Exploration, identification, and in vitro antagonism test of *Trichoderma* spp. against *Ganoderma* spp. at PT Bumitama Gunajaya Agro palm oil plantation, Central Kalimantan

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Abstract. *Trichoderma* spp. is one of the biocontrol agents that have important benefits for agriculture in Indonesia. Exploration is carried out at PT Bumitama Gunajaya Agro Oil Palm Plantations in high yield blocks. This study aims to identify and determine the isolates of *Trichoderma* spp. and to find out its in vitro antagonism against *Ganoderma* spp. Morphological identification of *Trichoderma* spp. carried out using a trinocular microscope. There were six isolates analyzed based on internal transcribed spacer sequences (ITS) in ribosomal DNA region using PCR technique with ITS 1 and ITS 4. The results of this research showed that *Trichoderma* spp. original from PT BGA Central Kalimantan has diverse characteristics. Sequencing analysis showed that six isolates were in one group with *Trichoderma asperellum* isolates T5 (Acc. No. MH809176), IIPR-80 (Acc. No. MK841018) and TV5 (Acc. No. MH393299). Based on the antagonism test, it was found that six isolates were able to inhibit the growth of *Ganoderma* spp. with an average percentage value is ≥50%.

Keywords: *Trichoderma* spp., morphological, molecular, antagonism test.

1. Introduction

*Trichoderma* spp. is a fungus that is abundant in all soil types and is considered a potential antagonist agent against soil-borne parasitic microorganisms [1]. The role of *Trichoderma* spp. on soil ecology is as a biocontrol agent so it is important to understand its biodiversity. The genus of *Trichoderma* spp. includes about 100 species that are widely distributed throughout the world due to their fast growth and their ability to use different substrates that can tolerate the presence of different pathogens and environmental conditions [2].

*Trichoderma* spp. isolates have different antagonistic mechanisms in inhibiting pathogens, namely through direct parasitism, nutritional competition, stimulator of plant health, or inducing systemic resistance of plants to various pathogens [3]. In inducing stress tolerance in host plants, this fungus works by increasing root and shoot development, inducing systemic resistance, inactivating pathogenic enzymes, dissolving and absorption of inorganic nutrients [4]. In addition, antagonistic activity of *Trichoderma* spp. against different plant pathogens occurs through different mechanisms also through
antibiosis, micro parasitism, and competition for nutrients and habitat [5]. Different abilities by each species of \textit{Trichoderma} spp. in controlling fungal pathogens, due to their different morphology and physiology, identification is needed which is an important factor in monitoring microorganisms used in the field.

Identification of \textit{Trichoderma} spp. currently carried out using morphological, cultural, molecular, and biochemical characterizations to obtain the correct species. Identification based on morphological characters to obtain species is difficult due to the many similarities in morphological characters and the increasing number of morphologically obscure species [7]. The main disadvantage of morphological identification is that it cannot be used to distinguish strains within a single species or species within the genus of \textit{Trichoderma} spp. [8]. Conventionally identification is highly dependent on the morphology and characterization of isolates, but not all fungi are capable of producing spores. Because of this, it is very difficult to identify only by using morphology.

Therefore, it is necessary to carry out further more accurate tests, namely molecular identification to overcome confusion in characterization. Internal sequence analysis of transcribed spacer regions 1 and 2 (ITS1 and ITS2) on ribosomal DNA can help to describe and characterize species in the genus \textit{Trichoderma} [9,10]. All of these analyses use PCR amplification because it can produce accurate strains, so it can be used in determining the species of \textit{Trichoderma} spp. [11].

According to Kubicek and Harman [12], the use of \textit{Trichoderma} spp. commercially must be preceded by accurate identification, appropriate formulation, and study of the synergism of various antagonistic mechanisms. This study aims to identify six isolates of \textit{Trichoderma} spp. from exploration results in the oil palm plantation of PT BGA Central Kalimantan and to test its in vitro antagonism against \textit{Ganoderma} spp. which will be used as biological control agents for \textit{Ganoderma} spp.

2. Materials and Methods

This study is experimental research at the Microbiology Laboratory of Research and Development Department at PT BGA with sterile and homogeneous conditions and using a completely randomized design. Six isolates obtained were regrown on PDA media and incubated for 7 days to obtain pure isolates. The six isolates that will be identified and tested for their antagonism, namely:

| No. | Isolate | Location          |
|-----|---------|-------------------|
| 1   | SAGE C22a | Sungai Agro Estate |
| 2   | PHRE C17a | Pantai Harapan Estate |
| 3   | KAGE I46a | Katari Agro Estate |
| 4   | KAGE K53a | Katari Agro Estate |
| 5   | KAGE G50a | Katari Agro Estate |
| 6   | KAGE J44a | Katari Agro Estate |

The morphological identification of the isolates was carried out macroscopically by observing morphological characteristics including colour of upper and lower surface colonies, shape of colony, and zoning. Meanwhile, microscopic identification includes shape of conidia and shape of conidiospores. Furthermore, molecular identification was carried out to determine species of six isolates of \textit{Trichoderma} spp. Microscopic identification of isolates was carried out by making microcultures (slide culture). Procedure in making microcultures for microscopic identification of fungi, are: petri dish is prepared and then given a tissue and dripped with sterile distilled water to provide optimum humidity for fungal growth. Above of tissue, an object-glass was placed, then one cork borer \textit{Trichoderma} spp. aged 7 days incubation from PDA was grown and then covered with a coverslip. The microculture was incubated at room temperature for 48 hours, observed using a trinocular microscope.

