

THE POTENTIAL OF ENDOPHYTIC *Trichoderma* FROM OIL PALM (*Elaeis guineensis* Jacq.) ROOTS OF NORTH SUMATRA, INDONESIA AGAINST *Ganoderma boninense*

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ABSTRACT

Utilisation of endophytic *Trichoderma* increases tremendously as an alternative control against *G. boninense*, causal pathogen of basal stem rot (BSR) disease of oil palm. However, investigation of endophytic *Trichoderma* from Indonesia is still very scarce. The aims of this study were to isolate, identify and investigate the potential of endophytic *Trichoderma* from oil palm roots. Three potential endophytic *Trichoderma* species were isolated and further identified using macroscopic, microscopic, and molecular methods. Antagonistic activities of endophytic *Trichoderma* were tested using dual culture agar and poison food agar assay. A molecular approach using DNA sequencing of 5.8S-ITS region successfully identified the endophytic *Trichoderma* isolate ET501 as *Trichoderma reesei* strain RHa, while isolates of endophytic ET523 and ET537 were identified as *Trichoderma asperellum* isolate F1 and *Trichoderma asperellum* strain Q1, respectively. *Trichoderma reesei* ET501 was the most aggressive isolate against *G. boninense* with PIRG of 95.1% compared to *T. asperellum* ET523 and *T. asperellum* ET537 with PIRG of 87.1% and 88.9%, respectively. Meanwhile, *T. reesei* ET501 showed the strongest antibiosis activity with 100% inhibition in 80% concentration, compared to *T. asperellum* ET523 and *T. asperellum* ET537 which gave 12.3% and 90.5% of inhibition, respectively.

Keywords: *Trichoderma reesei*, *Trichoderma asperellum*, *Ganoderma boninense*, basal stem rot, endophytic.

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INTRODUCTION

Basal stem rot (BSR) disease caused by a number of *Ganoderma* species, is the most destructive disease causing significant losses of oil palm yield in South-east Asia (Turner, 1981). The two largest palm oil producers in the world, Malaysia and Indonesia,

are being threatened by the BSR disease, which is caused by *Ganoderma boninense* (Cooper *et al.*, 2011; Turner, 1981). About 50% of the palms are lost with the majority of standing palms showing disease symptoms at the time of replanting after 25 years in North Sumatra (Cooper *et al.*, 2011).

There is no conclusive strategy or method to solve BSR disease effectively to date. However, currently, biological control has been widely implemented in Indonesia oil palm plantation as the main strategy to control and prevention method in integrated disease management (IDM). The demand for alternative control of plant pathogens has become stronger owing to concern about the safety and environmental impacts of chemicals (Schubert

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et al., 2008). Biological control is increasingly being considered as an alternative treatment in sustainable agriculture. *Trichoderma* spp. are one of the popular biological control agents that are widely used to control BSR disease.

Trichoderma is soilborne and associated with the roots of plants and commonly considered for their potential to control plant diseases with many aspects of endophytic association characteristic (Harman *et al.*, 2004; Vinale *et al.*, 2007). These fungi colonise the root epidermis and outer cortical layers and also release bioactive compounds that cause walling off of the *Trichoderma* thallus (Harman, 2006). Isolation of *Trichoderma* from different soils has revealed that several *Trichoderma* species are highly associated with plant and often as the predominant species in the plant root ecosystem (Kredics *et al.*, 2014).

Identification of *Trichoderma* strains that have potential as biocontrol agents can be preliminarily conducted using *in vitro* antagonistic assays (Larralde *et al.*, 2008). This assay is a predictive method to determine the antagonistic activity potential of the biocontrol agents before carrying out time-consuming and more expensive studies (Lo *et al.*, 1998). Identification of fungi species using morphological features is a less precise identification method compared to the molecular approach. Therefore, a molecular approach through polymerase chain reaction (PCR) amplification and sequence analysis are needed to complete a precise identification of *Trichoderma*.

Due to the importance of endophytic *Trichoderma* spp. as biocontrol agents against *G. boninense* in oil palm industry, exploration and investigation about endophytic *Trichoderma* need to be conducted. Precise identification and characterisation of *Trichoderma* are also important to optimise the full potential of *Trichoderma* in many specific needs (Lieckfeldt *et al.*, 1999). The aims of this study were to isolate, identify, and study the potential endophytic *Trichoderma* from North Sumatra that can be used as a biological control agent against *G. boninense*.

MATERIALS AND METHODS

Isolation of Endophytic *Trichoderma*

The endophytic *Trichoderma* isolates were isolated from roots of remaining healthy mature oil palm in high disease incidence area in Negeri Lama Estate, Asian Agri Group, North Sumatra, Indonesia in accordance to Elad *et al.* (1981) with some modifications. Roots sample were surface-sterilised through sequential immersion in 2% sodium hypochlorite (chlorine bleach), 70% ethanol and sterilised water followed by blotting dry on a sterile filter paper. Root samples were placed onto *Trichoderma* selective media (TSM) and incubated

for seven days at room temperature ($27^{\circ}\text{C} \pm 2$). Each distinct fungal colony was sub-cultured on potato dextrose agar (PDA) and incubated for seven days at room temperature.

