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Research Article
Antagonistic Potential of Endophytic Bacteria Against *Phytophthora palmivora* Causing Black Pod Rot Disease on Cacao (*Theobroma cacao* L.) In Indonesia

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Abstract

Background and Objective: The utilization of biological control agents using microorganisms is considered as one of the safest and most affordable strategies. This study was conducted to investigate the antagonistic potential of endophytic bacteria against *Phytophthora palmivora* causing black pod rot disease on cocoa in Indonesia. **Materials and Methods:** Endophytic bacteria were explored from healthy cocoa pods in Java, Sulawesi and Papua islands. Their antagonistic potential was screened using dual culture method. Bacterial isolates combating the growth of *P. palmivora* were grouped using rep-PCR technique (BOX1A, ERIC and REP primers). Their inhibition consistency was examined against *P. palmivora* using double layer technique. Histological assay on mycelial of pathogen was performed under SEM. The DNA of representative isolates was molecularly sequenced according to 16S rRNA and *gyrB* genes. The effectiveness of their antagonism under *in vivo* assay was observed on the *P. palmivora*-inoculated healthy cocoa pods. **Results:** The growth of *P. palmivora* was totally inhibited by 127 isolates. The clustering with rep-PCR assay revealed 12 groups of isolates which were independent on cacao clones, orchards and geographical origins. Several isolates showed the inhibition zone under double layer test. SEM viewed morphological abnormality as well as hyphal lysis, shrinking and wrinkling. The representative isolates were identified as members of *Achromobacter*, *Alcaligenes*, *Bacillus*, *Burkholderia* and *Sphingobium* genera. The optimum inhibition under *in vivo* experiment was exhibited by *B. subtilis*. **Conclusion:** The explored antagonists have possibility as alternative sustainable disease management strategy under appropriate formulation and application techniques as well as favourable environmental condition.

Key words: Endophytic bacteria, antagonism, black pod rot, cacao, *Phytophthora palmivora*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cacao (*Theobroma cacao* L.) is one of important commodities in Indonesia sharing about 15% of net export in the world[1] and contributing around 19.50 and 15.43% of global production in 2010 and 2011, respectively (as the second leading country in cocoa bean production after Ivory Coast)[2]. Indonesia exported 521,300 t (equal to US$ 1.3 billion) of cocoa products in 2009 so that they occupied the third revenue in plantation sector following oil palm and rubber[3]. However, Indonesia was listed as the third cocoa producing country after Ivory Coast and Ghana with decreasing[4] production from 740,513 t in 2012 to 659,800 t in 2017.

Black pod rot disease of cocoa caused by *Phytophthora palmivora* was considered as one of three main pests and diseases affecting cocoa production in Indonesia[5]. Many researchers have been conducted for management of black pod rot disease on cocoa in Indonesia, such as the use of antagonistic fungi under laboratory condition[6,7], application of phosphonate through trunk injection[8], liquid smoke of coconut shell[9] and the combination of urea and lime[10] as well as the screening on resistance cacao clones against *P. palmivora*[11-15].

The utilization of biological control agents using microorganisms is interesting approach since it is considered as one of the safest and most affordable strategies[16]. Several species of bacterial endophytes have been isolated from various parts of cacao tree as well as other crops and their antagonistic potential as biological control agents against *P. palmivora* and other cacao pathogens has been examined[17-25]. However, there are a few reports associating with the study of potential indigenous antagonistic bacteria over *P. palmivora*. Therefore, we studied the antagonistic potential of indigenous bacteria towards *P. palmivora* causing black pod rot disease on cocoa in Indonesia, primarily their consistency in inhibiting the pathogen under *in vitro* and *in vivo* conditions, genetic diversity and interaction with pathogen under electron microscope as well as molecular identification.

MATERIALS AND METHODS

The present study including isolation, culture of isolates, *in vitro* and *in vivo* assays as well as molecular activities were carried out in Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta (Indonesia) during the year 2018-2019. Meanwhile, histological test and DNA sequencing were performed in The Integrated Research and Testing Laboratory of Universitas Gadjah Mada, Yogyakarta (Indonesia).

Exploration, isolation and preparation of bacterial and pathogen isolates: The potential antagonistic bacteria were explored from healthy cocoa pods of several cocoa producing areas in Indonesia, such as Java, Sulawesi and Papua islands. Samples were packed in dry paper, put into plastic bag to maintain the freshness during the transportation prior to isolation.

Endophytic bacteria were isolated using serial dilution method[26]. Healthy cocoa pods were peeled and cut into small pieces. Ten gram of pod pieces were put into Erlenmeyer containing 100 mL of phosphate buffer (pH 7) added with 10 µL of tween 20, shaken for 1 h and diluted up to 10^{-8} dilution. The suspension of 10^{-6} to 10^{-8} dilutions was spread on tryptone soya agar (TSA) medium and incubated for 48 h. Growing colonies were counted.

The isolates were purified as the single colony from tested serial dilution on TSA medium using streak isolation method and incubated for 48 h under temperature of 28°C. They were then selected according to its morphological characteristics such as shape (form, elevation and margin) as well as colony colour and kept were kept in slant TSA medium for further assay.

The WAT1 isolate of *P. palmivora* was one of the collection isolates from Kulon Progo, Yogyakarta (Indonesia) which had been morphologically identified based on its distinctive characters and molecularly detected using PCR with species-specific primers[27]. It was considered as high virulent isolate under virulence test.

Screening of potential antagonistic bacteria under dual culture assay: *P. palmivora* isolate was cultured on the centre of petri dish containing potato dextrose agar (PDA) medium and different bacterial isolates were streaked on the four margin sides of same plates. The hyphal growth of pathogen was observed and measured after 1 week incubation at room temperature. The radial of mycelia toward the streak inoculation sites (R2) and the mycelial of pathogen on control (streaked with sterile distilled water/SDW) (R1) were measured and percentage of inhibition was calculated using following formula[25]:

$$\text{Inhibition (\%)} = \frac{R1 - R2}{R1} \times 100$$

The bacterial isolates showing optimal inhibition (indicated with no hyphal growth of pathogen) were selected for further genetic diversity analysis.

DNA extraction: Genomic DNA of bacterial isolates were extracted following procedure of Joko *et al.*[28] and Dwimartina *et al.*[29] with a slight modification. Bacteria were cultured on TSA medium for 48 h at 28°C. Their colonies were swapped and suspended into 1.5 mL tubes containing a half volume of SDW. The solution was centrifuged at 5,000 rpm for 2 min and supernatant was discarded. As much of 500 µL of tris-EDTA (TE) buffer and then homogenized using vortex. The solution was added with 30 µL of 10% sodium dodecyl sulphate (SDS) and then incubated at 37°C for 1 h. The next step was addition of 80 µL 5M NaCl and 60 µL CTAB/NaCl as well as incubation at 65°C for 10 min (inverting the tube several times every 5 min). As much of 700 µL of chloroform isoamyl alcohol (CIAA) (24:1) was added, homogenized, centrifuged at 10,000 rpm for 10 min. Upper part of solution was transferred into new 1.5 mL tubes. Total of 600 µL phenol CIAA (PCIAA) (25:24:1) was added and centrifuged at 10,000 rpm for 10 min. Again, upper part of solution (about 500 µL) was transferred into new 1.5 mL tubes. DNA was then precipitated with about 300 µL isopropanol, incubated at -20°C for 1 h and centrifuged at 10,000 rpm for 10 min. As much of 300 µL of 70% ethanol was added, homogenized and then centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was air-dried in the laminar air flow. The pellet was resuspended with 40 µL of TE buffer and kept under -20°C condition.

PCR assay using BOX, ERIC and REP primer sets: Primer sets of rep-PCR, namely BOX, ERIC and REP elements and their PCR condition were presented in Table 1. Each 25 µL of PCR reaction containing ddH_2O, PCR ready mix (Bioline, London, UK), 100 µM of forward and reverse primers and DNA template was performed using PEQSTAR XS (VWR International Ltd., Lutterworth, Leicestershire, UK). PCR products were employed for electrophoresis in 2.5% agarose gel (added with 2 µL of Greensafe Premium staining solution (Nzytech, Lisboa, Portugal)) at 100 V for 45 min using electrophoresis device of Powerpac Basic (Bio-Rad, Hercules, CA, USA). The gel was visualized under Bio-Rad UV Transilluminator (Bio-Rad).

