Diversity of endophytic fungi isolated from different plant parts of *Acacia mangium*, and antagonistic activity against *Ceratocystis fimbriata*, a causal agent of Ceratocystis wilt disease of *A. mangium* in Malaysia

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Acacia mangium is an important wood for commercial products especially pulp and medium-density fibreboard. However, it is susceptible to *Ceratocystis fimbriata* infection, leading to Ceratocystis wilt. Therefore, the present work aimed to (i) establish the diversity of endophytic fungi in different plant parts of *A. mangium* and (ii) evaluate the antifungal potentials of the isolated and identified endophytic fungi against *C. fimbriata*. Endophytic fungal identification was conducted by PCR amplification and sequencing of the internal transcribed spacer 1 (ITS1) and ITS4 regions of nuclear ribosomal DNA. A total of 66 endophytic fungi were successfully isolated from different parts of *A. mangium*: leaf (21), stem (13), petiole (12), root (9), flower (6), and fruit (5). The endophytic fungal isolates belonged to Ascomycota (95.5%) and Zygomycota (4.5%). For Ascomycota 13 genera were identified: *Trichoderma* (28.6%), *Nigrospora* (28.6%), *Pestalotiopsis* (12.7%), *Lasiodiplodia* (9.5%), *Aspergillus* (6.3%), *Sordariomycetes* (3%), and *Neopestalotiopsis*, *Pseudopestalotiopsis*, *Eutiarosporella*, *Curvularia*, *Fusarium*, *Penicillium*, and *Hypoxylon* each with a single isolate. For Zygomycota, only *Blakeslea* sp. (5%) was isolated. Against *C. fimbriata*, *Trichoderma koningiopsis* (AC 1S) from stem, *Nigrospora oryzae* (AC 7L) from leaf, *Nigrospora sphaerica* (AC 3F) from the flower, *Lasiodiplodia* sp. (AC 2U) from fruit, *Nigrospora sphaerica* (AC 4P) from petiole, and *Trichoderma* sp. (AC 9R) from root exhibited strong inhibition for...
C. fimbriata between 58.33 to 69.23%. Thus, it can be concluded that certain endophytic fungi of A. mangium have the potential to be harnessed as anti-Ceratocystis agent in future biotechnological applications.

**KEYWORDS**

Acacia mangium, endophytic fungi, Ceratocystis fimbriata, Ceratocystis wilt, antagonism

There are no specific methods or guidelines established on how to handle this disease in Malaysia yet up to now. But there were several actions that commonly are used by the plantation managers to prevent the infection of this disease. As Ceratocystis species penetrate and invade the trees by wounds, this problems can be prevent by avoid the occurrence of wound itself (Kile, 1993; Harrington, 2013; Nasution et al., 2019). Silviculture practice should be done in correct way and cautions. The timing of doing work for siliculture is also important to reduce the risk of disease development (Pilotti et al., 2016; Farid et al., 2018). Problems involved with wildlife in plantation areas also are count on in management such as establishment of wildlife management plan to overcome the conflicts occurred (Farid et al., 2018). Chemical control is one of application they used to delay the symptoms of the disease development and help the infected trees to live longer for at least 2 years (Blaedow, 2009; Nasution et al., 2019). Although the use of chemical fungicides are more preferred due to their rapid action, they are often associated with high production and application costs, human health hazards, restriction by domestic and international regulatory limits, trade bans, residual effects, environmental pollution, resistance development in pests, and potential elimination of beneficial natural enemies of the targeted pests (Yazid et al., 2020). Therefore, biological control is seen as a safer and cheaper alternative. Biological control is the use of living organisms (including microorganisms) to eliminate or reduce the density of pests / pathogens to safe levels (Wyckhuys et al., 2013). Often, indigenous organisms or microorganisms are utilised as biological control agent to minimise the risk of introducing foreign species that might grow uncontrollably and in turn become invasive. One such example of indigenous organisms or microorganisms is endophyte. The research is about using a microorganism (endophyte) to fight the pathogen (Ceratocystis fimbriata) which is one of biological control.

Like many other plant species, A. mangium is also associated with endophytes. Endophytes are usually bacteria or fungi that endosymbiotically live within a plant host without causing disease. These endophytes function to enhance the plant host growth and nutrient acquisition improve the plant host's ability to tolerate abiotic stresses or decrease biotic stresses by enhancing the plant host's resistance to infections (Farhat, 2020). Recently, an endophytic actinomycete of the genus Fodinicola was isolated from the roots of A. mangium, and has shown potential activity as
a beneficial plant-growth promoter and specialised secondary metabolite producer (Pham et al., 2020).

Despite endophytic fungi being regarded as new sources of novel bioactive compounds (Daoût et al., 1995; Cui et al., 2015), biological activities, and biotechnological developments, their true potential in controlling *A. mangium* diseases caused by *C. fimbriata* remains underexplored and underreported. Moreover, the leaf and root parts of *A. mangium* have been found to provide the habitats for various endophytic fungi (Mihara et al., 2005; Sarah Shafiei et al., 2017; Pham et al., 2020). Nevertheless, besides leaf and root, other plant parts of the species should also be explored for endophytic fungi which might offer novel species or strains that possess valuable bioactive compounds useful in controlling the Ceratocystis wilt disease. Therefore, the objectives of the present work were (i) to establish the diversity of endophytic fungi in different plant parts of *A. mangium*, and (ii) to evaluate the antifungal potentials of the isolated and identified endophytic fungi against *C. fimbriata*.

