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Antagonistic activity of indigenous endophytic bacteria from Cocoa plants against *Phytophthora palmivora* Bult the cause of black pod rot disease in Cocoa

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Black pod rot disease caused by *Phytophthora palmivora* Bult is one of the most important disease problems of Cocoa. This study aimed to screen the endophytic bacteria and to establish their potential as biological control agents against black pod rot disease in Cocoa. In-vitro testing through duel culture on V-juice media was carried out to select endophytic bacteria which had inhibitory ability against *P. palmivora* Bult PPKS isolates. The ten best isolates were selected for the activity and antagonistic mechanism against *P. palmivora* Bult isolates PPKU and PPKT. Endophytic bacterial isolates of 2BWB2, 2BRB, and 3BAE with a high activity of lytic enzymes (cellulase, protease and amylase), have the opportunity to be developed into biological agents to control black pod rot disease on cacao.

Keywords: antagonistic, black pod rot disease, cocoa, and endophytic bacteria,

INTRODUCTION

Black pod rot disease caused by *Phytophthora palmivora* Bult. is an important disease in cocoa plants in Indonesia. In general, loss of results due to attacks of cocoa pod rot is around 10% per year (Deberdt et al., 2008). In Java, this disease can reduce yields by 33-50% (McMahon et al., 2011), and in the dry season in the fields with large populations of ants, yield loss can reach 90% (Rosmana et al., 2018). *P. palmivora* can infect all parts of the cocoa plant, but the highest loss is caused by infection in the fruit (Damono et al., 2006).

Several approaches such as: chemical control, phytosanitary and cultural methods, genetic resistance, have been used to manage black pods, but they have not been effective in suppressing yield loss. The use of synthetic pesticides requires a high cost and can have a negative impact on the environment, killing non-target organisms, and endangering the health of consumers. Therefore, it is necessary to input environmentally friendly and safe pathogen control technology for consumers. At present, biological control using endophytic bacteria is widely researched and used to control plant

diseases because it is considered more effective, safer for the environment and consumers (Marwan et al., 2001; Nguyen et al., 2016; Ramli et al., 2016). Endophytic bacteria are intracellular bacteria that live in plant tissues and do not negatively affect the host plant (Lodewyckx et al., 2002; Miliute et al., 2015).

Endophytic bacteria have been reported to have antagonistic properties towards various types of pathogens, including being able to inhibit the development of *Ganoderma boninense* on oil palm (Buana et al., 2014; Ramli et al., 2016; Nasahi et. al., 2016), *Rigidoporus lignosus* on rubber plants (Hardiyanti et al., 2017), *Fusarium oxysporum* on pepper plants (Edward et al., 2013), *Colletotrichum gloeosporioides* on cocoa plants (Khaeruni et al., 2018a); bacterial pustule disease on soybeans (Habazar et al., 2015; Khaeruni et al., 2018b), and bacterial leaf blight on rice (Rahma et al., 2018; Serdani et al., 2018). Ability to colonize into tissues plants, causing endophytic bacteria to be more effective to be developed as plant pathogenic biological agents than other biological agents (Lodewyckx et al., 2002). As an internal colonizer of the root system, endophytes are able to compete within the vascular system, inhibiting pathogens to obtain both nutrients and space for its proliferation. This study aimed to isolate, characterize, test the activity and test the antagonistic mechanism of endophytic bacteria against *P. palmivora* causing black pod rot on cacao.

MATERIALS AND METHODS

Main Material

The plant materials used in this study were young pods and twigs of cocoa plants taken from each of the 4 cocoa planting locations in East Kolaka Regency, Southeast Sulawesi. From each location, 2 samples of healthy pods and twigs were taken from plants infected with *P. palmivora*. Three isolates of *P. palmivora* (PPKS, PPKT and PPKU isolates) used as target pathogens were obtained from the Phytopathology Laboratory of the Department of Plant Protection, Faculty of Agriculture, Halu Oleo University, Kendari.

