

Biological control agents potential and molecular identification of endophytic *Trichoderma* isolates originated from oil palm tissue against *Ganoderma boninense* Pat.

Potensi agen pengendali hayati dan identifikasi molekuler isolat *Trichoderma* endofit yang berasal dari jaringan tanaman kelapa sawit terhadap *Ganoderma boninense* Pat.

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ABSTRACT

This research aimed to determine the potential of endophytic *Trichoderma* sp. originated from oil palm plant tissue as a biological control agent and its molecular identification. The potency assay was carried out using a completely randomized design consisting of three isolates of *Trichoderma* sp. from different plant tissues (TR01 from root tissue, TS01 from stem tissue, and TM01 from midrib tissue) with inhibitory ability parameters. The inhibition of the growth of *G. boninense* was performed at the concentration of 15% and 20%, and the molecular identification of *Trichoderma* sp. endophyte isolates was analyzed using BLAST software. The results showed that *Trichoderma* sp. TM01 isolate had larger inhibitory than the other isolates on days 3 and 4 by 60% and 68%. TM01 isolate also showed inhibition to the growth of the *G. boninense* at 15% and 20%, with the percentage of inhibition reaching 71.79% and 82.05%. Based on phylogenetic analysis, the three endophytic isolates of *Trichoderma* sp. were closely related to *Trichoderma virens*.

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ABSTRAK

Penelitian ini bertujuan untuk mengetahui potensi *Trichoderma* sp. endofit yang berasal dari jaringan tanaman kelapa sawit sebagai agen pengendali hayati dan identifikasinya secara molekuler. Uji dilakukan dengan menggunakan rancangan acak lengkap yang terdiri dari tiga isolat *Trichoderma* sp. yang berasal dari jaringan tanaman yang berbeda (TR01 dari jaringan akar, TS01 dari jaringan batang, dan TM01 dari jaringan pelepah) dengan parameter kemampuan penghambatan. Penghambatan pertumbuhan *G. boninense* dilakukan pada konsentrasi 15% dan 20%, serta identifikasi molekuler isolat *Trichoderma* sp. endofit dianalisis menggunakan software BLAST. Hasil penelitian menunjukkan bahwa *Trichoderma* sp. isolat TM01 memiliki daya hambat yang lebih besar dibandingkan isolat lainnya pada hari ke-3 dan ke-4 sebesar 60% dan 68%. Isolat TM01 juga menunjukkan penghambatan terhadap pertumbuhan *G. boninense* pada konsentrasi 15% dan 20% dengan persentase penghambatan mencapai 71.79% dan 82.05%. Berdasarkan analisis filogenetik, ketiga isolat endofit *Trichoderma* sp. terkait erat dengan *Trichoderma virens*.

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INTRODUCTION

Stem rot disease caused by *Ganoderma boninense* is a significant disease in oil palm plantations in Indonesia. The pathogens attack plants that are already producing and attack the immature plants in the first generation, even in the nursery (Lisnawita et al., 2016; Suryanto et al., 2012). This disease incidence was reported to be higher in the peatlands with a higher attack rate and appeared earlier than on mineral soil (Rakib et al., 2017; Supriyanto et al., 2020). For example, Lisnawati et al. reported that basal stem rot disease incidence could reach 50% in the 17 years old oil palm plantation by the community in Bukit Kijang Village, Nort Sumatera (Lisnawita et al., 2016). Several decades ago, the disease height was only found in gardens with more than twice replanting, but now the disease is relatively high (Priwiratama et al., 2014).

Many efforts have been conducted to suppress the development of *Ganoderma boninense* in oil palm plantations. First, the technical culture has been applied, including sanitation of inoculum sources, hole-in-hole planting systems, surgery and backfilling, and construction of isolation trenches (Priwiratama et al., 2014). Another method uses fungicides such as trunk injection by hexaconazole (Mohammed et al., 2014). But this control needs to be done carefully; in the long term, the fungicides will have a negative impact on the environment, such as killing the non-pathogenic organisms, the occurrence of resistant pathogens, and the emergence of new physiological races (Chung et al., 2011; Living with Resistance project, 2018).