**Materials and methods**

**Plant materials**

Ten seedlings of *Acacia mangium* (≈30–50 cm in height) and 2 *A. mangium* trees (≈30 cm in diameter at breast height) free from disease and insect infestation were randomly sampled, and identified at Serdang, Selangor (coordinate E 101° 42.6333 N 2° 59.1833). The root, stem, petiole, and leaf from healthy *A. mangium* seedlings were sampled in three replicates, respectively. In addition, three replicates of flower and fruit were also sampled from mature trees, respectively. Each plant part was cut into five 0.5 cm² segments using a blade. These plant parts were washed thoroughly under running tap water to remove adherent debris on the surface.

**Isolation of endophytic fungi**

Plant part segments were surface-sterilised following the protocol suggested by Nuangmek et al. (2021). Briefly, the plant part segments were washed thoroughly under running tap water, immersed in 70% ethanol (Cerilliant Corporation, United States) for 1 min, rinsed thrice in sterile distilled water, and blot-dried using a sterile filter paper. Next, the surface-sterilised plant part segments were excised 1–2 mm from the edge, and explant-plated onto a Potato Dextrose Agar (PDA; Merck Milipore, Germany). The PDA plates were incubated at 27°C for 7 d. Single hyphae growing out from the cultivated plant part segments were sub-cultured onto fresh PDA. Pure cultures were grouped according to the six types of plant parts (root, stem, petiole, leaf, flower, and fruit). Isolates were group based on colour and morphology on PDA (Yoo and Eom, 2012). Cultures were maintained on PDA for 5 d before sub-cultured into Potato Dextrose Broth (PDB; Neogen®, United States) while shaken at 150 rpm at 26°C for 3–6 d. Following incubation, the culture supernatant was filtered through Whatman filter paper (Cytiva™ Sigma-Aldrich Chemie GmbH, Germany) before being used for genomic DNA extraction.

**DNA extraction and PCR amplification**

A total of 100 mg of fungal mycelia harvested from PDB was used for fungal genomic DNA extraction. Fungal genomic DNA was extracted as previously described by Landum et al. (2016), in accordance with the manufacturer’s instructions, using the FAVORGEN Fungi/ Yeast Genomic DNA Extraction Mini Kit (Taiwan). The nuclear ribosomal DNA internal transcribed spacer (ITS) of the fungal isolates were amplified using the forward primer, ITS-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and the reverse primer, ITS4 (5'-TCC TGC GGT TAT GCA TAT GC-3'); White et al., (1990). The final reaction volume was 25 μl, containing 12.5 μl of 2X PCRBio Tag Mix Red (PCR Biosystems, UK), 0.4 μM of forward and reverse primers, and 10mg of genomic DNA template. For negative control, the DNA was replaced with distilled water to verify the absence of contamination. The PCR was carried out using MyCycler™ (Bio-Rad, USA), programmed for 5 min at 95°C; 30 cycles for 30 s at 95°C, 30 s at 54.8°C, and 1 min at 72°C; and a final 10 min extension at 72°C. The PCR products were separated using 1% agarose gel in 1X TAE buffer (90mM Tris-acetate and 2mM EDTA, pH 8.0), stained with ethidium bromide (0.5 μg/ml), and visualised using FluorChem™ (Alpha Innotech, USA). The PCR products were sequenced by Apical Scientific Sdn. Bhd. (Malaysia). The sequences were deposited in NCBI GenBank, and compared with those already deposited in there via BLAST searches.

**Sequence and phylogenetic analyses**

The resulting DNA sequences were aligned using MUSCLE software embedded in MEGA software version 10.0.5 (Kumar et al., 2018), and manually trimmed and edited to obtain the complete sequences. Homology searches were carried out using the BLAST program against the NCBI GenBank database.¹ The Maximum Likelihood tree was constructed using MEGA software version 10.0.5 with all positions containing gaps and missing data were included for analysis. Clade supports were calculated based on 1,000 bootstrap replications. A total of 64 sequences of close relatives were downloaded from the NCBI GenBank, and combined with sequences of the 66 endophytic fungi isolated in the present work for phylogenetic tree construction. Two wood

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¹ [https://blast.ncbi.nlm.nih.gov/Blast.cgi](https://blast.ncbi.nlm.nih.gov/Blast.cgi)
decay macrofungi namely *Schizophyllum commune* (phylum Basidiomycota, family Schizophyllaceae) and *Phellinus gabonensis* (phylum Basidiomycota, family Hymenochaetaceae) were included as out-group.

