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Potential of Cocoa Derived-Endophytic Fungi in the Biocontrol of Phytophthora Palmivora

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ABSTRACT

Cocoa (*Theobroma cacao* L.) is an important commodity traded worldwide. Commonly cocoa is susceptible to various diseases that reduce its productivity. One of the important diseases affecting cocoa plants is fruit rot caused by *Phytophthora palmivora*. The study purpose is to explore endophytic fungi in the soil rhizosphere of cocoa plantations and investigate their existence as the potential biocontrol against fruit rot pathogen *P. palmivora* from different areas. Rhizosphere soil contains endophytic fungi collected from the rhizosphere soil of cocoa plants in Palolo and Labuan, Central Sulawesi, Indonesia. The microbes were successfully isolated. The samples were grouped based on their respective sampling areas. The laboratory test from rhizosphere samples from different areas used dual culture and volatile compound tests. Isolation results from the two areas obtained eight isolates from Palolo identified as: *Penicillium* (1), *Gliocladium* (2), *Trichoderma* (2), *Aspergillus* (2) and unidentified (1). Six isolates from Labuan identified isolates as *Trichoderma* (4) and *Aspergillus* (2). The results obtained in the dual culture test presenting the highest inhibitor for *P. palmivora* are isolate T1RP3 (78.46%). In the tests, isolate T1RP3 showed the highest inhibition against *P. palmivora* at 78.46% in the dual culture test, while isolate T5RL3 achieved the highest inhibition at 42.19% in the volatile compound test.

1. INTRODUCTION

Cocoa (*Theobroma cacao* L.) is an important plant with high economic value to be developed commercially. Cocoa plant products become export commodities as foreign exchange-earners. After palm oil and rubber, cocoa ranks third in the plantation commodity with high value. Another advantage, cocoa has a stable position with relatively high prices in the international market, and improvement in yield quality declares that cocoa remains an important commodity non-oil and gas. The average productivity of cocoa in smallholder plantations is only 696.9 kg/ha per year, while in large private plantations and government plantations can reach 1.5 to 2 tons/ha per year. The superior cocoa clones achieve productivity of 2.5 - 3.6 tons/ha per year [1].

One of the causes of low cocoa productivity in smallholder plantations is the presence of pests and diseases. Common important diseases that attack the cocoa plant are fruit rot, stem cancer, root disease, leaf stripes, swollen buds, broom disease, *Monilia* fruit rot, vascular streak dieback, and blight (*Marasmius* sp.) [2, 3]. One of the causes of low cocoa plant productivity is caused by fruit rot disease. Research conducted about fruit rot disease commonly occurs throughout the year on immature cocoa fruit and fruit near harvest. Its dynamic depending on the month and on certain clones [4, 5].

An important innovation in the management of disease on cocoa especially pod disease is endophytic fungi. This is a promising new agent in biological control that safety the environment. Endophytic fungi mean fungi living in the tissue of the host and not causing symptoms of diseases in the plant. Host plants are protected by endophytic fungi from other organisms and extreme conditions by resulting secondary metabolites in two ways: indirectly and directly. Fungi's endophytic ability to live in all parts of the plant, from the leaves to the roots. The area surrounding plant roots, along with the associated microbes that protect these parts, is known as the rhizosphere, which includes fungi. Endophytic fungi can defend their host plant from diseases and pests. All of the findings demonstrated the significance of fungal endophytes as a modern biological control agent for preventing harmful microbes causing cocoa diseases in plantations. The knowledge is needed for farmers in local cocoa plantations from developing countries [6, 7].

In general, controlling fruit rot disease in cocoa plants possibly uses endophytic fungi as important disease control agents. Endophytic fungi can improve plant resistance against pathogens and inhibit their development [8, 9]. Another research finds that endophytic fungi have the potential to control cocoa primary and destructive disease such as: *Phytophthora palmivora* caused black pod rot disease. In some cases, biocontrol of cocoa diseases is successful using endophytic fungi. Spreading endophytic fungi in the plant cell and not supporting the growth of the pathogen especially in cocoa at the field study and laboratory [10-12].