Molecular identification of six isolates analyzed based on internal transcribed spacer sequences (ITS) on the ribosomal DNA region using the PCR technique with ITS 1 and ITS 4. The
analysis method consists of: DNA extraction, Purification of PCR results, Sequencing, Sequencing analysis using Mega 6. The process of reading the nucleotide strand DNA is carried out by sending samples to the Microbiology Laboratory, Lampung University.

Antagonistic test of six *Trichoderma* spp. isolates using PDA media. Each isolate of *Trichoderma* spp. with same size were cultured from the opposite direction with *Ganoderma* spp. and incubated at room temperature 25 °C. The control used was *Ganoderma* spp. without *Trichoderma* spp.. Observations were made by measuring the colony growth of *Ganoderma* spp. radially. These data will be used to calculate percentage of antagonism *Trichoderma* spp. against *Ganoderma* spp. Percentage of antagonism was calculated according to the procedure of the Indonesian National Standard [13] regarding “Biological Control Agents (APH) part 3 *Trichoderma* spp.” APH quality requirements of *Trichoderma* spp. with the formula:

\[
Z = \frac{(r_1-r_2)}{r_1} \times 100
\]

Information:
- Z : Inhibition Percentage
- r1 : Radius *Ganoderma* sp. without *Trichoderma* sp.
- r2 : Radius *Ganoderma* sp. with *Trichoderma* sp.

3. Results

3.1 Identification of six isolates *Trichoderma* spp. BGA based on morphological characters

This study combines morphological and molecular identification approaches to provide more accurate results. Morphological identification was confirmed through macroscopic and microscopic observations through slide culture techniques which included spore morphology, colour, shape, zoning or wall texture, size, conidia formation and other related characteristics such as phialide pattern and conidiation [14]. Results of morphological characterization which included macroscopic and microscopic identification (Table 2) showed that six isolates of *Trichoderma* spp. when observed under microscope there was development, at beginning of growth mycelium was shaped like white cotton and then turned green and then became dark green with many conidia. On the seventh day growth of mycelium has filled the cup. Mycelium growth continues to grow until it forms a ring after mycelium is a full plate. This is in accordance with Stamets [15] which states that most saprophytic fungi initially have a white mycelium, then the colour can change when the mycelium matures. Suanda's research [16] also states that the genus *Trichoderma* has morphological characteristics; powdery texture with yellow-green colonies, no sclerotia and exudates.

Observation shape of conidia, conidiospores, and phialides used identification book Watanabe [17] with results of observations that conidia were round, conidiophores were insulated and branched upright, each branch contained 3-6 phialides with an oval shape like a pumpkin. According to Ghazanfar et al. [5] and Waghunde et al. [6], genus *Trichoderma* spp. has smooth or ellipsoidal conidia, branched conidiophores, insulated hyphae, and philaid shapes like pumpkins. Morphological characters obtained from microscopic and macroscopic observations showed that six isolates of *Trichoderma* spp. suspected to have different species with diverse morphological characters. Results of morphological observation cannot be used as a basis for determining species of *Trichoderma* spp. due to many subtle morphological similarities. According to Kullnig et al. [7], identification based on morphological characters to obtain species is difficult because of many similarities in morphological characters and the increasing number of morphologically obscure species. Further tests to determine species *Trichoderma* spp. is molecular identification.
Table 2. Morphology characters of six isolates *Trichoderma* spp. BGA (trinocular microscope with 100 times magnification).

| Fungal Isolates | Morphology Character | Documentation |
|-----------------|----------------------|---------------|
| SAGE C22        | Colonies Light green, smooth and thin surface like velvet, growing rapidly, spherical colonies spread in all directions. Hyphae, conidia are round, conidiophores are insulated and branched irregularly, each branch has 3-6 phialides | ![Documentation Image] |
| PHRE C17a       | Colonies dense dark green, growing rapidly, the surface of the colony is dense and smooth, and spreads irregularly in all directions. In old cultures there are white colonies. Hyphae, conidia round oval or cylindrical, conidiophores insulated and branched upright, each branch there are 1-2 phialides | ![Documentation Image] |
| KAGE I46        | Colonies dark green, smooth surface, growing rapidly, in the form of round colonies, there are like rings, in old cultures there are white colonies spreading in all directions. Hyphae, conidia are round, conidiophores are insulated and branched irregularly, each branch has a phialide | ![Documentation Image] |
Table 2. Morphology characters of six isolates *Trichoderma* spp. BGA (trinocular microscope with 100 times magnification) (Continued).