Pure cultures of endophytic *Trichoderma* were used for further study. A total of 20 endophytic *Trichoderma* isolates were obtained from Negeri Lama Estate and subjected to dual culture assay, a preliminary assay to screen mycoparasites potential through antagonistic activity against *G. boninense* (data not shown). From the preliminary assay, three most potential endophytic *Trichoderma* (ET501, ET523, and ET537) were selected for further identification and investigation to evaluate their aggressiveness against *G. boninense* using dual culture agar and antibiosis properties through poison food agar assay.

Identification of Endophytic *Trichoderma*

Morphological observation. Morphological observation of endophytic *Trichoderma* isolates was conducted by observing both macroscopic and microscopic fungal colonies. For macroscopic observation, the morphological feature was observed and the growth rate recorded up to five days from the measurement of five replicates. Meanwhile, for microscopic observation, mycelia from each isolate were taken from PDA plate and spread onto a clean slide mounted with a drop of water and observed under a light microscope using 1000X magnification. After that, the microscopic feature was compared to the monograph prepared by Rifai (1969).

Molecular Identification

DNA extraction and PCR amplification. Extraction of endophytic *Trichoderma* DNA was done using the mini protocol for purification of total DNA from fungi tissue with *Invitrogen* DNA isolation kit with modifications. Approximately, 100 mg of *Trichoderma* mycelia originally grown in PDA were further extracted following the DNA extraction protocol. PCR amplification of the *Trichoderma* DNA was done at ITS1 and ITS2 regions and the 5.8S gene using ITS 1 and ITS 4 primers. TAE agarose 1.5% (w/v) was used for the agarose gel electrophoresis with 100 V for 60 min with a size of PCR amplicon approximately 570 bp. The reaction was performed in 10 μl PCR mixes, 10 \times PCR buffer 1 μl , 0.1 μl dNTP, 0.5 μl forward and reverse primers, 0.1 μl *Taq* polymerase [New England Biolabs (NEB)], 1 μl of DNA template and 6.8 μl RNase-free water. The primer sequences used were ITS 1: 5'-TCC GTA GGT GAA CCT GCG G -3' as forward primer and ITS 4: 5'- TCC GCT TAT TGA TAT GC -3' as reverse primer with the initial denaturation was set at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min.

DNA sequencing and sequence analysis. DNA sequencing was done at Bioneer Laboratory (South Korea). The sequences obtained were further analysed using Basic Local Alignment Search Tool (BLAST) to obtain the closest matches sequence in the National Centre for Biotechnology Information (NCBI) GenBank database. A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysing (MEGA) software with Neighbour-Joining method and 1000 bootstraps to show the relationships among the homologous microorganisms.

Dual Culture Assay

A 6 mm diameter of *G. boninense* agar disc from Negeri Lama Estate that has been identified was taken from the edge of actively growing pure culture (five days) and placed on PDA 1 cm from the edge of the 9 cm petri dish. The *G. boninense* was allowed to grow for three days by which time the colony reached to ± 2 cm. After that, a 6 mm diameter disc of endophytic *Trichoderma* isolate was taken from the pure culture of isolated endophytic *Trichoderma*, then placed on the opposite side of the petri dish containing *G. boninense* separately, while control petri dish contained only *G. boninense*. The assay was incubated for six days. The antagonistic activity of the endophytic *Trichoderma* isolates was measured using the formula of percentage inhibition of radial growth (PIRG) following Bivi *et al.* (2010).

$$\text{PIRG} = \frac{R1 - R2}{R1} \times 100\%$$

where PIRG = percent inhibition of radial growth of *Ganoderma*, R1= represent the radial growth of *Ganoderma* in the absence of endophytic *Trichoderma*, while R2= represent the radial growth of *Ganoderma* in the presence of endophytic *Trichoderma*.

Poison Food Agar Assay

Five-day old endophytic *Trichoderma* (ET501, ET523 and ET537) conidia were harvested from cultures grown at room temperature on PDA by placing 10 ml of sterile distilled water on the plate surface and slowly agitating using a sterile glass rod. The concentrated endophytic *Trichoderma* spores suspension was collected and filtered through sterile glass wool for mycelium removal. The concentrated suspension was adjusted to 1×10^6 conidia using haemocytometer. About 200 ml potato dextrose broth (PDB) was prepared into a conical flask and inoculated with 1 ml of endophytic *Trichoderma* suspension and incubated in orbital shaker incubator with 0.78 RCF for seven days at room temperature. The mycelia were removed by filtration after seven days of growth and the filtrate was sterilised by passing through a 0.22 μm membrane disposable

unit (Sundram, 2013). The culture filtrate from conidia of endophytic *Trichoderma* was incorporated into PDA at 20%, 40%, 60%, and 80% concentration according to the method as described by Sundram (2013).

$$\text{PIMG} = \frac{R1 - R2}{R1} \times 100\%$$

PIMG is percent inhibition of mycelia growth. R1, radial growth of *Ganoderma* in the absence of antagonist fungal. R2, radial growth of *Ganoderma* in the presence of antagonist.

Scanning Electron Microscopy Observation

Scanning electron microscopy (SEM) observation was conducted in the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta in Indonesia. Zones of interaction between *G. boninense* and endophytic *Trichoderma* spp. on PDA medium after six days were prepared for observation using SEM. Each agar plate with fungal mycelia was first excised using a scalpel and trimmed to approximately 10 mm x 10 mm in size and as thin as possible to reduce the moisture. Samples were dried at 60°C for 90 min maximum. Samples were mounted on platinum formvar, coated with the platinum ion in JEOL JEC-3000FC auto fine coater for 1 min and vacuum until 2 pa of pressure, this treatment was replicated for two times. Samples were observed under a JEOL JSM 6510 LA SEM.