Phylogenetic analysis for rep-PCR assay: The band patterns were evaluated by recapitulating into 0-1 table (in which 0 for no appear band and 1 for appearing band). Dendrogram was constructed using NTSYS 2.10e program (Exeter Software, Setauket, New York, USA). For construction of dendrogram, the 0-1 table was set by putting the band arrangement in row and isolate number in column using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The table was saved in text

Table 1: Primer sets used in this study

Primers					
Annotation	Set	Sequence	PCR condition	Cycle	References[a]
BOX	BOX1A	5′-CTACGGCAAGGCGACGCTGACG-3′	95°C 7 min 94°C 1 min 53°C 1 min 65°C 8 min 65°C 16 min	30	Versalovic *et al.*[30], Masanto *et al.*[31]
ERIC	ERIC1R ERIC2	5′-ATGTAAGCTCCTGGGGATTCAC-3′ 5′-AAGTAAGTGACTGGGGTGAGCG-3′	95°C 7 min 94°C 1 min 52°C 1 min 65°C 8 min 65°C 16 min	30	Versalovic *et al.*[30], Masanto *et al.*[31], EPPO[32]
REP	REP1R-I REP2-I	5′-IIIICGICGICATCIGGC-3′ 5′-ICGICTTATCIGGCCTAC-3′	94°C 7 min 94°C 1 min 40°C 1 min 65°C 8 min 65°C 16 min	35	Masanto *et al.*[31], Versalovic *et al.*[33,34]
16S rRNA	27f 1492r	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-GGTTACCTTGTTACGACTT-3′	94°C 5 min 94°C 30 sec 55°C 30 sec 72°C 70 sec 72°C 5 min	35	Lane[35] and this study
gyrB	UP-IE AprU	5′-CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA-3′ 5′-TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA-3′	95°C 3 min 95°C 1 min 57°C 1 min 72°C 1 min 72°C 10 min	35	Yamamoto *et al.*[36] and this study

[a]Sequence of primer referred to previous researches, while PCR condition for 16S rRNA and *gyrB* were modified in this study

format of Microsoft (Microsoft Corporation) and then analysed using Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. Dendrogram was created and then saved in paint format of Microsoft (Microsoft Corporation).

Double layer test of potential antagonistic isolates: The isolates representing group or sub-group of genetic diversity were employed for using double-layer method of Gajbhiye *et al.*[37] with a slight modification. One microliter of bacterial suspension was spread on PDA plates. Previously, its optical density (OD) was measured and adjusted to be 0.1 under wavelength of 600 nm using spectrophotometer Genesys™ 10S UV-VIS (Thermo Fisher Scientific, Waltham, MA, USA). The mycelial disc of *P. palmivora* isolates was cultured on same plates after the suspension dried. The growth of pathogen and inhibition zone was observed for 1 week. The percentage inhibition was calculated using above formulation[25]. The representative isolates showing optimal inhibition against pathogen were continued for identification with DNA sequencing using 16S rRNA and gyrase subunit B (*gyrB*) genes.

Observation of inhibition activity under scanning electron microscope (SEM): The histological analysis was conducted according to the method of Jung *et al.*[38] and Mendez-Bravo *et al.*[39]. The plates of dual culture and double layer assays of *P. palmivora* and antagonistic bacterial isolates as well as untreated culture of pathogen were prepared for observation the antagonistic activity under scanning electron microscope (SEM) JSM-6510LA (JEOL Ltd., Akishima, Tokyo, Japan) at The Integrated Research and Testing Laboratory of Universitas Gadjah Mada, Yogyakarta (Indonesia).

DNA Sequencing using 16S rRNA and *gyrB* genes: Fragment of 16S rRNA and *gyrB* genes was amplified with corresponding universal primers and under PCR condition presented in Table 1. The 50 µL of PCR reaction containing ddH_2O, PCR ready mix, 100 µM of forward and reverse primers and DNA template was performed using T100 Thermal Cycler (Bio-Rad). PCR products were analyzed by electrophoresis on 1% agarose gel (added with 2 µL of Greensafe Premium staining solution (Nzytech, Lisboa, Portugal) in TBE buffer at 70 V for 45 min using electrophoresis device of Powerpac Basic and then visualized under Bio-Rad UV transilluminator. The amplified products were sequenced using ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis for identification of selected representative bacterial isolates: The consensus sequence was analysed using Mega 7.0 program[40] and then treasured with BLAST program at NCBI (www.ncbi.nlm.nih.gov) to find sequence homology for identification of bacterial taxonomy. The phylogenetic tree was constructed under maximum likelihood method with 1000 replicates of bootstrap using Mega 7.0 program[40]. An outgroup species was included for comparison.

***In vivo* antagonism test of identified antagonistic bacterial isolates on cocoa pod:** This assay was carried out following the method of Setyowati *et al.*[41] on cocoa pod of the most susceptible clone (RCC71)[12] collected from cocoa plantation of Segayung Unit, PT. Pagilaran Tbk (Batang, Central Java, Indonesia). Healthy cocoa pods were surface-sterilised using 70% alcohol and then rinsed with SDW. The pods were sprayed with suspension of 13 antagonistic bacterial isolates (10^8 CFU mL^{-1}) (12 isolates from this study and one isolate from previous research of Setyowati *et al.*[41] showing the highest inhibition level) prior to inoculation with high virulent isolate of *P. palmivora*. They were incubated for 3 days under room temperature and then the mycelial disc of pathogen was inoculated on wounded surface of the pods. The inoculated-pods were incubated at ambient temperature for a week. The uninoculated and bacterial untreated-pods was considered as negative control, while the inoculated ones with application of SDW was positive control. For comparison, the pods were sprayed with systemic fungicide (active ingredient of mefenoxam and mancozeb) (Ridomil Gold) (Syngenta International AG, Basel, Switzerland) on recommended dosage.

The lesion or necrotic area (cm^2) was measured using transparent millimetre block and the disease severity was calculated using the following formula[42]:

$$DS\ (\%) = \frac{\sum_{i=0}^{5}(ni \times vi)}{N \times V} \times 100$$

Where:

DS = Disease severity (%)
ni = Number of symptomatic pods on corresponding score
vi = Corresponding score of symptoms, i.e.
Score 0 = No symptom
Score 1 = Lesion or necrotic area between 0 and 20 cm^2

Score 2 = Lesion or necrotic area between 20 and 40 cm^2
Score 3 = Lesion or necrotic area between 40 and 60 cm^2
Score 4 = Lesion or necrotic area between 60 and 80 cm^2
score 5 = Lesion or necrotic area more than 80 cm^2
N = Total number of observed pods
V = Highest score

RESULTS

Exploration and isolation of bacterial isolates: The explored isolates were various among serial dilutions, i.e., 1-673 colonies (3-474 colonies in average) with number of colony type 1-4 in range (average of 1-3) (Table 2). The colony types were differentiated according to shape (form, elevation and margin) and colour. It was found circular and irregular form with raised, convex and umbonate elevation, entire, undulate and lobate margin as well as white, cream and light brown colour.

Several plates from Batang (Central Java), Nganjuk (East Java), North Minahasa (North Sulawesi), Papua and Ciamis (West Java) showed the reducing colony number following the decline of serial dilutions from 10^{-6} to 10^{-8}. However, such results were not consistently obtained from all cultures. Similarly, variation of colony type was not consistent among serial dilutions. Only some cultures from same geographical origins, excluding North Minahasa, revealed the decreasing colony type corresponding to diminishing serial dilutions.

Table 2: Bacterial isolates explored from healthy cocoa pods of cocoa growing areas of Indonesia

Geographical area	Clone of cocoa or orchard	Dilution level	Number of bacterial colony	Number of colony type	Isolation date
Nogosari Village, District of Rambipuji, Jember (East Java)	KKM22	10^{-6}	110.00	2.33	June 2018
		10^{-7}	57.67	2.67	
		10^{-8}	60.67	2.00	
	KSH2	10^{-6}	78.67	2.00	
		10^{-7}	263.00	2.33	
		10^{-8}	76.00	2.00	
	RCC73	10^{-6}	119.33	2.00	
		10^{-7}	192.33	1.67	
		10^{-8}	91.00	2.00	
	P7B	10^{-6}	159.33	2.00	
		10^{-7}	182.33	2.00	
		10^{-8}	126.67	2.33	
	DRC2	10^{-6}	152.67	2.00	
		10^{-7}	227.67	2.67	
		10^{-8}	207.67	2.00	
Simbangjati Village, District of Tulis, Batang (Central Java)	ICCRI3	10^{-6}	447.33	2.33	July 2018
		10^{-7}	179.00	2.33	
		10^{-8}	167.67	2.33	
	RCC70	10^{-6}	178.67	2.00	
		10^{-7}	235.00	2.33	
		10^{-8}	170.33	1.67	
	RCC71	10^{-6}	310.33	2.00	
		10^{-7}	306.33	1.67	
		10^{-8}	474.67	2.33	
	KKM	10^{-6}	331.00	2.33	
		10^{-7}	260.00	2.00	
		10^{-8}	145.00	2.33	
	RCC72	10^{-6}	182.00	2.33	
		10^{-7}	274.67	2.33	
		10^{-8}	199.00	2.00	
Kweden Village, District of Ngetos, Nganjuk (East Java)	Kadis 1	10^{-6}	26.33	3.00	August 2018
		10^{-7}	11.67	2.67	
		10^{-8}	10.00	2.00	
	Pardi 1	10^{-6}	43.67	2.00	
		10^{-7}	35.33	2.00	
		10^{-8}	27.00	2.33	
	Makruf 1	10^{-6}	82.33	2.00	
		10^{-7}	89.67	1.67	
		10^{-8}	128.00	1.67	
	Kadis 2	10^{-6}	96.00	1.67	
		10^{-7}	107.33	1.67	

