, and Hydrocotyle verticillata belonging to the genera (a) Colletotrichum, (b) Diaporthe, (c) Neocosmospora, and (d) Parenysodontium. A phylogenetic tree was constructed using the MEGA ver. 6 program. Sequences obtained in the study are shown in bold. The marks ET, HT, and T indicate epitype, holotype, and type strain, respectively. Numerical values (>50) on branches are the bootstrap values as a percentage of bootstrap replication from a 1,000 replicate analysis.
C. aeschynomenes. The species was found in leaves of the plants studied and occupied all of the plants. Colletotrichum were associated with endophytic fungi. The genus was the most frequent endophyte found in leaves of the plants studied.

### Table 4. Inhibition activity of fungal endophytes derived from the medicinal plants against *Fusarium oxysporum*

| Endophytic fungi               | Fungal code/IPBCC collection number | Host                          | % Inhibition^a |
|-------------------------------|------------------------------------|-------------------------------|----------------|
| Aspergillus pseudoterreus     | Tlr5 (IPBCC 11.758)                | Curcuma xanthorrhiza           | 44.8 e         |
| Aspergillus versicolor        | Pld3 (IPBCC 11.760)                | Centella asiatica             | 28.3 hi        |
| Aspergillus versicolor        | Jbd3 (IPBCC 11.749)                | Guazuma ulmifolia             | 41.2 f         |
| Colletotrichum aescynomenes   | Tlr2 (IPBCC 11.757)                | C. xanthorrhiza                | 41.0 f         |
| Colletotrichum siamense       | Pld6 (IPBCC 13.1092)               | C. asiatica                   | 30.5 h         |
| Colletotrichum tropicale      | Jbd1 (IPBCC 11.747)                | G. ulmifolia                  | 49.1 c         |
| Colletotrichum tropicale      | Pbd3 (IPBCC 11.752)                | Hydrocotyle verticillata      | 36.1 g         |
| Curvularia affinis            | Jbd14 (IPBCC 13.1088)              | G. ulmifolia                  | 60.0 b         |
| Diaporthe tectonae            | Jbd4 (IPBCC 11.750)                | G. ulmifolia                  | 45.8 de        |
| Didymella coffeae-arabicae    | Pbd2 (IPBCC 13.10895)              | H. verticillata               | 25.9 ij        |
| Lasiodiplodia cit mahajangana | Jbd7 (IPBCC 11.751)                | G. ulmifolia                  | 48.1 cd        |
| Muyocopron laterale           | Pbd6 (IPBCC 13.1097)               | H. verticillata               | 22.9 k         |
| Neocosmospora macrospora      | Pld1 (IPBCC 11.756)                | C. asiatica                   | 40.2 f         |
| Neocosmospora pseudensiforme  | Jbd2 (IPBCC 11.748)                | G. ulmifolia                  | 25.0 jk        |
| Paren glyodium album           | Jbd13 (IPBCC 11.755)               | G. ulmifolia                  | 6.01           |
| Speiropsis pedatospora        | Jbd11 (IPBCC 11.754)               | G. ulmifolia                  | 40.3 f         |
| Talaromyces trachyspermus     | Jbd10 (IPBCC 11.753)               | G. ulmifolia                  | 78.9 a         |

^aValues of % inhibition are means from 5 replications, means followed by the same letter are not significantly different in DMRT (p<0.05)

### 4. Discussion

#### 4.1. Diversity of Endophytic Fungi

All medicinally used organs of the plants studied were associated with endophytic fungi. The genus *Colletotrichum* was the most frequent endophyte found in this study and occupied all of the plants. The species *C. aescynomenes* was found in leaves of *C. xanthorrhiza*, *C. siamense* was found in leaves of *C. asiatica*, and *C. tropical* was found in leaves of *G. ulmifolia* and *H. verticillata*. The genus *Colletotrichum* is a ubiquitous endophyte and was isolated from roots, stems, branches, petioles, leaves, flowers, veins, bark, twig bark, twig xylem, intervein, and phloem of 73 medicinal plants (Rai et al. 2014). The genus *Colletotrichum* is reported as the dominant fungal endophyte in 16 out of 29 traditional Chinese medicinal plants (Huang et al. 2008) and *Zingiber officinale* (Ginting et al. 2013). The genus *Aspergillus* occupied all plants studied except *H. verticillata*. The fungus *A. pseudoterreus* was found to be associated with *C. xanthorrhiza*, whereas *A. versicolor* was associated with *C. asiatica* and *G. ulmifolia* in this study. The fungus *Neocosmospora* was found to be associated with *G. ulmifolia* and *C. asiatica* with different species for each plant. The species *N. pseudensiforme* was found to be associated with *G. ulmifolia*, while *N. macrospora* was associated with *C. asiatica* in this study.

