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The expression of pathogenicity-related genes in *Phytophthora palmivora* causing black pod rot disease on cacao (*Theobroma cacao* L.) in Indonesia

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ABSTRACT

Phytophthora palmivora has a broad range of host plants. The expression of its pathogenicity-related genes in causing black pod rot disease on cacao in Indonesia has not been studied yet. This research was conducted to recognize relative expression of those genes using qPCR analysis. In planta experiment was carried out by inoculating representative three isolates on model plant (*Nicotiana benthamiana*) at the incubation periods of 24, 48, 72, and 96 h. The expressed genes were reconfirmed using conventional PCR and quantitatively analyzed using qPCR technique. The results showed that among eight target genes, four genes were actively expressed on the inoculated *N. benthamiana*, including *CRN1*, *Pec1*, *Pec3*, and *RXLR5*. Furthermore, qPCR analysis revealed that those genes were upregulated on the inoculated plants during incubation periods. In WNO1 and WAT1 isolates, the optimum expression was documented on the first day of incubation time, and then it was decreasing on the following days. However, the delay expression was exhibited by the pathogenicity-related genes in isolate BTG1. We assumed it as the general pathway of pathogenicity mechanisms in *P. palmivora*, since prior screening pathogenicity assay categorized that isolate BTG1 into moderate pathogenic, isolate WAT1 into high pathogenic, and isolate WNO1 into less pathogenic.

Abbreviations: VSD: Vascular Streak Dieback; qPCR: Quantitative PCR; PCR: Polymerase Chain Reaction; Dpi: Days post-inoculation; CRN1: Crinkling and necrosis inducing proteins; *Pec1*: Pectinase; *Pec3*: Polygalacturonase; RXLR5: Avirulence homolog; HSP1A: HSP (Heat Shock Protein) 70-like protein; NPP1B: Necrosis inducing protein; RXLR1: RXLR (Arg-Xaa-Leu-Arg) effector; Eli1: Elicitin; β-Tub: Beta-tubulin; LP: Less pathogenic; MP: Moderate pathogenic; HP: High pathogenic; CWDEs: Cell wall degrading enzymes; PgeneMs: Putative gene models; DNA: Deoxyribonucleic acid; ddH2O: Double-distilled hydrogen oxide (water); RNA: Ribonucleic acid; cDNA: Complementary deoxyribonucleic acid; RB: Reaction buffer

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Expression; genes; level; *Phytophthora palmivora*; pathogenicity

1. Introduction

Phytophthora palmivora causing black pod rot disease is reported as one of the factors affecting cocoa production in Indonesia (McMahon and Purwantara 2004). The current incidence of such disease is higher compared to vascular streak dieback (VSD) caused by *Ceratobasidium theobromae* (syn. *Oncobasidium theobromae*) (Purwantara et al. 2015; Rosmana et al. 2019). Overall, the acreage of cocoa-growing area in this third cocoa-producing country is getting decrease for the last seven years (2012–2018), which has an impact on the production and export volume as well (Statistics of Indonesia 2019).

As one of the heterothallic *Phytophthora* species, the greater pathogenicity is possibly generated by genetic recombination of sexual reproduction in *P. palmivora* in addition to wider host range and formation of resistant oospores for survival (Ho et al. 1983). Initially, the pathogenicity of *P. palmivora* has been revealed by strains isolated from *Piper nigrum* (Turner 1973), as well as sweet orange, rough lemon, eggplant, and rosella (*Hibiscus sabdariffa*) (Boccas 1972), and azalea. Their pathogenicity ability from various host plants is documented correlating with the geographical region (Torres-Londono 2016).

Last one decade, isolation and pathogenicity test of *P. palmivora* isolates have been conducted from 21 regencies including 13 cocoa production provinces in Indonesia (Rubiyo and Sukamto 2008). The change of this pathogen isolates and the possibility in prevalence of new or more pathogenic isolates in the field need to be studied from time to time (Goodwin 1997; Rubiyo and Sukamto 2008). Sometimes, there is decreasing in the pathogenicity of *P. palmivora* under frequent transfer onto fresh agar medium in the laboratory (Miyage and Nagai 2019). Hence, it is important to examine their pathogenicity for current records.

The variability in aggressiveness and pathogenicity of *P. palmivora* directly influences the infection and disease severity and becomes important in understanding the disease cycle (de Oliviera et al. 2016). Recently, the pathogenicity-related genes on *P. palmivora* had been differentiated from those of *P. megakarya* isolated from cocoa pod in Ivory Coast and Cameroon, respectively, through the whole genome analysis (Ali et al. 2017). However, the expression of pathogenicity-related genes on *P. palmivora* isolates relating to infection time and different pathogenicity causing different severity of lesion has not been transcriptomically studied. Therefore, this study was conducted to determine the

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pathogenicity ability of *P. palmivora* isolates causing cocoa black pod rot disease in Indonesia, to detect their pathogenicity-related genes using the PCR method, and to figure out the expression of those genes under the condition of plant–pathogen interaction using the quantitative PCR (qPCR) technique. This transcriptomic analysis focused on *P. palmivora*, since *P. megakarya* is regulated as quarantine pest for Indonesia.

2. Materials and methods

2.1. Phytophthora palmivora *isolates*

This study used 55 isolates of *P. palmivora* (isolated from cocoa pods in 2017), which had been morphologically and sexually characterized as well as molecularly identified using species-specific primers (Masanto et al. 2019a). The culture of mycelial discs was maintained at −80°C within 2 ml microtube containing 50% of glycerol for long-term storage (2018–2019).

2.2. In vitro pathogenicity test using apple fruits

Firstly, the 5 mm-mycelial discs of representative isolate of *P. palmivora* (WAT3, Kulon Progo, D.I. Yogyakarta) (previously had been molecularly identified by Masanto et al. (2019a)) were inoculated on six varieties (Fuji, Gala, Manalagi, Premium, Red Delicious, and Rome Beauty) of healthy apple fruits for prescreening. The selected variety of apple fruits were then surface-sterilized using 70% alcohol and inoculated with all *P. palmivora* isolates. The inoculated fruits were incubated in plastic trays for one week at ambient temperature. The lesion severities caused by *P. palmivora* isolates were determined by measuring the latent period (day), lesion or necrotic area (cm^2), disease severity (%), and infection rate (Motulo 2008).

- Latent period (day)

This period was counted since inoculation time until the appearance of initial symptoms which was characterized with brown spot around the inoculated point on pod surface.

- Lesion or necrotic area (cm^2)

This area was calculated from the measurement of the diameter of lesion or necrotic using transparent millimeter block at the first day of appearance of symptom and the last day of full symptom.

- Disease severity (%)

This variable was calculated using the following formula:

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

where DS: disease severity (%); ni: number of symptomatic fruits on corresponding score; vi: corresponding score of symptoms, i.e. 0 = no symptom, 1 = lesion or necrotic area 0–20 cm^2, 2 = 20–40 cm^2, 3 = 40–60 cm^2, 4 = 60–80 cm^2, and 5 = more than 80 cm^2; N = total number of observed fruits; and V = highest score.