Table 2: Continue

Geographical area	Clone of cocoa or orchard	Dilution level	Number of bacterial colony	Number of colony type	Isolation date
		10^{-8}	62.00	1.33	
	Pardi 2	10^{-6}	66.33	1.33	
		10^{-7}	117.67	1.00	
		10^{-8}	156.67	1.67	
Talawaan Bantik Village, District of Wori, North Minahasa (North Sulawesi)	NS1	10^{-6}	88.33	2.33	August 2018
		10^{-7}	73.67	2.33	
		10^{-8}	27.67	3.00	
	NS2	10^{-6}	3.00	1.67	
		10^{-7}	5.00	1.67	
		10^{-8}	14.33	2.00	
Kaliharapan Village, District of Nabire, Nabire (Papua)	Papua 1	10^{-6}	82.33	1.33	September 2018
		10^{-7}	38.67	1.33	
		10^{-8}	48.33	1.00	
	Papua 2	10^{-6}	18.00	1.67	
		10^{-7}	22.33	1.33	
		10^{-8}	10.67	1.33	
	Papua 3	10^{-6}	16.67	1.00	
		10^{-7}	25.33	1.00	
		10^{-8}	17.00	1.00	
	Papua 4	10^{-6}	71.00	2.00	
		10^{-7}	107.33	2.00	
		10^{-8}	61.00	2.67	
	Papua 5	10^{-6}	31.00	2.67	
		10^{-7}	31.00	2.00	
		10^{-8}	30.33	2.00	
	Papua 6	10^{-6}	23.00	2.33	
		10^{-7}	11.67	1.33	
		10^{-8}	33.33	1.33	
Linggasari Village, District of Ciamis, Ciamis (West Java)	Ciamis 1	10^{-6}	131.33	1.00	October 2018
		10^{-7}	136.67	1.00	
		10^{-8}	188.00	1.00	
	Ciamis 2	10^{-6}	197.67	1.00	
		10^{-7}	170.67	1.00	
		10^{-8}	114.00	1.00	
Kertasari Village, District of Ciamis, Ciamis (West Java)	Ciamis 3	10^{-6}	122.67	1.00	October 2018
		10^{-7}	117.00	1.00	
		10^{-8}	130.00	1.00	
	Ciamis 4	10^{-6}	123.33	1.00	
		10^{-7}	108.67	1.00	
		10^{-8}	137.00	1.33	

Screening of potential indigenous antagonistic bacteria: From 362 screened isolates, the hyphal growth of *P. palmivora* was completely inhibited by 127 isolates (PI 100%), while around 2.5-38 mm (range of PI around 55.29-97.25%) of its hyphal growth was recorded in dual cultures with 72 bacterial isolates (Table 3). The remaining isolates were neglected since they could not grow under this antagonism assay, particularly those from North Sulawesi. The selected potential antagonistic bacterial isolates originated from West Java, Central Java, East Java and Papua (Table 4).

Genetic diversity of screened antagonistic bacteria using rep-PCR: There were 12 clusters of antagonistic bacteria within range of similarity index around 71-100% (Fig. 1). Those groups were independent on clone of cocoa, orchards and geographical origins. The group members varied from 1-37 isolates in which small groups consisted isolates from 1-3 geographical areas (i.e., group III, VI, VII, IX, XI and XII), while the remaining large clusters originated from 3-5 cocoa growing areas. According to their diversity within groups or sub-groups, a range of 1-14 isolates (total of 66 isolates) were selected from each cluster as representative isolates for double layer assay (Table 5).

Double layer test of potential antagonistic bacterial isolates: This second screening revealed that 46 isolates consistently showed maximum inhibition against *P. palmivora* with zero mycelial growth. Most isolates did not express clear

Table 3: *In vitro* screening of potential antagonistic bacteria isolates against *Phytophthora palmivora* using dual culture test

Geographical area	Clone of cocoa or orchard	Dilution level	Number of hyphal growth (mm)	Number of corresponding Isolations
Nogosari Village, District of Rambipuji, Jember (East Java)	KKM22	10^{-6}	6-7 (91.76-92.94)[a]	2
		10^{-7}	0 (100)	5
	KSH2	10^{-6}	0 (100)	4
		10^{-7}	0 (100)	5
		10^{-8}	0 (100)	6
	RCC73	10^{-6}	0 (100)	2
		10^{-7}	0 (100)	3
		10^{-8}	0 (100)	3
	P7B	10^{-6}	0 (100)	3
		10^{-7}	0 (100)	5
		10^{-8}	0 (100)	6
	DRC2	10^{-6}	0-4 (95.29-100)	1-4
Simbangjati Village, District of Tulis, Batang (Central Java)	ICCRI3	10^{-6}	0 (100)	6
		10^{-7}	0 (100)	6
		10^{-8}	0-2.5 (97.05-100)	1-5
	RCC70	10^{-6}	0 (100)	3
		10^{-7}	0-6 (91.76-100)	1-2
		10^{-8}	0 (100)	4
	RCC71	10^{-6}	0-2.7 (96.82-100)	1-2
		10^{-7}	0 (100)	3
		10^{-8}	0 (100)	6
	KKM	10^{-6}	0 (100)	2
		10^{-7}	0 (100)	2
		10^{-8}	0 (100)	1
	RCC72	10^{-6}	0-3 (96.47-100)	1-3
		10^{-8}	0 (100)	0
Kweden Village, District of Ngetos, Nganjuk (East Java)	Kadis1	10^{-6}	0 (100)	5
		10^{-7}	0-3 (96.47-100)	1-2
		10^{-8}	0-38 (55.29-100)	1
	Pardi1	10^{-6}	0-6.7 (92.11-100)	1
		10^{-7}	0-7 (91.76-100)	1-2
	Makruf 1	10^{-6}	7.3-14 (85.52-91.41)	1
		10^{-7}	0-10 (88.23-100)	1
		10^{-8}	6.7-8.7 (89.76-92.11)	1-2
	Kadis 2	10^{-6}	7-10.3 (87.88-91.76)	1
		10^{-7}	2.5-12 (85.88-97.05)	1
		10^{-8}	6.7-10.3 (87.88-92.11)	1-2
	Pardi 2	10^{-6}	8.3-9 (89.41-90.23)	1
		10^{-7}	9.7-16.7 (80.35-88.58)	1
		10^{-8}	7.7-9.3 (89.05-90.94)	1
Talawaan Bantik Village, District of Wori, North Minahasa (North Sulawesi)	NS1	10^{-6}	7.3-11.5 (86.47-91.41)	1-2
		10^{-8}	10 (88.23)	1
	NS2	10^{-6}	11.7 (86.23)	1
		10^{-7}	11.7 (86.23)	1
		10^{-8}	10.3-12.3 (85.52-87.88)	1
Kaliharapan Village, District of Nabire, Nabire (Papua)	Papua 1	10^{-6}	6 (92.94)	1
		10^{-7}	16 (81.17)	1
		10^{-8}	0 (100)	1
	Papua 2	10^{-7}	9 (89.41)	1
	Papua 3	10^{-8}	4 (95.29)	1
	Papua 4	10^{-7}	0 (100)	3
		10^{-8}	0-7 (91.76-100)	1-4
	Papua 5	10^{-6}	0 (100)	2
		10^{-7}	0-5 (94.11-100)	1-3
		10^{-8}	0 (100)	2
	Papua 6	10^{-6}	0 (100)	1

