Endophyte Microbes from Oil Palm (*Elaeis guineensis*) Tissues and Its Potential as a Biocontrol for *Ganoderma boninense In Vitro*

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Abstract

Endophyte microbes are microbes that colonize internal plant tissues without causing visible damage to their host plant and they can act as promising biological control agents in oil palm. The objective of this research is to obtain endophyte microbes which produce high chitinase activity and can inhibit *G. boninense* growth. Endophyte microbes are isolated from roots of oil palm planted in Padang Halaban estate, North Sumatera. Dual culture are performed in PDA medium using *G. boninense* from field. Chitinase assay was measured by spectrophotometry method. Potential isolates have been identified based on ITS rDNA using ITS1 and ITS4 primers for fungi and based on 16S rDNA using 9F and 1510R for bacteria. These endophyte fungi can inhibit *G. boninense* growth with inhibition ratio < 64%. T1 isolate has the highest activity of chitinase with 2.07 U/mL. There are four potential isolates of endophyte fungi: *Aspergillus, Fusarium, Hypocrea, and Trichoderma.* B2.1.2 isolate of bacteria has the highest inhibition ratio (67%) but B13.10.4 isolate has the highest chitinase activity with 3.43 U/mL. This result can be assumed that inhibition activity do not have any corelation with chitinase activity. The identification of potential isolates showed that they are *Serratia, Burkholderia, Acinetobacter*, and *Bacillus.*

Keywords: root-endophytes microbe, Ganoderma boninense, Elaeis guineensis, *chitinase activity, ITS rDNA, 16S rDNA*

Introduction

Basal stem rot (BSR) disease caused by *Ganoderma boninense* is the most destructive disease in oil palm industries (Flood *et al.* 2000). Mostly, visible disease symptoms of BSR would appear at a late stage. Development of BSR infection could happen at 6 until 12 months (Darmono, 1996). The infected oil palm would decay at basal tissue with the result that infected plant would fall down before the economical time. At the endemic location, plant could infected less than 2 years of planting period. Many strategies have been conducted to control BSR included cultural, chemical and clean clearing. Unfortunately, those strategies have not been successful, for example, the use of chemical fungicide in the field has not been proven as an effective control method (Sapak *et al*., 2008).

Biological control can be promising strategy to control BSR. One of microbes group which could use for biocontrol agent is endophyte microbes. Endophyte microbes is microbes that colonize internal plant tissues without causing visible damage to their host plant. Recent surveys of various host plants have demonstrated that endophyte microbes are ubiquitous in plant species (Shiomi *et al.*, 2006). As internal colonisers, therefore they are more able to compete within the vascular systems with capacity to prevent infection of *G. boninense*.

The objective of this study was to isolate the endophyte microbes for *G. boninense* biocontrol based on antagonistic characteristic and chitinase activity and also to identify endophyte microbes based on ribosomal DNA.

Materials and Methods

Root sampling and isolation of endophyte microbe

Oil palm roots were obtained from Padang Halaban Estate PT SMART, Tbk in North Sumatera, Indonesia. The age of palm was 28 years with symptomless BSR (Basal Stem Rot) at endemic areas. Random palms were sampled with the roots diameter 0.5 cm, taken about 1.0 m away from their bases at 25 – 30 cm depth.

Root samples were surface sterilized by dipping in 5.25 % sodium hypochlorite, and subsequently in 50, 70, and 90 % of ethanol then rinsed twice with sterilized water. Root section was transferred to Nutrient Agar (NA) for bacteria culture and Potato Dextrose Agar (PDA) for fungi. For bacteria were incubated at 37^{o}C and for fungi at 28^{o}C.

Microscopy observation of endophyte microbes

a. Bacteria Gram staining. One loop of bacteria was dropped onto a slide glass and air dried. The slide was stained with crystal violet for 1 min and then washed with alcohol, iodine for 1 min, and distilled water respectively. After that, the slide was added with safranin for one min and washed again with distilled water to remove any staining solution. The slide was examined under a bright-field microscope (Model Nikon Eclipse-50i, Japan).

b. Morphological and histological identification of fungal endophytes. Morphological identification of fungal isolates was according to colony or hyphal morphology of the fungal culture, surface and reverse colony color, and colony texture. Histological identification of fungal endophytes was carried out by observing the characteristics of the spores or conidia, and reproductive structures (sexual and asexual) under a bright-field microscope (Model Nikon Eclipse-50i, Japan).