- Infection rate

This rate was calculated using the formula of Zadoks and Schein (1980):

$$r = \frac{2.3}{t2 - t1}\left(\log\frac{x2}{1 - x2} - \log\frac{x1}{1 - x1}\right)$$

where r = infection rate, t_1 = time observation of disease severity at x_1, t_2 = time observation of disease severity at x_2, x_1 = disease severity at time t_1, x_2 = disease severity at time t_2. Criteria for determining the pathogenicity ability were described in Table 1. However, the most determinant parameter was infection rate, since there was assumption that the more pathogenic isolate was, the higher the infection rate it had (Nirwanto 2007).

2.3. Culture of isolates for DNA extraction

The isolates were cultured on V8 juice agar medium as well as incubated for 5–7 days in growth chamber at 25°C, and their DNA was extracted using Prepman Ultra Reagent under the protocol of manufacturer (Applied Biosystems, Foster City, CA, USA) as described in previous experiment (Masanto et al. 2019a). These DNA templates were then used for further assay.

2.4. Detection of pathogenicity-related genes on Phytophthora palmivora *using the PCR technique*

The presence of reference genes (β-tubulin) and some pathogenicity-related genes, such as pectinases, elicitins, *HSPs* (heat shock proteins), *CRNs* (crinkling and necrosis inducing proteins), *NPPs* (necrosis inducing *Phytophthora* proteins), and *RXLRs*, was comprehensively detected on the DNA of all *P. palmivora* isolates using eight primer sets (Table 2) under the PCR condition following the instruction of manufacture for PCR ready mix (Bioline, London, UK). A total of 10 µl of PCR reaction consisting of PCR ready mix (Bioline), 100 µM of forward and reverse primers, DNA template, as well as ddH_2O was demonstrated using T100 Thermal Cycler (Bio-Rad, Tokyo, Japan). PCR products were run for electrophoresis in 1% agarose gel added with 2 µl of Greensafe Premium staining solution (Nzytech, Lisboa, Portugal) at 70 V for 50 min using electrophoresis device of Powerpac Basic (Bio-Rad). The gel was visualized under Bio-Rad UV Transilluminator (Bio-Rad).

2.5. In planta experiment for inoculation of Phytophthora palmivora *on model plant*

Three *P. palmivora* isolates with three category pathogenicity (less/LP, common/MP, and highly pathogenic/HP) were cultured on PDA and incubated at room temperature for a week. Their mycelial discs were then aseptically inoculated on leaves of healthy model plant (*Nicotiana benthamiana*).

Table 1. Criteria for determining the pathogenicity level of *P. palmivora*.

Latent period (day)	Disease severity (%)	Infection rate	Pathogenicity level
More than 7	$0 < DS \leq 10$	$r = 0$	Non pathogenic
Between 5 and equal to 7	$10 < DS \leq 20$	$0 < r \leq 0.2$	Less pathogenic
Between 3 and equal to 5	$20 < DS \leq 30$	$0.2 < r \leq 0.4$	Moderate pathogenic
Between 1 and equal to 3	$DS > 30$	$r > 0.4$	High pathogenic

Table 2. Primer sets corresponding to pathogenicity-related genes of *P. palmivora*.

Annotation	Gene/ Primer name	Primer sequence (5′–3′)	Tm (°C)	References	PCR condition
Phytophthora palmivora β-tubulin	Pp β-tubF	GTGGATCCCCAACAACATCA	55.1	Gumtow et al. (2018)	Pre-denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 10 s; and final extension at 72°C for 5 min
	Pp β-tubR	ACATCTCCTGGATGGCAGT	55.9		
Crinkler	Pp CRN1F	TGGTTCCTACTTGTGGGTGA	55.7		
	Pp CRN1R	GCCAAACGCTGTAATTTCCT	53.8		
Elicitin	Pp Eli1F	TGCAAGTCGCTGATTGAGTC	55.4		
	Pp Eli1R	GTCTCCGTAGTCGTCGTGGT	58.5		
Hsp70-like protein	Pp HSP1AF	TATCATCGACTGCGAGCAAA	54.1		
	Pp HSP1AR	GGTGTCTGCAACACGTCAAA	56.0		
Necrosis-inducing protein	Pp NPP1BF	GGATTCACCGTCCTCAGGTA	56.0		
	Pp NPP1BR	CCGGCCAGTTACTCTCGTAG	57.0	Ali et al. (2017)	
Pectinase	Pp Pec1F	GTTGAACCACCACCTCGTCT	57.2		
	Pp Pec1R	TGATGGTCATCGAGTCGGTA	55.1		
Polygalacturonase	Pp Pec3F	TGCACTCCGATTACAGCAAG	55.2		
	Pp Pec3R	CTTCGGGTTCACCTCAATGT	55.0		
RXLR effector	Pp RxLR1F	GTTTCGTCGATGCCAAAGAT	53.4		
	Pp RxLR1R	AAGTCCAACCAGAGCCAAAA	54.7		
Avirulence homolog	Pp RxLR5F	ACGTGTTCCGAAGATGGAAA	54.3		
	Pp RxLR5R	ACCTCTCGTGACCGTATTGG	56.5		

The model plant was more than two months old having more than five leaves. The bottom and subsequent upper leaves of each model plant were inoculated with different category of pathogenic isolates and then incubated for 24, 48, 72, and 96 h, respectively. Unwounded and wounded leaves without any inoculation of pathogen were considered as negative and positive controls, respectively.

2.6. RNA extraction and cDNA synthesis from control and inoculated model plant

The RNA of infected leaves of model plant (at 24, 48, 72, and 96 after inoculation) were extracted using Total RNA Mini Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) following the protocol of manufacture. A total of 0.1 g fresh tissue sample was weighed and then put into mortar. Prior to immediate grinding using pestle, 500 µl of RB buffer was added. The grinded samples were transferred into 1.5 ml microcentrifuge tube, added with 5 µl of β-mercaptoethanol, and mixed by vortex. The lysis step was initiated by incubating the sample at 60°C for 5 min. The sample mixture was then transferred into the filter column, which was previously prepared in a 2 ml collection tube. They were centrifuged at 1000 × g for 1 min using HERMLE Z 216 MK (HERMLE Labortechnik, Wehingen, Germany), and then the filter column was discarded. The clarified filtrate was carefully transferred into a new 1.5 ml microcentrifuge tube, added with a half volume of absolute ethanol, and then vigorously shaken. The mixture was transferred into the RB column, which was formerly placed in a 2 ml collection tube and then centrifuged at 15,000 × g for 1 min. The flow-through was discarded, and the RB column was put back in the 2 ml collection tube. The collected RNA was washed by adding 400 µl of W1 buffer into the center of the RB column and centrifuging at 15,000 × g for 30 s. The flow-through was discarded, and the RB column was placed back in the 2 ml collection tube. The next step was two times washing using 600 µl of wash buffer (previously has been added with ethanol) and centrifugation at 15,000 × g for 30 s and 1 min, respectively. The RNA was dried by centrifugation at 15,000 × g for 3 min. The dried RB column was then placed in a clean 1.5 ml microcentrifuge tube, added with 50 µl of RNAse-free water to the center of the column matrix, and incubated at room temperature for 2 min to ensure that the RNAse-free water was completely absorbed. The purified RNA was eluted by centrifugation at 15,000 × g for 1 min. The step might be continued with RNA quantification using Nano Drop machine or storage at −80°C.

