Isolation and Screening of Antagonistic Diazotrophic Endophytic Bacteria from Oil Palm Roots against *Ganoderma boninense*

Yurnaliza[1], Meylisa Purba[1], Dita Isnaini Rambe[1], Listra Sarimunggu[1], Isnaini Nurwahyuni[1] and Sovia Lenny[2]

[1]*Departement of Biology, Faculty Matematics and Natural Sciences, Jl. Bioteknologi No 1, Universitas Sumatera Utara, Medan, 20155, Indonesia.*

[2]*Departement of Chemistry, Faculty Matematics and Natural Sciences, Jl. Bioteknologi No 1, Universitas Sumatera Utara , Medan, 20155, Indonesia.*

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Abstract: *Ganoderma boninense* was pathogenic fungi affected rot disease on the basal stem of oil palm. Strategies to control *G.boninense* should be increased to reduced disease incidence. The aims of the study were to obtain isolates of bacterial endophyte diazotroph from oil palm roots and to examine their growth inhibiting activity to *G. boninense*. Oil palm roots were collected from three locations of oil palm plantation, namely Universitas Sumatera Utara (USU), PTPN IV Adolina, and community's oil palm plantations at Desa Bingkat, Serdang Bedagai. Bacterial endophytes was isolated from sterile roots and cultivated on Nutrien Agar and Ashby's specific medium. Dual culture methods were used as antagonistic test to *G.boninense*. Potential isolates were identified by Vitek test analyzer. The results showed that population of the bacteria were abundant in non-commercial plantation (USU and community plantation). Screening of bacterial isolates to inhibit *G.boninense* colony resulted 13 isolates of bacterial with various of percentage inhibition growth ranged from 11 to 62.5%. One of potential isolate of bacteria performed red color and by Vitek analyzer was identified as *Seratia marcencens*.

1 INTRODUCTION

Oil palm is most planted plant as producer of vegetable oils in the word. The function of oil palm crops is not only in the food products manufacture (Murphy, 2014), but also in wood industry for improving wood density in construction (Sulaiman *et al*., 2012). For the reason, the government support to keep the growth and productivity of oil palm. The major current problem in oil palm plantation is the high rate disease attack due to *Ganoderma boninense*. The fungi cause diseases in all stage of oil palm growth even when plant in nursery stage (Ariffin *et al*., 2000).

Some intensive controlling efforts were continued to be done, physically, chemically (Jee *et al*., 2015) or used bio-control agent as fungi (Yurnaliza *et al*., 2014) or bacteria (Bivi *et al*., 2010). The controlling results are still needed to be improved. The previous research about bio-control activity of fungal endophytes to protect oil palm against *G.boninense* infection showed that some species of fungal endophytes were able to produced antifungal, lytic enzymes (Yurnaliza *et al*., 2014) and induced plant resistance (Yurnaliza, 2015) and (Yurnaliza *et al*., 2017) to protected oil palm from *G.boninense* infection.

This study is focused to search a new bacteria for oil palm protection against *G.boninense*. Bacterial antagonist are expected to have antifungal and lytic enzyme activity, plant growth promoting compound or the ability to induce their host resistance. Diazotrophic bacteria were the potential candidate bacteria that have ability as growth promoting of plant and also as biological control agent Diazotrophic are the nitrogen-fixing bacteria from atmosphere that live symbiotically or free. This bacteria live abundant in rhizosphere or as endophytes in the tissues plant. The diazotrophic endophytes are very important to improve of plant growth, because of the plant will get converting product of nitrogen by bacteria directly (Aryantha *et al*., 2018). Besides that, some diazotroph bacteria also produce indole-3-acetic acid for stimulating

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plant growth (Zega *et al*., 2018), antifungal compound or lytic enzymes to control pathogenic fungi (Ji *et al*., 2014). Therefore, as a endophytes, the diazotroph bacteria are promising to be expanded as a bio-control agent candidate to control *G.boninense*. This research is expected to obtain diazotroph endophytic bacteria from oil palm root as bio-control agent of *G.boninense*.

2 MATERIAL AND METHODS

2.1 Samples of Oil Palm Roots

The roots of oil palm were obtained from Universitas Sumatera Utara (USU), state-owned enterprise of PTPN IV Adolina, and small holder of community oil palm plantations at Desa Bingkat, Serdang Bedagai.

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2.2 Isolation Bacteria

Isolation of bacterial endophyte from oil palm roots were started from surface sterilization according to procedure of Yurnaliza *et al*., (2014) with modification. Oil palm roots (10 g) were washed with running tap water, and surface sterilized respectively using ethanol 70% v/v (2 min), sodium hypochlorite 5.25% (5 min), ethanol 70% (30 sec) and last rinsed with sterile distilled water (3 times each). The sterile roots were homogenized by mortar and pestle and dissolved with 10 ml of sterile distilled water. The root suspension was diluted by serial dilution until 10^{-3} and the dilution suspension were spread onto Ashby's and Nutrient Agar (NA) medium. The culture was incubated for 24-48 hrs at temperature ± 28ºC. The rising colony bacteria on the medium were calculated the total colony and sub-cultured to the new medium for purification. The single culture as pure culture were characterized the morphology of colony, Gram stain and several biochemical characters.