The leading cause of these pathogens being difficult to control is their ability to survive in extreme conditions in the soil in the form of structural breaks or chlamydospores even when the host crop is unavailable (Loyd et al., 2019). *Ganoderma boninense's* capability to survive in the soil can reach ten years. Furthermore, the fungus *G. boninense* has different genetic variability with different pathogenicity against plants. It may be because parts of the genome have suffered duplication or deletion during the evolution of various strains (Bharudin et al., 2022). The population structure is likely a reflection of the high genetic variability of the *G. boninense* population. It can be explained by the existence of crosses between basidiospores of *G. boninense* in various regions of the world and the regional adaptation of various pathogenic genotypes to different palm oil hosts (Wong et al., 2021). Therefore, the application of biological control agents is needed, such as *Streptomyces* sp. (Sujarit et al., 2020), *Pseudomonas aeruginosa*, and *Trichoderma asperellum* (Muniroh et al., 2019). *Trichoderma* sp is one of the most widely used biological control agents. Some species of *Trichoderma* endophytic have shown biocontrol activity so that it can protect the host plant. *Trichoderma* endophyte also reported having activity as plant growth promoting fungi (Al-Askar et al., 2022; Shentu et al., 2013).

Basic information about the endophytic *Trichoderma virens* to control *G. boninense* is still limited until now. So, the study on exploring potential biological agents of *Trichoderma* endophyte as an antifungal against *G. boninense* should be carried out. This study aimed to determine the potential of endophytic *Trichoderma* sp. originated from oil palm plant tissue as a biological control agent and its molecular identification.

METHODS AND MATERIALS

Material research

This research was conducted in the Genetics and Plant Pathology Laboratory, Universitas Riau. This study used endophytic *Trichoderma* sp. isolated from root tissues (TR01), stem tissues (TS01), and midrib tissues (TM01) of oil palm plantations (*Elaeis guineensis* Jacq) in Riau, pathogenic fungi *Ganoderma boninense* (collection of the Business Biopesticides and Biofertilizer Unit of Agriculture Faculty, Universitas Riau), ITS4 reversed primer (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 forward primer (5'-GAAAAAAY-3').

Inhibition Assay of endophytic *Trichoderma* sp. against *Ganoderma boninense* pathogens

The purified pathogenic isolate of *G. boninense* and endophytic fungi obtained were tested using dual culture methods

on Potato Dextrose Agar (PDA) in vitro. Furthermore, incubated at room temperature for 3-4 days with three replications. Calculation of the inhibition using the formula as follows:

$$\text{Inhibition zone} = \frac{(r_1 - r_2)}{r_1} \times 100\% \quad (1)$$

where,

r_1 = radius colony hyphae of pathogens that grow in contrast to endophyte and r_2 = radius colony hyphae of pathogens that grow toward endophyte

Crude metabolites Assay of Trichoderma sp. Endophyte

Fungal endophyte metabolites were set up endophytic fungal isolates, taking three pieces for each isolate using a cork borer, and putting it into the Erlenmeyer containing 100 ml of medium Potato Dextrose Broth (PDB). Furthermore, it was shaken at 100 rpm for 2 weeks at room temperature and centrifuged at 6000 rpm for 20 minutes to obtain the supernatant. The supernatant was filtered with a syringe filter of 0.2 μm . With some modification, this method followed Achmad (1997) in Sukapiring et al. (2016). Furthermore, each distillate metabolite by 20 mL to 80 mL mixed with PDA (for the 20% dilution), and subsequently dilution concentration of 15%. PDA mix containing the metabolite was poured on a petri dish, added with pathogenic fungi *G. boninense* in the middle, and then incubated at room temperature for 3-7 days. The growth of pathogenic fungal colonies was observed, and the inhibition of pathogen growth was measured. Pathogenic fungus grown on PDA was used as a control. The percentage of inhibition was calculated using the formula:

$$\text{Inhibition ability} = \frac{(D_1 - D_2)}{D_1} \times 100\% \quad (2)$$

where,

D_1 = diameter the pathogenic fungal hyphae as control (cm), D_2 = diameter pathogenic fungal hyphae as treatments (cm)