The extracted RNA was quantified using DNA RNA Protein Quantification Spectrophotometer MN-913A MaestroNano Pro (Maestrogen Inc., Hsinchu City, Taiwan). The device was set up for blank assessment using 1 µl of RNAse-free water. A total of 1 µl of RNA sample was drop for sample quantification. Several parameters were measured and calculated, such as absorbance values of A230, A260, A80, ratio A260/A230, and A260/A280, as well as concentration.

The quantified RNA was run for reverse transcription PCR to synthesize cDNA using T100 Thermal Cycler (Bio-Rad) with SensiFAST™ cDNA Synthesis Kit (Bioline). A total of 20 µl reaction solution consisted of 4 µl of 5 × TransAmp buffer, 1 µl of Reverse Transcriptase, 1 µl of RNA sample (200 ng/µl), and finalized with 14 µl of RNAse-free H_2O. Reverse transcription condition was set up as follows: primer annealing at 25°C for 10 min, reverse transcription at 42°C for 15 min, inactivation at 85°C for 5 min, and storage stage at 4°C. The cDNA product might be quantified using nanodrop device and then kept in −20°C. Further analysis was a reconfirmation of expressed pathogenicity-related genes using conventional PCR or quantitative analysis using qPCR.

2.7. Expression of pathogenicity-related genes of P. palmivora using the conventional PCR method

The expression of those pathogenicity-related genes of *P. palmivora* after RNA extraction and cDNA synthesis from control and inoculated model plant was amplified with the similar above method of conventional PCR.

2.8. Analysis of qPCR on expressed pathogenicity-related genes of Phytophthora palmivora

A total of 20 µl of PCR reaction contained ddH_2O, 2 × SensiFAST™ SYBR® No-ROX One-Step Kit (Bioline) PCR ready mix, 400 nM of forward and reverse primers, reverse

transcriptase, ribosafe RNAse inhibitor, and RNA template. The mixture was run under following condition: reverse transcriptase at 45°C for 10 min; polymerase activation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 10 s and extension at 72°C for 5 s; and final extension at 72°C for 5 min. Meanwhile, melt profile was adjusted to the instrument of Bio-Rad CFX96 (Bio-Rad).

2.9. Data analysis

Mean comparison analysis was applied for the diameter of lesion or necrotic (since other parameters were calculated from this basic data) with the analysis of variance (ANOVA) and the further test of Tukey using RStudio program (Massachusetts, USA). The $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001) was employed to analyze the relative gene expression data. The expression of pathogenicity-related genes was quantitatively compared with housekeeping gene (β-tubulin). Their relative expression was illustrated in graph Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and heat map comparison using ClustVis web tool (Metsalu and Vilo 2015).

3. Results

3.1. Selection of apple fruits for further pathogenicity test

The necrotic symptom on six varieties of apple fruits appeared at 3 dpi with the range of initial diameter, necrotic area, and disease severity around 3.53–5.87 cm, 10.77–27.02 cm^2, and 20–40%, respectively (Table 3). At the last incubation (5 dpi since the full symptoms occurred on tested apple fruit), those parameters increased up to 6.10–8.57 cm, 29.33–57.86 cm^2, and 40–66.67% in range, respectively. The disease developed with the range of infection rate about 0.24–0.60.

Statistically, there was not a significant difference in initial and final diameters of necrotic or lesion on Manalagi and Red Delicious varieties showing high infection rates of 0.56 and 0.60, respectively. However, the variety of Manalagi was selected for the further pathogenicity test due to the clear and distinguishable symptoms on its green skin in addition to high final disease severity and infection rate.

3.2. Pathogenicity test on selected apple fruit

Further pathogenicity assay revealed that distinct symptoms appeared mostly at 1 dpi with the range of initial diameter, lesion area, and disease severity around 0.23–1.60 cm, 0.10–2.08 cm^2, and 6.67–20%, respectively (Table 4). On the final incubation (5 dpi since the full symptoms occurred on tested apple fruit), their increment was 3.30–7.53 cm, 13.39–44.72 cm^2, and 20–60% in range, respectively. Those isolates could cause disease with the infection rate about 0.12–0.45 in range. According to those parameters, they could be categorized into high (three isolates from Cianjur, Kulon Progo, and Saumlaki), less (three isolates from Bangka Belitung and Gunung Kidul), and moderate pathogenic isolates (other 49 isolates from various geographical origins).

Statistically, there was found a significant difference in the initial diameter of necrotic caused by less, moderate, and high pathogenic isolates. Meanwhile, the significant difference in the final diameter of necrotic occurred among high pathogenic isolates. There was no significant difference in the final diameter of necrotic among less, moderate, and high pathogenic isolates. We selected the WNO1, BTG1, and WAT1 isolates to represent the less, moderate, and high pathogenic categories, respectively, for in planta experiment.

3.3. Detection of pathogenicity-related genes on *Phytophthora palmivora* isolates using the PCR technique

All eight primer sets of pathogenicity genes were able to detect the related genes on all isolates tested with band size ranged at 125–250 bp (Table 5). The lowest band size was revealed by *RXLR5*, while the highest band size was amplified by *Pec3* primers.

3.4. Expression of pathogenicity-related genes under in planta experiment using the PCR method

Among eight used primer sets, only four of them could be positively reconfirmed on inoculated *N. benthamiana*, those were *CRN1, Pec1, Pec3* and *RXLR5*. They were consistently detected on *N. benthamiana* at 24 and 48 h post-inoculation (Table 6). Two genes, i.e. *HSP1A* and *NPP1B,* might not be expressed during interaction of pathogen with model plant. The other two genes might not be specific as pathogenicity genes of *P. palmivora* since they were positively detected with single and double bands on *RXLR1* and *Eli1*, respectively. No gene expression was found on either control wounded or unwounded plant samples.

3.5. Quantitative examination of expressed pathogenicity genes using the qPCR analysis

The analysis of qPCR revealed that those genes of *CRN1*, *Pec1*, *Pec3* and *RXLR5* were upregulated on the inoculated plants during incubation periods. The *CRN1* and *RXLR5*

Table 3. Pathogenicity test of *Phytophthora palmivora* on six varieties of apple fruits.