Endophytic fungi give more benefits against the pathogens caused by disease in the plantation. Also, the useful fungi are able to induce secondary metabolites against pathogens, help the absorption of nutrients then increase plant health [13-16]. Based on the benefit of endophytic fungi increasing plant health, especially the antagonism attack *Phytophthora palmivora*. The study purpose is to explore endophytic fungi in the soil rhizosphere of cocoa plantation and investigate their existence as the potential biocontrol against fruit rot pathogen *P. palmivora* from different areas. Using endophytic fungi in the research field against *Phytophthora palmivora* will provide valuable information and contribute to increasing the productivity of cocoa in different areas.

2. MATERIAL AND METHOD

2.1 Rhizosphere soil sample

Sampling was conducted randomly in healthy cocoa plantations in two different areas namely: Palolo, Sigi Regency, and Labuan, Donggala Regency, Central Sulawesi Indonesia. Criteria of a healthy cocoa plantation are: farmers intensive management of natural enemies, using prominent cocoa clones, minimizing chemical pesticide application, and producing high-quality cocoa before research. Rhizosphere soil samples were taken in the soil depth of the cocoa plantation about 10 - 20 cm diagonally at 10 points with distances every point about 20 m. Each soil sample taken as much as 10 to 20 g soil then combined as a composite. Testing of rhizosphere soil sample was held in the Laboratory of the Faculty of Agriculture, Alkhairaat University, Palu Central Sulawesi.

2.2 Collection and isolation of rhizosphere fungi

Rhizosphere fungi were collected and isolated from the soil in two different areas of the cocoa plantation (total of 20 samples). Every sample was treated using the serial dilution method until reaching a dilution of 10^{-6}. Fungi from soil rhizosphere were grown on PDA (Potato Dextrose Agar) medium through scatter method. The samples were incubated at room temperature for 48 hours. After couple of hours, the fungi grown in different characteristics are purified for further identification and used as stock for testing.

Isolation of pathogenic fungi P. palmivora

P. palmivora was isolated from cocoa fruit showed symptoms of fruit rot. The diseased and healthy parts of the fruitnwere cut into 1 - 2 cm long pieces, then surface sterilization was used alcohol. The clean cocoa part is grown on a V8 juice medium. The fungi that grow are isolated and moved to Potato Dextrose Agar medium then used as testing stock.

Antagonism Test (*Dual Culture*)

The purpose of the test is to identify the antagonism between endophytic fungi and *P. palmivora* caused fruit rot disease in cocoa from two plantations. The test was conducted in the dual culture method, in which colonies of endophytic fungi and pathogens with a diameter of 0.5 cm, placed on Potato Dextrose Agar medium in the petri dishes. The distance of 3 cm between colonies of rhizosphere fungi and *P. palmivora*. There are 10 replicates for every area of rhizosphere fungi. The fungi in the petri dishes were incubated at the laboratory. The observations were made by measuring colony growth in petri dishes. Measurement of the percentage inhibition based on the ability of each isolate showed the existence of fungi in the inhibition zone on petri dishes. Percentage of inhibition of endophytic fungi from rhizosphere used the formula [17]:

$$\mathrm{P} = \frac{r1 - r2}{r1} \times 100\% \quad (1)$$

where, P=Percentage of inhibition (%); r1=Diameter of pathogen colonies from rhizosphere fungi isolates; r2=Diameter of *P. palmivora* colonies near the rhizosphere fungi isolate.

Volatile compound test

Volatile compound testing was carried out on isolates of rhizosphere fungi and rot disease pathogen *P. palmivora* used cork borer (0.5 cm) in the center of Potato Dextrose Agar medium separately in the petri dish. Both cups were then glued with plastic wrapping and stacked on top of each other then incubated at room temperature. There are 10 replicates from each area of cocoa plantation. Observations were made by measuring the colony diameter of the pathogenic fungi *P. palmivora* every 24 hours until the pathogenic fungi culture filled the petri dish. Calculating the volatile compound test using a modified method [18]:

$$\mathrm{V} = \frac{v_0 - v_1}{v_0} \times 100\% \quad (2)$$

where, V=Inhibition; v_0=Diameter of control colonies; v_1=Average diameter of treatment colonies.

