



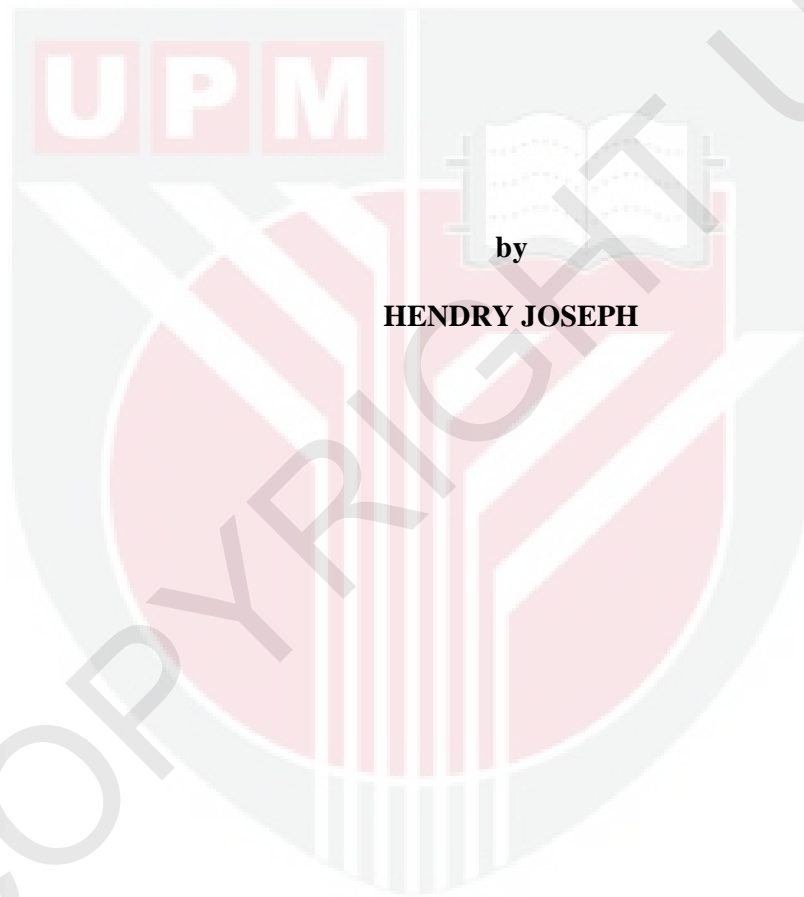
UNIVERSITI PUTRA MALAYSIA

**CHARACTERIZATION AND PATHOGENICITY OF *COCONUT*
CADANGCADANG VIROID VARIANTS IN OIL PALM
(*Elaeis guineensis* Jacq.) SEEDLINGS**

HENDRY JOSEPH

ITA 2012 1

**CHARACTERIZATION AND PATHOGENICITY OF COCONUT CADANG-
CADANG VIROID VARIANTS IN OIL PALM (*Elaeis guineensis* Jacq.)
SEEDLINGS**



by

HENDRY JOSEPH

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirements for the Degree of
Doctor of Philosophy**

January 2012

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for Doctor of Philosophy

CHARACTERIZATION AND PATHOGENICITY OF *COCONUT CADANG-CADANG VIROID* VARIANTS IN OIL PALM (*Elaeis guineensis* Jacq.) SEEDLINGS

By

HENDRY JOSEPH

January 2012

Chairman : Ganesan Vadamalai, PhD

Institute : Tropical Agriculture

Coconut cadang-cadang viroid is considered a threat to the oil palm industry. It is associated with an orange spotting (OS) disorder in oil palm but its pathogenicity has never been confirmed. Therefore this study was to establish Koch's postulate of CCCVd variants characterized from oil palm. Detection of CCCVd-like RNAs in oil palm was enhanced with optimized Sodium Chloride EDTA Tris-HCL mercaptoethanol (NETME) extraction and dot blot hybridization using full-length digoxigenin (DIG)-labelled CCCVd₂₄₆ cRNA probe. Nucleic acid sequenced from a known CCCVd positive symptomatic (SRD6) and asymptomatic (SRH2) oil palm from Selangor contained a variant of 246 nt (CCCVd OP₂₄₆). The presence of oil palm CCCVd variant was also confirmed in Sabah when a symptomatic oil palm (SBH4) was found with CCCVd OP₂₄₆. Sequence analysis showed that CCCVd OP₂₄₆ obtained from Selangor (SRD6, SRH2) and Sabah (SBH4) were identical and