### Antagonism assay

Endophytic fungal isolates were cultivated on PDA plates at 26°C for 7 days. The antagonistic activity was evaluated through the dual culture assay against *C. fimbriata*. The pathogenic *C. fimbriata* (FRIM1162) isolate used in this study was isolated from an infected *Acacia mangium* (Syzwan et al., 2021) and maintained at 27°C on PDA media at the Mycology & Pathology Unit, Forest Research Institute Malaysia (FRIM). Briefly, a fungal disc of 5 mm in diameter was taken from *C. fimbriata*, and placed 3 cm from the margin of the PDA plate (9 cm in diameter). Next, a 5 mm disc of the endophytic fungus was placed 3 cm from the margin of the PDA plate, and directly opposite of the *C. fimbriata* disc. Inoculated PDA plates were incubated at room temperature for 7 days. PDA plates inoculated with *C. fimbriata* in the absence of endophytic fungus served as negative controls. The assay was performed in triplicates. Observations were carried out for 6 days, after which the mycelial radial growth of test pathogen (*C. fimbriata*) on a control plate (rl) and in the presence of the antagonistic fungus (r2) were measured, and the percentage inhibition (I%) in mycelial growth was calculated as: $I\% = \left( \frac{r1 - r2}{r1} \right) \times 100$ (Hajieghrari et al., 2008). The I% data were analysed statistically with ANOVA using the SAS statistical software. To examine the significance between endophytic fungal isolates, Fisher’s LSD was performed at $p \leq 0.05$.

### Results

#### Identification of endophytic fungi

A total of 66 endophytic fungal isolates were successfully isolated from different parts of healthy *A. mangium* (Table 1); 21 from leaf, 12 from petiole, 13 from stem, nine from root, six from flower, and five from fruit. Correspondingly, 66 isolates were successfully amplified using primers ITS1 and ITS4. The endophytic fungal isolates mostly belonged to Ascomycota (95.5%) followed by Zygomycota (4.5%) based on the BLAST searches analysis (Table 2). For Ascomycota, 13 genera were identified; *Trichoderma* (28.6%), *Nigrospora* (28.6%), *Pestalotiopsis* (12.7%), *Lasidiopodia* (9.5%), *Aspergillus* (6.3%), *Sordariomycetes* (3%), and genera that were represented by a single isolate were *Neopestalotiopsis*, *Pseudopestalotiopsis*, *Eutiarosporella*, *Curvularia*, *Fusarium*, *Penicillium*, and *Hypoxylon*. Only *Blakeslea* sp. (4.5%) of Zygomycota was identified in the present work (Table 1). All the fungal ITS rDNA sequences exhibited high

| Plant part | Individual number | Total |
|------------|-------------------|-------|
| Fruit      | 2                 | 1     |
| Flower     | 2                 | 1     |
| Leaf       | 4                 | 2     |
| Petiole    | 1                 | 1     |
| Stem       | 1                 | 1     |
| Root       | 1                 | 1     |
| Total      | 8                 | 1     |

| Genus             | Isolates |
|-------------------|----------|
| *Trichoderma*     | 19       |
| *Nigrospora*      | 19       |
| *Pestalotiopsis*  | 8        |
| *Lasidiopodia*    | 6        |
| *Aspergillus*     | 4        |
| *Sordariomycetes* | 3        |
| *Neopestalotiopsis* | 1    |
| *Pseudopestalotiopsis* | 1  |
| *Eutiarosporella* | 1        |
| *Curvularia*      | 1        |
| *Fusarium*        | 1        |
| *Penicillium*     | 1        |
| *Hypoxylon*       | 1        |
| *Blakeslea* sp.   | 3        |
TABLE 2 Percentage of identity matches of 66 fungal isolates from different plant parts of Acacia mangium based on ITS sequences using BLAST analyses, and their percentage of inhibition against Ceratocystis fimbriata.

| No. | Endophytic isolate ID | Plant part | Inhibition activities (%) (mean ± standard error) | GenBank Accession number | ITS region | Phylum, Class, Family |
|-----|-----------------------|------------|----------------------------------------------------|--------------------------|------------|-----------------------|
| 1   | AC 1R                 | Root       | 55 ± 0.58                                          | MW254902                 | 99.28      | 0                     | Blakeslea trispora |
|     |                       |            |                                                    |                          |            | Zygomycota, Zygomycetes, Choanephoraceae |
| 2   | AC 2R                 | Root       | 0 ± 0.00                                           | MW254903                 | 99.63      | 0                     | Trichoderma gamsii |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Hypocreaceae |
| 3   | AC 3R                 | Root       | 0 ± 0.00                                           | MW254904                 | 100        | 0                     | Aspergillus aculeatinus |
|     |                       |            |                                                    |                          |            | Ascomycota, Eurotiomycetes, Trichosphaeriales |
| 4   | AC 4R                 | Root       | 44 ± 2.08                                          | MW254905                 | 99.38      | 0                     | Nigrospora sphaerica |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 5   | AC 5R                 | Root       | 20 ± 0.00                                          | MW254913                 | 99.21      | 0                     | Aspergillus niger   |
|     |                       |            |                                                    |                          |            | Ascomycota, Eurotiomycetes, Trichosphaeriales |
| 6   | AC 6R                 | Root       | 8.88 ± 0.66                                        | MW254916                 | 99.63      | 0                     | Trichoderma spirale |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Hypocreaceae |
| 7   | AC 7R                 | Root       | 14.28 ± 0.43                                       | MW254942                 | 99.17      | 0                     | Sordariomycetes sp. |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Hypocreaceae |
| 8   | AC 8R                 | Root       | 25 ± 2.89                                          | MW254956                 | 99.58      | 0                     | Nigrospora oryzae   |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 9   | AC 9R                 | Root       | 58.33 ± 5.02 ×10<15                                 | MW254964                 | 99.81      | 0                     | Trichoderma sp.     |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Hypocreaceae |
| 10  | AC 1S                 | Stem       | 58.33 ± 5.02 ×10<15                                 | MW254907                 | 99.81      | 0                     | Trichoderma koningiopsis |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Hypocreaceae |
| 11  | AC 2S                 | Stem       | 33.33 ± 0.29                                       | MW254909                 | 99.79      | 0                     | Nigrospora sphaerica |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 12  | AC 3S                 | Stem       | 0 ± 0.00                                           | MW254914                 | 99.63      | 0                     | Pestalotiopsis viasiae |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Sporocadaceae |
| 13  | AC 4S                 | Stem       | 0 ± 0.00                                           | MW254920                 | 99.81      | 0                     | Pestalotiopsis sp.   |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Sporocadaceae |
| 14  | AC 5S                 | Stem       | 45.45 ± 5.02 ×10<15                                 | MW254924                 | 99.45      | 0                     | Trichoderma sp.     |
|     |                       |            |                                                    |                          |            | Ascomycota, Sordariomycetes, Hypocreaceae |
| 15  | AC 6S                 | Stem       | 20 ± 3.61                                          | MW254925                 | 99.15      | 0                     | Lasiodiplodia theobromae |
|     |                       |            |                                                    |                          |            | Ascomycota, Dothideomycetes, Botryosphaeraceae |