RESULTS AND DISCUSSION

Endophytic *Trichoderma* Isolates

All colonies of endophytic *Trichoderma* emerged from TSM after three to seven days of incubation at room temperature (Figure 1). Samuels (2004) reported that incubation temperature is an important taxonomic criterion for *Trichoderma* identification, but most of the endophytic *Trichoderma* species grow optimally at 25°C-30°C. Initial mycelium of endophytic *Trichoderma* morphological characteristics on PDA appeared as white hyphal growth followed by green conidiation on the fourth to fifth day after plating. The same result was also reported by Sundram (2013), where young *Trichoderma* morphological characteristics also appeared as faint white hyphal growth followed by green conidiation initiating from the centre of the plate.

Morphological Characteristics

Colony characteristics of endophytic *Trichoderma*. The morphological characteristics of endophytic

Trichoderma species have been used to characterise and distinguish the species of isolates (Gams and Bissett, 1998). Certain colony characters such as growth rate, colony appearance, and pigmentation can be the main characteristics of a species. Preliminary identification was carried out which showed that all isolates were endophytic *Trichoderma* species. Growth rate results for all endophytic *Trichoderma* isolates are shown in Table 1. The isolate of endophytic *Trichoderma* ET501 grew as the fastest isolate which fully colonised the petri dish in four days of incubation time, while endophytic *Trichoderma* ET523 and ET537 isolates fully colonised the petri dish in five days of incubation.

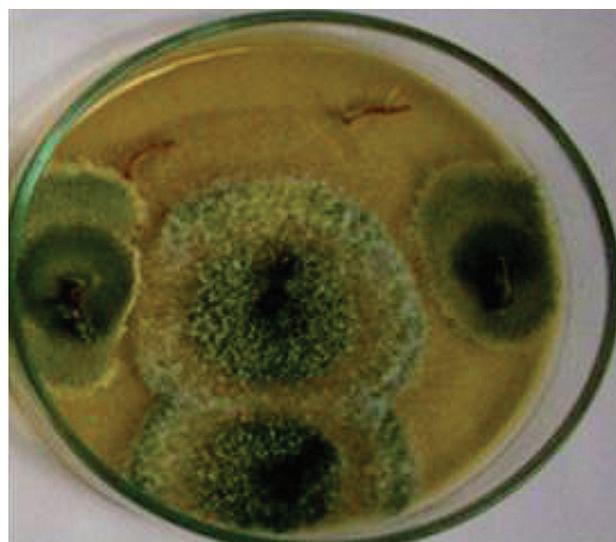


Figure 1. Endophytic *Trichoderma* isolates from oil palm roots on *Trichoderma* selection media (TSM).

The conidia were dispersed and formed concentric rings pattern in all isolates within five days of incubation (Figure 2). Samuels (2004) reported that conidia of most of *Trichoderma* species will form within a week of incubation. Concentric ring of endophytic *Trichoderma* ET523 isolate was growing densely compared to endophytic *Trichoderma* ET537 and ET501 isolates. The endophytic *Trichoderma* ET537 isolate appearance was more cottony than endophytic *Trichoderma* ET523 and ET501 isolates. The same result was also reported by Shah *et al.* (2012) where some of *Trichoderma* species showed white cottony mycelium with green conidiation towards the margins. The colour of mature conidia of endophytic *Trichoderma* ET501 isolate was yellowish green (Figure 2a), while conidia of endophytic *Trichoderma* ET523 and ET537 were greyish green colour (Figures 2b and 2c, respectively). Sekhar *et al.* (2017) also reported that *Trichoderma* spp. conidia vary in colour from dark green to pale yellowish. The endophytic *Trichoderma* ET501 was found to produce diffusible yellow pigments and caused the PDA to turn yellowish. These diffusible yellow pigments were not found in other isolates. *Trichoderma* strains within *Longibrachiatum* species typically have conspicuous bright greenish yellow pigments, at least when first isolated (Samuels, 2004).

Microscopic features of endophytic *Trichoderma* spp. The microscopic features of endophytic *Trichoderma* revealed different characteristic between the isolates. The shapes and sizes of conidia were presented in Table 2. The endophytic *Trichoderma*

TABLE 1. THE GROWTH RATE OF ENDOPHYTIC *Trichoderma* ON PDA AT ROOM TEMPERATURE

| <i>Trichoderma</i> isolate | Average mycelium growth/days (cm) | | | | | |
|----------------------------|-----------------------------------|-----|-----|-----|-----|----|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| ET501 | 0 | 1.0 | 4 | 7 | FC | FC |
| ET523 | 0 | 0.9 | 2.9 | 5.1 | 7.3 | FC |
| ET537 | 0 | 0.9 | 3.0 | 5.1 | 7.2 | FC |

Note: FC – fully colonised in 9 cm petri dish; PDA - potato dextrose agar.

