

TABLE OF CONTENTS

	Page
TITLE PAGE	i
ENDORSEMENT PAGE	ii
STATEMENT PAGE	iii
PREFACE	iv
TABLE OF CONTENTS	vi
LIST OF TABLES	x
LIST OF FIGURES	xii
LIST OF APPENDICES	xiii
ABSTRACT	xiv
INTISARI	xv
 CHAPTER	
I. INTRODUCTION	
A. Background	1
B. Statement of Problems.....	6
C. General Objectives.....	8
D. Expected Outcomes.....	9
E. Originality.....	9
 II. LITERATURE REVIEW AND THEORICAL BASIS	
A. Literature Review	11
1. Cacao	11
1.1. History	11
1.2. Current Status	12
1.3. Taxonomy and Botany	12
1.3.1. Taxonomy	12
1.3.2. Botany	13
1.4. Ecology	14
1.5. Importance of Economy.....	15
2. Black Pod Rot Disease	16
3. <i>Phytophthora palmivora</i>	18
4. Heterothallism in <i>P. palmivora</i>	23
5. Genetic Diversity of <i>P. palmivora</i>	25

6. Virulence Level of <i>P. palmivora</i>	26
7. Virulence-Related Genes of <i>P. palmivora</i>	27
8. Current Management Strategies for <i>P. palmivora</i> on Cacao.....	27
9. Indigenous Antagonistic Bacteria.....	29
B. Theory Basis	31
C. Hypothesis	32

III. RESEARCH METHODOLOGY

A. Materials and Equipment	34
B. Time and Location	39
C. Procedures.....	39
1. Experiment 1 (Morphological and molecular characteristics of <i>P. palmivora</i>).....	39
1.1. Sampling of diseased cocoa pods.....	39
1.2. Isolation of plant pathogenic <i>Phytophthora</i> isolates.....	39
1.3. Asexual characteristics of <i>Phytophthora</i> isolates.....	40
1.4. DNA extraction of <i>Phytophthora</i> isolates.....	40
1.5. Molecular identification of <i>Phytophthora palmivora</i>	40
1.6. Sexual characteristics of <i>Phytophthora palmivora</i>	44
1.7. Data analysis	44
2. Experiment 2 (Genetic diversity of <i>Phytophthora palmivora</i>).....	44
2.1. Analysis of genetic diversity using rep-PCR technique.....	44
2.2. Analysis of genetic diversity using microsatellite markers.....	45
2.2.1. Screening of primer sets.....	45
2.2.2. Sequence analysis.....	46
2.2.3. Fragment analysis.....	48
2.3. Data analysis.....	49
3. Experiment 3 (Expression of virulence-related genes of <i>P. palmivora</i>).....	50
3.1. Virulence test of <i>P. palmivora</i> on apple fruits.....	50
3.2. Detection of virulence-related genes in genomic DNA of <i>P. palmivora</i> isolates using PCR technique	51
3.3. <i>In planta</i> test for inoculation of <i>P. palmivora</i> on a model plant	52
3.4. RNA extraction	54
3.5. Expression of virulence-related genes of <i>P. palmivora</i> using	

conventional PCR method	55
3.5. Analysis of qPCR	55
3.6. Data analysis	55
4. Experiment 4 (Exploration of endophytic bacteria on healthy cocoa pod)	56
4.1. Sampling of healthy cocoa pods.....	56
4.2. Isolation of potential indigenous endophytic bacteria	56
4.3. Antagonistic test using dual culture method.....	56
4.4. DNA extraction of antagonistic bacteria.....	57
4.5. Clustering of antagonistic bacteria using rep-PCR technique	58
4.6. Double layer test.....	59
4.7. Cytological assay of antagonistic activity under Scanning Electron Microscope (SEM).....	59
4.8. Molecular identification of representative antagonistic bacteria	59
4.9. <i>In vivo</i> antagonistic test	60
4.10. Data analysis	61
D. Data Collection	62