Isolation of Endophytic Bacteria from cocoa pods and twigs

Cocoa pod and twig samples were first cleaned of dirt attached to running water. Sample pods and twigs were shaved off the surface with a sterile knife and then cut into small pieces with a size of 2 cm. The sample pieces were washed again with sterile distilled water before surface sterilization by soaking in 70% ethanol for 30 seconds and continued with soaking in 0.1% $HgCl_2$ for 2 minutes (Hung and Annapurna, 2004). Endophytic bacteria were isolated using a scour method and dilution technique as stated by Melnik et al. (2011). The suspension of the scoured sample was diluted serially in 0.8% physiological salts. A total of 100 µl of suspension was spread over Trypticase Soy Agar (TSA) medium. As a control, the pieces of pods and twigs that were sterilized on the surface were placed on the TSA media. All plates were incubated for several days at 28^{o}C. The bacterial colonies that grew then were purified in TSA medium and coded based on the source and location of the sampling.

Selection of Antagonist Endophytic Bacteria

The initial selection of endophytic bacteria through inhibitory tests was carried out according to the modified method of (Agustiyani et al., 2014). The test pathogen used at this stage was *P. palmivora* PPKS. Test of the antagonistic activity of endophytic bacteria against *P. palmivora* used a cross-plug with two replications. One agar block of P. palmivora aged 7 days and 5 mm in diameter was grown in the middle of V8 juice medium in a petri dish and incubated for 2 days at room temperature.

After 2 days of incubation period, 1 ose of pure culture of endophytic bacteria was scratched on three sides of the cup, 2 cm from the edge of the cup and 1 side was emptied as a control. Each side was scratched with different isolates and each isolate was repeated twice. After 7 days of incubation, the inhibition of pathogen growth from bacterial activity was observed and calculated using the formula: Inhibition (I) = ((CT) / C) x 100%, where C is the radius of *P. palmivora* without endophytic bacteria and T is the finger *P. palmivora* finger with treatment of endophytic bacteria (Nourozian et al., 2006). Isolates that showed growth inhibition of *P. palmivora* ≥ 50% were marked as +++, isolates whose inhibition was 50% < X ≥ 25% were marked as + +, and inhibition <25% was marked +.

Test of Inhibitory of Selected Endophytic Bacteria against P. palmivora PPKU and PPKT

In this stage, 10 isolates of endophytic bacteria were selected which had the best activity against *P. palmivora* inhibitors on Phase I testing, while the target pathogen used two *P. palmivora* isolates, namely PPKU isolates isolated from cocoa plants from North Kolaka and PPKT

isolates isolated from East Kolaka (Collection of Phytopathology Laboratory of Plant Protection Department). Testing of inhibitory ability was carried out using the cross-plug method (Agustiyani et al., 2014) on V8 Juice media. One agar block of *P. palmivora* aged 7 days and 5 mm in diameter was grown in V8 Juice medium, 3 cm from the inside of the petri dish and incubated for 2 days at room temperature.

After 2 days the incubation period, 1 ose of pure culture of endophytic bacteria was scratched in front of the test pathogen culture with a distance of 3 cm. Each endophytic bacteria was tested 3 times as a test. After 7 days of incubation, the inhibition formed from the activity of endophytic bacteria was observed. The percentage of inhibition is calculated according to the formula proposed by Nourozian et al., (2006).

Morphological and Physiological Characteristics of Selected Endophytic Bacteria

The morphological characters of the ten endophytic bacteria observed included Gram staining, shape, elevation, and margin, and by the number of results of physiological test including motility, utilization citrate, and the ability to ferment glucose and mannitol. The tests carried out followed the standard protocols (Yulvizar, 2013; Zahida et al., 2013).

Test of Lytic Enzymes Activity

Activity test of lytic enzymes included cellulase, protease, and amylose. Cellulase activity was detected according to Agustiyani et al. (2014) in Nutrient Agar supplemented with *carboxymethyl cellulose* 2% (w/v). Protease activity was detected in Nutrient Agar supplemented with 2.5% (w/v) skimmed milk according to Perneel et al. (2007), while amylase activity was detected on 0.002% starch agar plates and incubated at 55°C for 24 hours. After incubation, the plates were flooded by Gram's Iodine (2% I2 and 0.2% KI) to produce starch-iodine complex which is visualized by deep blue color (Gazali and Suwastika, 2001). The qualitative activity of the three enzymes was determined based on the index value of the total zone diameter divided by the diameter of the bacterial colony (Agustiyani et al., 2014).