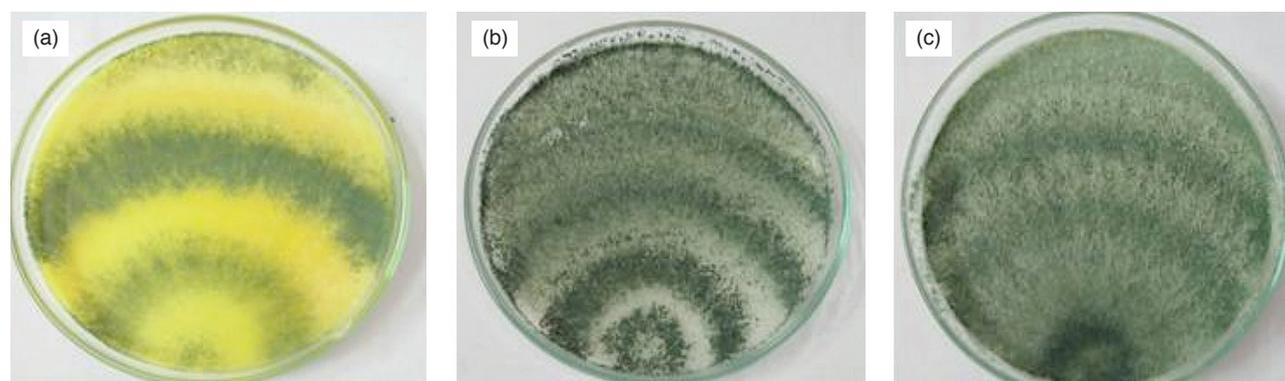


Figure 2. Endophytic *Trichoderma* isolates. (a) ET501; (b) ET523; (c) ET537.

TABLE 2. MORPHOLOGICAL CHARACTERISTICS OF ENDOPHYTIC *Trichoderma* ISOLATES

| <i>Trichoderma</i> Isolate | Shape | Conidial size | |
|----------------------------|-------------|---------------|------------|
| | | Length (µm) | Width (µm) |
| ET501 | Ellipsoidal | 3.95 | 2.65 |
| ET523 | Globose | 3.30 | 3.30 |
| ET537 | Globose | 3.10 | 3.10 |

ET501 has an ellipsoidal shape of conidia. On the other hand, both of endophytic *Trichoderma* ET523 and ET537 isolates showed globose conidia shape (Figures 3). These results are in accordance with Sekhar *et al.* (2017) who reported the variation of *Trichoderma* conidia shape including ellipsoidal, sub-cylindrical, obovoid, globose, and narrow ellipsoid. The endophytic *Trichoderma* ET501 has conidia with the average diameters of 3.95 µm in length and 2.65 µm in width (Figure 3a). Meanwhile, the conidial size of endophytic *Trichoderma* ET523 and ET537 isolates were almost similar, where endophytic *Trichoderma* ET523 conidia tended to be slightly bigger than endophytic *Trichoderma* ET537 conidia (Figures 3b and 3c, respectively). Isolate of endophytic *Trichoderma* ET523 has average size conidia of 3.3 µm, while that of endophytic *Trichoderma* ET537 3.1 µm.

regions of the rDNA diverged at different rates (Bruns *et al.*, 1991; Latifah *et al.*, 2002). Hence, ITS region becomes the main focus for further molecular identification.

Although the PCR products size among the samples were approximately the same, it may not fully represent the sequence similarity nor phylogenetic relationship. Therefore, the genomic sequence obtained must be further distinguished using analysing tools such as the restriction amplification fragment length polymorphisms (RAFLP), random amplified polymorphic DNA (RAPD), or genomic sequence analysis (*e.g.* BLAST, phylogenetic tree, *etc.*). The sequences, thus, obtained were further analysed using BLAST to identify the homologous sequences.

Sequence analyses of endophytic *Trichoderma* spp. DNA. ITS region sequences of each endophytic *Trichoderma* isolates were further compared to the five possible homologous sequences from NCBI GenBank database. Direct sequencing of ITS region combined with BLAST search has proved to be effective and reliable for the identification of fungi directly from wood in construction (Hogberg and Land, 2004). The five most possible homologous sequences of endophytic *Trichoderma* spp. from NCBI GenBank database are shown in Table 3. In Table 3a,

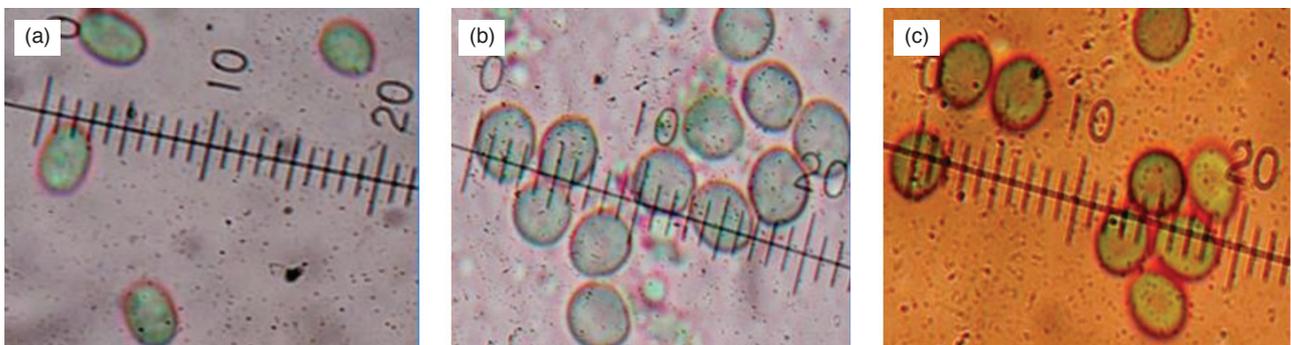


Figure 3. Conidia of endophytic *Trichoderma* under light microscope with 1000X magnification. (a) ET501; (b) ET523; (c) ET537.