Some endophytic fungi are associated with specific hosts, and some species are associated with more than one host (Suryanarayanan et al. 2002). In this study, *C. affinis*, *D. tectonae*, *L. mahajangana*, *P. album*, *S. pedatospora*, and *T. trachyspermus* were found only in *G. ulmifolia*; while *D. coffeae-arabicae* and *M. laterale* were found in *H. verticillata* (Table 3). Host-specificity, host-recurrence, host selectivity, or host-preference is the relationship of fungal endophytes with single or multiple plant hosts (Cohen 2006). Some endophytic fungi are even tissue specific, fungi of different species occupy different tissues of a single plant (Ganley and Newcombe 2006).

#### 4.2. Antifungal Activity of Endophytic Fungi

The inhibition activity of endophytic fungi against *F. oxysporum* can be grouped into low, medium, and high inhibition activity. Low inhibition activity was represented by the inhibition activity of <30%, the moderate inhibition activity by 30% to 59%, and the high inhibition by >60% (Table 4). From these isolates, five isolates (29.4%) showed low, 10 isolates (58.8%) showed moderate, and two isolates (11.8%) showed high inhibition activity against *F. oxysporum*. The endophytic fungi obtained from *G. ulmifolia* ranged from low to high inhibition activity. The isolates derived from rhizomes of *C. xanthorrhiza* showed moderate inhibition activity,
and the isolates from leaves of *C. asiatica* and *H. verticillata* showed low to moderate activity against *F. oxysporum*. The endophytic fungi *T. trachyspermus* JBd10 and *C. affinis* JBd14 derived from leaves of *G. ulmifolia* showed high inhibition activity. Strobel et al. (2007) reported that *Muscodor albus* isolated from *G. ulmifolia* produced unusual biochemical and biological properties.

The endophytic fungi *T. trachyspermus* and *C. affinis* derived from leaves of *G. ulmifolia* showed the highest biocontrol activities against *F. oxysporum*. Chomcheon et al. (2010) reported that *Talaromyces* sp. derived from mangrove could produce antimicrobial metabolites (7-epiaustadiol, stemphyperylenol, and secalonic acid A) to control *Pseudomonas aeruginosa*. *Talaromyces* is a teleomorph stage of *Penicillium*. Devi et al. (2012) isolated endophytic *Penicillium* sp. from *C. asiatica*, and it produced alkaloids, phenols, flavonoids, tannin, and glycosides. Furthermore, Shiozawa et al. (1994) reported that the species *T. trachyspermus* SANK 12191 produced trachyspic acid, a new metabolite that inhibited tumor cell's heparanase. *Curvularia affinis* isolated from the stem of *Zingiber officinale* had high antagonistic activity against *F. oxysporum* with a percentage inhibition value of 68.8% (Ginting et al. 2013). *Curvularia affinis* isolated from soil could produce the secondary metabolites pyrenocine J, pyrenochaetic acid D, pyrenocine A, and pyrenochaetic acid A. The metabolite pyrenocine J showed cytotoxic activity against human hepatic cancer (Zhang et al. 2012). In symbiotic interactions inside the host plants, the role of endophytic fungi is to protect the host plants from fungal pathogenic attack by direct and indirect mechanisms. The direct mechanism occurs through interaction between endophytes with fungal pathogens occupying the ecological niche, while the indirect mechanism is by inducing plant resistance. In the direct mechanism, endophytic fungi produce antibiotics and lytic enzymes that suppress the growth or kill pathogens. Various reports have documented that endophytic fungi grown in synthetic medium produce secondary metabolites that are powerful against pathogenic bacteria and fungi, including plant fungal pathogens (Gunatilaka 2006). These endophytic fungi are potential sources of antifungal compounds, particularly for controlling *F. oxysporum*.