(Continued)
| No. | Endophytic isolate ID | Plant part | Inhibition activities (%) (mean ± standard error) | GenBank Accession number | ITS region | Match identity (%) | E-value | Identification in GenBank | BLAST match in GenBank | Phylum, Class, Family |
|-----|-----------------------|------------|--------------------------------------------------|--------------------------|------------|--------------------|---------|--------------------------|-----------------------|----------------------|
| 16  | AC 7S                 | Stem       | 45 ± 0.00                                        | MW254931                 | KX009501   | Ascomycota,        | 99.25   | Trichoderma gamsii       | Ascomycota, Sordariomycetes, Hypocreaceae |
| 17  | AC 8S                 | Stem       | 40 ± 0.00                                        | MW254937                 | MJ38228    | Ascomycota,        | 99.59   | Nigrospora oryzae        | Ascomycota, Sordariomycetes, Hypocreaceae |
| 18  | AC 9S                 | Stem       | 0 ± 0.00                                         | MW254940                 | FJ442652   | Ascomycota,        | 99.63   | Trichoderma ovalisporum  | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 19  | AC 10S                | Stem       | 0 ± 0.00                                         | MW254944                 | MN599950   | Ascomycota,        | 99.8    | Aspergillus niger        | Ascomycota, Eurotiomycetes, Trichosphaeriales |
| 20  | AC 11S                | Stem       | 45.45 ± 2.60                                     | MW254951                 | KC178665   | Ascomycota,        | 100     | Sordariomycetes sp.      | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 21  | AC 12S                | Stem       | 14.28 ± 0.30                                     | MW254954                 | KX446132   | Ascomycota,        | 97.98   | Eutiarosporella sp.      | Ascomycota, Sordariomycetes, Dothideomycetes, Botryosphaeriales |
| 22  | AC 13S                | Stem       | 0 ± 0.00                                         | MW254959                 | MT556677   | Ascomycota,        | 100     | Nigrospora sp.           | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 23  | AC 11                  | Leaf       | 55.55 ± 5.02 ×10⁻¹⁵                             | MW254906                 | KM103313   | Ascomycota,        | 99.63   | Trichoderma gamsii       | Ascomycota, Sordariomycetes, Hypocreaceae |
| 24  | AC 21                  | Leaf       | 37.5 ± 1.44                                      | MW254908                 | MN625838   | Ascomycota,        | 99.38   | Nigrospora sphaerica     | Ascomycota, Sordariomycetes, Hypocreaceae |
| 25  | AC 31                  | Leaf       | 45.45 ± 0.75                                     | MW254910                 | KX009501   | Ascomycota,        | 99.81   | Trichoderma gamsii       | Ascomycota, Sordariomycetes, Hypocreaceae |
| 26  | AC 41                  | Leaf       | 16.67 ± 9.53                                     | MW254918                 | MH275056   | Ascomycota,        | 100     | Curvularia pandanicola   | Ascomycota, Sordariomycetes, Hypocreaceae |
| 27  | AC 51                  | Leaf       | 16.67 ± 0.00                                     | MW254919                 | MT597837   | Ascomycota,        | 99.63   | Pestalotiopsis microspora| Ascomycota, Sordariomycetes, Sporocadaceae |
| 28  | AC 61                  | Leaf       | 45.45 ± 1.16                                     | MW254921                 | EU137910   | Ascomycota,        | 99.81   | Pestalotiopsis microspora| Ascomycota, Sordariomycetes, Sporocadaceae |
| 29  | AC 71                  | Leaf       | 58.3 ± 5.02 ×10⁻¹⁵                               | MW254922                 | MN182281   | Ascomycota,        | 98.77   | Nigrospora oryzae        | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 30  | AC 81                  | Leaf       | 28.57 ± 2.51 ×10⁻¹⁵                              | MW254923                 | MT448890   | Ascomycota,        | 99.58   | Fusarium chlamydosporum  | Ascomycota, Sordariomycetes, Nectriaceae |