RESULTS

Endophytic Bacteria from Cocoa Pod and Plant Twigs

Isolation of endophytic bacteria from pods and young twigs of cocoa which was preceded by sterilization of plant tissue surface as described by Hung and Annapurna (2004), resulted in 50 isolates of endophytic bacteria: 21 isolates obtained from pod tissue and 29 isolates obtained from young twig tissue. The efficiency of disinfection method was checked. There was no growth of bacteria on surface-disinfected plates, uncut stem pieces. The performance of endophytic bacterial colonies obtained from cocoa pod and leaf samples is presented in Figure 1

Screening Antagonistic Endophytic Bacteria against *P. Palmivora*

The results of screening of 50 isolates of endophytic bacteria obtained 23 isolates were antagonistic against *P. palmivora* PPKS, 19 isolates were isolated from pods and 4 isolates were isolated from cacao twigs. Of the 23 isolates, 15 isolates had strong inhibitory ability, and 8 isolates had moderate inhibition. The codes and origin of the 23 endophytic bacteria isolates are shown in Table 2

Antagonistic Activity of Endophytic Bacteria against *P. palmivora* PPKT & PPKU

The testing results of antagonistic activity of 16 endophytic bacterial isolates against *P. palmivora* obtained 8 isolates of endophytic bacteria which were able to inhibit ≥50% of the growth of P. palmivora PPKT and P. palmivora PPKU *in vitro* (Table 3)

As shown in Table 3, endophytic bacterial 1BRB1, 2BRB, 5BRB3, 2RPR1, 4RRB, 3BAE, 2RPR1 and 2BWB2 isolates inhibited more than 50% growth of both *P. palmivora* PPKT and PPKU. Both 2BRB and 5BRB3 caused similar inhibition percentages in PPK (60 and 60.59%, respectively) as well as in PPKU with inhibition of 53.57 and 53.33%, respectively.

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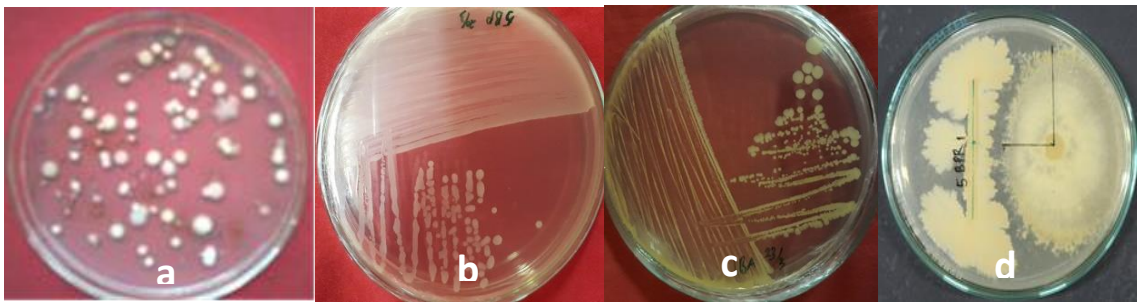

Figure 1. Single colony from isolation results (a), samples of endophytic bacterial isolates purified by Gram positive (b), Gram negative (c), antagonistic activity of endophytic bacteria against *P. palmivora* in-vitro (d), Eb: endophytic bacteria, Pp: *P. palmivora*

Table 1. Total endophytic bacteria isolated from cacao plantation at East Kolaka District

No	Origin Location (Village/Sub District)	Number Isolates of Endophytic Bacteria		Total
		Pods	twigs	
1	Rubia/Aere	4	9	13
2	Aere/Aere	4	7	11
3	Pombaria/Lambadia	7	6	13
4	Wanuaambuteo/Lambadia	6	7	13
	Total	21	29	50

Table 2. Endophytic bacterial isolated from cacao pod and young branch that are antagonistic to *P. palmivora*