5. Conclusion

There were 9 isolates of endophytic fungi obtained from the leaves of *G. ulmifolia*, 3 isolates each from the leaves of *C. asiatica* and *H. verticillata*, and 2 isolates from the rhizomes of *C. xanthorrhiza*. The genus *Colletotrichum* occupied all of the plants studied. The fungi *C. affinis*, *D. tectonae*, *L. mahajangana*, *P. album*, *T. trachyspermus*, and *S. pedatospora* were found only in *G. ulmifolia*; while *D. coffeae-arabicae* and *M. laterale* were found only in *H. verticillata*. Endophytic fungi derived from medicinally used organs of *G. ulmifolia*, *C. asiatica*, *H. verticillata*, and *C. xanthorrhiza* had inhibition activity against *F. oxysporum* with inhibition values ranging 6.0-78.9%. *T. trachyspermus* JBd10 and *C. affinis* JBd14 had the highest inhibition values.

Acknowledgements

The research was funded by the Indonesian Agency for Agricultural Research and Development through KKP3T program (agreement No: 1998.9/LB.620/L.1/5/2011 and No: 1142/LB.620/L.1/3/2012) and Research Center for Bioresources and Biotechnology, Bogor Agricultural University–Nara Institute of Science and Technology for cooperation among Young Researcher. Part of this research was also partially funded by the Indonesian Ministry of Research, Technology and Higher Education Republic of Indonesia for the World Class Professor Program Scheme A (agreement No: 123.12/D2.3/KP/2018) to Tropical Biopharmaca Research Center IPB. We thank the Indonesian Medicinal and Aromatic Crops Research Institute, Bogor, Indonesia for providing the plant samples for fungal isolation.

References

Abdollahzadeh J et al. 2010. Phylogeny and morphology of four new species of Lasiodiplodia from Iran. *Persoonia* 25:1-10.

Adhikari M et al. 2015. Discovery of two new *Talaromyces* species from crop field soil in Korea. *Mycobiology* 43:402-407.

Barnett HL, Hunter BB. 1998. *Illustrated Genera of Imperfect Fungi*. 4th ed. USA: Prentice-Hall Inc.

Cannon PF et al. 2012. *Colletotrichum*-current status and future directions. *Stud Mycol* 73:181-213.

Chen Q et al. 2017. Didymellaceae revisited. *Stud Mycol* 87:105-159.
Chomcheon P et al. 2010. Curvularides A-E: antifungal hybrid peptide-polypeptides from an endophytic fungus Curvularia geniculata. Chem Eur J 16:11178-11185.

Cohen SD. 2006. Host selectivity and genetic variation of Discula umbrinella isolates from two oak species: analyses of intergenic spacer region sequences of ribosomal DNA. Microb Ecol 52:463-469.

Cook RK. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. Annu Rev Phytopathol 31:53-80.

Devi NN et al. 2012. Phytochemical analysis and enzyme analysis of antifungal endophytes from Centella asiatica. Asian Pac J Trop Biomed 2:1280-1284.

Dissanayake Aj et al. 2017. The current status of species in Diaporthe Mycosphere 8:1106–1156.

Ganley RJ, Newcombe G. 2006. Fungal endophytes in seeds and needles of Pinus monticola. Mycol Res 110:318–327.

Ginting RCB et al. 2013. Endophytic fungi harbored in Guazuma ulmifolia sp. nov. a new fungus associated with hazelnut fruit development in Italy. Mycol Prog 19:317-328.

Hall TA. 1999. BioEdit: a user-friendly biological sequence analysis program for windows 95/98/NT. Nucleic Acids Sym Ser 41:95-98.

Hallmann J et al. 2007. Isolation procedures for endophytic microorganisms. In: Schulz B, Boyle C, Sieber TN (Eds.). Soil biology Vol. 9. New York: Springer-Verlag Berlin Heidelberg. pp. 299-319.