Identification of Trichoderma sp. endophyte

Fungal endophyte isolates TR01, TS01, and TM01 were reisolated on an SDA medium. Chromosomal DNA was isolated using enzymes lyticase and Wizard Genomic Purification Kit. The DNA obtained was then amplified by PCR using ITS4 and ITS5 primers. PCR amplification of ITS-1 and ITS-2 rDNA region was carried out by using primer pair ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS-5 (5'-GAAAAAAAAAAAAAY-3') with temperature of annealing 45°C. Total volume was 50 μL , consisting of 3 μL DNA isolates, 10 μL primer ITS4 and ITS5, 5 μL of 2 mM dNTP, 5 μL Total GoTaq Colourless, 3 μL MgCl_2 , 8.75 μL sterile distilled water and 0.25 μL Taq DNA polymerase. The amplification reaction took place in three stages for each circle. The hot reaction started at 95 °C for 5 minutes. The first stage was the process of denaturation at 94°C for 1.5 min. The second stage was the process of annealing at 45 °C for 1 minute. The third stage was an extension of chain DNA at 72°C for 3 minutes. PCR amplification occurred in as many as 35 cycles. PCR amplification product was analyzed using 1.2% agarose gel electrophoresis and soaked in ethidium bromide, and the DNA bands were observed with the aid of UV light using a UV Transilluminator.

Data analysis

The PCR products were then sequenced by Eijkman Molecular Biology Institute. The sequencing results were analyzed with BLAST (Basic Local Alignment Search Tool) software contained on the website National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) to determine the level of the closest kinship with the fungi in the Gen Bank (NCBI) database. The following sequence was taken for analysis of phylogenetic trees. Multiple Sequence Alignment and phylogenetic tree construction were analyzed using the MEGA6 program. Phylogenetic tree prepared by the neighbor-joining algorithm, the stability of the grouping using analysis bootstrap with 1000 replications (Saputra et al., 2015).

RESULT AND DISCUSSIONS

Inhibition ability of Trichoderma sp. endophyte against Ganoderma boninense pathogens

The results showed that three isolates of *Trichoderma* sp. endophyte were able to inhibit the growth of *G. boninense* in vitro. In Table 1, three isolates of *Trichoderma* sp. had no significant results each other. The three isolates of *Trichoderma* sp. endophyte from different oil palm tissue showed their potential as biological control agents by inhibiting the two test cultures of 59-68%. The high inhibition was demonstrated by endophytic *Trichoderma* sp. TM01 isolate (68%) on day 4 (Figure 1 and 2).

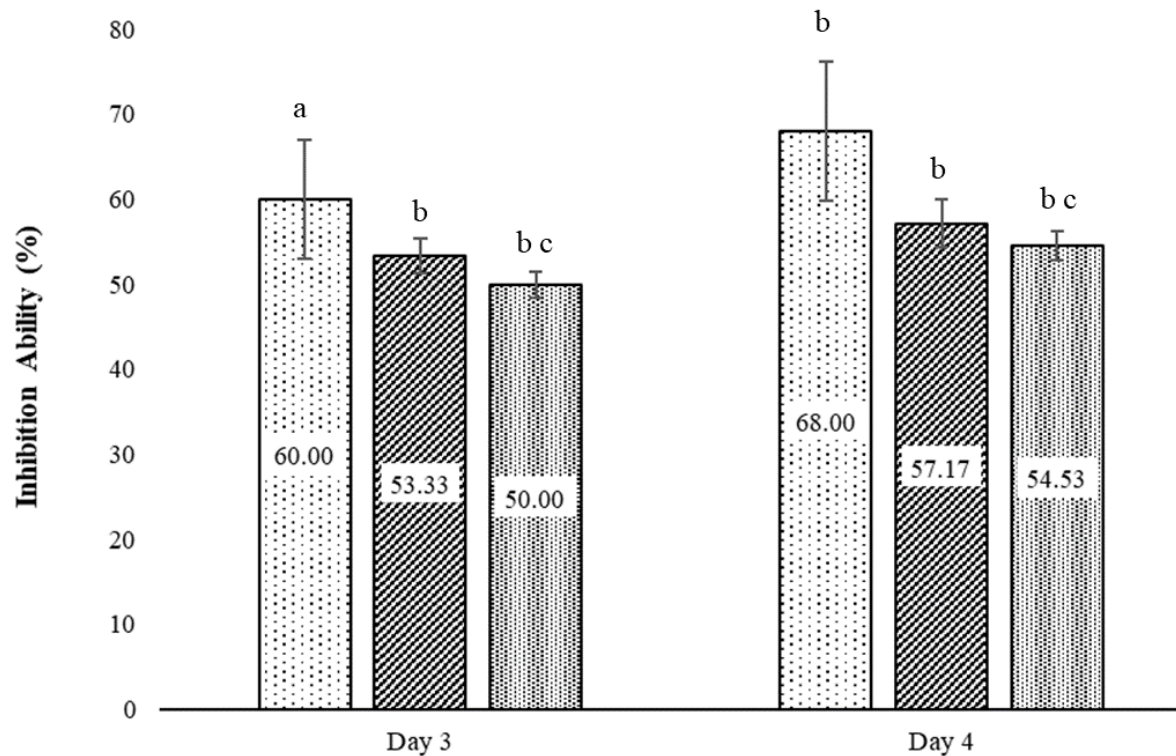


Figure 1. Inhibition ability of *Trichoderma* sp. endophytes against fungal *G. boninense* growth. *Note.* Figures followed by different lowercase in the same column were significantly different according to DNMRT test at 5%, which were □ for TR01, ▨ for TS01, and ▩ for TM01.