| Fungal Isolates | Morphology Character | Documentation |
|-----------------|----------------------|---------------|
| KAGE K53        | Colonies, light green whitish, smooth and thin surface, growing rapidly, in the form of round colonies, there are like rings spreading in all directions, in old cultures there are yellowish white colonies. Hifa hialin, conidia are round, conidiophores are insulated and branched upright, each branch has 3-6 phialides | ![Documentation](image1) |
| KAGE G50        | Colonies dark green, smooth and thin surface, growing rapidly, in the form of round colonies, there are like rings spreading in all directions, in old cultures there are solid white colonies. Hifa hialin, conidia oval or cylindrical in shape, conidiospores insulated and branched irregularly long, each branch has 3-6 phialaid. | ![Documentation](image2) |
| KAGE J43a       | Colonies light green, yellowish white, smooth and thin surface growing fast, round colonies, there are like rings spreading in all directions, in old cultures there is a yellowish white colour. Hifa hialin, not septate, conidia spherical. | ![Documentation](image3) |

Information: *Trichoderma* spp. showing Conidiophores (Cp), Phialides (P), Conidia (C).
3.2 Molecular identification of six isolates Trichoderma spp. BGA
Identification was carried out after morphological observations to confirm the initial assumption that six species of isolates were differenced. Six isolates of *Trichoderma* spp. analyzed using internal transcribed spacer sequences (ITS) in ribosomal DNA region using PCR technique with ITS 1 and ITS 4 with purification analysis method of PCR results, sequencing analysis using Mega 6. Electrophoresis results showed that PCR using ITS 1 and ITS 4 produced amplicon approximately 500 bp (Figure 1).

**Figure 1.** PCR products were electrophoresed using 0.8% agarose in 1% TBE, the amount of DNA concentration used was 0.2 ug/ uL.

PCR product was electrophoresed using 0.8% agarose in 1% TBE, amount of DNA concentration used was 0.2 ug/uL. DNA Ladder used is 1kb in size with band size starting from bottom as follows (pb): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, 1000. Sequence analysis is described through Dendogram was created based on results of gene sequence analysis using Mega 6 with neighbor-joining method [22]. Neighbor joining method is an accurate method for trees that have short branches. Long branches tend to reduce character resemblance.

The genetic distance in the phylogenetic tree shows a value of 0.05, where this value indicates a difference of 5 bases in every 100 bases of each sequence. In dendogram using neighbor joining method and bootstrap analysis (10000 repetitions) and produces a value of 98% which means the isolate sequence is significantly different from outgroup because it has a base pair sequence. Results of sequencing analysis showed that six fungal isolates analyzed were in the same group with *Trichoderma asperellum* isolates T5 (Acc. No. MH809176), IIPR-80 (Acc. No. MK841018), and TV5 (Acc. No. MH393299) as depicted in Fig. dendogram (Figure 2). So that, initial assumption that six isolates species were different was rejected because the results of molecular identification of six isolates were in the same group as *Trichoderma asperellum*. *Trichoderma asperellum* is a group of *Trichoderma* fungi that have been widely used to control plant pathogens with various mechanisms [18]. *T. asperellum* (GDFS1009) has a high mycelium growth rate, high sporulation capacity, and very high inhibition against pathogens that cause fusarium cucumber wilt and corn stem rot [19]. According to research by Agustina et al. [20] *T. asperellum* was able to inhibit the growth of *Botryodiplodia theobromae* by 78.67%. In addition, several isolates of *T. asperellum* were reported as potential endophytic fungi.
3.3 Antagonism test six isolates Trichoderma spp. BGA against Ganoderma spp.

One of the requirements for an organism to be considered as a biological agent is to have the ability of antagonism to inhibit the growth of pathogenic fungi. Trichoderma spp., has an active metabolism that produces a large number of enzymes that act as a powerful weapon against other unfavorable fungi (Li et.al., 2016). In this study, six isolates of Trichoderma spp. which belong to the same group as T. asperellum but has varying antagonism with an average inhibitory value of ≥50 % (Figure 3).

The difference in inhibition describes the different abilities of each isolate to inhibit the growth of competing microorganisms [16]. This difference is thought to be influenced by the type, amount, and...
quality of antibiotics or other substances produced by Trichoderma spp. which can inhibit the growth of pathogens ([24]. Six of 30 study isolates of T. asperellum showed high antagonistic activity against Fusarium oxysporum because it produces rich volatile and secondary metabolites which are effective biocontrol agents against pathogens such as F. oxysporum, R. solani and P. ultimum [25].

4. Conclusion

Six isolates of Trichoderma spp. BGA plantations had different morphological characteristics. Based on molecular identification, six isolates of Trichoderma spp. were in the same group as Trichoderma asperellum T5 (Acc. No. MH809176), IIPR-80 (Acc. No. MK841018), and TV5 (Acc. No. MH393299). Antagonism of six isolates of Trichoderma spp. BGA plantations belonging to the same group as T. asperellum have various antagonism abilities with an average inhibition value of ≥50 %. One interesting conclusion in this study was that T. asperellum was described with varying morphology and antagonism in six isolates of Trichoderma spp. BGA oil palm plantation.

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