(Continued)
TABLE 2 (Continued)

| No. | Endophytic isolate ID | Plant part | Inhibition activities (%) (mean ± standard error) | GenBank Accession number | Match identity (%) | E-value | Identification in GenBank | BLAST match in GenBank | Phylum, Class, Family |
|-----|------------------------|------------|---------------------------------------------------|--------------------------|-------------------|--------|--------------------------|----------------------|----------------------|
|     |                        |            |                                                   |                          |                   |        |                          |                      |                      |
| 31  | AC 9 l                 | Leaf       | 0 ± 0.00                                          | MW254926                 | 99.38             | 0      | Nigrospora sphaerica     | MN366004             | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 32  | AC 10 l                | Leaf       | 30 ± 5.77                                         | MW254934                 | 99.57             | 0      | Lasiodiplodia theobromae | KP293981             | Ascomycota, Dothideomycetes, Botryosphaeriaceae |
| 33  | AC 11 l                | Leaf       | 12.5 ± 6.93                                       | MW254936                 | 99.63             | 0      | Trichoderma koningiopsis | JQ617301             | Ascomycota, Sordariomycetes, Hypocreaceae |
| 34  | AC 12 l                | Leaf       | 0 ± 0.00                                          | MW254938                 | 99.44             | 0      | Pestalotiopsis neglecta | MN006391             | Ascomycota, Sordariomycetes, Sporocadaceae |
| 35  | AC 13 l                | Leaf       | 22.22 ± 0.00                                      | MW254939                 | 99.62             | 0      | Trichoderma gamsii       | KX009581             | Ascomycota, Sordariomycetes, Hypocreaceae |
| 36  | AC 14 l                | Leaf       | 0 ± 0.00                                          | MW254943                 | 99.58             | 0      | Nigrospora oryzae        | JX966549             | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 37  | AC 15 l                | Leaf       | 33.33 ± 1.59                                      | MW254945                 | 99.79             | 0      | Nigrospora sp.           | MT561433             | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 38  | AC 16 l                | Leaf       | 45.45 ± 5.02 ×1010                                 | MW254946                 | 99.58             | 0      | Lasiodiplodia theobromae | MK690643             | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 39  | AC 17 l                | Leaf       | 40 ± 5.77                                         | MW254948                 | 99.43             | 0      | Pestalotiopsis visiae    | KP747709             | Ascomycota, Sordariomycetes, Sporocadaceae |
| 40  | AC 18 l                | Leaf       | 25 ± 0.00                                         | MW254949                 | 99.59             | 0      | Nigrospora sphaerica     | MT043797             | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 41  | AC 19 l                | Leaf       | 0 ± 0.00                                          | MW254950                 | 99.8              | 0      | Aspergillus aculeatus    | KJ605160             | Ascomycota, Eurotiomycetes, Trichocomaceae |
| 42  | AC 20 l                | Leaf       | 40 ± 5.77                                         | MW254962                 | 99.59             | 0      | Nigrospora sphaerica     | MH368102             | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 43  | AC 21 l                | Leaf       | 0 ± 0.00                                          | MW254963                 | 99.58             | 0      | Nigrospora sphaerica     | MT561433             | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 44  | AC 1P                  | Petiole    | 0 ± 0.00                                          | MW254917                 | 99.81             | 0      | Trichoderma crustum      | MK911703             | Ascomycota, Sordariomycetes, Hypocreaceae |
| 45  | AC 2P                  | Petiole    | 0 ± 0.00                                          | MW254932                 | 99.79             | 0      | Nigrospora sphaerica     | MT561433             | Ascomycota, Sordariomycetes, Trichosphaeriales |

(Continued)
| No. | Endophytic isolate ID | Plant part | Inhibition activities (%) (mean ± standard error) | GenBank Accession number | ITS region | Phylum, Class, Family |
|-----|----------------------|------------|--------------------------------------------------|--------------------------|------------|----------------------|
|     |                      |            |                                                  |                          |            |                      |
| 46  | AC 3P                | Petiole    | 50 ± 4.91                                       | MW254933                 | 97.68      | Nigrospora sphaerica | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 47  | AC 4P                | Petiole    | 58.33 ± 5.02 ×10^-15                             | MW254935                 | 99.38      | Nigrospora sphaerica | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 48  | AC 5P                | Petiole    | 45.45 ± 5.02 ×10^-15                             | MW254947                 | 99.79      | Nigrospora sphaerica | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 49  | AC 6P                | Petiole    | 45 ± 5.77                                       | MW254952                 | 99.8       | Penicillium rolfii  | Ascomycota, Sordariomycetes, Eurotiomycetes, Trichocomaceae |
| 50  | AC 7P                | Petiole    | 20 ± 5.77                                       | MW254957                 | 100        | Trichoderma longibrachiatum | Ascomycota, Sordariomycetes, Hypocreaceae |
| 51  | AC 8P                | Petiole    | 30 ± 5.77                                       | MW254958                 | 99.37      | Neopestalotiopsis cubana | Ascomycota, Sordariomycetes, Pestalotiopsidaceae |
| 52  | AC 9P                | Petiole    | 45.45 ± 5.02 ×10^-15                             | MW254961                 | 99.79      | Pestalotiopsis sp.  | Ascomycota, Sordariomycetes, Pestalotiopsidaceae |
| 53  | AC 10P               | Petiole    | 45.45 ± 5.02 ×10^-15                            | MW254965                 | 99.57      | Lasiodiplodia theobromae | Ascomycota, Sordariomycetes, Sporocadaceae |
| 54  | AC 11P               | Petiole    | 14.28 ± 0                                       | MW254966                 | 70.1       | Trichoderma sp.     | Ascomycota, Sordariomycetes, Hypocreaceae |
| 55  | AC 12P               | Petiole    | 20 ± 5.77                                       | MW254967                 | 99.44      | Trichoderma koningiopsis | Ascomycota, Sordariomycetes, Hypocreaceae |
| 56  | AC 1F                | Flower     | 8.88 ± 0.00                                     | MW254911                 | 99.28      | Blakeslea trispora  | Ascomycota, Zygomycetes, Chonanusporaceae |
| 57  | AC 2F                | Flower     | 8.88 ± 5.14                                     | MW254915                 | 94.87      | Hypoxylon monticulosum | Ascomycota, Sordariomycetes, Hypoxylaceae |
| 58  | AC 3F                | Flower     | 58.33 ± 5.02 ×10^-15                             | MW254927                 | 100        | Nigrospora sphaerica | Ascomycota, Sordariomycetes, Trichosphaeriales |
| 59  | AC 4F                | Flower     | 40 ± 0.00                                       | MW254941                 | 99.62      | Trichoderma longibrachiatum | Ascomycota, Sordariomycetes, Hypocreaceae |
| 60  | AC 5F                | Flower     | 45.45 ± 2.60                                    | MW254953                 | 99.59      | Pseudopestalotiopsis theae | Ascomycota, Sordariomycetes, Pestalotiopsidaceae |