IV. RESULTS

1. Experiment 1 (Morphological and molecular characteristics of <i>P. palmivora</i>)	64
1.1. Sampling of diseased cocoa pods.....	64
1.2. Isolation of plant pathogenic <i>Phytophthora</i> isolates.....	64
1.3. Asexual characteristics of <i>Phytophthora</i> isolates.....	68
1.4. Molecular identification of <i>Phytophthora palmivora</i>	69
1.5. Sexual characteristics of <i>Phytophthora palmivora</i>	74
1.6. Morphometric variation	74
2. Experiment 2 (Genetic diversity of <i>Phytophthora palmivora</i>)	79
2.1. Analysis of genetic diversity using rep-PCR technique	79
2.2. Analysis of genetic diversity using microsatellite markers.....	83
3. Experiment 3 (Expression of virulence genes of <i>Phytophthora palmivora</i>)	88
3.1. Virulence test of <i>Phytophthora palmivora</i> on apple fruits.....	88
3.2. Detection of virulence-related genes in genomic DNA of <i>P. palmivora</i> isolates using PCR technique	94

3.3. <i>In planta</i> test and expression of virulence-related genes of <i>Phytophthora palmivora</i> on inoculated model plant	94
3.4. Analysis of qPCR.....	94
4. Experiment 4 (Exploration of endophytic bacteria on healthy cocoa pod)	100
4.1. Sampling of healthy cocoa pods.....	100
4.2. Isolation of potential indigenous endophytic bacteria	101
4.3. Antagonistic test using dual culture method.....	105
4.4. Clustering of antagonistic bacteria using rep-PCR technique	110
4.5. Double layer test.....	114
4.6. Cytological assay of antagonistic activity under Scanning Electron Microscope (SEM).....	118
4.7. Molecular identification of representative antagonistic bacteria	120
4.8. <i>In vivo</i> antagonistic test	123
V. DISCUSSION	125
V. CONCLUSION AND SUGGESTIONS	153
A. CONCLUSION	153
B. SUGGESTIONS	153
SUMMARY	155
RINGKASAN	157
REFERENCES.....	159
APPENDICES A	190
APPENDICES B	196
APPENDICES C	222

LIST OF TABLES

	Page
Table 1.1 Indonesian export from fresh and processed cocoa products	2
Table 2.1 Beneficial factors supporting the successfulness of <i>P. palmivora</i> as most important plant pathogen in tropical regions	22
Table 3.1 Required materials and equipment corresponding to research activities	35
Table 3.2 Primer sets used for identification of pathogenic agent, rep-PCR analysis and identification of antagonistic bacteria.....	42
Table 3.3 Degenerate primers used for screening step of microsatellite.....	45
Table 3.4 Criteria for determining the virulence level of <i>P. palmivora</i>	51
Table 3.5 Primer sets corresponding to virulence-related genes of <i>P. palmivora</i>	53
Table 3.6 Collected data corresponding to research activities	62
Table 4.1 The isolates of <i>Phytophthora</i> collected in this experiment 1.....	64
Table 4.2 Asexual features of isolates collected from cocoa pod rots on cacao growing areas in Indonesia	70
Table 4.3 Sexual structures of isolates collected from cocoa pod rots on cacao growing areas in Indonesia	75
Table 4.4 List of <i>P. palmivora</i> isolates at RBRC collection used in this experiment 2	80
Table 4.5 List of degenerated SSR primers prior to selection of single thick PCR bands	84
Table 4.6 Performance of bands from selected colonies of cloned <i>E. coli</i>	85
Table 4.7 Results of sequence analysis on seven selected primers from cloned <i>E. coli</i>	85
Table 4.8 Virulence test of <i>Phytophthora palmivora</i> on six varieties of apple fruits	88
Table 4.9 Virulence test of all isolates of <i>Phytophthora palmivora</i> on selected apple fruits	90
Table 4.10 Detection of virulence-related genes in genomic DNA of <i>Phytophthora palmivora</i> isolates using PCR technique	95
Table 4.11 Expression of virulence-related genes of <i>P. palmivora</i> on inoculated <i>Nicotiana benthamiana</i> using conventional PCR method	98
Table 4.12 Exploration of endophytic bacteria from healthy cocoa pod in Indonesia	102
Table 4.13 <i>In vitro</i> screening of potential antagonistic bacteria isolates	106
Table 4.14 Selected isolates for further clustering using rep-PCR technique.....	107
Table 4.15 Selected representative bacterial isolates from each cluster for further <i>in vitro</i> antagonistic test against <i>P. palmivora</i>	112
Table 4.16 Double layer test of selected potential antagonistic bacteria isolates against <i>Phytophthora palmivora</i>	115
Table 4.17 Molecular identification of representative antagonistic bacterial isolates corresponding consistent inhibition using 16S rRNA and <i>gyrB</i> genes	122
Table 4.18 <i>In vivo</i> assay on inhibition of black pod rot disease on detached cocoa pod with the application of potential antagonistic bacteria	124
Table 5.1 Comparison of asexual features and sexual structures of <i>Phytophthora palmivora</i> among several studies	127
Table 5.2 Group of isolates corresponding to asexual features, mating type and sexual structures	129
Table 5.3 Transmovement of cacao seeds/seedlings in Indonesia (2013-2017)	134