had 97% sequence similarity with CCCVd₂₄₆ of coconut. Partially purified nucleic acid extract of SRD6 and SRH2 were inoculated into oil palm pre-germinated seedlings and tissue culture plantlets. CCCVd-like RNAs were detected by dot blot hybridization, six months after inoculation in all pre-germinated seedlings and in tissue culture plantlets except for seedling P2-5 (pre-germinated seedling) and control (Milli-Q water inoculated) seedlings and plantlets. Pre-germinated seedlings and tissue culture plantlets that were inoculated with SRD6 nucleic acid extract were shorter with more fronds compared to those inoculated with SRH2 nucleic acid extract and control (Milli-Q water). All ten pre-germinated seedlings inoculated with SRD6 nucleic acid extract showed OS symptom on the leaf, 9 months after inoculation meanwhile 2 of 10 pre-germinated seedlings inoculated with SRH2 nucleic acid extract showed OS symptoms at 12 months after inoculation. All three tissue culture plantlets that were inoculated with SRD6 nucleic acid showed OS symptom on the leaf at 6 months after inoculation Meanwhile all five tissue culture plantlets inoculated with SRH2 nucleic acid extract did not show any OS symptom, raising the possibility of host resistance against CCCVd variants especially in tissue culture plantlets as pre-germinated seedlings inoculated with SRH2 nucleic acid extract showed OS symptoms in two seedlings. Symptom appearance was faster in tissue culture plantlet compared to pre-germinated seedling. RT-PCR, cloning and sequencing at 12 months after inoculation revealed that the old and young frond of the inoculated oil palm pre-germinated seedlings and tissue culture plantlets contained CCCVd OP₂₄₆ identical in sequence to the symptomatic (SRD6) and asymptomatic (SRH2) oil palms from the field that were used as inoculums in this study, therefore fulfilling Koch's postulate. Lack of sequence variation between field and inoculated CCCVd OP₂₄₆ from both symptomatic (SRD6) and asymptomatic

(SRH2) oil palms suggests that sequence variation may not be vital in symptom expression of CCCVd oil palm variant. This reports for the first time that oil palm CCCVd OP₂₄₆ is the causal agent of OS in oil palm and replicates autonomously in its host either expressing symptoms or in a symptomless manner.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk mendapat Ijazah Doktor Falsafah

PENCIRIAN DAN KEPATOGENAN VARIAN-VARIAN *COCONUT CADANG-CADANG VIROID* PADA ANAK-ANAK BENIH KELAPA SAWIT (*Elaeis guineensis* Jacq.)

Oleh

HENDRY JOSEPH

Januari 2012

Pengerusi : Ganesan Vadamalai, PhD

Institut : Pertanian Tropika

Coconut cadang-cadang viroid dianggap ancaman kepada industri kelapa sawit. Ia dikaitkan dengan penyakit *orange spotting (OS)* kelapa sawit tetapi kepatogennanya belum disahkan oleh itu kajian ini bertujuan untuk mendirikan dalil Koch ke atas anak benih sawit menggunakan varian CCCVd OP₂₄₆ yang dicirikan daripada kelapa sawit. Pengesanan kehadiran RNA menyerupai CCCVd dipertingkatkan dengan pengoptimuman kaedah pengekstrakan Natrium Chloride EDTA Tris-HCL Mercaptoethanol (NETME) dan dot blot hybridization menggunakan *full-length digoxigenin (DIG)-labelled CCCVd₂₄₆ cRNA probe*. Penjujukan DNA mencirikan asid nukleik daripada pokok sawit bersimtom (SRD6) dan tidak bersimtom (SRH2) daripada Selangor sebagai CCCVd OP₂₄₆. Kehadiran varian CCCVd di Sabah disahkan apabila pokok sawit bersimtom (SBH4) didapati mengandungi CCCVd

OP₂₄₆. Analisa jujukan menunjukkan bahawa jujukan CCCVd OP₂₄₆ daripada SRD6, SRH2 dan SBH4 adalah seiras dan mempunyai 97% persamaan dengan CCCVd₂₄₆ pokok kelapa. Ekstrak asid nukleik separa tulen daripada pokok sawit SRD6 dan SRH2 digunakan sebagai inokulum di dalam kajian patogenesis ini dan diinokulat pada anak benih sawit dan planlet kultur tisu menggunakan penyuntik tekanan tinggi. RNA menyerupai CCCVd dikesan terkandung didalam kesemua anak benih sawit dan planlet kultur tisu dengan *dot blot hybridization* 6 bulan selepas diinokulasi kecuali P2-5 (anak benih sawit) serta anak benih sawit dan planlet kultur tisu kawalan (diinokulat dengan air Milli-Q). Anak benih sawit dan planlet kultur tisu yang diinokulat dengan asid nukleik pokok sawit SRD6 agak terbantut dengan jumlah pelepah lebih banyak berbanding dengan yang diinokulat dengan asid nukleik pokok sawit SRH2 dan air Milli-Q. Kesemua sepuluh anak benih sawit diinokulat dengan asid nukleik pokok sawit SRD6 menunjukkan simptom OS pada daun 9 bulan selepas diinokulasi manakala 2 daripada 10 anak benih sawit diinokulat dengan asid nukleik pokok sawit SRH2 menunjukkan simptom OS 12 bulan selepas diinokulasi. Ketiga-tiga planlet kultur tisu diinokulat dengan asid nukleik pokok sawit SRD6 menunjukkan simptom OS 6 bulan selepas diinokulasi. Manakala, kesemua 5 planlet kultur tisu diinokulat dengan asid nukleik pokok sawit SRH2 tidak menunjukkan sebarang simptom OS menyingkap kemungkinan wujud kerintangan hos terutamanya oleh planlet kultur tisu kerana terdapat 2 anak benih sawit yang diinokulat dengan asid nukleik pokok sawit SRH2 menunjukkan simptom OS. Didapati simptom OS muncul lebih cepat pada planlet kultur tisu berbanding anak benih sawit. Ujian RT-PCR, pengklonan dan penjujukan 12 bulan selepas diinokulasi menunjukkan bahawa pelepah tua dan muda anak benih sawit dan planlet kultur tisu mengandungi CCCVd OP₂₄₆ menyerupai jujukan pokok sawit bersimtom (SRD6) dan tidak bersimtom