	Diameter of necrotic (cm)		Necrotic area (cm^2)		Disease severity (%)		
Variety	Initial[a]	Final[b]	Initial	Final	Initial	Final	Infection rate
Manalagi	5.13ab[c]	7.03ab	20.77	39.04	26.67	53.33	0.56
Gala	5.87a	7.17ab	27.02	40.88	40	53.33	0.24
Fuji	4.33ab	6.10b	14.79	29.33	20	40	0.49
Red Delicious	3.53b	6.70ab	10.77	35.96	20	46.67	0.60
Rome Beauty	4.57ab	6.73ab	16.63	35.87	26.67	46.67	0.43
Premium	5.4a	8.57a	22.89	57.86	40	66.67	0.51

[a]Initial data were collected at 3 days after inoculation.
[b]Final data were collected at 5 days after inoculation.
[c]Same letters following the number were not significantly different (at α = 5%).

Table 4. Pathogenicity test of all isolates of *Phytophthora palmivora* on selected apple fruits.

Isolates	Geographical origin	Latent period (day)	Diameter of necrotic (cm)		Necrotic area (cm^2)		Disease severity (%)		Infection rate	Pathogenicity level	Mating type
			Initial[a]	Final[b]	Initial	Final	Initial	Final			
LBP1	Deli Serdang, North Sumatra	1	0.53efghij[c]	5.73ab	0.22	25.90	20.00	40.00	0.24	Moderate	A1
BKN1	Kampar, Riau	1	0.80abcdefghij	5.97ab	0.54	28.13	20.00	40.00	0.24	Moderate	A2
BSK1	Tanah Datar, West Sumatra	1	0.80 abcdefghij	6.23ab	0.54	30.52	20.00	40.00	0.24	Moderate	A1
CRP1	Rejang Lebong, Bengkulu	1	0.57defghij	5.93ab	0.26	27.72	20.00	40.00	0.24	Moderate	A2
MRD1	Ogan Komering Ulu Selatan, South Sumatra	1	1.50ab	6.80ab	1.76	36.37	20.00	46.67	0.31	Moderate	A2
KOT1	Tanggamus, Lampung	1	0.30j	5.57ab	0.10	24.37	13.33	40.00	0.37	Moderate	A1
GDT1	Pesawaran, Lampung	1	0.73bcdefghij	5.93ab	0.43	27.68	20.00	40.00	0.24	Moderate	A1
KBA1	Central Bangka, Bangka Belitung	1	0.30j	4.77ab	0.11	17.84	13.33	20.00	0.12	Less	A2
PDG1	Pandeglang, Banten	1	0.70bcdefghij	6.00ab	0.46	28.76	20.00	40.00	0.24	Moderate	A2
CJR1	Cianjur, West Java	1	1.03abcdefghij	7.53a	0.87	44.60	20.00	60.00	0.44	High	A2
CJR2	Cianjur, West Java	1	1.17abcdefghi	6.67ab	1.07	35.22	20.00	46.67	0.31	Moderate	A2
CJR3	Cianjur, West Java	1	1.20abcdefgh	6.70ab	1.13	35.33	20.00	40.00	0.24	Moderate	A2
SMD1	Sumedang, West Java	1	0.50efghij	4.50ab	0.29	23.85	13.33	26.67	0.22	Moderate	A2
SMD2	Sumedang, West Java	1	0.73bcdefghij	6.70ab	0.42	35.27	20.00	40.00	0.24	Moderate	A2
PWT1	Purwokerto, Central Java	1	0.80abcdefghij	6.40ab	0.52	32.31	20.00	40.00	0.24	Moderate	A2
BTG1	Batang, Central Java	1	0.83abcdefghij	6.47ab	0.54	32.85	20.00	40.00	0.24	Moderate	A2
BTG2	Batang, Central Java	1	0.40ghij	6.07ab	0.19	28.91	13.33	40.00	0.37	Moderate	A2
WSB1	Wonosobo, Central Java	1	0.53efghij	6.13ab	0.35	29.82	13.33	40.00	0.37	Moderate	A2
TMG1	Temanggung, Central Java	1	0.63cdefghij	5.63ab	0.32	24.93	20.00	40.00	0.24	Moderate	A2
UNR1	Semarang, Central Java	1	0.87abcdefghij	6.63ab	0.60	34.55	20.00	40.00	0.24	Moderate	A2
WAT1	Kulon Progo, D.I. Yogyakarta	1	1.30abcde	7.47a	1.34	44.72	20.00	60.00	0.45	High	A2
WAT2	Kulon Progo, D.I. Yogyakarta	1	0.43fghij	6.07ab	0.23	29.06	13.33	40.00	0.37	Moderate	A2
WAT3	Kulon Progo, D.I. Yogyakarta	1	0.83abcdefghij	5.77ab	0.81	28.71	13.33	40.00	0.37	Moderate	A2
WAT4	Kulon Progo, D.I. Yogyakarta	1	1.43abc	5.67ab	1.61	25.33	20.00	40.00	0.24	Moderate	A2
SMN1	Sleman, D.I. Yogyakarta	1	0.77abcdefghij	6.17ab	0.47	29.98	20.00	40.00	0.24	Moderate	A2
SMN2	Sleman, D.I. Yogyakarta	1	0.80abcdefghij	6.47ab	0.52	32.89	20.00	40.00	0.24	Moderate	A2
WNO1	Gunung Kidul, D.I. Yogyakarta	1	0.53efghij	5.27ab	0.24	21.81	20.00	33.33	0.17	Less	A1
WNO2	Gunung Kidul, D.I. Yogyakarta	1	0.53efghij	6.23ab	0.25	31.19	20.00	33.33	0.17	Less	A1
WNO3	Gunung Kidul, D.I. Yogyakarta	1	1.60a	6.27ab	2.08	32.84	20.00	46.67	0.31	Moderate	A1
WNO4	Gunung Kidul, D.I. Yogyakarta	1	1.40abcd	6.83ab	1.60	36.73	20.00	46.67	0.31	Moderate	A1
WNO5	Gunung Kidul, D.I. Yogyakarta	1	0.53efghij	6.50ab	0.22	33.19	20.00	40.00	0.24	Moderate	A1
JMR1	Jember, East Java	1	0.57defghij	6.27ab	0.25	30.85	20.00	40.00	0.24	Moderate	ND[d]
JMR2	Jember, East Java	1	0.47efghij	6.17ab	0.20	30.27	20.00	40.00	0.24	Moderate	A2
BYW1	Banyuwangi, East Java	1	0.67bcdefghij	5.77ab	0.34	26.20	20.00	40.00	0.24	Moderate	A2
SAG1	Sanggau, West Kalimantan	1	0.33ij	4.17ab	0.13	20.45	13.33	26.67	0.22	Moderate	A1
SMR1	Samarinda, East Kalimantan	1	0.30j	6.27ab	0.10	30.83	13.33	40.00	0.37	Moderate	A2
PKY1	Mamuju Utara, West Sulawesi	1	1.17abcdefghij	6.23ab	1.07	31.25	20.00	40.00	0.24	Moderate	A2
MKS1	Makassar, South Sulawesi	1	1.23abcdefg	6.63ab	1.19	34.64	20.00	40.00	0.24	Moderate	A2
LSS1	Kolaka Utara, Southeast Sulawesi	1	0.37hij	6.60ab	0.17	34.21	13.33	40.00	0.37	Moderate	A2
KKA1	Kolaka, Southeast Sulawesi	1	0.80abcdefghij	6.33ab	0.51	31.61	20.00	40.00	0.24	Moderate	A2
KKA2	Kolaka, Southeast Sulawesi	1	0.70bcdefghij	7.30a	0.38	41.89	20.00	53.33	0.37	Moderate	A2
RMB1	Bombana, Southeast Sulawesi	1	0.43fghij	6.07ab	0.15	29.05	20.00	40.00	0.24	Moderate	A2
KDI1	Kendari, Southeast Sulawesi	1	0.67bcdefghij	6.33ab	0.34	31.62	20.00	40.00	0.24	Moderate	A2
BNG1	Buton Utara, Southeast Sulawesi	1	0.77abcdefghij	6.57ab	0.46	33.92	20.00	40.00	0.24	Moderate	A2
KTG1	Kotamobagu, North Sulawesi	1	0.50efghij	6.73ab	0.23	35.59	20.00	40.00	0.24	Moderate	A2
MME1	Sikka, East Nusa Tenggara	1	1.27abcdef	7.23a	1.26	41.10	20.00	53.33	0.37	Moderate	A2
AMB1	Ambon, Maluku	1	0.77abcdefghij	6.93ab	0.52	37.89	20.00	53.33	0.37	Moderate	A2
SML1	Saumlaki, Maluku	1	0.60cdefghij	6.37ab	0.30	31.83	20.00	40.00	0.24	Moderate	A2
SML2	Saumlaki, Maluku	2	0.23j	3.3b	0.12	13.39	6.67	20.00	0.44	High	A2
TTE1	Ternate, North Maluku	1	1.00abcdefghij	6.20ab	0.80	30.37	20.00	40.00	0.24	Moderate	A2
MNK1	Manokwari, West Papua	1	0.57defghij	7.17a	0.25	40.97	20.00	46.67	0.31	Moderate	A2
TIM1	Mimika, Papua	1	0.77abcdefghij	6.60ab	0.47	34.52	20.00	46.67	0.31	Moderate	A2
TIM2	Mimika, Papua	1	0.77abcdefghij	6.57ab	0.46	34.00	20.00	40.00	0.24	Moderate	A2
TIM3	Mimika, Papua	1	1.17abcdefghi	5.90ab	1.07	27.43	20.00	40.00	0.24	Moderate	A2
JAP1	Jayapura, Papua	1	0.80abcdefghij	6.03ab	0.53	31.29	20.00	46.67	0.31	Moderate	A2