Molecular identification

Molecular identification only conducted on 2 isolates of *Trichoderma* sp. with different morphological identification results and the highest inhibition in each different isolate origin, namely isolate T1RP3 and T5RL3. Molecular identification was carried out by Genetika Science Indonesia Company. DNA extraction was performed based on the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005) (B/7.2.1/KP/001). The DNA extracted was PCR amplified using universal primers, namely ITS1 (5'-TCCGTAGGTGAACCTGCGG - 3') and ITS1 (5'-TCCGTAGGTGAACCTGCGG - 3') and ITS4 (5' - TCCTCCGCTTATTGATATGC - 3'). The amplification results analyzed by electrophoresis and observed in the UV transilluminator. DNA PCR results were processed by the MEGA-X program [19]. Sequencing was carried out to determine the level of homologous cyclicity in GenBank with the BLAST program (https://www.ncbi.nlm.nih.gov/pmc) National Center for Biotechnology Information (NCBI). The relationships known by looking at the percentage of homology, while for phylogeny trees used to see the relationship between sequences [20].

3. RESULT AND DISCUSSION

Pathogenic fungi P. palmivora

P. palmivora is known as the destructive plant pathogen to cocoa pods. Morphological observations 7th days of *P.*

palmivora colonies from cocoa pods from plantation were made macroscopic and microscopic (Figure 1). In macroscopic, the colonies on Potato Dextrose Agar medium appeared white in color resembling cotton. Microscopic observations showed that *P. palmivora* has non-concentrated hyphae. The sporangiophores are hyaline and also non-concentrated. The sporangium is shaped like a lemon with papillae at the tip. Result of identification based on study [17] found that beside living organism, *P. palmivora* is able grow in the artificial medium to enrich nutrition.

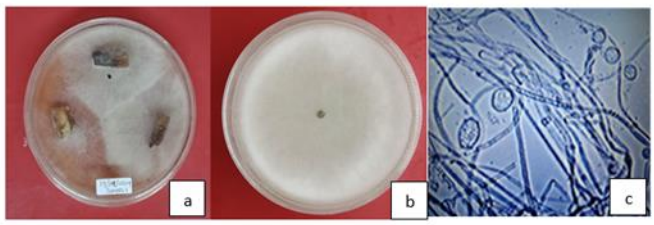

Figure 1. Planting cocoa rot samples on V8 Juice media (a), colonies of *P. palmivora* (b), microscopic visualization of *P. palmivora* (c)

Rhizosphere fungi from Palolo and Labuan

The natural rhizosphere fungi spread in the specific agricultural land in Central Sulawesi. For example, Palolo and Labuan is the important areas of cocoa planting in Central Sulawesi. The results of the isolation of rhizosphere fungi from two areas in the initial stage obtained about 30 isolates from Palolo and 22 isolates from Labuan. Visual observations were made of isolates that showed potential as antagonists. Based on the preliminary observation, 8 isolates have potential as antagonists originating from Palolo and 6 isolates from Labuan.

The results of antagonistic tests in the dual culture of 8 isolates of endophytic fungi from rhizosphere showed 4 isolates (PRP10, T1RP3, T2RP1, A2RP12) able to inhibit the development of pathogenic fungi *P. palmivora* more than 50%. The inhibition of endophytic fungi against the *P. palmivora* varies between isolates. The average inhibitor ability of 8 endophytic fungi isolates was showed in Table 1.

Table 1. Percentage of inhibition dual culture isolate from endophytic fungi against *P. Palmivora*

Isolate	Origin	Inhibition (%)
Penicillium sp. (P)		
PRP10	Palolo	71.22
Gliocladium sp. (G)		
G1RP7	Palolo	42.97
G2RP5	Palolo	67.66
Trichoderma sp. (T)		
T1RP3	Palolo	74.86
T2RP1	Palolo	74.40
T3RL1	Labuan	41.26
T4RL2	Labuan	25.90
T5RL3	Labuan	42.43
T6RL7	Labuan	27.65
Aspergillus sp. (A)		
A1RP9	Palolo	65.28
A2RP12	Palolo	74.31
A3RL5	Labuan	26.24
A4RL8	Labuan	33.46
Unidentified (U)		
URP17	Palolo	66.34