Molecular Identification of Endophytic *Trichoderma* spp.

PCR amplification. PCR amplification using ITS1 and ITS4 as primers showed that endophytic *Trichoderma* isolated from oil palm roots produced PCR product of about 570 bp (Figure 4). The same result was reported by Hermosa *et al.* (2000) where PCR products of *Trichoderma* spp. ranged from 560-600 bp. Fungal internal transcribed spacer (ITS) region combined the highest resolving power for discriminating closely related species with a high PCR and sequencing success rate across a broad range of fungi (Schoch *et al.*, 2011). Different taxonomic levels could be exploited due to different

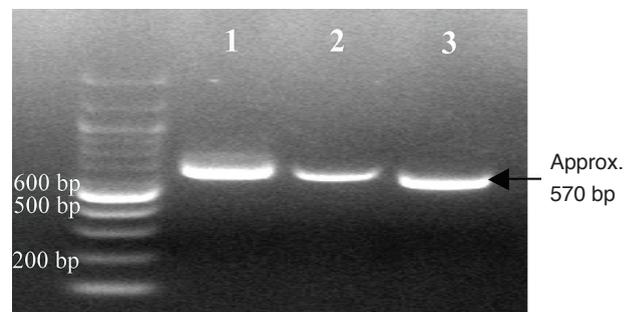


Figure 4. Polymerase chain reaction (PCR) products of the most potential endophytic *Trichoderma* isolates against *G. boninense*. 1 = ET501, 2 = ET523, and 3 = ET537. The target band size is about 570 bp, M = 100 bp ladder (NEB).

it was suggested that the endophytic *Trichoderma* ET501 was identified as *T. reesei* since the most similar homolog to the sequence is *T. reesei* with 99% of similarity based on BLAST maximum score. Both of endophytic *Trichoderma* ET523 and ET537 isolates were identified as *T. asperellum* species as shown in Tables 3b and 3c with 99% of the maximum identity for all BLAST results. The DNA sequence data gave a detailed and precise information that is highly relevant to endeavours such as evolutionary studies of the origin of organism groups and their geographical distribution, taxonomic classification, and the detection and identification of species in various substrates and habitats (Felsenstein, 2004; Shenoy *et al.*, 2007; Stajich *et al.*, 2009; Nilsson *et al.*, 2011).

The higher similarity rank of homologous sequences from BLAST GenBank database showed the phylogenetic relationship closeness of the isolate. The phylogenetic trees were constructed on the relationships among the homologous microorganisms based on DNA sequence homology (Figure 5). Based on the phylogenetic tree that was constructed using MEGA 5.0 software,

endophytic *Trichoderma* ET501 isolate has the closest relationship with *T. reesei* strain RHa (Figure 5a). Meanwhile, endophytic *Trichoderma* ET523 and *Trichoderma* ET537 isolates showed the closest relationship with *T. asperellum* isolate F1 (Figure 5b) and *Trichoderma* strain Q1 (Figure 5c), respectively. The phylogenetic trees give stronger evidence of the three endophytic *Trichoderma* isolates (ET501, ET523, and ET537) to be classified as *Trichoderma* species. Identification of *Trichoderma* species, traditionally based on morphological aspects, has changed to the use of molecular data, which includes a wide use of ITS regions and ribosomal RNA 5.8S regions amplification (Maymon *et al.*, 2004; Watanabe *et al.*, 2005). The DNA-based method has been used to build the microbial modern taxonomy and widely used in species identification in most fungi (Petti, 2007; Tsui *et al.*, 2011). Identification of the endophytic *Trichoderma* strains used in the present work, is a key point, which will allow a better future investigation to evaluate bioactivity under different growing conditions and their mode(s) of action. This information, will allow a better microorganism selection, which could be susceptible to a better use

TABLE 3a. THE FIVE MOST HOMOLOGOUS MICROORGANISMS FROM THE NATIONAL CENTRE FOR BIOTECHNOLOGY INFORMATION (NCBI) GENBANK DATABASE IN COMPARISON TO THE ET501 ISOLATE

| Accession No. | Description | Max. score | Total score | Query value (%) | Max. ident (%) |
|---------------|--|------------|-------------|-----------------|----------------|
| KM246746.1 | <i>Trichoderma reesei</i> strain RHa 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 134 | 1 134 | 99 | 99 |
| KT876619.1 | <i>Trichoderma reesei</i> strain IPBCC93_260 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 131 | 1 131 | 100 | 99 |
| KT336514.1 | <i>Trichoderma reesei</i> isolate M10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 129 | 1 129 | 99 | 99 |
| KU729028.1 | <i>Trichoderma reesei</i> strain ATCC 26921 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence | 1 120 | 1 120 | 98 | 99 |
| KP263684.1 | <i>Trichoderma reesei</i> isolate TV219 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 118 | 1 118 | 97 | 99 |