[a]Initial data were collected at 1 and 2 days after inoculation.
[b]Final data were collected at 5 days after inoculation.
[c]Same letters following the number were not significantly different (at $\alpha = 5\%$).
[d]ND indicated that this isolate was positively reacted with both standard isolates (A1- and A2-mating types) (Masanto et al. 2019a).

Table 5. Detection of pathogenicity-related genes on *Phytophthora palmivora* using the PCR technique with eight primer sets.

	Detection of pathogenicity-related genes using eight primer sets							
Isolates	*CRN1* (±150 bp)	*Eli1* (±150 bp)	*HSP1A* (±200 bp)	*NPP1B* (±200 bp)	*Pec1* (±175 bp)	*Pec3* (±250 bp)	*RXLR1* (±225 bp)	*RXLR5* (±125 bp)
LBP1	+[a]	+	+	+	+	+	+	+
BKN1	+	+	+	+	+	+	+	+
BSK1	+	+	+	+	+	+	+	+
CRP1	+	+	+	+	+	+	+	+
MRD1	+	+	+	+	+	+	+	+
KOT1	+	+	+	+	+	+	+	+
GDT1	+	+	+	+	+	+	+	+
KBA1	+	+	+	+	+	+	+	+
PDG1	+	+	+	+	+	+	+	+
CJR1	+	+	+	+	+	+	+	+
CJR2	+	+	+	+	+	+	+	+
CJR3	+	+	+	+	+	+	+	+
SMD1	+	+	+	+	+	+	+	+
SMD2	+	+	+	+	+	+	+	+
PWT1	+	+	+	+	+	+	+	+
BTG1	+	+	+	+	+	+	+	+
BTG2	+	+	+	+	+	+	+	+
WSB1	+	+	+	+	+	+	+	+
TMG1	+	+	+	+	+	+	+	+
UNR1	+	+	+	+	+	+	+	+
WAT1	+	+	+	+	+	+	+	+
WAT2	+	+	+	+	+	+	+	+
WAT3	+	+	+	+	+	+	+	+
WAT4	+	+	+	+	+	+	+	+
SMN1	+	+	+	+	+	+	+	+
SMN2	+	+	+	+	+	+	+	+
WNO1	+	+	+	+	+	+	+	+
WNO2	+	+	+	+	+	+	+	+
WNO3	+	+	+	+	+	+	+	+
WNO4	+	+	+	+	+	+	+	+
WNO5	+	+	+	+	+	+	+	+
JMR1	+	+	+	+	+	+	+	+
JMR2	+	+	+	+	+	+	+	+
BYW1	+	+	+	+	+	+	+	+
SAG1	+	+	+	+	+	+	+	+
SMR1	+	+	+	+	+	+	+	+
PKY1	+	+	+	+	+	+	+	+
MKS1	+	+	+	+	+	+	+	+
LSS1	+	+	+	+	+	+	+	+
KKA1	+	+	+	+	+	+	+	+
KKA2	+	+	+	+	+	+	+	+
RMB1	+	+	+	+	+	+	+	+
KDI1	+	+	+	+	+	+	+	+
BNG1	+	+	+	+	+	+	+	+
KTG1	+	+	+	+	+	+	+	+
MME1	+	+	+	+	+	+	+	+
AMB1	+	+	+	+	+	+	+	+
SML1	+	+	+	+	+	+	+	+
SML2	+	+	+	+	+	+	+	+
TTE1	+	+	+	+	+	+	+	+
MNK1	+	+	+	+	+	+	+	+
TIM1	+	+	+	+	+	+	+	+
TIM2	+	+	+	+	+	+	+	+
TIM3	+	+	+	+	+	+	+	+
JAP1	+	+	+	+	+	+	+	+

[a]Symbol '+' means the isolates were positively detected with corresponding primers sets.

tended to be decreasingly expressed following the incubation period from more than twofold change to less than a half-fold change, while the fluctuating appearance was found on *Pec1* and *Pec3* genes of less and high pathogenic isolates (Figure 1). In less and high pathogenic isolates, the optimum expression was documented on the first day of incubation time (24 h), and then it was decreasing on the following days. However, the delay expression was exhibited on the second day by the pathogenicity-related genes in moderate pathogenic isolate (Figure 2).