(SRH2) dari ladang maka ini memenuhi dalil Koch. Ketiadaan variasi pada jujukan CCCVd OP₂₄₆ ladang dengan yang diinokulat daripada pokok sawit bersimtom (SRD6) dan tidak bersimtom (SRH2) menunjukkan variasi jujukan mungkin tidak berperanan dalam ekspresi simtom. Penemuan ini julungkalinya melaporkan bahawa varian CCCVd OP₂₄₆ kelapa sawit adalah agen penyebab kepada penyakit OS kelapa sawit dan membiak di dalam hos samada menunjukkan simtom ataupun tidak.



ACKNOWLEDGEMENTS

Thank you to God Almighty for giving me the strength to complete this study.

I wish to thank my supervisor, Dr. Ganesan Vadamalai for his support and guidance throughout my study, and also to my internal co-supervisory members Associate Professor Datin Dr. Siti Nor Akmar Abdullah and Dr. Lau Wei Hong for their assistance and for providing me a place to work at the early stage of my study. I am very grateful to my external co-supervisor Professor Dr. John W. Randles of the University of Adelaide, Australia for his invaluable comments, suggestions and training. I am indebted to Dr. Dagmar Hanold also from the University of Adelaide for her advice on the tissue culture part of my pathogenicity study. Not forgetting Associate Professor Dr. Uma Rani Sanniah and her postgraduate student, Mr. Suran from the Department of Crop Science, UPM for their kind help in getting tissue culture equipments and materials.

I am thankful to the Institute of Tropical Agriculture (ITA), UPM for providing postgraduate students conducive working environment. My acknowledgement to Professor Dr. Zulkifli Idrus (current Director of ITA), Professor Dr. Mohamed Hanafi Musa, Hj. Zahardin Zulkifli and all ITA central laboratory staffs for the excellent service and especially to Professor Dr. Sariah Meon (former Director of ITA) who have supported the CCCVd project. I am blessed to have Dr. Kong, Dr. Nagmeh, Ying Hooi, Li Chu, Reza, Sathis, Roslina, Aisyah, Joey and Sarina in Dr. Ganesan's viroid research group who had provided me the help, support and friendship that are much needed. To my caring lab mates, Lee Chuen, Carmen, Jacky, Tze Ling, Rani,

Hanif, Chee Yong and Roger, I will cherish all the good memories. Not forgetting Dr. Jose, Mr. Fabian, Dr. Sahidullah, Jainatul and all other international students, post-doctoral and fellow researchers for their international friendship.

I also wish to thank Dr. Hj. Abdul Kadir Hj. Rosline and Associate Professor Datuk Dr. Worrان Hj. Kabul (current and former Rector of Universiti Teknologi MARA Cawangan Sabah) and Dato' Professor Dr. Hj. Nasuddin Othman, Dean of Faculty of Plantation and Agrotechnology for their support. Thanks also to my colleagues in the Faculty of Plantation and Agrotechnology UiTM Sabah and Shah Alam namely Sabki, Alexius, Fitri, Hamjah, Viduriati, Hazmi, Hairiah, Norsidek and Hafezan for the support and encouragements. Not forgetting to all my ex-students who are now successful with their own careers. My special thanks to Dr. Geoffrey H. Tanakinjal, Peter Lintar, Florence Ginibun, Neni Kartini, David Moy and to all that I did not mention here for their friendship.

I am grateful to the Government of Malaysia for a scholarship, and Universiti Teknologi MARA my employer for granting me study leave and to Sime Darby Plantation and Sawit Kinabalu Bhd for providing the leaf materials and Dr. Idris Abu Seman of MPOB for his advice and support in sampling arrangement.