In vitro screening of endophyte microbe against *G.boninense*

The endophytes microbe isolates were screened for characteristic to inhibit *G. boninese in vitro* by dual culture test and chitinolytic activity assay.

Dual culture test (Jinantana & Syariah 1997). A 5 mm diameter agar disc was taken from a week-old PDA culture of *G. boninense*. For bacteria, agar disc with *G. boninense* was placed in central PDA and 6 cm diameter filter paper with 24 hours old of endophyte bacteria was placed around of *G. boninense* isolate. For fungi, agar disc with *G. boninense* was placed 6 cm away from agar disc with fungi isolate. For control, agar disc *Ganoderma* were inoculated without endophyte microbes. All of the antagonistic were conducted triplicate and incubated in 28 ^{o}C. The ability of the endophyte microbes to inhibit the growth of *G. boninense* was determined after 7 days incubation by measuring the diameter of the *G. boninense* colony in control plate (R1) compare with the diameter of the *G. boninense* colony in plate with endophyte microbe (R2).

Chitinolytic activity assay. Bacteria and fungi isolates were inoculated to liquid chitin medium with 0.3 % chitin (w/v) and incubated at 37 ^{o}C with orbital shaking 150 rpm. The chitinolytic assay was measured after 1, 2, 3, 4, 5, 6, and 7 days after incubation using Spektrophotometer UV-Vis at 420 nm. One unit of chitinase was represented as nmol N-acetylglucosamine min^{-1} mL^{-1} protein. N-acetylglucosamine was used as a standard with variation concentration 0-200 ppm.

Identification of endophyte microbes

Isolation 16s rRNA gene for bacteria and ITS rRNA gene for fungi. Single colony of 24 hours bacteria from NA was inoculated to 5 mL Luria Broth (LB) and incubated for 18 hours at 37 ^{o}C with orbital shaking 150 rpm. The culture was centrifuged at 6000 rpm for 5 min. The pellet was used for the isolation of DNA using Wizard Genomic (Promega) by manufacture procedure. One of 5-day-old fungi colony from PDA was inoculated into 25 mL Potato Dextrose Broth (PDB) and incubated for 5 days at room temperature. The mycelium was used for the isolation DNA using Plant Genomic DNA (Sigma) following the manufacture's procedure. The 1500 bp of *16S rRNA*

gene for bacteria was amplified using 9F (GAG TTT GAT CCT GGC TCA G) and 1510R (GGT TAC CTT GTT ACG ACT T) primers and 600 bp of *ITS rRNA* gene for fungi was amplified using ITS 1 (TCC GTA GGT GAA CCT GCG G) and ITS 4 (TCC TCC GCT TAT TGA TAT GC) primers. Polymerase chain reaction was carried out in a thermal cycler (Applied Bio system Verity) in a total volume of 50 uL containing 2.5 mM MgCl2, 5uL of 10X Taq Buffer, 200 mM dNTPs, 50 pmoles of each forward and reverse primer, Dream *Taq* Polymerase (Fermentas), and 10 ng DNA template. PCR was performed by initial denaturation at 95 ^{o}C for 4 min, followed by 35 cycles of denaturation at 95 ^{o}C for 1 min, annealing at 55 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min, with final extension at 72 ^{o}C for 10 min.