(Continued)
similarity with existing sequences in the NCBI database (Table 1).

The ITS sequences obtained in the present work were deposited in the NCBI GenBank (MW254902 - MW254967) for future reference. A total of 66 sequences of close relatives were downloaded from the NCBI GenBank, and combined with sequences of the 66 endophytic fungi for phylogenetic tree construction (Figure 1). Nine different orders were observed, of which six belonged to Ascomycota (Amphisphaeriales, Brotryosphaeriales, Eurotiales, Hypocreales, Pleosporales and Trichosphaeriales), one belonged to Zygomycota, and two belonged to Basidiomycota (out-group). Most of the endophytic fungal isolates clustered under the order Trichosphaeriales (20 isolates) belonged to genus Nigrospora, and under the order Hypocreales (19 isolates) belonged to genera Fusarium and Trichoderma. Tables 3 and 4 summarises these results.

**Antagonism assay**

All 66 endophytic fungal isolates were tested in the antagonism assay against C. fimbriata. After 5 days of incubation, six fungal isolates namely Trichoderma koningiopsis (AC 1S) stem, Nigrospora oryzae (AC 7L) leaf, Nigrospora sphaerica (AC 3F) flower, Lasiodiplodia sp. (AC 2 U) fruit, Nigrospora sphaerica (AC 4P) petiole, and Trichoderma sp. (AC 9R) root were observed to exhibit stronger inhibition where the mycelia of the antagonists had breached into C. fimbriata colony (Figure 2). Of these, four fungal isolates namely T. koningiopsis (AC 1S) stem, Lasiodiplodia sp. (AC 2 U) fruit, N. sphaerica (AC 4P) petiole, and Trichoderma sp. (AC 9R) root colonised almost 99% of the culture plate. Although N. sphaerica (AC 7L) leaf and N. sphaerica (AC 3F) flower did not colonise the entire culture plate, there was no growth of C. fimbriata observed. The inhibition percentages (I%) of endophytic fungi against the pathogen C. fimbriata in dual culture assay are shown in Figure 3. Lasiodiplodia sp. (AC 2 U) isolated from fruit recorded the highest I% (69.23%), followed by Trichoderma sp. (AC 9R) isolated from root, Nigrospora sphaerica (AC 4P) isolated from flower, Trichoderma koningiopsis (AC 1S) isolated from stem, and Nigrospora oryzae (AC 7L) isolated from leaf with value 58.33%, respectively.

Thirteen endophytic fungi from various plant parts of A. mangium showed no inhibition against C. fimbriata (Figure 4) namely A. aculeatinus (AC 3R) isolated from root, A. aculeatus (AC 19L) isolated from leaf, A. niger (AC 10S) isolated from stem, N. oryzae (AC 14L) isolated from leaf, Nigrospora sp. (AC 13S) isolated from stem, N. sphaerica (AC 2P) isolated from petiole, P. neglecta (AC 12L) isolated from leaf, Pestalotiopsis sp. (AC 4S) isolated from stem,
P. visiae (AC 3S) isolated from stem, T. crissum (AC 1P) isolated from petiole, T. gamsii (AC 2R) isolated from root, and T. ovalisporum (AC 9S) isolated from stem.

**Diversity of endophytic fungi**

Endophytic fungi are ubiquitous, and every plant species examined to date have been found colonised by them (Arnold et al., 2001). A single plant species may harbour hundreds of endophytes which may inhabit all available tissues, including leaves, petioles, stems, twigs, barks, xylems, roots, fruits, flowers, and seeds (Chapela and Boddy, 1988; Fisher et al., 1993; Saikkonen et al., 1998; Jena and Tayung, 2013). In the present work, endophytic fungi were isolated from different plant parts of A. mangium with the highest number of isolates found in leaf and dominated by the genera *Trichoderma* and Nigrospora. *Trichoderma* spp. were present in all plant parts,
while Nigrospora spp. were present in all but fruit. In total, 66 endophytic fungal isolates were obtained from different plant parts of A. mangium.