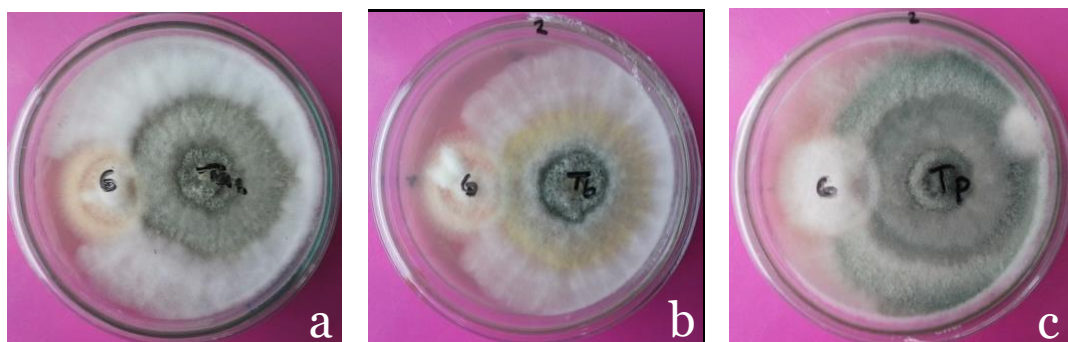


Figure 2. Inhibition of fungal pathogens *G. boninense* by isolates of *Trichoderma* sp. endophyte oil palm plant tissues: a. Isolates TR01 (root), b. Isolates TS01 (stem), and c. isolates TM01 (midrib)

Figure 1 and 2 show that the antagonist ability of all the *Trichoderma* sp. endophyte isolates was higher than *T. virens* isolates from the study by Anil Kumar et al. (2013), in which the inhibition ability was only 55.58-60.74% in inhibiting

Fusarium oxysporum f.sp. *lycopersici* (FOL). According to Silva et al. (2019), the main mechanisms controlling plant pathogens using *Trichoderma* sp. can be in several ways: mycoparasites (hyperparasite), antibiosis, competition, and lysis. Mycoparasite (hyperparasite) occurs where the mycelium fungus *Trichoderma* sp. parasitizes another fungal mycelium by penetrating the cell wall and into the cells of pathogenic fungi to take nutrients from the cell so that the pathogenic fungus dies. Antibiotics are a mechanism of inhibition of *Trichoderma* sp. by producing antibiotic compounds that can evaporate, such as alanatechin, paracelsin and trichotoxin to destroy the fungal cells by damaging their cell membrane permeability. The competition is the ability of *Trichoderma* sp. in a place to live and food sources. The lysis mechanism in *Trichoderma* sp. was the ability to perform interference hyphae to produce chitinase enzyme that could lead to lysis of the cell wall of fungal pathogens (Nusaibah and Musa, 2019).

Inhibition ability of Trichoderma sp. endophyte crude metabolites

In Figure 3, the crude metabolites of three *Trichoderma* sp. isolates inhibit the growth of *G. boninense*. Among the three isolates, the addition of 15% of crude metabolites of isolate TM01 performed maximum inhibition of 71.79% compared to other isolates of 24.24% (TS01) and 9.09% (TR01)—however, the provision of crude metabolites by 20%, *Trichoderma* sp. TM01 and TS01 isolates demonstrated the inhibition of the *G. boninense* highly, respectively 82.05% and 78.78% compared to the crude metabolites of *Trichoderma* sp. TR01 isolate.