Indefinite thanks to my late father, mother, brothers, sisters and relatives for their never ending concern, love and support throughout my life journey. Last but not least, I am very grateful to my wife Ai Di Yen for her understanding, compassions and love and for taking care of our son Nathaniel Samson when I was away.

I certify that a Thesis Examination Committee has met on 6th January 2012 to conduct the final examination of Hendry Joseph on thesis entitled “**Characterization and Pathogenicity of Coconut cadang-cadang viroid Variants in Oil Palm (*Elaeis guineensis* Jacq.) Seedlings**” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15th March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Sariah Meon, PhD

Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Jugah Kadir, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Zainal Abidin Mior Ahmad, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Ricardo Flores, PhD

Professor
Universidad Politécnica de Valencia
Valencia, Spain
(External Examiner)

SEOW HENG FONG, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Ganesan Vadamalai, PhD

Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Datin Siti Nor Akmar Abdullah, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Lau Wei Hong, PhD

Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

John W. Randles, PhD

Professor
School of Agriculture, Food & Wine
University of Adelaide, Australia
(Member)

BUJANG BIN KIM HUAT, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

HENDRY JOSEPH

Date: 6th January 2012



TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	vi
ACKNOWLEDGEMENTS	viii
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xvi
LIST OF FIGURES	xviii
LIST OF ABBREVIATIONS	xxv
CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	4
2.1 Orange spotting	4
2.1.1 Symptoms and effect of growth and yield	5
2.1.2 Infectivity and spread	7
2.2 Viroids	7
2.2.1 Possible spread of viroids	9
2.2.2 Classification and structure	9
2.2.3 Replication	12
2.2.4 Movement	14
2.2.5 Transmission and symptomatology	14
2.2.6 Pathogenesis	15
2.2.7 Pathogenicity study	17
2.3 Coconut cadang-cadang viroid (CCCVd)	18
2.3.1 Variations of CCCVd molecules	18
2.4 CCCVd in oil palm	19
III ISOLATION AND CHARACTERIZATION OF CCCVd VARIANTS FROM OIL PALM	21
3.1 Introduction	21
3.2 Materials and Methods	23
3.2.1 Collection of samples	23
3.2.2 Nucleic acid extraction with NETME extraction method	23
3.2.3 Hybridization assay	24
3.2.4 Dot blot hybridization	25
3.2.5 Northern blot hybridization	25
3.2.6 Characterization with RT-PCR, cloning and sequencing	26
3.3 Results	33
3.3.1 Dot blot hybridization	33
3.3.2 Northern blot hybridization	34

3.3.3	Characterization with RT-PCR, cloning and sequencing	38
3.3.4	Sizes of cloned insert	45
3.3.5	Sequence analysis	45
3.4	Discussion	53
3.5	Conclusion	58

IV

	PATHOGENICITY AND THE RELATIONSHIP OF SEQUENCE VARIATION IN OIL PALM <i>COCONUT CADANG-CADANG VIROID</i> VARIANTS WITH REPLICATION AND SYMPTOM EXPRESSION IN OIL PALM SEEDLINGS	59
4.1	Introduction	59
4.2	Materials and Methods	62
4.2.1	Source of seeds for pathogenicity study on pre-germinated oil palm seedlings	62
4.2.2	Source of plantlets for pathogenicity study on oil palm tissue culture seedlings	62
4.2.3	CCCVd inoculum	63
4.2.4	Inoculation	64
4.2.5	Seedlings arrangement and data recording	67
4.2.6	Detection of CCCVd-like RNAs replication in the inoculated oil palm seedlings with dot blot hybridization	70
4.2.7	Characterization of CCCVd-like RNAs from the Inoculated oil palm seedlings	70
4.2.8	The relationship of sequence variations in CCCVd variants with replication and symptom expression in oil palm seedlings	71
4.3	Results	72
4.3.1	Data analysis of the inoculated pre-germinated oil palm seedlings	72
4.3.2	Data analysis of the inoculated oil palm tissue culture seedlings	74
4.3.3	Symptom appearance	75
4.3.4	Detection of CCCVd-like RNAs replication in the inoculated oil palm seedlings with dot blot hybridization	80
4.3.5	Characterization of CCCVd-like RNAs from the Inoculated Pre-germinated Oil Palm Seedlings	82
4.3.6	Characterization of CCCVd-like RNAs from the Inoculated Tissue Culture Oil Palm Seedlings	91
4.3.7	The relationship of sequence variation in oil palm CCCVd variants with replication and symptom expression in oil palm seedlings	100
4.4	Discussion	119
4.5	Conclusions	129

V	SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH	130
	REFERENCES	135
	APPENDICES	146
	BIODATA OF STUDENT	169
	LIST OF PUBLICATIONS	171