TABLE 3b. THE FIVE MOST HOMOLOGOUS MICROORGANISMS FROM THE NATIONAL CENTRE FOR BIOTECHNOLOGY INFORMATION (NCBI) GENBANK DATABASE IN COMPARISON TO THE ET523 ISOLATE

| Accession No. | Description | Max. score | Total score | Query value (%) | Max. ident (%) |
|---------------|--|------------|-------------|-----------------|----------------|
| KU987251.1 | <i>Trichoderma asperellum</i> isolate SDLA28 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence | 1 024 | 1 024 | 98 | 99 |
| KP281701.1 | <i>Trichoderma asperellum</i> isolate F1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 022 | 1 022 | 98 | 99 |
| KU987250.1 | <i>Trichoderma asperellum</i> isolate SDLA27 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence | 1 020 | 1 020 | 97 | 99 |
| KT876619.1 | <i>Trichoderma asperellum</i> isolate CRT2-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence | 1 018 | 1 018 | 97 | 99 |
| KP281704.1 | <i>Trichoderma asperellum</i> isolate F4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 016 | 1 016 | 97 | 99 |

TABLE 3c. THE FIVE MOST HOMOLOGOUS MICROORGANISMS FROM THE NATIONAL CENTRE FOR BIOLOGY INFORMATION (NCBI) GENBANK DATABASE IN COMPARISON TO THE ET537 ISOLATE

| Accession No. | Description | Max. score | Total score | Query value (%) | Max. ident (%) |
|---------------|---|------------|-------------|-----------------|----------------|
| KM268676.1 | <i>Trichoderma asperellum</i> strain DWG3(1) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 029 | 1 029 | 97 | 99 |
| KC576729.1 | <i>Trichoderma asperellum</i> strain CEN768 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence | 1 027 | 1 027 | 97 | 99 |
| HQ293149.1 | <i>Trichoderma asperellum</i> strain Q1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 026 | 1 026 | 97 | 99 |
| KP263700.1 | <i>Trichoderma asperellum</i> isolate TV239 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 024 | 1 024 | 97 | 99 |
| KC403957.1 | <i>Trichoderma asperellum</i> strain TA2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1 022 | 1 022 | 97 | 99 |

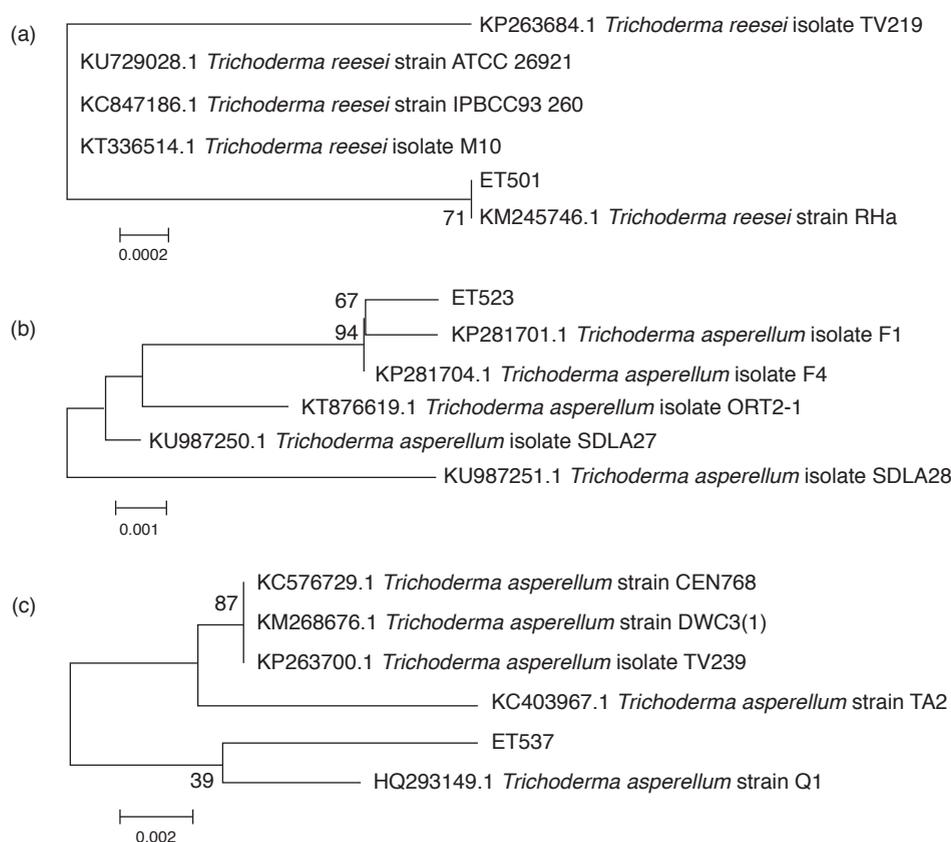


Figure 5. Phylogenetic tree of endophytic *Trichoderma* spp. isolates with other five homologous microorganisms. (a) ET501 isolate [with National Centre Biology Information (NCBI) query ID: lcl\141685]; (b) ET523 isolate [with National Centre for Biology Information (NCBI) query ID: lcl\61097]; (c) ET537 isolate [with National Centre Biology Information (NCBI) query ID: lcl\72211].

for plant pathogen control (Guigón-López *et al.*, 2010).