Trichoderma and Nigrospora have also been reported as endophytes in other plants such as Rauvolfia serpentine, Prosopis cineraria, and Piper nigrum (Gehlot et al., 2008; Dutta et al., 2014; Sopialena et al., 2018). Trichoderma is also found in many ecosystems, and can reduce the severity of plant diseases by inhibiting the plant pathogens in the soil through their highly potent antagonistic and mycoparasitic activities (Hermosa et al.,

| No. | ID     | GenBank Accession no. | Plant part | Amphisphaeriales                        |
|-----|--------|-----------------------|------------|----------------------------------------|
| 1   | AC 3S  | MW254914              | Stem       | Pestalotiopsis vismiae (94% bootstrap) |
| 2   | AC 4S  | MW254920              | Stem       | Pestalotiopsis sp.                     |
| 3   | AC 9P  | MW254961              | Petiole     | Pestalotiopsis sp.                     |
| 4   | AC 5L  | MW254919              | Leaf       | Pestalotiopsis microspora              |
| 5   | AC 6L  | MW254921              | Leaf       | Pestalotiopsis microspora              |
| 6   | AC 12L | MW254938              | Leaf       | Pestalotiopsis neglecta                |
| 7   | AC 1U  | MW254912              | Fruit      | Pestalotiopsis microspora              |
| 8   | AC 5F  | MW254953              | Flower     | Pseudopestalotiopsis theae             |
| 9   | AC 17L | MW254948              | Leaf       | Pestalotiopsis vismiae (77% bootstrap) |
| 10  | AC 8P  | MW254958              | Petiole     | Neopestalotiopsis cubana Brotryosphaeriales (77% bootstrap) |
| 11  | AC 6S  | MW254925              | Stem       | Lasiodiplodia theobromae (97% bootstrap) |
| 12  | AC 10P | MW254965              | Petiole     | Lasiodiplodia theobromae              |
| 13  | AC 10L | MW254934              | Leaf       | Lasiodiplodia theobromae              |
| 14  | AC 16L | MW254946              | Leaf       | Lasiodiplodia theobromae              |
| 15  | AC 2U  | MW254928              | Fruit      | Lasiodiplodia theobromae              |
| 16  | AC 4U  | MW254930              | Fruit      | Lasiodiplodia venezuelensis            |
| 17  | AC 12S | MW254954              | Stem       | Eutiarosporella sp. Eurotiales (97% bootstrap) |
| 18  | AC 10S | MW254944              | Stem       | Aspergillus niger (97% bootstrap)      |
| 19  | AC 3R  | MW254904              | Root       | Aspergillus aculeatinus                |
| 20  | AC 5R  | MW254913              | Root       | Aspergillus niger                      |
| 21  | AC 19L | MW254950              | Leaf       | Aspergillus aculeatus                  |
| 22  | AC 6P  | MW254952              | Petiole     | Penicillium rolfsii Hypocreales (97% bootstrap) |
| 23  | AC 2R  | MW254903              | Root       | Trichoderma gamss (95% bootstrap)      |
| 24  | AC 6R  | MW254916              | Root       | Trichoderma spirale                    |
| 25  | AC 9R  | MW254964              | Root       | Trichoderma sp.                        |
| 26  | AC 1S  | MW254907              | Stem       | Trichoderma koningiopsis               |
| 27  | AC 5S  | MW254924              | Stem       | Trichoderma sp.                        |
| 28  | AC 7S  | MW254931              | Stem       | Trichoderma gamss                      |
| 29  | AC 9S  | MW254940              | Stem       | Trichoderma ovalisporum                |
| 30  | AC 1P  | MW254917              | Petiole     | Trichoderma crisuum                    |
| 31  | AC 7P  | MW254957              | Petiole     | Trichoderma longibrachiatum            |
| 32  | AC 11P | MW254966              | Petiole     | Trichoderma sp.                        |
| 33  | AC 12P | MW254967              | Petiole     | Trichoderma koningiopsis               |
| 34  | AC 1L  | MW254906              | Leaf       | Trichoderma gamss                      |
| 35  | AC 3L  | MW254910              | Leaf       | Trichoderma gamss                      |
| 36  | AC 11L | MW254936              | Leaf       | Trichoderma koningiopsis               |
| 37  | AC 13L | MW254939              | Leaf       | Trichoderma gamss                      |
| 38  | AC 4F  | MW254941              | Flower     | Trichoderma longibrachiatum            |
| 39  | AC 5U  | MW254960              | Fruit      | Trichoderma harzianum                  |
| 40  | AC 6F  | MW254955              | Fruit      | Trichoderma koningiopsis               |
| 41  | AC 8L  | MW254923              | Leaf       | Fusarium chlamydosporom Fleosporales (95% bootstrap) |
| 42  | AC 4L  | MW254918              | Leaf       | Curvularia pandanicola Hypocreales (95% bootstrap) |