Hammerschmidt L et al. 2015. Two new metabolites from the endophytic fungus Xylaria sp. isolated from the medicinal plant Curcuma xanthorrhiza. Tetrahedron Lett 56:1193-1197.

Harold PR et al. 2015. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol Mol Biol Rev 79:293-320.

Huang WY et al. 2008. Biodiversity of endophyte fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers 33:61-75.

Kusari P et al. 2013. Endophytic fungi harbored in Cannabis sativa L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. Fungal Divers 60:137-151.

Marin-Felix Y et al. 2020. Multi-locus phylogeny of the genus Curvularia and description of ten new species. Mycol Progress 19:559-588.

MycoBank database. 2021. Fungal Databases, Nomenclature and Species Banks. Available at https://www.mycobank.org. [Date accessed: 5 March 2021]

Morton DT, Stroube NH. 1955. Antagonistic and stimulatory effects of microorganisms upon Sclerotium rolfsii. Phytopathology 45:417-420.

Nei M, Kumar S. 2000. Molecular evolution and phylogenetics. New York: Oxford University Press.

Rai M et al. 2014. Multiple applications of endophytic Colletotrichum species occurring in medicinal plants. In: Gurib-Fakim A (Eds.). Novel plant bioresources: applications in food, medicine, and cosmetics. Chichester: Wiley. pp. 227-236.

Rakotominiana EF et al. 2008. Endophytic fungi from leaves of Centella asiatica: occurrence and potential interactions within leaves. Antonie van Leeuwenhoek 93:27-36.

Russell RJ et al. 2011. Biodegradation of polyester polyurethane by endophytic fungi. Appl Environ Microbiol 77:6076-6084.

Sambrook J, Russel DW. 2000. Molecular Cloning: A Laboratory Manual. 3rd ed. Vol 3. Cold Spring Harbor: Laboratory Press.

Samoza RA et al. 2014. Phylogeney, identification, and nomenclature of the genus Aspergillus. Stud Mycol 78:141-173.

Sandoval-Denis M, Crous PW. 2018. Removing chaos from confusion: assigning names to common human and animal pathogens in Neocosmospora. Persoonia 41:109–129.

Scarpini M et al. 2020. Didymella corticola sp. nov., a new fungus associated with hazelnut fruit development in Italy. Mycol Prog 19:317-328.

Septiana E et al. 2017. Endophytic fungi associated with turmeric (Curcuma longa L.) can inhibit histamine-forming bacteria in fish. Hayati J Biosci 24:46-52.

Shaw JJ et al. 2015. Biosynthesis and genomic analysis of medium-chain hydrocarbon production by the endophytic fungal isolate Nigrograna mackinnoni E5202H. Appl Microbiol Biotechnol 99:3715–3728.

Shiozawa H et al. 1994. Trachyspic acid, a new metabolite produced by Talaromyces trachyspermus that inhibits tumor cell heparanase: taxonomy of the producing strain, fermentation, isolation, structural elucidation, and biological activity. J Antibiot 48:357-362.

Strobel GA et al. 2007. Muscodor albus E-6, an endophyte of Guazuma ulmifolia making volatile antibiotics: isolation, characterization, and experimental establishment in the host plant. Microbiology 153:2613-2620.

Strobel GA, Daisy B. 2003. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67:491-502.

Suryanarayanan TS et al. 2002. Occurrence and distribution of fungal endophytes in tropical forests across a rainfall gradient. Can J Bot 80:818-826.

Tamura K et al. 2013. Mega6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725-2729.

Thompson JD et al. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. Nucleic Acids Res 22:4673-4680.

Tsang et al. 2016. Cutaneous hyalohyphomycosis due to Parenigondium album gen. et comb. nov. Med Mycol 54:699-713.

White TJ et al. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JS, White TJ, (Eds.), PCR Protocols: a Guide to Methods and Applications. New York: USA, Academic Press. pp. 315-322.

Zhang H et al. 2012. Two new metabolites from a soil fungus Curvularia affinis strain HS-FG-196. J Asian Nat Prod Res 14:1078-1083.