Table 3: Continue

Geographical area	Clone of cocoa or orchard	Dilution level	Number of hyphal growth (mm)	Number of corresponding Isolations
		10^{-7}	0-7 (91.76-100)	1-3
		10^{-8}	0-8.3 (90.23-100)	1-3
Linggasari Village, District of Ciamis, Ciamis (West Java)	Ciamis 1	10^{-6}	8.3 (90.23)	1
		10^{-8}	4-4.5 (94.70-95.29)	1
	Ciamis 2	10^{-6}	0 (100)	1
		10^{-8}	5.5 (93.52)	1
Kertasari Village, District of Ciamis, Ciamis (West Java)	Ciamis 3	10^{-7}	3.5 (95.88)	1
	Ciamis 4	10^{-6}	3.3 (96.11)	1
		10^{-8}	3-6.5 (92.35-96.47)	1

[a]Percentage of inhibition (%) of antagonistic bacterial isolates against *P. palmivora* in which the mycelial growth of *P. palmivora* as control is about 85 mm

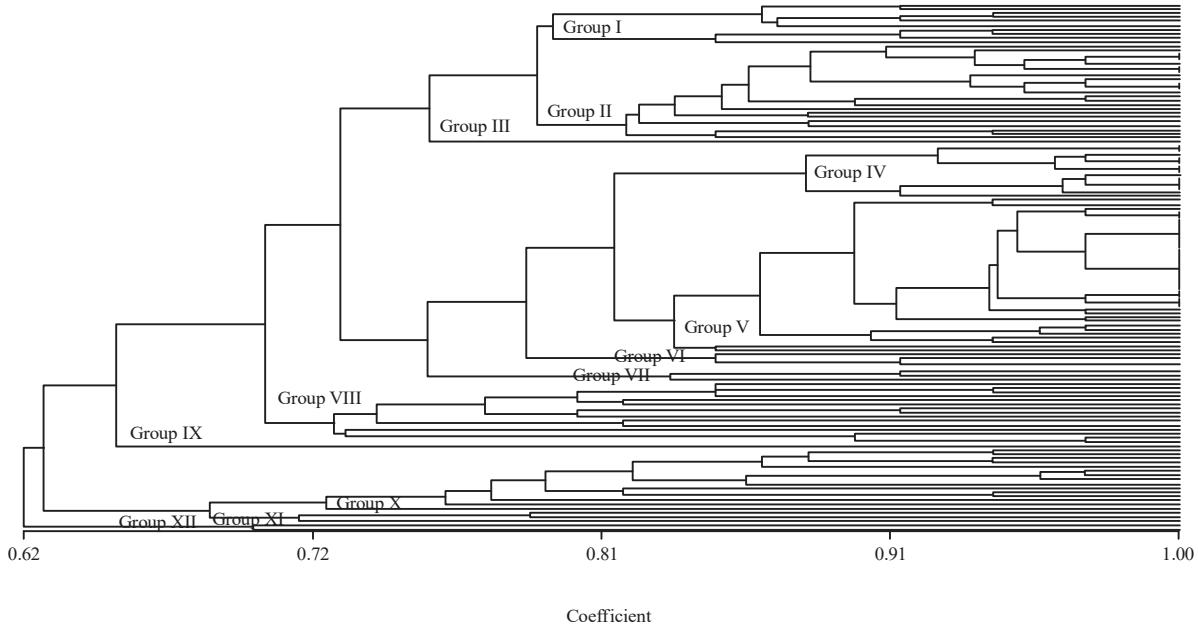

Fig. 1: Dendrogram of genetic diversity with rep-PCR elements clustering the potential antagonistic of endophytic bacteria after *in vitro* dual-culture test

inhibition zone, while 19 isolates demonstrated consistent, quite consistent and inconsistent inhibition zone with range of diameter approximately 4.7-12.7 mm, 1.3-5.3 mm and 0.6-2.7 mm, respectively (Table 6).

A quite consistent inhibition was shown by other 5 isolates with colony diameter of *P. palmivora* about 0.7-2.0 mm (PI around 97.33-99.06% in range) and diameter of inconsistent inhibition zone about 0.6-13.3 mm in range. The growth of pathogen was also inconsistently inhibited by 4 isolates (colony diameter about 1.3-8.0 mm or PI around 78.88-98.27% in range) and even 11 isolates were the most inconsistent in hampering the growth of *P. palmivora* (colony diameter around 1.7-46.7 mm or PI about 37.73-97.73% in range).

These quite and inconsistent inhibiting isolates performed inconsistent inhibition zone with range of diameter approximately 0.6-13.3 mm. Meanwhile, there was no any inhibition zone which was exhibited by most of inconsistent and whole most inconsistent isolates.

The consistency of inhibition within bacterial isolates was independent on group. Only one-member clusters, such as cluster III, IX and XII were found consistently inhibiting the growth of *P. palmivora* with consistent inhibition zone, consistent inhibition without any inhibition zone and quite consistent inhibition with inconsistent inhibition zone, respectively.

Observation of inhibition activity under scanning electronic microscope (SEM): The scanning electron micrograph showed the morphological abnormality as well as shrinking and lysis indicating the damage of *P. palmivora* hyphae under confrontation with antagonistic bacterial isolates both on dual culture (Fig. 2a) and double layer tests

Table 4: Selected isolates for further genetic diversity analysis using rep-PCR technique

Isolate codes	Geographical origin	DNA isolation date
KKM221071a	Jember (East Java)	October 2018
KKM221071b	Jember (East Java)	October 2018
KKM221071c	Jember (East Java)	October 2018
KKM221072a	Jember (East Java)	October 2018
KKM221072b	Jember (East Java)	October 2018
KSH21062a	Jember (East Java)	October 2018
KSH21062b	Jember (East Java)	October 2018
KSH21063a	Jember (East Java)	October 2018
KSH21063b	Jember (East Java)	October 2018
KSH21071a	Jember (East Java)	October 2018
KSH21071b	Jember (East Java)	October 2018
KSH21072a	Jember (East Java)	October 2018
KSH21072b	Jember (East Java)	October 2018
KSH21073c	Jember (East Java)	October 2018
KSH21081a	Jember (East Java)	October 2018
KSH21081b	Jember (East Java)	October 2018
KSH21082a	Jember (East Java)	October 2018
KSH21082b	Jember (East Java)	October 2018
KSH21083a	Jember (East Java)	October 2018
KSH21083b	Jember (East Java)	October 2018
RCC731061	Jember (East Java)	October 2018
RCC731062	Jember (East Java)	October 2018
RCC731071a	Jember (East Java)	October 2018
RCC731071b	Jember (East Java)	October 2018
RCC731072	Jember (East Java)	October 2018
RCC731082b	Jember (East Java)	October 2018
RCC731083a	Jember (East Java)	October 2018
RCC731083b	Jember (East Java)	October 2018
P7B1061b	Jember (East Java)	October 2018
P7B1062a	Jember (East Java)	October 2018
P7B1072a	Jember (East Java)	October 2018
P7B1072b	Jember (East Java)	October 2018
P7B1073a	Jember (East Java)	October 2018
P7B1073b	Jember (East Java)	October 2018
P7B1073c	Jember (East Java)	October 2018
P7B1081a	Jember (East Java)	October 2018
P7B1081b	Jember (East Java)	October 2018
P7B1082a	Jember (East Java)	October 2018
P7B1082b	Jember (East Java)	October 2018
P7B1083a	Jember (East Java)	October 2018
P7B1083b	Jember (East Java)	November 2018
DRC21061a	Jember (East Java)	November 2018
DRC21061b	Jember (East Java)	November 2018
DRC21063a	Jember (East Java)	November 2018
DRC21063b	Jember (East Java)	November 2018
ICCRI31061b	Jember (East Java)	November 2018
ICCRI31061c	Batang (Central Java)	November 2018
ICCRI31062a	Batang (Central Java)	November 2018
ICCRI31062b	Batang (Central Java)	November 2018
ICCRI31063a	Batang (Central Java)	November 2018
ICCRI31063b	Batang (Central Java)	November 2018
ICCRI31071a	Batang (Central Java)	November 2018
ICCRI31071b	Batang (Central Java)	November 2018
ICCRI31072a	Batang (Central Java)	November 2018
ICCRI31072b	Batang (Central Java)	November 2018
ICCRI31073a	Batang (Central Java)	November 2018
ICCRI31073b	Batang (Central Java)	November 2018
ICCRI31081a	Batang (Central Java)	November 2018
ICCRI31082a	Batang (Central Java)	November 2018
ICCRI31082b	Batang (Central Java)	November 2018
ICCRI31083a	Batang (Central Java)	November 2018
ICCRI31083b	Batang (Central Java)	November 2018
RCC701061a	Batang (Central Java)	November 2018