4. Discussion

The pathogenicity experiment revealed that there were three categories of pathogenic levels *P. palmivora* isolates, which were dominated by moderate pathogenic isolates. All test isolates were positively detected using the PCR method with eight primer sets of pathogenicity-related genes. However, the result of *in planta* assay found that *CRN1*, *Pec1*, *Pec3*, and *RXLR5* were expressed at 24 and 48 h after inoculation. Meanwhile, the other four genes expressions were not specifically detected on model plant.

Despite those four targeted genes and one reference genes were expressed under the qPCR analysis at all incubation periods, the prior detection of those genes using conventional PCR revealed their presence only at 24 and 48 h post-infection. Such a situation might be due to the higher sensitivity of SYBR green-based qPCR. This method had been previously recognized as a more sensitive technique compared to conventional PCR for the detection of wheat

Table 6. Reconfirmation of expressed pathogenicity-related genes on inoculated *Nicotiana benthamiana* using the conventional PCR method.

Sample Source	Code	Detection of pathogenicity-related genes using some primer sets								
		β-Tub	*CRN1*	*Eli1*	*HSP1A*	*NPP1B*	*Pec1*	*Pec3*	*RXLR1*	*RXLR5*
Pathogen	LP[a]	+[c]	+	+	+	+	+	+	+	+
	MP	+	+	+	+	+	+	+	+	+
	HP	+	+	+	+	+	+	+	+	+
Control	+	-[d]	-	+[e]	-	-	-	-	+	-
	-	-	-	+	-	-	-	-	+	-
Treatment	LP24[b]	+	+	+	-	-	+	+	+	+
	MP24	+	+	+	-	-	+	+	+	+
	HP24	+	+	+	-	-	+	+	+	+
	LP48	+	+	+	-	-	+	+	+	+
	MP48	+	+	+	-	-	+	+	+	+
	HP48	+	+	+	-	-	+	+	+	+
	LP72	-	-	+	-	-	-	-	+	-
	MP72	-	-	+	-	-	-	-	+	-
	HP72	-	-	+	-	-	-	-	+	-
	LP96	-	-	+	-	-	-	-	+	-
	MP96	-	-	+	-	-	-	-	+	-
	HP96	-	-	+	-	-	-	-	+	-

[a]LP, MP, and HP referred to less, moderate, and high pathogenic isolates, respectively.
[b]Number followed those abbreviations represented the incubation period (h).
[c]Symbol '+' indicated samples were positively detected by corresponding primer sets with a single band.
[d]Symbol '–' indicated samples were negatively detected by corresponding primer sets.
[e]Symbol '++' indicated samples were positively detected by corresponding primer sets with a double band.

pathogenic fungi (Kuzdraliński et al. 2017). The measurement of fluorescence in single PCR well by qPCR produced higher sensitivity than staining and gel-based detection of conventional PCR products under ultraviolet light (Xia et al. 2018).

The insight of different pathogenic level from various geographical origins might be beneficial as basic judgement in preventing the transportation of cocoa seedlings or seeds from or to different pathogenic level areas in Indonesia. Meanwhile, the understanding of expressed pathogenicity-related genes could be helpful as principle information to develop resistant clones of cacao having plant defense genes for modulating those pathogenicity genes.

The discovery of pathogenicity grouping in the current project demonstrated that their pathogenic level was independent of previous groupings (morphometric variation and genetic diversity) as well as geographical origins and mating types (Motulo 2008; Masanto et al. 2019a, 2019b). However, high pathogenic isolates consistently belonged to A2 mating types (Table 2). This phenomenon did not comply with the research of Motulo (2008), reporting that high pathogenic *P. palmivora* isolates from cacao and coconut were A1- and A2-mating types, respectively.

The independence of pathogenic level in *P. palmivora* on geographical areas for current work was similar to the report of Rubiyo and Sukamto (2008). These findings indicated cross-transportation and wide-spread of various host plants for *P. palmivora* in Indonesia.

The use of model fruit for the pathogenicity test of *P. palmivora* was also performed on prior studies (Torres-Londono 2016; Azni et al. 2017). This phenomenon was common due to its wide variety of host plants and its capability in cross-infection. Apple was noted as one of the host plants for *P. palmivora* (Erwin and Ribeiro 1996). Slamet (1991) reported that *P. palmivora* isolates from cacao could generate small and medium spot symptoms on coconut and black pepper, respectively.

The utilization of model plant (*Nicotiana benthamiana*) in the transcriptomic study of *P. palmivora* had been also reported (Khunjan et al. 2016; Evangelisti et al. 2017). This plant was preferred due to its susceptibility to a wide range of plant pathogenic agents including oomycetes (Goodin et al. 2008).

This research detected eight genes (Table 4), which were commonly and potentially involved in the pathogenesis of other *Phytophthora* species (Tyler et al. 2006). The expression of four genes in this study was also parallel to that of Ali et al. (2017), finding that the *RXLR* and pectinase genes families were specifically induced in planta experiment.

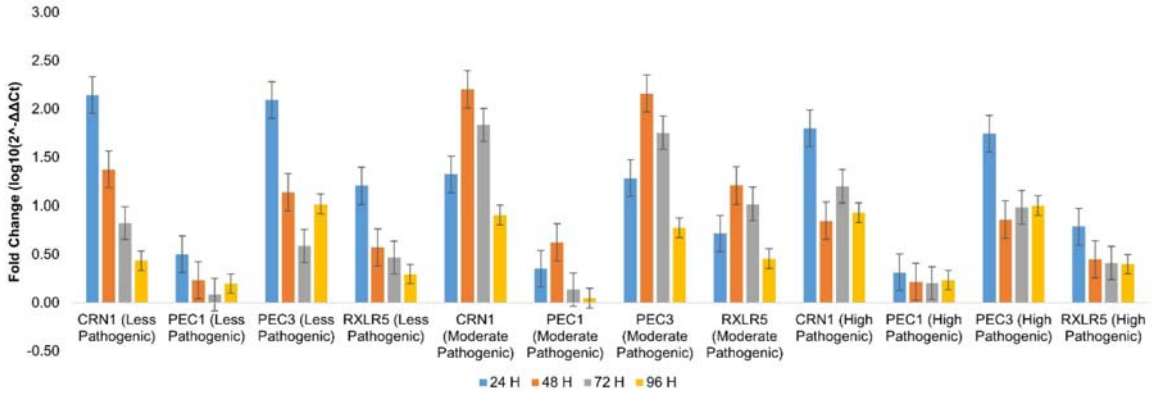

Figure 1. Relative expression of pathogenicity-related genes in less, moderate, and high pathogenic isolates of *Phytophthora palmivora* for 4 dpi.