(Continued)
Moreover, as revealed by research in recent decades, some *Trichoderma* strains can interact directly with roots, thus increasing plant growth potential, resistance to disease, and tolerance to abiotic stresses (Mastouri et al., 2010; Hermosa et al., 2012; Brotman et al., 2013). *Nigrospora* is also a beneficial member of the foliar endophytic community due to its mutualistic existence with their host plants, and having a potential for biological control strategies (Zakaria et al., 2016). Other than *Nigrospora*, *Pestalotiopsis* also is a beneficial member of the foliar endophytic community due to its ability to switch its nutritional mode, thus able to stay as an endophyte or switch to saprophyte when necessary (Douanla-Meli et al., 2013; Hamzah et al., 2018). Besides *Trichoderma*, *Nigrospora*, and *Pestalotiopsis*, other fungal genera such as *Lasiodiplodia*, *Sordariomycetes*, and *Aspergillus* have also been reported as predominant endophytic fungi in other plants species (Li et al., 2012; del Castillo et al., 2016), and have an antagonism ability (Chen et al., 2010). *Fusarium* too is a common endophytic fungal genus found in trees (Zakaria et al., 2010). Although it is widely available in most tropical plants investigated in past studies (Warman and Aitken, 2018), we recorded a low isolation frequency of *Fusarium*. Our finding also revealed lesser-known fungal genera, namely *Eutiarosporella*, *Curvularia*, *Glomerella*, and *Hypoxylon* in *A. mangium*.

In the present work, ITS sequences identified 63 endophytic fungal isolates from the phylum Ascomycota, and three from Zygomycota. The phylum Ascomycota has been reported to be the most common endophytic fungal phylum when isolated using standard isolation protocols (Koukol et al., 2012; Hamzah et al., 2018). Fungi from the phylum Zygomycota have been reported to be culture-method dependent (Crozier et al., 2006; Hamzah et al., 2018), which might explain the small isolate number reported in the present work. Comparative studies also show that only a small fraction of microorganisms in nature can be cultured using conventional microbiological techniques (Amann et al., 1995). There are many factors that can affect the microbial viability under laboratory conditions, for example the lack of knowledge about their nutritional requirements.

### Antagonism activities against Ceratocystis fimbriata

Fungal antagonism can manifest in many ways such as nutrition competition, niche exclusion, mycoparasitism, and the
production of extracellular metabolites (Siameto et al., 2010). These metabolites, especially antibiotics and lytic enzymes, have been widely applied in various fields like crop-pathogen controls. Endophytic microorganisms isolated from plants can produce various novel bioactive metabolites (Ramasamy et al., 2010). The bioactive metabolites produced by plants, microorganisms, and organisms are useful for the discovery and development of new drugs.

In the present work, Lasiodiplodia sp., T. koningiopsis, N. sphaerica and Trichoderma sp. successfully inhibited the pathogen C. fimbriata in the dual culture assay. The ability to out-grow the pathogen in vitro suggested that these fungi competed
for the space and nutrient with the pathogen. In theory, biological agents with antifungal properties are known to secrete certain enzymes which break down their competitors’ cell wall, thus restricting their growth (Sharon et al., 2001). The antagonism displayed by Lasiodiplodia sp. was more aggressive as compared to other endophytic fungi (Figure 3). This could be attributed to the production of lytic enzymes by Lasiodiplodia sp. (Anitha and Rabeeth, 2010). The antagonism displayed by Lasiodiplodia sp., T. koningiopsis, N. sphaerica and Trichoderma sp. could also be explained by their secretion of secondary metabolites into the growth medium, as well as nutrient depletion in the growth medium (Robinson et al., 2014). The antagonism displayed might also be influenced by the antibiotics or hydrolytic enzymes they produced (Kamala and Indira, 2011). The difference in antagonism
magnitude observed in the present work could also be dependent on specific fungal species (Kai et al., 2007). Previously, Lasiodiplodia sp. from the flower of Viscum coloratum also exhibited antimicrobial activity which could be due to the presence of cyclo-(Trp-Ala), ICA, indole-3-carbaldehyde, mullein, and 2-phenylethanol in their extract (Qian et al., 2014). Lasiodiplodia sp. isolated from the twig of Aegle marmelos has also been shown to have in vitro fibrinolytic activities (Meshram and Saxena, 2016). Another plant parts such as bark and leaf of Terminalia sp. has also been isolated with Lasiodiplodia sp. which not only exhibited antimicrobial and antioxidant activities, but also aided the plant to withstand stressful environmental conditions (Patil et al., 2014).

Conclusion

Diversity of endophytic fungi were successfully isolated from different parts of A. mangium, with Trichoderma spp. being the most prevalent, and were isolated from all six plant parts. Against C. fimbriata, the crude extracts from Trichoderma spp., N. sphaeraica, and Lasiodiplodia sp. exhibited strong inhibition in the dual culture assay. Thus, it can be concluded that certain endophytic fungi of A. mangium have the potential to be harnessed as anti-Ceratocystis agent in future biotechnological applications.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Author contributions

RT designed the study, collected, identified plant materials, and edited the manuscript. RT and RZ conducted the experiments, drafted, and revised the manuscript. Data analysis performed by RT, MA, MM, NS, WAW-M-A, and AH. MH assisted in DNA extraction. RT, MA, MM, NS, and AH supervised. RT acquired funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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