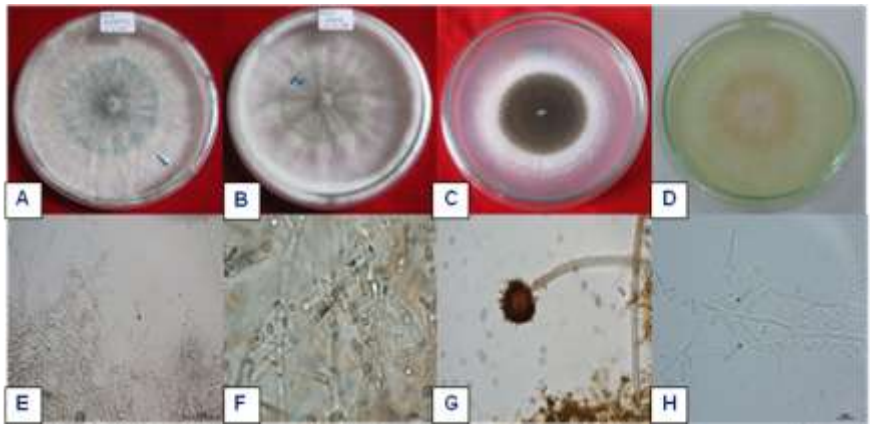
Cloning and transformation. *Escherichia coli* DH5α cells were transformed with PCR-amplified *16S rRNA* genes and *ITS rDNA* genes ligated in pGEM easy vector plasmid (Promega) using Fermentas DNA ligation kit (Fermentas). The plasmid was transformed to *E. coli* DH5α using heat shock treatment. The transformed cells (100 uL) were spread on LA plates containing X-Gal (50 ug/ml), IPTG (100 ug/ml) and ampicillin (50 ug/ml). The plates were incubated at 37^{o}C for 16 hours to screen blue and white colonies. Positive result was confirmed by PCR using M13F and M13R primers of white colonies.

Gene sequencing. The *16s rRNA* and *ITS rRNA* gene was sequenced to determine the homology with the known sequences in the NCBI database. DNA sequencing was done on FirstBase using ***M13*** forward ***sequencing primer*** (GTAAAACGACGGCCAGT).

Results and Discussion

Seventeen of endophyte bacterias were isolated from oil palm root with chitinolytic index ranged from 1.00 to 3.43 (data not showed). The ability of bacteria to form clear zones was measured qualitatively through chitinase activity of bacteria. The chitinase enzymes produced by bacteria are able to diffuse to the media. It can be seen from the presence of clear zones on the media. Most of the bacteria are Gram-negative (66%). According to Bell *et al*.,(1995), the abundance of Gram-negative endophytic bacteria in the oil palm roots was supported by population endophytes in other plant

Endophyte fungi of T1 and T13 isolates sp. are white color colony and scattered greenish patches become visible as the conidia are formed (Figure 1A). Microscopic of T1 and T13 isolates showed that the hyphae are septate and hyaline. Phialides are branched, flask-shaped, and attached to the conidiophores. Conidia have round or ellipsoidal form and have green color with 3 µm average diameter (Figure 1E). V2 and V3 isolates have white colony form in PDA initially, but typically become green (Figure 1B). Phialides are branched and tapered at tips. Spherical conidia gathered at tip of phialides in a tight, and ball-shaped (Figure 1F). A1 and A2 isolates have varies colony color from black to pale (Figure 1C). A1 and A2 isolate showed that hyphae are septate and hyaline. Conidiophores arise from the basal foot cell found at the supporting hyphae and terminate in a vesicle at the tip (Figure 1G). Mycelium morphology of F1 and F2 isolates are mostly white, but sometimes can be purple (Figure 1D). Some species also produce distinctly different conidia in the aerial mycelium (referred to as microconidia). Aerial mycelium is the growth of hyphae above the agar surface and often form a convex shape (Figure 1H).



(A,E: T1 and T3 isolates; B,F: V2 and V3 isolates; C,G: A1 and A2 isolates; D, H: F1 and F2 isolates)

Figure 1. Morphological and histological of fungal endophytes.