No	Code	Antagonistic activity	Sample Source	Origin Location
1	1BRB1*	+++	Pod	Rubia/Aere
2	2BRB*	+++	Pod	Rubia/Aere
3	4BRB*	+++	Pod	Rubia/Aere
4	5BRB1*	+++	Pod	Rubia/Aere
5	5BRB3*	+++	Pod	Rubia/Aere
6	5BRB4*	+++	Pod	Rubia/Aere
7	1RRB2	++	Twing	Rubia/Aere
8	4RRB*	+++	Twing	Rubia/Aere
9	2BAE	++	Pod	Aere/ Aere
10	3BAE*	+++	Pod	Aere/ Aere
11	5BAE*	+++	Pod	Aere/Aere
12	3BPR1*	+++	Pod	Pombaria/ Lambandia
13	3BPR2	++	Pod	Pombaria/Lambandia
14	3BPR3	++	Pod	Pombaria/ Lambandia
15	5BPR2*	+++	Pod	Pombaria/Lambandia
16	2BPR	++	Pod	Pombaria/Lambandia
17	2RPR1*	+++	Twing	Pombaria/ Lambandia
18	2BWB1	++	Pod	Wonuambuteo/Lambandia
19	3BWB	++	Pod	Wonuambuteo/Lambandia
20	3BWB	++	Pod	Wonuambuteo/Lambandia
21	4BWB1*	+++	Pod	Wonuambuteo/Lambandia
22	2BWB2*	+++	Pod	Wonuambuteo/Lambandia
23	1RWB*	+++	Twing	Wonuambuteo/Lambandia

Notes: +++) isolates of endophytic bacteria that have strong inhibitory activity, ++) isolates of endophytic bacteria with moderate inhibitory activity, *) isolates selected for further testing.

Table 3. Antagonistic activity of 16 bacterial endophytic, isolated from cacao pod and young

branch, against *P. palmivora*

No	Code of Isolates	Inhibition (%) to *P. palmivora*	
		PPKT isolate	PPKU isolate
1	1BRB1**	50,92	50,71
2	2BRB**	60,00	53,57
3	4BRB	38,33	37,14
4	5BRB1	39,76	33,92
5	5BRB3**	60,59	53,33
6	5BRB4	41,54	50,00
7	2RPR1**	50,00	55,05
8	4RRB**	50,25	50,00
9	3BAE**	52,92	51,66
10	5BAE	39,85	42,22
11	3BPR1	36,66	40,00
12	5BPR2	41,19	40,31
13	2RPR1**	56,31	50,00
14	4BWB1	43,80	47,00
15	2BWB2**	50,71	50,00
16	1RWB	44,52	41,42

Note : **) Selected isolates for further tests

Table 4. Selected morphological features of bacterial endophytic isolated from cacao pod and young branch

Code of Isolates	Colony Characteristic (color, form, elevation, margin)	Cell Shape	Variant Group
1 BRB 1	White, irregular, flat, entire	Rod-shaped	I
2 BRB	Cream-yellowish, spindle, flat, entire	Rod-shaped	II
5 BRB 3	Cream-yellowish, spindle, flat, entire	Rod-shaped	II
4 RRB	Cream transparent, spindle, convex, wavy	Rod-shaped	III
3 BAE	White, circular, convex, erose	Rod-shaped	IV
2 RPR 1	Cream transparent, circular, flat, entire	Rod-shaped	V
2 RWB 2	Cream transparent, circular, flat, entire	Rod-shaped	V
2 BWB 2	Cream-yellowish, spindle, flat, entire	Rod-shaped	VI

Table 5. Selected physiological features of bacterial endophytes isolated from cacao pods and swings

Code of Isolates	Gram Staining	Motility	Citrate Utilization	Glucose Fermentation	Mannitol Fermentation
1 BRB 1	-	+	-	+	-
2 BRB	+	+	-	+	-
5 BRB 3	+	+	-	+	-
4 RRB	+	+	-	+	-
3 BAE	+	+	-	+	-
2 RPR 1	+	+	-	+	-
2 RWB 2	-	+	-	+	-
2 BWB 2	-	+	-	+	-

Note : “+”, positive reaction; “-“, negative reaction

Table 6. Index activity of lytic enzymes of bacterial endophytes from cacao pods and young twigs on the substrate.

No.	Isolate Code	Index of Enzyme Activities		
		Cellulase	Protease	Amylase
1	1 BRB1	2.0	-	2.3
2	2 BRB	2.0	1.8	1.2
3	5 BRB3	2.0	2.2	-
4	4 RRB	-	1.8	-
5	3 BAE	2.5	2.1	2.4
6	2 RPR1	-	-	-
7	2 BWB2	2.4	2.3	1.3