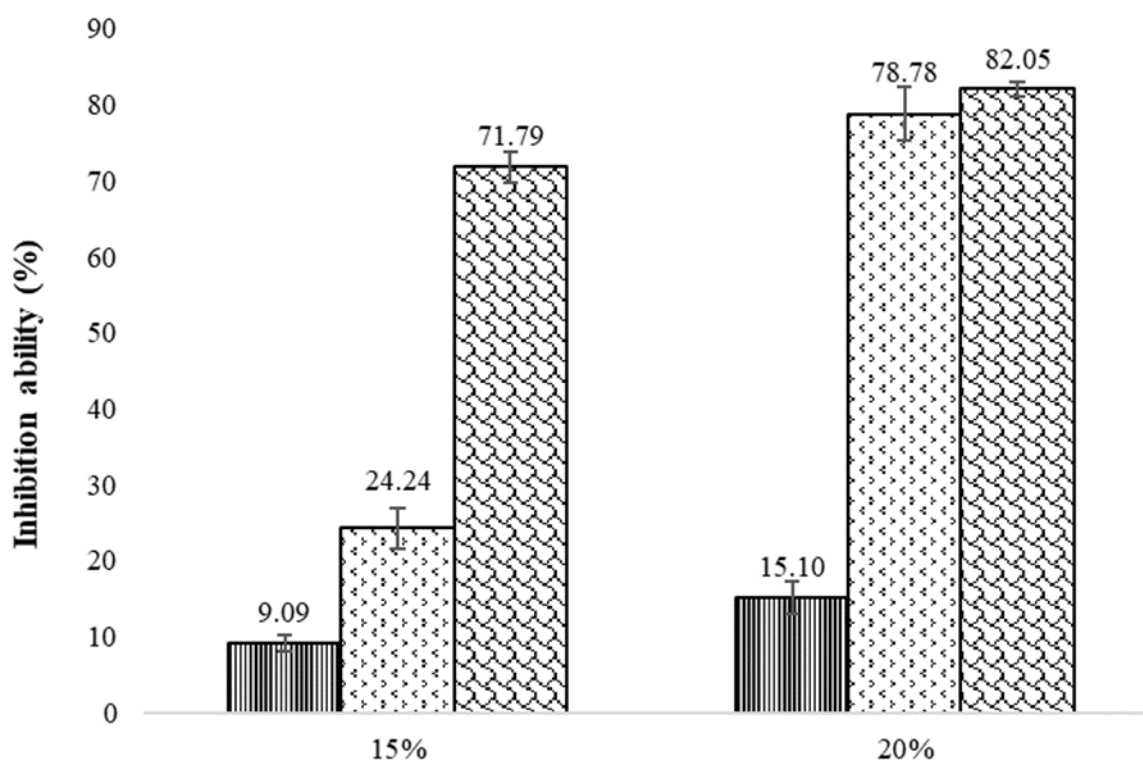


Figure 3. Inhibition of *G. boninense* by crude metabolites of *Trichoderma* sp. endophytes from oil palm plant tissue, which were for ■ TR01, ▨ for TS01, and ▤ for TM01.

Crude metabolites of the three isolates were able to inhibit the growth of fungus *G. boninense* with percentage inhibition of 9.09 to 71.79% by adding 15% of metabolites and from 15.10 to 82.05% in increments of 20% of metabolites in PDA media. The high pathogenic fungi growth inhibition of *G. boninense* could be due to the crude metabolites of *Trichoderma* sp. Several studies show that the endophytic fungus *Trichoderma* can produce antifungal activity metabolites. Novel endophytic *Trichoderma longibrachiatum* WKA55 could provide various benefits to host plants through various mechanisms, including the synthesis of antimicrobial substances (cellulase, protease, and polygalacturonase) and phytohormones (citric acid) peptides that could be reducing the mycotoxigenic fungi dan

promoting germination on peanut seed (Al-Askar et al., 2022). Endophytic *Trichoderma brevicompactum* from garlic could produce trichodermin and had inhibitory activity on *Rhizoctonia solani*, *Botrytis cinerea*, and *Colletotrichum lindemuthianum* (Shentu et al., 2013). Endophytic *Trichoderma atroviridae* and *Trichoderma koningii* from Cupressaceae foliar tissues also produce the secondary metabolites and suppress the growth of several fungus as a model target, such as *Pyricularia oryzae*, *Diplodia seriata*, *Phaeobotryon cupressi* and *Spenceri martinsii viticola* (Hosseyni-Moghaddam and Soltani, 2014).

Identification of *Trichoderma* sp. endophyte

DNA from *Trichoderma* sp. endophyte isolates was successfully amplified from ITS-1 and ITS-2 rDNA using a primer ITS 4 and ITS 5 at a annealing temperature of 45°C. Then sequencing was performed to find out the type. From a phylogenetic tree drawn by the *neighbor-joining algorithm* method (Figure 4), the third isolates of *Trichoderma* sp. endophyte isolates TR01, TM01, and TS01 are closely related to each other.