Antagonistic test was used to measure the ability of bacteria in inhibiting the growth of *Ganoderma in vitro*. Inhibition ratio of bacteria and fungi against *G. boninese* has different ranges. For bacteria, B2.1.2 isolate has the highest inhibition ratio (67%), while B24.1.7 and B28.5.3 isolate has the lowest inhibition ratio (26 %) and V3 isolate has the highest inhibition ratio (64 %) while the F2 has the lowest inhibition ratio (33 %) (Table 1). There are two possible mechanisms to inhibit growth of *G. boninense*, both are antifungal compound and chitinase activity. The chitinase could lysis cell wall of *G. boninense*. In addition, bacteria and fungi also capable to produce secondary metabolites, these can inhibit the growth of pathogenic fungi. According to Sidduqque *et al*., (2009), *Trichoderma* can produce volatile compounds with marked gas formation and capable to inhibit the growth of *G. boninense* up to 70% by dual culture method . A compound produced by *Trichoderma* is known as 6 - pentyl-alpha-pyrone (6PAP) which is a secondary metabolites compound (Coney *et al*. 1997).

Table 1 Antagonistic potential of endophyte microbe in dual culture test

Microbe isolate	Inhibition ratio (%)
a. Bacteria	
B2.1.2	67 a
B93.22.83	37 b
B28.5.3	26 d
B50.1.3	34 c
B24.1.7	26 d
B13.10.4	34 c
b. Fungi	
T1	58 b
T13	61 ab
V2	63 a
V3	64 a
A1	45 c
A1	37 d
F1	38 cd
F2	33 d

Means in the same column with different alphabet(s) are significantly different ($p < 0.05$) according to Duncan test.

All microbe isolates could produce chitinase enzyme activity although with different pattern (Table 2). B13.10.4 isolate of bacteria has the highest chitinase activity (3.43 U/mL at 5 days of incubation).Whilst, the highest enzyme activity of chitinase in fungi was T1 isolate (2.07 U /mL at 7 days of incubation). Although B2.1.2 and V3 isolates have the highest inhibition ratio, they did not produce enzyme with highest activity. According to Aktuganov (2003), chitinase activity was not related to antifungal activity. The bacteria with high antagonist activity is not always have a high chitinase activity.

Synergism between the antifungal compounds and enzymes of bacteria can enhance the role of bacteria as bio-control pathogen (Sheri *et al*., 2002). Some studies suggest that the enzyme chitinase production of the genus *Trichoderma* spp. is more effective than chitinase enzyme produced by other organisms, to inhibit a various plant pathogenic fungi (Lorito *et al*., 1994). Several studies are also reported that *Trichoderma* was capable in producing chitinase enzymes that play a role in several fungal pathogens including *Sclerotium rolfsii* and *Rhizoctonia solani*, (Haran *et al*., 1996; Harman *et al*., 1993).

Table 2 Chitinase activity enzyme from chitinolytic microbe

Microbe isolate	Enzyme activity (U/mL)	Time incubation (days)
a. Bacteria		
B2.1.2	1.54 c	5
B93.22.83	1.51 c	6
B28.5.3	1.44 cd	7
B50.1.3	1.28 d	5
B24.1.7	2.08 b	2
B13.10.4	3.43 a	5
b. Fungi		
T1	2.07 a	7
T13	1.75 b	6
V2	1.51 c	2
V3	1.16 de	2
A1	0.99 e	6
A2	0.93 e	6
F1	1.56 c	6
F2	1.30 d	6

Means in the same column with different alphabet(s) are significantly different ($p < 0.05$) according to Duncan test.

Table 3 Identification of selected microbe based on ribosomal DNA

Isolate	Identity	Homology (%)
B2.1.2	*Burkholderia sp*	99
B93.22.83	*Serratia sp*	99
B24.1.7	*Acinetobacter sp*	99
B13.10.4	*Bacillus cereus*	99
T1	*Trichoderma asperellum*	99
V3	*Hyphocrea virens*	99
A1	*Aspergillus sp*	99
F1	*Fusarium oxysporum*	99

The fungi and bacteria were identified base on their ribosomal DNA. Identification of fungi showed that there are *Trichoderma, Hypocrea, Aspergillus*, and *Fusarium* (Table 3). These four fungi are known to be a natural bio-control of fungal pathogens (Krupke *et a*l. 2003; Rubini *et al*., 2005; Adriana & Sergio 2001). Identification of potential isolates showed that they are *Serratia,*

Burkholderia, Acinetobacter, and *Bacillus* (Table 3). These results proved that several bacteria and fungi are potential as bio-fungicide because of their high inhibition ratio and activity of chitinase.

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