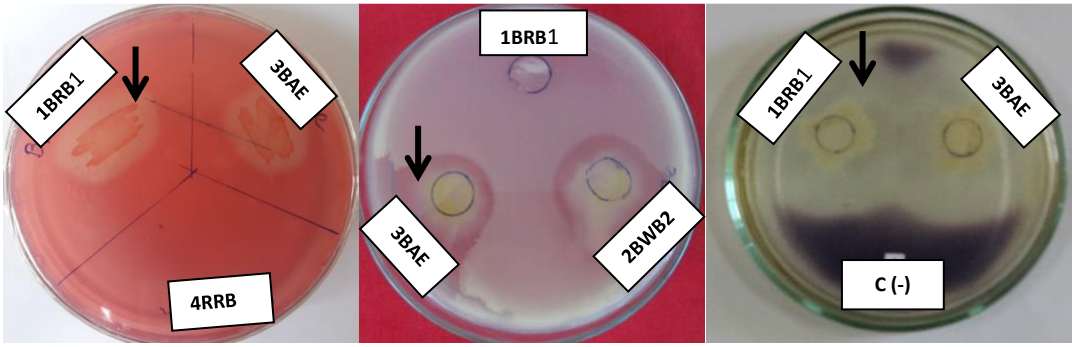

Figure 2. Lytic enzymes activity of endophytic bacterial from cacao plant. Activity of cellulase(a), protease(b), dan amylase (c) was indicated by the formation of clear zones (arrows) on the substrate.

Morphological dan Physiological Characteristics of Selected Endophytic Bacteria

Morphological characterization shows that the morphological characteristics of endophytic bacterial colonies that have strong antagonistic abilities were quite diverse. Of the 8 isolates tested there were 6 different variations of colony morphology characters, but the 8 isolates had the same cell shape i.e. rod shape shown in Table 4. Data in Table 4 show that although all cells of endophytic bacterial isolates isolated from young pods and twigs of cocoa were rod-shaped, the morphological characteristics were quite diverseOf the 8 isolates tested, there were 5 different groups of morphological characteristics. On the other hand, the physiological characteristics diversity was low because they differed only from Gram staining, but they were not different in the use of citrate, glucose and mannitol. The diversity of physiological characteristics of low endophytic bacteria was also reported by Goryluk et al. (2009). Of the 12 isolates of endophytic bacteria isolated from the *Chelidonium majus* L plant, 11 isolates had the same physiological characteristics.

Testing of physiological characteristics showed that selected endophytic bacteria were Gram positive (5 of 8 tested isolates). All cells were motile and able to ferment glucose. However, all tested endophytic isolates were unable to use citrate and unable to ferment mannitol (Table 5).

Lytic Enzymes Activity

Observations on degrading enzyme activity (cellulase, amylase, and proteinase) are shown in Table 6. While the degrading enzyme activity tested in vitro on media containing cellulase substrate, protein and starch is shown in Figure 2. In Table 6, there are 3 isolates of endophytic bacteria: 2BRB, 3BAE, and 2BWB2 which had strong inhibitory ability against *P. palmivora* also had the ability to secrete 3 types of enzymes, namely: cellulase, protease and amylase at once. The results of this test have proven that endophytic bacterial isolates that had strong inhibitory activity against *P. palmivora* were closely related to the production of pathogenic cell wall degrading enzymes. Acebo-Guererro et al.

(2015) reported that CP07 and CP30 rhizobacteria which had > 50% inhibitory effect on *P. palmivora* also had lytic enzyme activity such as high protease and lipase. Gao et al. (2010) suggested that lytic enzymes such as chitinase and cellulase produced by antagonistic microbes played a role in degrading cell walls of pathogens such as fungi, nematodes, and oomycet whose cell components can be chitin or cellulose. Therefore, endophytic bacteria isolated from 2BWB2, 2BRB, and 3 BAE were potential to be developed as biological agents to control black pod rot disease in cocoa plants.

CONCLUSION

There were 8 isolates of endophytic bacteria from pods and young branches of cocoa which had strong inhibitory ability (> 50%) against 3 *P. palmivora* isolates. The 8 endophytic bacterial isolates have high diversity of colony morphological characteristics but low diversity of physiological characteristics. Among the 8 endophytic bacteria that are antagonistic isolates, isolates 2BWB2, 2BRB, and 3BAE with a high activity of lytic enzymes (cellulase, protease and amylase), have the opportunity to be developed into biological agents to control black pod rot disease on cacao.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

AK designed and performed the experiments and also wrote the manuscript. Darmansyah and RA collected the data. TW and GAKS performed laboratory experiment, data analysis and wrote the manuscript. All authors read and approved the final version.

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