The results observations of antagonistic tests in dual culture against *P. palmivora* in the laboratory condition showed that all isolates successfully inhibited with percentage inhibition from 25.90% to 74.86%. All fungi isolates have space and nutrient competition mechanisms that inhibit grown of *P. palmivora* on Potato Dextrose Agar medium in controlling room temperature. This is the direct observation from the laboratory and gives best result of endophytic fungi against the pathogens. The similar result reported by studies [21-23] that endophytic fungi with fast growth can suppress the growth of pathogenic fungi. Antagonistic fungi can compete with plant pathogens because grow faster and inhibit the development of pathogens. Then, studies [24, 25] stated that the competition mechanism arises because two competing microorganisms directly require the same space and nutrients. The methods are very clear and useful for observing the activity of beneficial fungi to inhibit pathogens that cause plant diseases. A benefit of endophytic fungi is their ability to grow in a non-obligate artificial medium.

The innovation-based environmentally friendly of agroecosystem management is very important. For example, the research explored two local cocoa plantations in Central Sulawesi (Palolo and Labuan). In these places, the ecological factor has many contributions to the pathogens development caused plant diseases in cocoa. The temperature, humidity and microclimate around the cocoa plantation give more opportunities for pathogens to develop. Also, genetics from cocoa impact to resistance of plants attacked by pathogens. More farmers believe that chemical pesticides or fungicides effectively handle the presence of black-pod disease in their plants. In contrast, more chemical application to the farm enhances habitat pollution, especially in soil and water. The rhizosphere fungi are very sensitive to the presence of the chemical in soil. The microbes from the rhizosphere can spread passively through water, soil particles and agricultural tools. The presence of beneficial microbes can be supported by an environment free from chemical compounds. The result in Table 1 gives the best opportunity to farmer increase their potential for cocoa-based environmentally friendly.

Volatile compound test

Biocontrol agents such as beneficial fungi to be the important alternative reduce chemical applications supporting management of pathogen-caused plant diseases and save the environment. Among biocontrol agents, for example *Trichoderma* is the one of effective agent controlling plant disease. *Trichoderma* is able inhibit soil-borne plant pathogens development. Every pathogen of plant diseases or antagonistic fungi can produce different volatile compounds based on their purpose. Commonly antagonistic fungi produce volatile compounds as the defense for itself and inhibit plant pathogens attacking crops. Then toxicity of volatile compounds varies based on the age of antagonistic fungi. Based on findings research, these in vitro results indicated that endophytic fungi able to suppress development of *P. palmivora*.

The same investigations clarify the capability of endophytic fungi to control plant disease pathogens. The specific volatile compound can be detected and identified by the test. The results of the study on the test of volatile compounds against the growth of *P. palmivora* showed varies suppression of the colonies development of the pathogenic fungi. The level of inhibition varies greatly depending on the isolate used in the treatment (Table 2).

Table 2. Percentage inhibition of volatile compound test from endophytic fungi against *P. palmivora*

Isolate	Origin	Inhibition (%)
Penicillium sp. (P)		
PRP10	Palolo	9.22
Gliocladium sp. (G)		
G1RP7	Palolo	10.00
G2RP5	Palolo	10.30
Trichoderma sp. (T)		
T1RP3	Palolo	13.33
T2RP1	Palolo	12.50
T3RL1	Labuan	19.70
T4RL2	Labuan	20.56
T5RL3	Labuan	42.19
T6RL7	Labuan	29.28
Aspergillus sp. (A)		
A1RP9	Palolo	8.88
A2RP12	Palolo	9.67
A3RL5	Labuan	35.45
A4RL8	Labuan	34.31
Unidentified (U)		
URP17	Palolo	6.67

The result test of volatile compounds showed the unique material produced by isolates were different ranging from 6.67 - 42.19%. This indicate that the volatile compounds produced by all isolates able to inhibit growth of *P. palmivora*. It seems endophytic fungi from the rhizosphere soil working very well against pathogens. In their natural habitat contain healthy cocoa plantation of two areas meaning that farmers have been reduce of chemical fungicides and giving opportunity the antagonism fungi against the cocoa pathogens.