2.3 Selection Antagonistic Bacterial against *Ganoderma boninense*

Antagonistic activity of bacterial endophytes diazotroph were selected by dual test assay. The bacteria was co-cultured with *G.boninense* on the extract potato medium (PDA, Merck®). The *G.boninense* was cultured in medium at the center of dish (9 cm in the diameter). The endophytic bacteria were cultured besides of *G.boninense* at distance 3 cm from the center of inoculated fungus. The culture plates were stored on dark room at room temperature during 5 days.The percentage inhibition of colony *G.boninense* by bacterial endophytes were calculated as formulated (Yurnaliza *et al*., 2014)

$$\%CGI = \left(\frac{r1-r2}{r1}\right) \times 100\% \quad (1)$$

Noted: CGI is radial colony growth inhibited , r1and r2 were respectively radius growth of *G.boninense* colonies to control (no bacterial) and test bacterial.

2.4 Characterization of Bacteria

The bacteria were characterized morphology colony, Gram stain type, motility and catalase activity. The high potential bacteria was identified by Vitek analyser (bioMerieux, UK). The procedure identification is accomplished by biochemical methods.

3 RESULTS AND DISCUSSION

3.1 Population of Bacterial Endophytes Diazotoph

Total population of endophytic bacteria from sterile roots of oil palm segments were dissimilar in each sample location and type medium for cultivation. Oil palm roots from Universitas Sumatera Utara had more abundant bacteria than other locations. Percentage of population bacterial diazotrophic endophytes indicated that in un-commercial locations (USU and community plantation) the population dizotrophic bacteria were more higher than PTPN IV. PTPN IV Adolina was a larger plantation state-owned enterprise in agribusiness. Normally, application of chemical fertilizers in company management was a standard procedure to improve productivity of plant growth. Presence of diazotrophic bacteria as endophytes in oil palm roots were affected by utilization of chemical fertilizer. Communities of diazotroph and total bacteria were effected by fertility management of the land (Orr *et al*., 2012). Ayuni *et al*., (2015) said the population diazotroph bacteria of *Stenotrophomonas maltophila* and it's nitrogenase enzyme were affected significantly by urea-N treatment to rice plant.

Table 1. Population of bacterial endophytes from roots of oil palm from 3 locations.

Location	Total bacteria (10^5)	Diazotroph bacteria (10^5)	Percentage of diazotroph bacteria/10g oil palm root (%)
University of Sumatera Utara (USU)	4.64	2.19	47,2
PTPN IV Adolina	4.06	1.08	27
Community plantation	1.08	0.075	6,9

Counting of total population bacterial endophytes in oil palm roots using nutrient agar indicated the total population generally, but specific for diazotrophic bacteria were calculated on Ashby's medium. Ashby's medium is a selective medium without N-source for isolating and cultivating diazotrophic bacteria (Stella *et al*., 2012). Only bacteria that have nitrogenase enzyme will grow in this medium. The highest percentage of diazotrophic bacteria in 10 g root sample were in community plantation (Table 1).

Based on morphological colony of bacterial growth on Ashby's medium were selected as much as 30 isolates. Next selected bacteria was based on their activity to inhibited Ganoderma mycelium growth in dual culture assay test.

3.2 Characterization Bacteria and Antagonistic Activity Assay

Dual culture assays between 30 isolates of bacterial endophytes diazotroph and *G.boninense* resulted 13 potential isolates with %CGI 11.1-62.5 %. Almost all isolates were coccus Gram negative. Bacterial endophytes from oil plam roots were isolated by Ramli *et al*., (2016) and Bivi *et al*., (2010) also dominated by Gram negative bacteria. The motility tests showed several bacteria motile and all isolates were unable to reduce hydrogen peroxide (catalase negative) (Table 2). The colour of colony was variable from white, yellow, yellowish, orange and red. The red colour bacteria was usually Genus of Serratia. One of the red colour bacteria (AP 35) had the highest %CGI (62.5%) (Figure 1).

All of endophytic diazotroph bacterial isolates has different abilities in inhibiting *G.boninense*. From dual assay was resulted two model inhibition mycelium type, inhibited and inhibited with depletion of mycelium (Figure 1). Inhibited of radial growth mycelium probably caused by antifungal activity of bacteria.