Table 4: Continue

Isolate codes	Geographical origin	DNA isolation date
RCC701061b	Batang (Central Java)	November 2018
RCC701062	Batang (Central Java)	November 2018
RCC701071b	Batang (Central Java)	November 2018
RCC701073	Batang (Central Java)	November 2018
RCC701081	Batang (Central Java)	November 2018
RCC701082	Batang (Central Java)	November 2018
RCC701083a	Batang (Central Java)	November 2018
RCC701083b	Batang (Central Java)	November 2018
RCC711061a	Batang (Central Java)	November 2018
RCC711063b	Batang (Central Java)	November 2018
RCC711071	Batang (Central Java)	November 2018
RCC711072	Batang (Central Java)	November 2018
RCC711073b	Batang (Central Java)	November 2018
RCC711081a	Batang (Central Java)	November 2018
RCC711081b	Batang (Central Java)	November 2018
RCC711082a	Batang (Central Java)	November 2018
RCC711082b	Batang (Central Java)	November 2018
RCC711083a	Batang (Central Java)	November 2018
RCC711083b	Batang (Central Java)	November 2018
KKM1063a	Batang (Central Java)	November 2018
KKM1063b	Batang (Central Java)	November 2018
KKM1071a	Batang (Central Java)	November 2018
KKM1071b	Batang (Central Java)	November 2018
KKM1081b	Batang (Central Java)	November 2018
RCC721061b	Batang (Central Java)	November 2018
RCC721062b	Batang (Central Java)	November 2018
RCC721062c	Batang (Central Java)	November 2018
RCC721081a	Batang (Central Java)	November 2018
RCC721081b	Batang (Central Java)	November 2018
Kadis11061c	Batang (Central Java)	November 2018
Kadis11062c	Nganjuk (East Java)	November 2018
Kadis11063a	Nganjuk (East Java)	November 2018
Kadis11063b	Nganjuk (East Java)	November 2018
Kadis11063c	Nganjuk (East Java)	November 2018
Kadis11073b	Nganjuk (East Java)	November 2018
Kadis11073c	Nganjuk (East Java)	November 2018
Kadis11082a	Nganjuk (East Java)	November 2018
Pardi11063	Nganjuk (East Java)	November 2018
Pardi11071c	Nganjuk (East Java)	November 2018
Pardi11072c	Nganjuk (East Java)	November 2018
Makruf11073	Nganjuk (East Java)	November 2018
Papua11081	Nganjuk (East Java)	November 2018
Papua41071b	Nabire (Papua)	November 2018
Papua41071c	Nabire (Papua)	November 2018
Papua41073a	Nabire (Papua)	November 2018
Papua41081c	Nabire (Papua)	November 2018
Papua41082c	Nabire (Papua)	November 2018
Papua41083a	Nabire (Papua)	November 2018
Papua41083b	Nabire (Papua)	November 2018
Papua51062a	Nabire (Papua)	November 2018
Papua51063a	Nabire (Papua)	November 2018
Papua51071b	Nabire (Papua)	November 2018
Papua51073a	Nabire (Papua)	November 2018
Papua51073c	Nabire (Papua)	November 2018
Papua51081a	Nabire (Papua)	November 2018
Papua51083a	Nabire (Papua)	November 2018
Papua61063a	Nabire (Papua)	November 2018
Papua61072a	Nabire (Papua)	November 2018
Papua61073a	Nabire (Papua)	November 2018
Papua61073b	Nabire (Papua)	November 2018
Papua61081b	Nabire (Papua)	November 2018
Papua61082	Nabire (Papua)	November 2018
Papua61083	Nabire (Papua)	November 2018
Ciamis21062	Ciamis (West Java)	November 2018

Table 5: Representative isolates corresponding to their diversity clusters for double layer test

Cluster	Isolate member	Geographical origins	Representative isolates for double layer test	
			Isolate numbers	Isolate codes
I	10 isolates (1, 9-11, 14-15, 87, 103, 115-116)	Jember, Batang, Nganjuk, Nabire	8 isolates (1, 9, 10, 14, 15, 87, 103, 115)	KKM221071a, KSH21063b, KSH21071a, KSH21073c, KSH21081a, KKM1081b, Pardi11072c, Papua51071b
II	23 isolates (2-3, 17-24, 28, 30-31, 71, 94, 99, 102, 106, 113, 118, 121, 124-125)	Jember, Batang, Nganjuk, Nabire	14 isolates (2, 3, 19, 22, 23, 28, 30, 71, 94, 99, 106, 113, 121, 124)	KKM221071b, KKM221071c, KSH21083a, RCC731062, RCC731071a, RCC731083b, P7B1062a, RCC701083b, Kadis11062c, Kadis11073c, Papua41071b, Papua51062a, Papua61072a, Papua61081b
III	1 isolate (13)	Jember	1 isolate (13)	KSH21072b
IV	13 isolates (32-33, 64-66, 68-69, 73-78)	Jember, Batang	6 isolates (32, 64, 69, 73, 74, 78)	P7B1072b, RCC701061b, RCC701082, RCC711061b, RCC711071, RCC711081b
V	37 isolates (34-39, 43, 48-61, 63, 67, 80-81, 83-86, 88-92, 96-98)	Jember, Batang, Nganjuk	10 isolates (34, 39, 43, 56, 60, 63, 88, 91, 96, 97)	P7B1073b, P7B1082b, DRC21061b, ICCRI31073a, ICCRI31082b; RCC701061a, RCC721061b, RCC721081a, Kadis11063b, Kadis11063c
VI	4 isolates (40-42, 44)	Jember	2 isolates (40, 44)	P7B1083a, DRC21063a
VII	3 isolates (45, 47, 95)	Jember, Batang, Nganjuk	1 isolate (45)	DRC21063b
VIII	15 isolates (4, 62, 100-101, 104-105, 107-112, 114, 117, 126)	Jember, Batang, Nganjuk, Nabire	9 isolates (4, 104, 105, 107, 109, 110, 112, 114, 126)	KKM221072a, Makruf11073, Papua11081, Papua41071c, Papua41081c, Papua41082c, Papua41083b, Papua51063a, Papua61083
IX	1 isolate (82)	Batang	1 isolate (82)	RCC711083b
X	15 isolates (5-6, 8, 12, 16, 25-27, 29, 46, 79, 93, 120, 123, 127)	Jember, Batang, Nganjuk, Nabire, Ciamis	11 isolates (5, 6, 8, 16, 25, 27, 46, 79, 93, 120, 127)	KKM221072b, KSH21062a, KSH21063a KSH21081b, RCC731072, RCC731083a, ICCRI31061b, RCC711082a, Kadis11061c, Papua61063a, Ciamis21062
XI	3 isolates (7, 119, 122)	Jember, Nabire	2 isolates (7, 122)	KSH21062b, Papua61073a
XII	2 isolates (70, 72)	Batang	1 isolate (70)	RCC701083a

Table 6: Double layer test of selected potential antagonistic bacteria isolates against *Phytophthora palmivora*

Isolate codes	Geographical origins	Clone/Orchard	Clusters	Inhibition of mycelial growth			Formation of inhibition zone (mm)	
				Mycelial growth (mm)	PI (%)			
KKM221071a	Jember, East Java	KKM22	I	0	100[a]	Consistent[b]	0	-
KKM221071b	Jember, East Java	KKM22	II	0	100	Consistent	0	-
KKM221071c	Jember, East Java	KKM22	II	0	100	Consistent	5.3	Consistent[c]
KKM221072a	Jember, East Java	KKM22	VIII	0	100	Consistent	0	-
KKM221072b	Jember, East Java	KKM22	X	0.7	99.06	Quite consistent	13.3	Inconsistent
KSH21062a	Jember, East Java	KSH2	X	0	100	Consistent	8	Consistent
KSH21062b	Jember, East Java	KSH2	XI	0	100	Consistent	0	-
KSH21063a	Jember, East Java	KSH2	X	0	100	Consistent	0	-
KSH21063b	Jember, East Java	KSH2	I	0	100	Consistent	0	-
KSH21071a	Jember, East Java	KSH2	I	0	100	Consistent	5.3	Quite consistent
KSH21072b	Jember, East Java	KSH2	III	0	100	Consistent	4.7	Consistent
KSH21073c	Jember, East Java	KSH2	I	0	100	Consistent	12.7	Consistent
KSH21081a	Jember, East Java	KSH2	I	0	100	Consistent	0	-
KSH21081b	Jember, East Java	KSH2	X	5.3	92.93	Most inconsistent	0	-
KSH21083a	Jember, East Java	KSH2	II	7.3	79.64	Most inconsistent	0	-
RCC731062	Jember, East Java	RCC73	II	4	83.52	Most inconsistent	1.3	Inconsistent
RCC731071a	Jember, East Java	RCC73	II	4	83.52	Most inconsistent	0	-
RCC731072	Jember, East Java	RCC73	X	8	78.88	Inconsistent	0	-
RCC731083a	Jember, East Java	RCC73	X	0	100	Consistent	1.3	Inconsistent
RCC731083b	Jember, East Java	RCC73	II	0	100	Consistent	0	-
P7B1062a	Jember, East Java	P7B	II	0	100)	Consistent	0	-
P7B1072b	Jember, East Java	P7B	IV	0	100	Consistent	2.7	Inconsistent
P7B1073b	Jember, East Java	P7B	V	1.3	98.27	Inconsistent	0	-
P7B1082b	Jember, East Java	P7B	V	6	92.00	Inconsistent	0	-