Table 5.4	Comparison of primer sets used for genetic diversity analysis of <i>P. palmivora</i> in this study	139
Table 5.5	Comparison of documented inhibitory percentage on similar previous reports with this study	149

LIST OF FIGURES

	Page
Figure 1.1 The overall volume and value of cocoa export from Indonesia during seven consecutive years (2012-2018)	3
Figure 1.2 The acreage and production of cacao in Indonesia during seven consecutive years (2012-2018)	3
Figure 3.1. Diagram for determination of R1 and R2 under dual culture method of antagonistic test	57
Figure 4.1 Symptoms of black rot disease on cocoa pod	64
Figure 4.2 Geographical areas for sampling of <i>Phytophthora palmivora</i> in Indonesia	67
Figure 4.3 Colony performance of <i>Phytophthora</i> isolated from symptomatic cocoa pod rot on solid agar	68
Figure 4.4 Asexual characteristics of <i>Phytophthora</i> isolated from symptomatic cocoa pod rot in cacao growing areas of Indonesia	68
Figure 4.5 Performance of DNA bands from all <i>Phytophthora</i> isolates under molecular assay using multiplex PCR method with species-specific primers at 150 bp in size	73
Figure 4.6 Sexual structures produced by representative <i>Phytophthora palmivora</i> isolates from different mating types.....	74
Figure 4.7 Dendrogram of clustering the <i>Phytophthora palmivora</i> isolates based on variation of asexual and sexual features	77
Figure 4.8 Principle component analysis clustering <i>P. palmivora</i> isolates based on variation of asexual and sexual features	78
Figure 4.9 Geographical areas for sampling of <i>P. palmivora</i> in Japan	82
Figure 4.10 Phylogenetic tree degenerated from combination of rep-PCR primers	83
Figure 4.11 Phylogenetic tree degenerated from microsatellite markers	87
Figure 4.12 Relative expression of virulence-related genes in low, moderate and high virulent isolates of <i>Phytophthora palmivora</i> for 4 days post inoculation	99
Figure 4.13 Heatmap comparison of expressed virulence-related genes in low, moderate and high virulent isolates of <i>Phytophthora palmivora</i> for 4 days post inoculation.....	100
Figure 4.14 Healthy cocoa pods for exploration of endophytic bacteria	101
Figure 4.15 Performance of explored bacterial colony corresponding to serial dilution	101
Figure 4.16 The inhibition of mycelial growth of <i>Phytophthora palmivora</i> under dual culture test using the explored bacterial isolates	105
Figure 4.17 Colony performance of antagonistic bacteria on Nutrient Agar (NA) media isolated from healthy cocoa pods	106
Figure 4.18 Dendrogram of genetic diversity with rep-PCR elements clustering the potential antagonistic of endophytic bacteria after <i>in vitro</i> dual-culture test	111
Figure 4.19 Scanning electron micrographs visualizing antagonistic activity of endophytic bacteria against <i>P. palmivora</i>	119
Figure 4.20 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	121

LIST OF APPENDICES

	Page
Appendices A Supplementary figures supporting the research	190
Appendices B Supplementary data supporting the research	196
Appendices C Preparation of solutions and instructions supporting the research....	222