Dual Culture Assay of Potential Endophytic *Trichoderma*

The biocontrol activity of endophytic *Trichoderma* spp. was tested against *G. boninense* in dual culture assay. All the endophytic *Trichoderma* isolates were aggressive mycoparasites with highly antagonistic activity against *G. boninense*. Isolate of endophytic *T. reesei* ET501 showed to be the most aggressive isolate compared to other isolates according to the pathogen inhibition ability with 95.1% inhibition, while endophytic *T. asperellum* ET523 showed 87.1%

and *T. asperellum* ET537 showed 88.9% inhibition. Different mycoparasitic activities of each endophytic *Trichoderma* samples through the PIRG values are shown in Table 4. All the endophytic *Trichoderma* isolates showed significant inhibitory effect on *G. boninense* growth. According to Musa *et al.* (2017), this effect could be most likely due to the production of antibiotic, secondary metabolite compounds or lytic enzymes which contributed to the direct antagonistic degrading effect on the *G. boninense* cell wall and mycoparasitic activities.

The contact zones between endophytic *Trichoderma* and *G. boninense* in all dual culture plates was curved, with concavity oriented towards the *G. boninense* mycelium (Figure 6). Isolate of

TABLE 4. ANTAGONISTIC ACTIVITY OF POTENTIAL ENDOPHYTIC *Trichoderma* AGAINST *G. boninense* THROUGH DUAL CULTURE AND POISON FOOD AGAR ASSAYS

| Treatment | Percentage inhibition of radial growth (PIRG) | Percentage inhibition mycelium growth (PIMG %) | | | |
|----------------------------|---|--|-------------------|-------------------|-------------------|
| | | 20% | 40% | 60% | 80% |
| Control | 0 ^a | 0 ^a | 0 ^a | 0 ^a | 0 ^a |
| <i>T. reesei</i> ET501 | 95.1 ^b | 18.7 ^b | 30.1 ^c | 81.3 ^s | 100 ^k |
| <i>T. asperellum</i> ET523 | 87.1 ^c | 2.5 ^c | 3.4 ^c | 7.5 ^h | 12.3 ^d |
| <i>T. asperellum</i> ET537 | 88.9 ^c | 12.3 ^d | 23.4 ^f | 70.5 ⁱ | 90.5 ^l |

Note: Means within the same column followed by the superscript of same letters are not significantly different at $P < 0.05$ with Duncan ($n=5$).

endophytic *T. reesei* ET501 mycelium was in contact with the *G. boninense* mycelium in three days of incubation, while other isolates revealed no mycelia contact with *G. boninense* at the same number of days of incubation (Figure 6a). The endophytic *T. reesei* ET501 growth could fully covered the *G. boninense* mycelium in 10 days of incubation, while other isolates only made contact with *G. boninense* mycelium in 10 days of incubation without covering the *G. boninense* (Figure 6b).

Antibiosis Properties Poison Food Agar Assay of Potential Endophytic *Trichoderma*

The endophytic *Trichoderma* isolates which have promising potential to inhibit *G. boninense* growth through *in vitro* assay were then subjected to the poison food agar assay. All isolates have the potential to inhibit the growth of *G. boninense* in poison food agar assay compared to control (Figure 7). The endophytic *T. reesei* ET501 culture filtrate completely inhibited *G. boninense* growth (100% of PIMG) with 80% incorporation of the culture filtrate into the media, while *T. asperellum* ET537 and *T. asperellum* ET523 gave 90.5% and 12.3% of PIMG, respectively (Table 4). Endophytic *T. reesei* ET501 isolate also gave higher inhibition percentage when observed in all concentration of culture filtrate compared to endophytic *T. asperellum* ET523 and *T. asperellum* ET537 isolates. Miettinen-Oinonen (2004) reported that *T. reesei* is one of cellulolytic best-known organisms, producing readily and in large

quantities a complete set of extracellular cellulolytic enzymes. *T. reesei* also reportedly produces at least three extracellular α -1,3-glucanases (Budiarti *et al.*, 2009), which is the key enzyme in the lysis of cell walls during their mycoparasitic action against phytopathogenic fungi (De La Cruz *et al.*, 1995). Meanwhile, *T. asperellum* biological activity has commonly been related to chitinolytic enzyme production (Viterbo *et al.*, 2002), α -1,3-glucanase and α 1,6-glucanase production (Marcello *et al.*, 2010), cellulases and proteases too (Sanz *et al.*, 2004). El-Komy *et al.* (2015) also reported that *T. asperellum* isolates significantly reduced the mycelial growth of plant pathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Endophytes may be a better and effective alternative as biocontrol agents as they are buffered from environmental changes which are very important for rhizosphere competence of a biocontrol agent (Sundram, 2013). Therefore, the potential use of endophytic *T. reesei* and *T. asperellum* as biocontrol agents against *G. boninense* may be the answer for enhanced biocontrol strategy to overcome BSR.

Morphological Changes of *Ganoderma boninense* After Interaction with Potential Endophytic *Trichoderma*

Healthy *G. boninense* mycelium as control can be seen in Figure 8a. Healthy mycelium of *G. boninense* was cultured on PDA medium without the presence of *Trichoderma* spp. The mycelium was

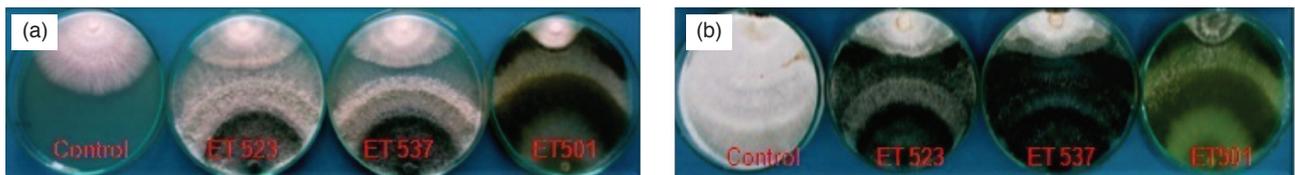


Figure 6. Dual culture assay of endophytic *Trichoderma* spp. against *Ganoderma*. (a) Six days of incubation time; (b) Ten days of incubation time.