Table 6: Continue

Isolate code	Geographical origin	Clone/Orchard	Cluster	Inhibition of mycelial growth			Formation of inhibition zone (mm)	
				Mycelial growth (mm)	PI (%)			
P7B1083a	Jember, East Java	P7B	VI	0	100	Consistent	0	-
DRC21061b	Jember, East Java	DRC2	V	0	100	Consistent	0	-
DRC21063a	Jember, East Java	DRC2	VI	0	100	Consistent	2.7	Inconsistent
DRC21063b	Jember, East Java	DRC2	VII	0	100	Consistent	0	-
ICCRI31061b	Batang, Central Java	ICCRI3	X	0	100	Consistent	0	-
ICCRI31073a	Batang, Central Java	ICCRI3	V	0	100	Consistent	2	Inconsistent
ICCRI31082b	Batang, Central Java	ICCRI3	V	0	100	Consistent	1.3	Quite consistent
RCC701061a	Batang, Central Java	RCC70	V	0	100	Consistent	2	Inconsistent
RCC701061b	Batang, Central Java	RCC70	IV	0	100	Consistent	0	-
RCC701082	Batang, Central Java	RCC70	IV	0	100	Consistent	2	Inconsistent
RCC701083a	Batang, Central Java	RCC70	XII	0.7	99.06	Quite consistent	1.3	Inconsistent
RCC701083b	Batang, Central Java	RCC70	II	0	100	Consistent	2.7	Inconsistent
RCC711063b	Batang, Central Java	RCC71	IV	0	100	Consistent	2	Quite consistent
RCC711071	Batang, Central Java	RCC71	IV	0.7	99.06	Quite consistent	4.7	Inconsistent
RCC711081b	Batang, Central Java	RCC71	IV	0	100	Consistent	1.3	Quite consistent
RCC711082a	Batang, Central Java	RCC71	X	0	100	Consistent	0	-
RCC711083b	Batang, Central Java	RCC71	IX	0	100	Consistent	0	-
KKM1081b	Batang, Central Java	KKM	I	0	100	Consistent	2	Quite consistent
RCC721061b	Batang, Central Java	RCC72	V	0	100	Consistent	0.6	Inconsistent
RCC721081a	Batang, Central Java	RCC72	V	0	100	Consistent	0	-
Kadis11061c	Nganjuk, East Java	Kadis1	X	0	100	Consistent	0.6	Inconsistent
Kadis11062c	Nganjuk, East Java	Kadis1	II	1.3	98.27	Quite consistent	0	-
Kadis11063b	Nganjuk, East Java	Kadis1	V	11.3	84.93	Most inconsistent	0	-
Kadis11063c	Nganjuk, East Java	Kadis1	V	9.3	87.60	Most inconsistent	0	-
Kadis11073c	Nganjuk, East Java	Kadis1	II	0	100	Consistent	0	-
Pardi11072c	Nganjuk, East Java	Pardi1	I	8.7	88.40	Most inconsistent	0	-
Makruf11073	Nganjuk, East Java	Makruf1	VIII	0	100	Consistent	0	-
Papua11081	Nabire, Papua	Papua1	VIII	46.7	37.73	Most inconsistent	0	-
Papua41071b	Nabire, Papua	Papua4	II	0	100	Consistent	0	-
Papua41071c	Nabire, Papua	Papua4	VIII	2	97.33	Quite consistent	0.6	Inconsistent
Papua41081c	Nabire, Papua	Papua4	VIII	0	100	Consistent	0	-
Papua41082c	Nabire, Papua	Papua4	VIII	1.7	97.73	Most inconsistent	0	-
Papua41083b	Nabire, Papua	Papua4	VIII	31.3	58.27	Most inconsistent	0	-
Papua51062a	Nabire, Papua	Papua5	II	14	81.33	Most inconsistent	0	-
Papua51063a	Nabire, Papua	Papua5	VIII	0	100	Consistent	0	-
Papua51071b	Nabire, Papua	Papua5	I	0	100	Consistent	0	-
Papua61063a	Nabire, Papua	Papua6	X	34.7	53.73	Most inconsistent	0	-
Papua61072a	Nabire, Papua	Papua6	II	0	100	Consistent	0	-
Papua61073a	Nabire, Papua	Papua6	XI	0	100	Consistent	1.3	Inconsistent
Papua61081b	Nabire, Papua	Papua6	II	0	100	Consistent	0	-
Papua61083	Nabire, Papua	Papua6	VIII	0	100	Consistent	0	-
Ciamis21062	Ciamis, West Java	Ciamis2	X	0	100	Consistent	0	-

Percentage of inhibition (%) of antagonistic isolates against *P. palmivora* in which the mycelial growth of *P. palmivora* as control is about 75 mm, [b]Their consistency in inhibiting mycelial growth is compared with the previous dual culture test and among three replications of double layer test, [a]Their consistency in producing inhibition zone is compared among three replications of double layer test

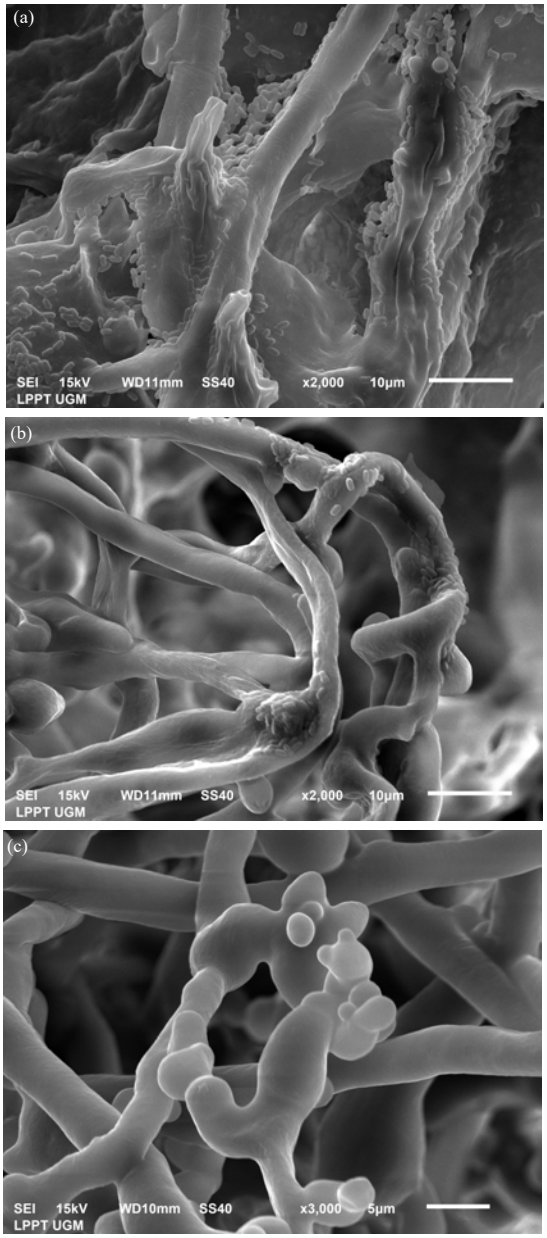

Fig. 2(a-c): (a) Scanning electron micrographs visualizing the antagonistic action of endophytic bacteria against *P. palmivora* under dual culture, (b) Double layer assays at 2,000X magnification and (c) Normal hyphal growth of *P. palmivora* without any lysis and bacterial cells adhering the mycelium showed by control at 3,000X magnification

(Fig. 2b). The lysis was extremely more severe and the adhering rod-shape bacteria were more abundant under dual culture than double layer tests. The intact and normal growing hyphae without any lysis was revealed on *P. palmivora* under control without any antagonist treatment (Fig. 2c).