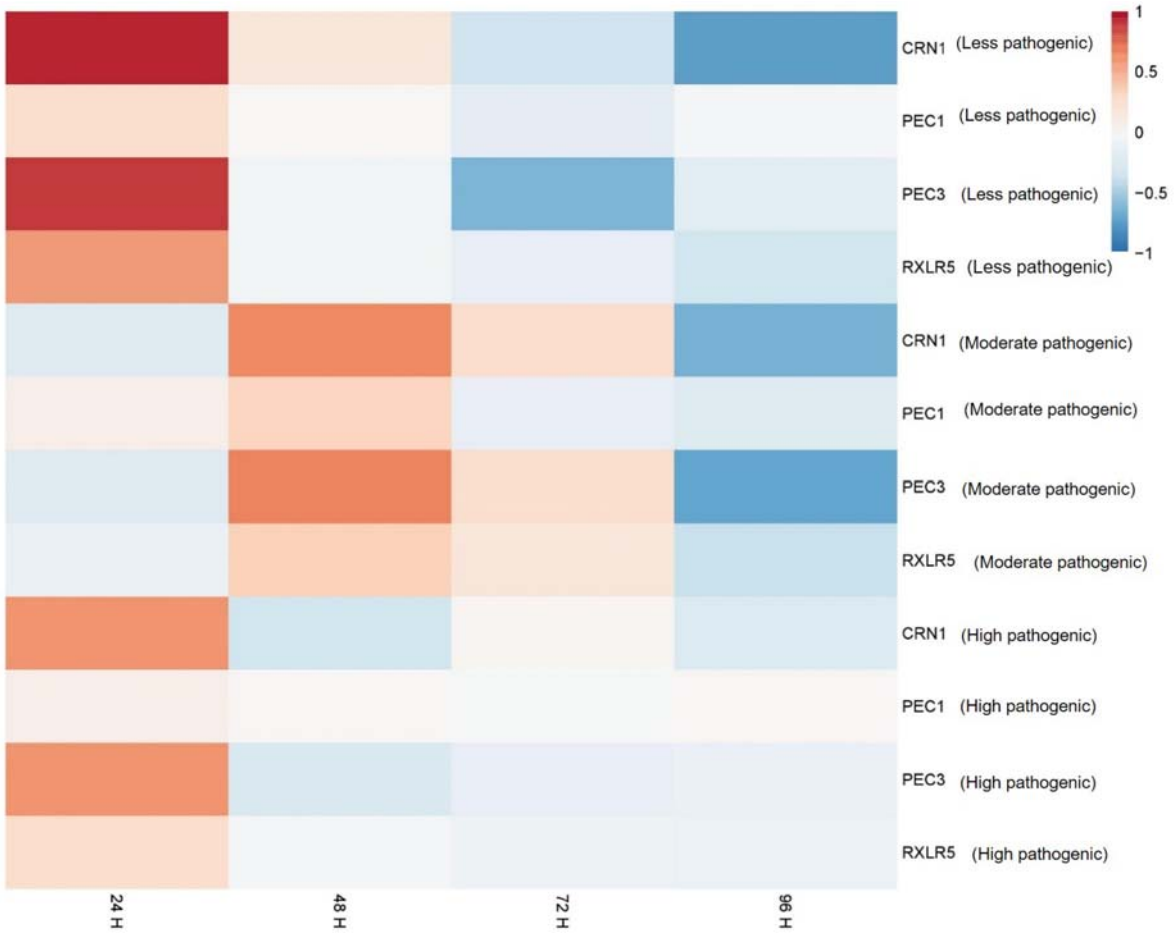

Figure 2. Heatmap comparison of expressed pathogenicity-related genes in less, moderate, and high pathogenic isolates of *Phytophthora palmivora* for 4 dpi.

The *CRNs* were first discovered in *P. infestans* (Torto et al. 2003) and known as a major class of well-studied cytoplasmic effectors in *Phytophthora* species (Jiang et al. 2008). Previously, Ali et al. (2017) found that mostly constitutive expression was revealed by *CRNs*. A fluctuating expression of *CRN* domains from initial observation until 72 h post-infection (Figures 1 and 2) was also documented in *P. capsici* infecting tomato (Stam et al. 2013). The lowest expression of *CRN* effectors at 96 h post-infection in this study was also found in *P. infestans* (Zuluaga et al. 2016). Meanwhile, the highest expression of *CRN* proteins in this experiment was supported by Judelson (2007) summarizing their induction on crinkling and necrosis of *N. benthamiana*.

The delay expression of pathogenicity-related genes by moderate pathogenic isolates in this research was assumed as the general pathway of pathogenicity mechanisms in *P. palmivora*, since prior screening assay on pathogenic level recorded that 49 of 55 isolates were categorized into moderate pathogenic. As the common category, moderate pathogenic isolates were documented in Sumatra, Java, Sulawesi, Kalimantan, as well as Papua islands and dominated by A2-mating type isolates. Prior research of Motulo (2008) revealed that moderate pathogenic isolates of *P. palmivora* from coconut and cacao were predominated A1- and A2-mating types, respectively.

The explanation for fluctuating expression of pectinase genes in current work might be due to the interference of certain factors. Akinferon (1968– 1969) had successfully identified suitable media as well as nutritional (carbon and nitrogen sources) and extrinsic (incubation period, temperature, and pH) factors affecting the in vitro production of extracellular pectolytic enzymes by *P. palmivora*.

The high expression of pathogenicity-related genes in less pathogenic isolates exhibited that their maximum efforts still did not generate the severe symptoms like moderate and high pathogenic isolates did. The generated final disease severity and infection rate of tested less pathogenic isolate (WNO1) at five days incubation were 33.33% and 0.17, compared to that of moderate pathogenic with 40% and 0.21, respectively (Table 4). The unique phenomena on less pathogenic isolates suggested the role of other factors in alternating the common mode of action in the pathogenicity of *P. palmivora*. One of them could be sexual characteristics. Two of three less pathogenic isolates were characterized with A1-mating types in our prior study (Masanto et al. 2019a). Previously, Motulo (2008) found that less pathogenic isolates of *P. palmivora* from cacao and coconut were A2- and A1-mating types, respectively.

The small expression of pathogenicity-related genes on high pathogenic isolates indicated how extremely pathogenic they were in affecting severe disease on the plant. These isolates were recorded causing the maximum disease severity of 60% within five days incubation with the highest infection rate of 0.45. Such data might illustrate distinct transcriptomic characteristics of pathogenicity-related genes in high pathogenic isolates that should be further studied.

Our previous reports grouped the representative isolates for this in planta experiment into clusters I and II of morphometric variations with A1- and A2-mating types for

less and moderate as well as high pathogenic, respectively (Masanto et al. 2019a), whereas their genetic diversity was categorized into groups I and III using microsatellite and repetitive markers, respectively (Masanto et al. 2019b). Those were large groups comprising most of the isolates from Java island (Masanto et al. 2019a, 2019b). Such information might provide the consistent relationship of representative tested isolates in morphological, sexual, and genetic aspects. Therefore, further genomic characteristics of those isolates could be investigated in future study.

The upregulated expression of *RXLR* effectors at 24 and 48 h post-infection was similar to the previous research of *P. palmivora* (Carella et al. 2018). However, they were detected earlier, i.e. 0 and 3 h as well as 12 h after inoculation on *P. infestans* (Schoina et al. 2019) and *P. sojae* (Wang et al. 2011), respectively. *P. palmivora* isolated from infected papaya showed the high expression of *PpalEPICs* genes at first- and second-day post-inoculation (Gumtow et al. 2018). Meanwhile, life stage-related genes (such as *Hmp1* and *Cdc14*) of *P. palmivora* isolated from oil palm and mango were expressed at 3 h as well as 24 and 48 h after inoculation (Le Fevre et al. 2016; Evangelisti et al. 2017; Carella et al. 2018). These different findings illustrated that the time expression of genes was various with the type of genes, host plants, and *Phytophthora* species.