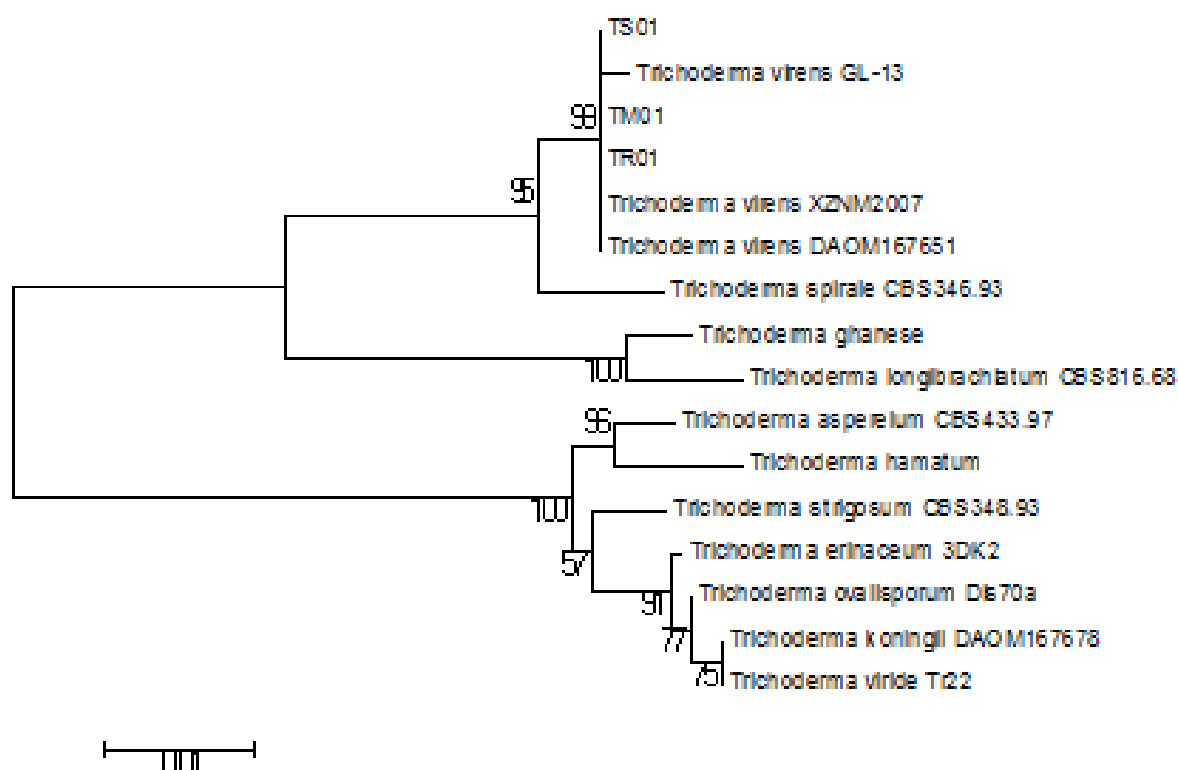


Fig 4. Phylogenetic tree of *Trichoderma* sp. endophytic isolates TR01 (from root tissues), TM01 (from midrib), TS01 (from the stem) and a relative of the *Trichoderma* other based on gene sequences of ITS-1 and ITS-2 rDNA. The bootstrap value indicated on each node.

All three isolates were similar to *Trichoderma virens* with a bootstrap value 99 in the 1000 replications. Sundram (2013) reported about 40 isolates of *Trichoderma* sp. from the root and stem of oil palm, but none were isolated from rachis and stem. This research found *Trichoderma virens* isolates not only from root and stem, but also from the midrib of oil palm. Based on the observations, the three known isolates closely related to each other show different inhibitory ability against the growth of the pathogen *G. boninense* on the direct test inhibition of multiple cultures from the three isolates.

CONCLUSIONS

Trichoderma sp. TM01 isolates had larger inhibitory than the other isolates on days 3 and 4 by 60% and 68%, respectively. TM01 isolates also inhibited the growth of the *G. boninense* at a concentration of 15% and 20%, with the

percentage of inhibition reaching 71.79% and 82.05%. Based on phylogenetic analysis, three endophytic isolates of *Trichoderma* sp. were closely related to *Trichoderma virens*.

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