Identification of selected representative isolates: Twelve isolates representing those with consistent inhibition, 4 geographical areas and ten clusters were proceeded to molecular identification. They were amplified with 16S rRNA and *gyrB* genes at approximately 1,475 bp and 940 bp, respectively (Table 7). All representative isolates were positively detected with 16S rRNA primers, while nine isolates were reacted with *gyrB* primers. Based on their DNA sequencing, they were identified as *Achromobacter xylosoxydans*, *Alcaligenes faecalis* subsp. *faecalis, A. pakistanensis, Bacillus altitudinis, B. amyloliquefaciens, B. cereus, B. siamensis, B. subtilis, B. velezensis, Burkholderia cepacia, B. ptereochthonis* and *Sphingobium yanoikuyae* with the percentage of identity around 81.12-100% (Fig. 3).

***In vivo* antagonism test of identified antagonistic bacterial isolates on cocoa pod:** The initial disease symptom on bacterial-treated cocoa pods were recorded on 3rd day after inoculation with disease severity around 44-100% in range after a week incubation (Table 8). Meanwhile, fungicide treatment could delay the symptom appearance on the 5th day after inoculation and disease severity about 12% on the last incubation day. The lowest severity of disease was revealed by isolate number 1 corresponding to *B. subtilis*, whereas another four isolates (number 45, 99, 109 and 127 which were identified as *Bacillus* spp. and *S. yanoikuyae*) generated the highest one.

DISCUSSION

This study explored the bacterial on healthy cocoa pod collected from various cocoa clones and orchards in different geographical origins of Indonesia due to the common infection of *P. palmivora* on pod and little or no information of microorganisms for biological control from cocoa pod[17]. The resistance level of some cocoa clones in this experiment has been reported[12,45]. It was found that screened bacteria from those cocoa clones expressing antagonistic potential were around 5-17 isolates (Table 4). The availability of various microbes associating with cacao played important role in its resistance against pathogen[12,25]. However, current research did not elaborate the correlation of clonal resistance on cocoa with the number of screened antagonistic isolates. It may be investigated in further study.

None of antagonistic bacterial isolates from Sulawesi in this study was parallel to former findings[17,22] and might be caused by high disease incidence in the field, i.e., 70-80%[46]. Future research is required to update novel prevalence of

Table 7: Molecular identification of representative isolates corresponding consistent inhibition using 16S rRNA and *gyrB* genes

			Primer				
Isolate code	Geographical origin	Cluster	16S rRNA (±1,475 bp)	*gyrB* (±940 bp)	Closest strain at NCBI	Identity (%)	Accession number
KKM221071a	Jember, East Java	I	+	+	*Bacillus subtilis*	100.00	MK346244 (India)
KKM221071c	Jember, East Java	II	+	-	*Alcaligenes pakistanensis*	93.33	LC001699 (Pakistan)
KSH21072b	Jember, East Java	III	+	+	*Bacillus siamensis*	100.00	MK382639 (China)
P7B1072b	Jember, East Jawa	IV	+	-	*Alcaligenes faecalis* subsp. *faecalis*	95.92	LC001703 (Pakistan)
DRC21063b	Jember, East Java	VII	+	+	*Bacillus amyloliquefaciens*	99.47	MH521167 (China)
ICCRI31061b	Batang, Central Java	X	+	-	*Achromobacter xylosoxidans*	81.12	MK370558 (Ethiopia)
ICCRI31082b	Batang, Central Java	V	+	+	*Burkholderia ptereochthonis*	83.52	LT158637 (Belgium)
RCC711063b	Batang, Central Java	IV	+	+	*Bacillus velezensis*	100.00	MK263025 (China)
RCC711083b	Batang, Central Java	IX	+	+	*Burkholderia cepacia*	91.01	DQ288141 (Italy)
Kadis11073c	Nganjuk, East Java	II	+	+	*Bacillus cereus*	100.00	MK346118 (China)
Papua41081c	Nabire, Papua	VIII	+	+	*Sphingobium yanoikuyae*	100.00	KX507143 (USA)
Ciamis21062	Ciamis, West Java	X	+	+	*Bacillus altitudinis*	100.00	MH305357 (Brazil)

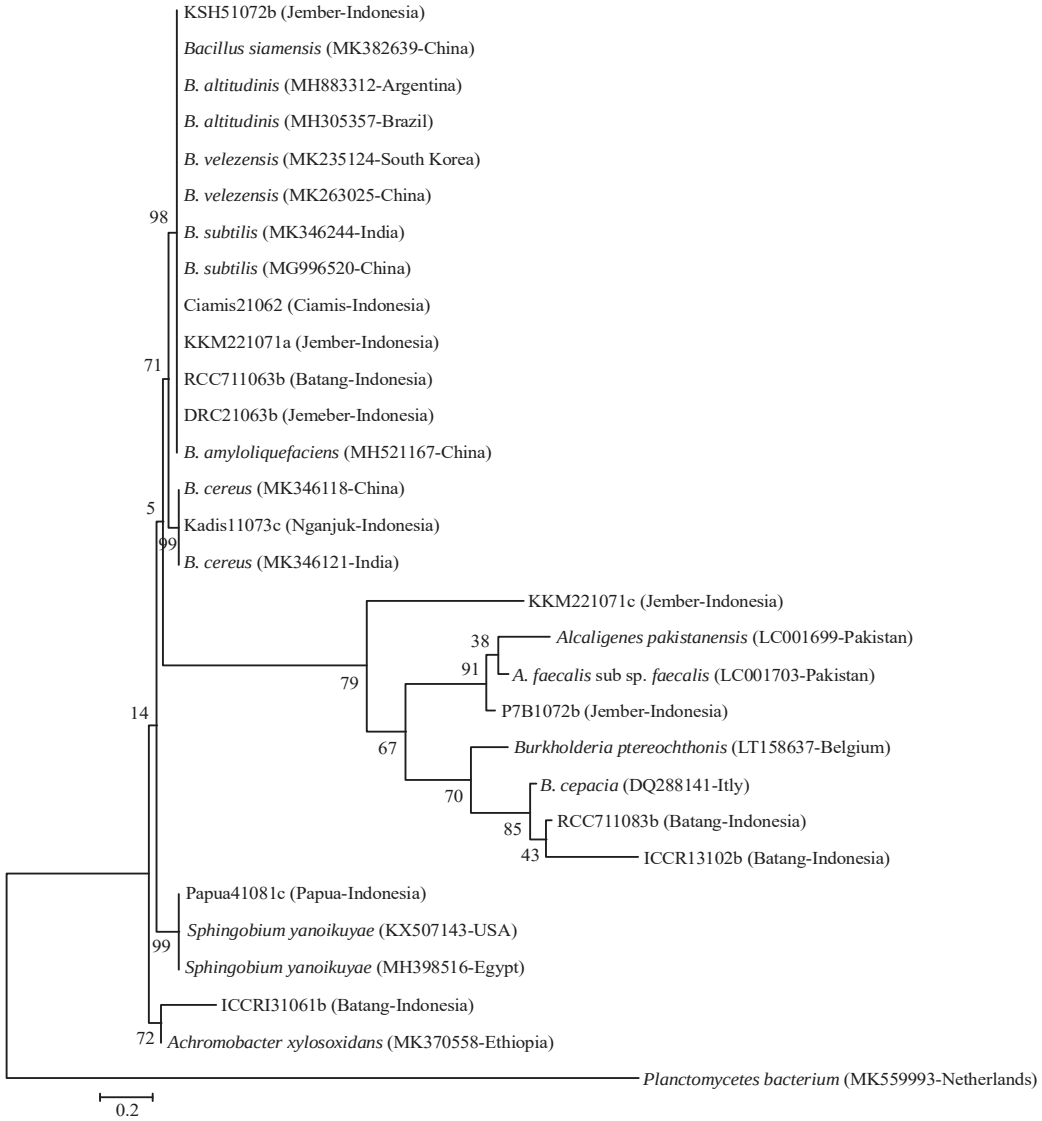

Fig. 3: Phylogenetic tree constructed under maximum likelihood method with 1000 replicates of bootstrap using Mega 7.0 program for referring the representative potential antagonistic bacterial isolates to the closest bacterial strain at NCBI. *Planctomycetes bacterium* was considered as out group species

Table 8: *In vivo* assay on inhibition of black pod rot disease on detached cocoa pod with the application of potential antagonistic bacteria

Treatments	Appearance of initial symptom (day after inoculation)	Lesion or necrotic diameter (cm)		Lesion or necrotic area (cm^2)		Disease severity (%)	
		Initial	Final	Initial	Final	Initial	Final
KKM221071a	3	0.66	6.14	0.86	43.22	8	44
KKM221071c	3	0.26	8.76	0.09	77.66	12	64
KSH51072b	3	1.04	11.00	0.95	98.27	20	88
P7B1072b	3	1.00	7.72	1.07	60.70	16	64
DRC21063b	3	0.94	9.26	1.22	68.55	12	80
ICCRI31061b	3	0.26	13.90	0.17	152.48	8	100
ICCRI31082b	3	0.68	12.02	0.74	146.01	12	80
RCC711063b	3	1.00	7.80	0.99	48.09	16	56
RCC711083b	3	1.04	10.48	1.41	115.42	12	72
Kadis11073c	3	0.80	13.04	0.84	134.84	12	100
Papua41081c	3	1.26	12.78	1.65	130.56	16	100
Ciamis21062	3	1.38	17.48	1.53	241.36	20	100
B26	3	0.12	7.64	0.05	46.80	4	56
Fungicide	5	0.30	1.16	0.18	4.30	8	12
Control (+)	3	0.42	21.30	0.69	361.44	4	100
Control (-)	0	0.00	0.00	0.00	0.00	0	0

black pod rot disease in Indonesia and its correlation with the existence of beneficial endophytic microbes.