The variation in expression of pathogenicity-related genes of *P. palmivora* might be due to the difference in their specific roles as hemibiotrophic plant–pathogen. It was assumed to generate gene products for concealing the responses of plant defense during the process of obtaining nutrients from living tissue (biotrophic stage) and deliver those for defeating plant tissue during its necrotrophic phase (Le Fevre et al. 2016; Ali et al. 2017). Such the last stage for *P. infestans* was observed at 144 h (6 dpi) (Zuluaga et al. 2016). Therefore, further study on such phenomenon in *P. palmivora* is required.

The *RXLR* and *CRN* effectors were the most notable cytoplasmic proteins, which were translocated into the plant cell (Bozkurt et al. 2012). The *RXLR* effector contributed to pathogenicity as both activator and suppressor of plant immunity (Hardham and Cahill 2010; Oh et al. 2010; Anderson et al. 2015) by preventing the secretion of the immune protease and triggering cell death on the host plant (Bozkurt et al. 2011). One of the responses in plant immunity against such effectors was the biosynthesis of defense-related enzymes and secondary metabolites (Deenamo et al. 2018). However, the current study did not investigate such response in tested plant. Such presumptions and other modes of action, as well as the appearance of plant response against pathogenicity-related genes in *P. palmivora*, might be investigated intensively in future experiment.

The *RXLR* effectors had rapid evolutionary rates facilitating the modulation of oomycetes on the host process during infection (Lamour et al. 2007), characterized by nonpathogenic activity on plant species with cognate R genes (Morgan and Kamoun 2007; Aguilera-Galvez et al. 2015), and were presumed to have a common evolutionary origin for oomycetes (Dou et al. 2008). They were recognized as the largest and most diverse family of pathogenicity-related genes in *P. palmivora* (Ali et al. 2017). They were, respectively, expressed with 61 (from 137 putative gene models/PgeneMs) and 58 (162 PgeneMs) (Ali et al. 2017). The given information might support their consistent expression in present work.

The expression of most pectinase gene families (*Pec1* and *Pec3*) in this research indicated the role of pectinase in breaking down pectin and confirmed the previous report of Spence (1961) investigating the role of pectolytic enzymes causing the blackening rot of cocoa pod. It was considered as a component of plant primary cell wall and middle lamella as well as affected much broader host range of *P. palmivora* (Ali et al. 2017). These CWDEs were produced during the early infection of pathogen on the plant (D'Ovidio et al. 2004). Among 72 expressed pectinases of 100 PgeneMs for predicted pectinases in *P. palmivora*, 52 proteins were reported to estimate secretion signal peptides (Ali et al. 2017).

The unexpressed pathogenicity-related genes were probably due to different used host plants (Judelson 2012). Other apoplastic effectors, Kazal-like extracellular protease inhibitor, were induced from *P. palmivora* infecting *Hevea brasiliensis* (Ekchaweng et al. 2017). This research employed a model plant of *N. benthamiana*, while the referred experiment of Ali et al. (2017) directly applied cocoa pod.

In addition to those eight pathogenicity-related genes, there were some other genes having a role in the pathogenicity of *P. palmivora* on other host plants. This research studied the expression of pathogenicity-related genes representing eight groups of *P. palmivora* on cacao reported by Ali et al. (2017). Other pathogenicity-related genes of *P. palmivora* were *PpEPI10* (Kazal-like extracellular protease inhibitor 10, an apoplastic effector suppressing the protease of *Hevea brasiliensis*) (Ekchaweng et al. 2017) and *PpalEPICs* (extracellular cystatin-like cysteine protease inhibitors) (Gumtow et al. 2018). *P. palmivora* also had life stage-related genes, such as *PpHmp1* and *PpCdc1* genes relating to haustoria membrane protein and sporulation-specific cell cycle, respectively (Khunjan et al. 2016; Le Fevre et al. 2016; Carella et al. 2018). The presence and expression of those genes in *P. palmivora* isolated from cacao are important to investigate in future study.

The *NPPs* were produced under plant–pathogen interaction during the alteration from the biotrophic to necrotrophic stages (Fellbrich et al. 2002). However, this experiment did not record their expression possibly due to requiring more incubation time for the shift of *P. palmivora* from biotrophic to necrotrophic phases.

Distinct expression was revealed by *Eli1* (elicitin). It was noted to be negative in the correlation between in vitro production and the pathogenicity of *Phytophthora* isolates (Yu 1995) with little or no secretion in pathogenic isolates of *Phytophthora* spp. on tobacco (Grant et al. 1996). Khairum et al. (2018) presumed that its optimum production depended on certain medium in vitro condition. Similarly, Ali et al. (2017) reported that the elicitin gene family did not also express a preferential pattern. Meanwhile, the unexpressed *RXLR1* genes were presumably due to suppression by avirulence activities of *RXLR5* effectors in which this phenomenon was also observed by Wang et al. (2011) in *P. sojae*. The intrinsic disorder as common characteristics of oomycete RXLR proteins might also affect their expression in this research (Shen et al. 2017).

These findings could provide a valuable information for molecular plant–microbe interaction. Whisson et al. (2007) summarized that one of the most important unanswered problems in plant pathology was the mechanism of plant pathogenic fungi and oomycetes in manipulating host metabolism and defense for establishing disease. Various

expression of pathogenicity-related genes of *P. palmivora* in this study agreed with the points of Kamoun (2006) summarizing a complex array of effector proteins in plant pathogenic oomycetes for modulating host cell defenses.

5. Conclusions

The present study categorized *P. palmivora* isolates in Indonesia into three pathogenic level, i.e. less, moderate, and high pathogenic according to lesion or necrotic area, disease severity, and infection rate. Despite eight pathogenicity-related genes were able to be detected on those isolates using the PCR technique, only four genes were expressed under in planta test on model plant. The delay expression on the second-day post-inoculation of those genes was assumed as the common pathogenicity pathway in this pathogen compared to that of the earlier expression of those genes on the first day after inoculation of less and high pathogenic isolates. The insight on the important role of those expressed genes may be investigated in the future, especially on the main host plant, in order to develop resistance clones of cacao against *P. palmivora* in Indonesia.

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Disclosure statement

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Author contributions

Masanto, A. Wibowo, and S. Subandiyah designed and planned the experiments. Masanto, N.F. Ridwan, and W.D. Sawitri performed on the technical experiments and analyzed the data. Masanto drafted the manuscript. A. Wibowo, W.D. Sawitri, K. Kageyama, and S. Subandiyah performed editing and corrections as well as approved the final version of manuscript before submitted by S. Subandiyah.

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