The base material unit in the biogenesis of the alkaloids contains dominant amino acids. Commonly alkaloids are the derivative of protein producing by microorganisms. This is the reactive substances contain biological activity in the low dosage [26, 27]. Another strain of microorganisms contains alkaloids. In illustration, *Trichoderma* produces volatile compound such as: alkaloids, elimoklavine and festuclavine. *Penicillium* produced other alkaloids namely agroklavine and ergometrine playing function as the antifungal activity against *Botrytis cinerea*, *Fusarium solani* and *Alternaria tenuis.* The compound is known as well as antibacterial action able suppress the development of several pathogenic bacteria contribution on the death of living cell [23, 28, 29]. Result of studies [30-32] revealed that endophytic fungi can produce volatile antibiotic or alkaloid compounds. Volatile compounds are also included in secondary metabolites that exhibit antibiosis mechanisms. Antibiosis is an antagonistic mechanism that involves the secondary metabolites production in the form of antibiotic namely: lyase enzymes, siderophores and other toxic substances.

Molecular test of Trichoderma sp.

Commonly infections in plants are associated with phytopathogens very destructive to crops and affected by temperature and humidity as important environmental factors. The plant pathogens are responsible for about 20–40% of losses in total production in the field. *Trichoderma* develop by spores and attack plant pathogen microbes. Besides temperature and humidity, *Trichoderma* colonization is influenced by the soil conditions and strain type. The common methods of detecting the presence plant pathogens and their antagonist fungi activities using molecular tests [33-35].

The results of electrophoresis for two isolates T1RP3 (AO3) and isolate T5RL3 (NK3) using ITS1 and ITS4 primer pairs were amplified at the target band -700bp. The results of cyclic analysis of the BLAST program to two isolates of *Trichoderma* sp. based on data from Gene Bank (Table 3).

Table 3. Cyclic analysis isolates of *Trichoderma* sp.

Isolates	Organism	Similarity (%)	Number Access
T1RP3	*T. asperellum* strain NECC30406	100	MH153622.1
T5RL3	*T. asperellum* strain NG125	100	MW287256.1

The results of the sequence analysis of *T. asperellum* strain NECC30406 have a similarity of 100%, while for *T. asperellum* strain NG125 has a similarity of 100%, although both are identified as *T. Asperellum*. In fact, *T. asperellum* isolates from Palolo have different morphological characters with *T. asperellum* isolates from Labuan. The *T.* asperellum from Labuan can be proven from the total score of the sequencing analysis results with total value of *T. asperellum* from Palolo (T1RP3) of 1068, while the total value for *T. asperellum* from Labuan (T5RL3) is 1066, in slightly lower by 2 points. Another researcher [36, 37] stated that every fungi has a specific character for identification more difficult if used manual techniques. Then, studies [38, 39] stated that the application of molecular technology will prepare a piece of useful information about different endophytic fungi from the same genus.

Environmental Protection Agency (EPA) have registered few biocontrol products find the microorganisms in the listed products contain fungi of the genus *Trichoderma* spp., mainly *T. lignorum*, *T. viride*, and *T. harzianum* [40, 41]. As the antagonist fungi, *Trichoderma* is known very effectively used in agriculture based on their action mechanisms such as mycoparasitism, antibiosis, competition, and resulting volatile compounds able to inhibit plant pathogens infections [42, 43]. For the example, studies molecular showed controlled *Alternaria alternata*, *Colletotrichum gloeosporioides*, and *Penicillium digitatum* in orange by *Trichoderma* spp. [44]; similar beneficial fungi controlled *Botrytis cinerea* in strawberry [41]; apple attacked by *Fusarium proliferatum* [42, 45]; *Fusarium incarnatum* attacked muskmelon [43]; banana attacked by *Fusarium oxysporum* banana [46]; mangrove attacked by *Colletotrichum* sp. and *Fusarium* sp. in mangrove [47]; also others economic plant in the worldwide.