Present study screened more bacterial isolates (362 isolates) from cocoa pods compared to previous works[17,20,21,24,25,41,44] because of using serial dilution on common agar medium. This abundance finding was comparable to former investigation implementing same isolation technique and solid medium[47,48]. Higher dilutions of 10^{-3} to 10^{-5} could explore 114-511 colonies of epiphytic bacteria from healthy green cacao pods[25]. Then, the given technique could be recommended to isolate considerable useful indigenous microorganisms.

This experiment revealed the highest *in vitro* inhibition of antagonistic bacteria against *P. palmivora* among the previous investigations[17,22-25,41,43,44,49-51] (Table 9). This suggested that the exploration of endophytic isolates from healthy pod might be considered as an essential screening stage of antagonistic bacteria for biological control.

Using rep-PCR assay, the present study could classify more than a hundred antagonistic bacterial isolates in short time and a few steps because its consistency and reliability in assessing the genetic diversity as well as specific region of targeted-PCR primers[52]. More distinct and more informative band profiles found in this investigation was also supported by the previous research on *Anoxybacillus* species[53]. It could be noticed that these primer sets were still relevant as rapid and appropriate tools for antagonistic screening.

This study might be considered as the first utilisation of rep-PCR methods in clustering the antagonistic endophytes from cocoa pods. The current findings of antagonistic bacteria reflected high degree genotypic diversity among them on healthy cocoa pods and they complied with former study[54]. It was assumed that those high genetic variability and independent clusters showed high adaptability of antagonistic bacteria to their environment and expressed the abundance of their hereditary capacity in the long-term evolution process.

Variation in consistency of inhibition performed by screened bacterial endophytes in this study indicated their dynamic antagonistic ability under different *in vitro* culture conditions. The consistent performance of inhibition under two cultural methods reflected the stability on antagonistic capability of the microbial endophytes under laboratory assay.

Similar antagonistic phenomena under SEM on phytopathogenic fungi were also reported[55-61]. However, current investigation did not find inhibition of zoospore production and sporangial breakdown since the *in vitro* antagonism assay on common artificial agar medium was more suitable for mycelial growth rather than the production of those asexual organs. The comprehensive observation is required in future to recognise the effect of antagonistic microbes on the development of reproduction features of pathogen.

Beyond the plant pathology, 16S rRNA and *gyrB* genes had been used for analysis of microbial community compositions[62], recovering the results of long-established procedures[63] and for comparative taxonomic analyses[64]. Hence, these housekeeping genes might be recommended for molecular identification of bacterial isolates using DNA sequencing approach.

Table 9: Comparison of documented inhibitory percentage on similar previous reports with this study

Source of bacterial isolates	Host plant	Bacterial species	Percentage of inhibition (%)	References
Healthy pod	Cocoa (*Theobroma cacao* L.)	*Alkaligenes* spp., *Achromobacter xylosoxidans*, *Bacillus* spp., *Burkholderia* spp., *Sphingobium yanoikuyae*	37.73-100	This study
Healthy pods and twigs	Cocoa (*Theobroma cacao* L.)	ND[a]	33.92-60.59	Khaeruni *et al.*[24]
Healthy pod	Cocoa (*Theobroma cacao* L.)	ND	12.51-74.76	Setyowati *et al.*[41]
Healthy leaves, branches and pods	Cocoa (*Theobroma cacao* L.)	*Bacillus altitudinis*, *Pseudomonas aeruginosa*, *Chryseobacterium proteolyticum*	60.91-82.41	Alsultan *et al.*[44]
Healthy pod	Cocoa (*Theobroma cacao* L.)	*Neisseria* sp., *Enterobacter* sp., *Pseudomonas putida*	0.01-69.7	Akrofi *et al.*[25]
Healthy twigs and leaves	Cocoa (*Theobroma cacao* L.)	*B. amyloliquefaciens*	35.77	Hamzah *et al.*[43]
Rhizosphere	Cocoa (*Theobroma cacao* L.)	*Streptomyces* spp.	27.37-68.17	Purnomo *et al.*[49]
Rhizosphere	Cocoa (*Theobroma cacao* L.)	*P. chlororaphis*	36.7-51.7	Acebo-Guerero *et al.*[23]
Rhizosphere	Potato (*Solanum tuberosum*)	*B. subtilis*, *P. fluorescence*	69.5-72.8	Pratama *et al.*[51]
Roots and rhizosphere	Cocoa (*Theobroma cacao* L.)	*Bacillus* spp., *Pseudomonas* spp.	48-78	Thomas *et al.*[22]
Pod	Cocoa (*Theobroma cacao* L.)	*Streptomyces* spp.	0-25	Macagnan *et al.*[17]
Pod	Cocoa (*Theobroma cacao* L.)	*Burkholderia* spp., *Pseudomonas* spp.	66-82.1	Jaaffar[50]

[a]ND means the species of antagonistic bacteria is not determined yet

The dominance of *Bacillus* species as potential antagonistic endophytes against black pod rot pathogen on cocoa had been also documented[18,65]. The capability of those five genera of endophytic bacteria against fungal pathogens on cocoa and other crops had been recorded[66-75]. It convinced that they were potential as beneficial microorganisms for future strategy of sustainable crop disease management. Nevertheless, the scientific justification documenting antagonistic records of *A. pakistanensis* and *B. ptereochthonis* against phytopathogenic microorganisms could not be found. Their low identity percentages probably required more accurate and proper molecular identification technique using specific gene region.

The maximum and consistent inhibition of *B. subiltis* under *in vivo* test had been previously documented[49,51,76-80]. Surprisingly, inconsistent results of *in vitro* and *in vivo* assays using other antagonists in this experiment were parallel to previous reports[41,81] but in disagreement with other works[25,50]. Such inconsistencies were possibly due to the dependency of *in vitro* test on interaction of competing microorganisms on rich-nutrient agar medium, the incubation under controlled conditions and the absence of host-plant tissue.

This fundamental study did not implement the antagonistic isolates under the field conditions as the estimated results could be reflected by the *in vivo* assay. Macagnan *et al.*[17] presumed that variation of environmental conditions and competition amongst microflora of the pods could affect the effectiveness of biological control against cacao pathogens in the field and they suggested to investigate the population dynamics of these antagonists for minimizing the failure of field experiments. The dosage and composition of production medium for antagonist were also reported to affect the effectiveness of biological control in the field assays[82]. The advanced work is required to determine proper formulation to provide favourable environmental circumstances for optimal activity of microbes in the field.

CONCLUSION

Some endophytic bacteria had been successfully explored from healthy cocoa pods in Indonesia with antagonistic potential against *P. palmivora* causing black pod rod disease. They have possibility as alternative sustainable disease management strategy under appropriate formulation and application techniques as well as favourable environmental condition.

SIGNIFICANT STATEMENT

This study focused on exploration of bacterial isolates on healthy cocoa pods collected from various cocoa clones and orchards in different geographical origins of Indonesia, since the common infection of *P. palmivora* was found on pod. Comprehensively, this experiment screened the antagonistic endophytic bacteria through two *in vitro* assays, clustered them using rep-PCR technique and examined their *in vivo* inhibition on detached healthy cocoa pods. Furthermore, the representative isolates were then molecularly identified using DNA sequencing of 16S rRNA and *gyrB* genes. Such screening steps might be expected generates the most effective isolates as biological control agent of *P. palmivora* on cacao.

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