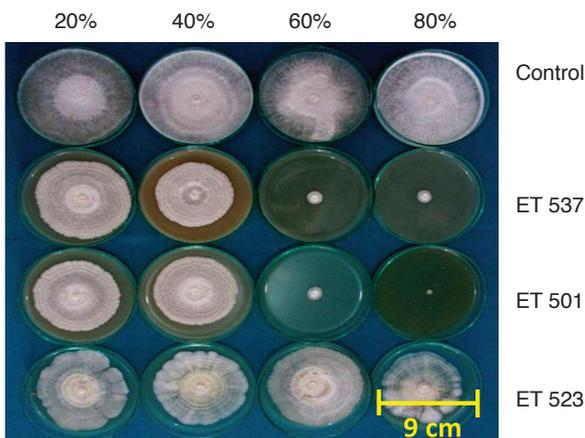


Figure 7. Antibiosis properties via poison food agar assay of endophytic *Trichoderma* culture filtrate incorporated into growth media at 20%, 40%, 60% and 80% concentration.

tight and packed with branches of hyphae strands appearing normal with no deformity. The presence of endophytic *Trichoderma* significantly caused disruption to *Ganoderma* mycelium. Alexander *et al.* (2015) also reported that observation under SEM showed that *Trichoderma* spp. and *Bacillus* spp. induced stripping of *G. boninense* hyphal structure by destroying the cellular structure. In this research, endophytic *T. reesei* ET501 and endophytic *T. asperellum* ET537 gave the most destructive effect to *Ganoderma* mycelium in six days of incubation. While, endophytic *T. asperellum* ET523 caused the least destructive effect to *G. boninense* mycelium. Disrupted *G. boninense* mycelium is characterised by flattened and shrivelled hyphal structure compared to healthy *G. boninense* hyphae (Figure 8b).

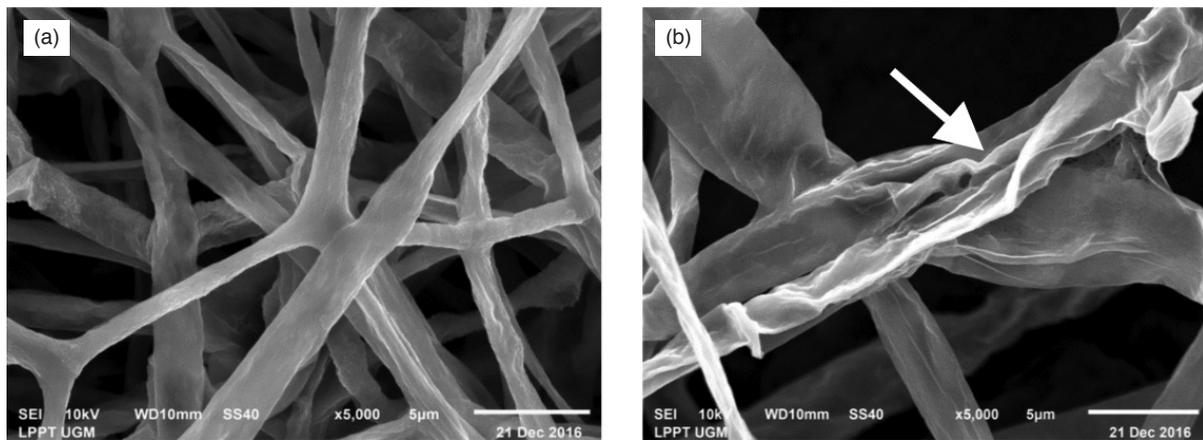


Figure 8. Six-day old *G. boninense* mycelium observed under scanning electron microscopy (SEM). (a) Healthy *G. boninense* mycelium at 5000X magnification; (b) *G. boninense* mycelium destroyed by endophytic *T. reesei* ET501 at 5000X magnification (arrowed).

CONCLUSION

Three most potential endophytic *Trichoderma* isolates from oil palm roots were successfully isolated with different characteristics and identified by DNA sequence analysis of 5.8S-ITS region. The endophytic ET501 isolate was identified as *Trichoderma reesei* strain RHa, while two other isolates namely endophytic ET523 and ET537 were identified as *Trichoderma asperellum* isolate F1 and *Trichoderma asperellum* strain Q1, respectively. All endophytic *Trichoderma* isolates in this study could give a promising inhibition effect to *G. boninense*. Endophytic *T. reesei* ET501 was the most aggressive isolate against *G. boninense* compared to endophytic *T. asperellum* ET523 and *T. asperellum* ET537 with PIRG inhibitory effect of 95.1%, 87.1% and 88.9%, respectively. Meanwhile, endophytic *T. reesei* ET501 also showed the strongest antibiosis activity with 100% inhibition in 80% concentration, followed by *T. asperellum* ET537 (90.5%) and *T. asperellum* ET537 (12.3%). Both endophytic *T. reesei* ET501 and *T. asperellum* ET537 gave the most destructed effect to *G. boninense* mycelium, while the endophytic *T. asperellum* ET523 caused the least destruction to *G. boninense* mycelium.

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