Related to the result of Table 3, the molecular analysis tests of *Trichoderma*'s activities, *T. asperellum* isolates from Palolo showed morphological characters different from *T. asperellum* isolates from Labuan. Commonly different types of lytic enzymes are produce from *Trichoderma* form in the different types. It means that material has been differing in their efficiency and activity destroyed pathogenic fungi cell walls. Research of studies [42, 43, 48] showed commonly antagonist fungi produce important enzymes built by proteins such as glucanases and chitinases able to destroy components of pathogens cell walls. Enzymes from *Trichoderma* are able kill many cells of pathogens caused by plant disease. The endophytic fungi has ability penetrate and destroyed the cell wall. Many researchers believe that *Trichoderma* plays an important role as the biocontrol agent that safety environment. They were compete with plant pathogens used many ways such as, taking benefit of small space, nutrients, antagonistic

mechanisms, production of secondary metabolites and specific enzymes. *Trichoderma* able to destroy the cell walls and kill the pathogens. The unique mechanism, fungi can confront through inducing resistance, increasing plant defense reaction and mycoparasitism.

Besides antagonists fungi, *Trichoderma* has ability as plant growth-promoting properties and biocontrol agents. *Trichoderma* produced many secondary metabolites such as: heptanes, viridin, harzianolides, 2,4-ditert-butyl phenol, 6-pentyl-2H-pyran-2-one (6-PP) and propenyl phenyl methyl ester. Metabolites antifungal against various plant pathogens from *Trichoderma* showed by 6-pentyl-2H-pyran-2-one. Also, *T. asperellum* produces many isolate such as: hydrolytic enzymes: protease, cellulase, β-1,3-glucanases, β-1,4-glucanases and chitinase. Each isolate able destroy the cell wall components of the various plant pathogens with antifungal material [49-54].

Based on phylogenetic analysis aligned with the database at NCBI isolate T1RP3 and isolate T5RL3 have a closer relationship to *T. asperellum* (Figure 2).

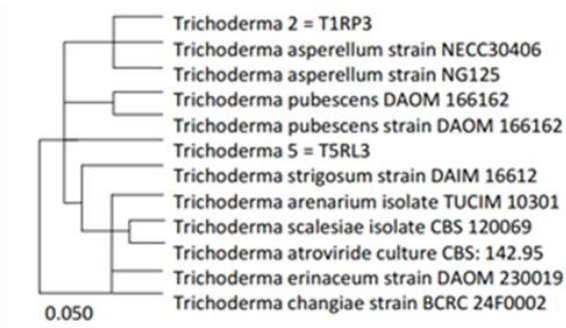

Figure 2. Phylogenetic analysis isolate of *Trichoderma* sp. used MEGA-XCavity geometry

The very important understanding about *Trichoderma* colonization patterns in inhibit the development of pathogens. This is very crucial factor supporting commercial production of beneficial organism. Stummer et al. [55] reported that *T. gamsii, T. afroharzianum,* and *T. harzianum* showed persistence and colonization in wheat rhizosphere soils and other crops. The efficacy of *T. gamsii, T. afroharzianum,* and *T. harzianum* attacked *F. pseudograminearum* was recorded and assessed. The quantitative PCR (qPCR) for monitoring and detecting the mechanism of *Trichoderma* strains reported indicated highly pathogen suppression activity, colonization and wheat biomass [55-58]. Ratnawati et al. [59] reported that agricultural land at Central Sulawesi has highly indigenous rhizosphere microbes including beneficial fungi. *The Trichoderma* collected from the shallot rhizosphere in Palu Valley is able to inhibit the development of *Alternaria porrii*. The *Trichoderma* colonization focused on the soil conditions as their natural habitat could supporting future industrialization, commercialization efforts increasing agricultural production, *Trichoderma* colonization is effective as the prerequisite for biocontrol agent to exert its effects to plant pathogens. This result indicated soil in Central Sulawesi potentially produces safe agricultural products for consumers.

4. CONCLUSIONS

The conclusions of the research are:

1. Based on the selection results, the potential endophytic fungi as biological agents against *P. pamivora* were 8 isolates from Palolo and 6 isolates from Labuan. It is very important result for the development of cocoa plantation especially in Central Sulawesi.

2. The results of identification and characterization in macroscopic and microscopic morphology, found *Aspergillus* sp., *Penicillium* sp., *Gliocladium* sp., *Trichoderma* sp., and unidentified.

3. Isolate T1RP3 showed the highest inhibition, at 78.46%, in the dual culture test, while isolate T5RL3 demonstrated the highest inhibition in the volatile compound test, at 42.19%.

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