The depletion of mycelium was occurred when the fungal hypa was contact and close to bacterial colony. It's probably caused by lytic enzyme that was produced by bacteria. The enzymes such as chitinase and glucanase used chitin and glucan from cell wall of *G.boninense* as a substrate. Chitin and glucan components which are the major constituents of the cell wallof the fungus. Bacterial endophyte diazotroph in this research (AP 39) are possibly able to produce bothenzyme. The activity of lytic

Table 2: Gram stain, cell type, morphology colony and percentage of colony growth inhibition (CGI) activity bacterial endophytes diazotroph against *G. boninense*

Code	Gram	Cell shape	Morphology of colony				CGI (%)	Motility	Catalase test
			Shape	elevation	margin	colour			
AU 6	-	Coccus	iregular	Raised	Curled	yellow	40.0	-	-
AU 30	-	Coccus	Circular	Flat	Lobate	white	11.1	+	-
AU 33	-	Coccus	Iregular	Flat	Lobate	white	22.0	+	-
AP 23	-	Coccus	Circular	Flat	entire	white	44.4	-	-
AP 24	+	Coccus	Circular	Flat	Entire	white	48.8	-	-
AP 25	+	Coccus	Iregular	Raised	Undulate	yellowish	11.1	+	-
AP 32	-	Coccus	Circular	Convex	Undulate	white	20.0	+	-
AP 33	+	Coccus	Iregular	Convex	Undulate	Orange	22.2	-	-
AP 34	-	rod	Iregular	Raised	Lobate	red	22.2	+	-
AP 35	-	rod	Iregular	Flat	Lobate	red	62.5	+	-
AP 36	-	rod	Circular	Raised	Undulate	white	15.0	-	-
AP 39	-	Coccus	Iregular	Flat	Curled	pink	44.4	-	-
AK 25	+	rod	Circular	Flat	entire	white	16.6	-	-

enzyme made the colony thinning and the growth inhibited.

Bacterila diazotroph not only be able to produce chemical compound to induce plant growth, but also produce antifungal (Weber *et al*., 2007) and fungal mycelium lysis enzymes. Ji *et al*., (2014) showed that the diazotroph endophytic bacteria were able to inhibited mycelium of *Fusarium oxysporum* and *Rhizoctonia solani* in dual culture test. According to Bivi *et al*., (2010) in addition to enzyme activity, the use of endophytic bacteria as a biocontrol agent of *G.boninense* is an effective means. That's because endophytic bacteria are indigenous bacteria that colonize the oil palm root vascular system, so it has better nutrition and space competitiveness than *G.boninense*, so the growth of *G.boninense* will be depressed.

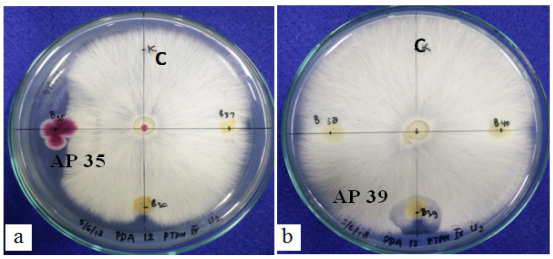

Figure 1: Morphological characteristics of inhibited colony G.boninense when antagonistic activity assay with bacterial endophytes diazotroph (a). Inhibited growth and (b) depletion mycelium, C (no bacteria)

Identification of the red one of diazotrophic bacteria by Vitek analyzer resulted species of *Serratia marcencens* with probability number was 99%. The biochemical detail in Table 3.

Table 3. Biochemical test results of isolate AP 35 using Gram Negative (GN) Card by Vitek analyzer (bioMerieux, UK)

Well	Test	Results	Well	Test	Results
2	Ala-Phe-Pro-Arylamidase	-	33	Saccharose/Sucrose	+
3	Adonitol	+	34	D-Tagatose	-
4	L-yrrolydonyl-Arylamidase	+	35	D-Trehalose	+
5	L-arabitol	+	36	Citrat (sodium)	+
7	D-cellobiose	-	37	Malonate	-
9	Beta Galactosidase	-	39	5- Keto Gluconate	-
10	H2S Production	-	40	L-Lactate alkalinisation	+
11	Beta-N-Acetyl-Glucosaminidase	+	41	Alpha Glucosidase	-
12	Glutamyl Arylamidase pNA	-	42	Succinate alkalinisation	-
13	D-Glucose	+	43	Beta-N-acetyl-Galactosaminidase	-
14	Gamma-Glutamyl-Transferase	+	44	Alpha Galactosidase	-
15	Fermentation Glucose	+	45	Phosphatase	+
17	Beta-Glucosidase	+	46	Glycine Arylamidase	-
18	D-maltose	+	47	Ornithine Decarboxylase	+
19	D-Manitol	+	48	Lysine Decarboxylase	+
20	D-Mannose	+	52	Decarboxylase Base	-
21	Beta-Xylosidase	-	53	L-Histidine assimilation	-
22	Beta-Alanine arylamidase pNA	-	56	Coumarate	+
23	L-Proline Arylamidase	+	57	Beta-Glucoronidase	-
26	Lipase	-	58	o/129 resistance [comp vibrio]	+
27	Palatinose	-	59	Glu-Gly-Arg-Arylamidase	+
29	Tyrosine Arylamidase	-	61	L-Malate assimilation	-
31	Urease	-	62	Ellman	-
32	D-sorbitol	+	64	L-Lactate assimilation	-

4 CONCLUSIONS

The thirteen isolates of bacterial endophytes diazotroph from oil pam roots inhibited of *G.boninense* mycelium with percentage colony growth inhibition was 11-62,5%.Diazotroph bacteria were dominated by Gram negative bacteria with the coccus cell shape. The potential isolate was a red colony bacteria and identified as *Serratia marcencens* by Vitek analyzer (bioMerieux, UK).

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