UNIVERSITI PUTRA MALAYSIA

RAPID DETECTION, ACCUMULATION AND TRANSLOCATION OF COCONUT CADANG-CADANG VIROID VARIANTS IN OIL PALM

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ITA 2014 9

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By

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

September 2014

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

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September 2014

Chairman: Ganesan Vadamalai, PhD
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Coconut cadang-cadang viroid (CCCVd) is the causal agent of the lethal Coconut cadang-cadang disease in the Philippines. CCCVd variants were also found in Malaysian oil palm, but in low concentration and difficult to detect. Palms infected with CCCVd variants were associated with orange spotting (OS) disorder and it was estimated that the average yield from the OS-affected palms was 25-50% lower than the healthy palms. Existing diagnostic methods such as hybridization assay and Reverse transcription polymerase chain reaction (RT-PCR) methods are able to detect CCCVd variants in oil palms; however they are laborious, insensitive and time-consuming. In addition, the relationship between CCCVd sequence variation and viroid accumulation has not been studied. In view of this, the present research was undertaken to produce a simple and rapid detection of CCCVd variants using reverse transcription Loop-mediated isothermal amplification (RT-LAMP) method. Besides that, CCCVd accumulation between two different variants together with movement in inoculated oil palm seedlings was studied using real-time PCR. Simultaneously, the pathogenicity of the two different variants was observed in the inoculated seedlings. CCCVd variants were successfully detected in infected samples as early as 60 min at 60 °C using the primer C2.1. Positive reactions of RT-LAMP showed colour change from orange to green after addition of a fluorescent reagent. The RT-LAMP products generated a laddering pattern on 2% agarose gel electrophoresis with bands of different sizes. The optimal condition for RT-LAMP amplification of CCCVd RNAs from oil palm was at 60 °C for 60 min with 6.0 mM $MgSO_4$, 0.8 M betaine, 2.4 mM dNTPs, 1.6 µM of each inner primer FIP and BIP, 0.4 µM each of outer primer B3 and F3 and 8U *Bst* DNA polymerase. The sensitivity of both RT-PCR and RT-LAMP was found to be equal. The RT-LAMP primers were specific in detecting CCCVd since they did not amplify other viroids that were used in the specificity test. CCCVd were also detected in some of the random field samples collected. The BLAST program results showed that the amplified product of RT-LAMP was indeed a variant of $CCCVd_{246OP}$. For viroid accumulation study, two variants of CCCVd ($CCCVd_{246OP}$ and $CCCVd_{293OP}$) were inoculated into 10 three-

month old oil palm seedlings each, with 10 control seedlings. A RT-PCR and sequencing was carried out to characterise the CCCVd variants obtained from the inoculated seedlings at different time intervals. CCCVd was detected three months after inoculation in low concentrations. The viroid titre showed that the accumulation of the CCCVd variant in the inoculated seedlings fluctuated without any distinct increasing or decreasing pattern. The results of sequencing analysis showed that $CCCVd_{246OP}$ (Genbank: HQ608513.1) was characterised from seedlings inoculated with $CCCVd_{293OP}$ plasmids. In addition, a 246-nt sequence was also recovered from the symptomatic (OS) seedling with 99% sequence similarity to $CCCVd_{246OP}$, which confirmed the pathogenicity of this 246-nt CCCVd variant. In viroid movement study, the $CCCVd_{246OP}$ variant was inoculated into 12 seedlings. The seedlings were sampled every three months with three replicates; separating the leaves, stems and roots. CCCVd was detected in the leaves, stems and roots of the oil palm seedlings after three, six, nine and twelve months of inoculation. The quantification showed that CCCVd load varied in different parts of the seedlings, with higher concentrations in the stems and leaves as compared to the roots.

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

PENGESANAN PANTAS, PENGUMPULAN DAN TRANSLOKASI VARIAN *COCONUT CADANG-CADANG VIROID* DALAM KELAPA SAWIT

Oleh

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Coconut cadang-cadang viroid (CCCVd) merupakan ejen penyebab bagi penyakit *Coconut cadang-cadang* di Filipina. Varian CCCVd juga dijumpai di pokok kelapa sawit di Malaysia tetapi dalam kepekatan yang rendah dan sukar untuk dikesan. Pokok kelapa sawit yang dijangkiti oleh varian CCCVd yang dikaitkan dengan penyakit *orange spotting* (OS) dianggarkan memperolehi hasil purata sebanyak 25-30% lebih rendah berbanding pokok yang sihat. Kaedah diagnostik sedia ada seperti penghibridan dan *Reverse transcription polymerase chain reaction* (RT-PCR) berjaya mengesan varian CCCVd di kelapa sawit namun ianya rumit, kurang sensitif dan memakan masa.Tambahan pula, hubungan antara jujukan CCCVd dan pengumpulan viroid belum pernah dikaji. Oleh itu, kajian ini bertujuan untuk menghasilkan kaedah pengesanan varian CCCVd yang mudah dan pantas menggunakan *Reverse Transcription Loop-mediated isothermal amplification* (RT-LAMP). Selain itu, pengumpulan CCCVd diantara dua varian dan juga pergerakan CCCVd di dalam anak pokok kelapa sawit yang telah disuntik dikaji menggunakan kaedah *real-time PCR*. Pada masa yang sama, kepatogenan bagi dua varian tersebut dalam anak pokok yang disuntik dikaji. Varian CCCVd berjaya dikesan dalam sampel yang dijangkiti selepas 60 minit pada 60 °C menggunakan primer C2.1. Reaksi positif RT-LAMP menunjukkan perubahan warna dari jingga ke hijau setelah ditambah dengan reagen pendarfluor. Produk RT-LAMP yang dianalisis atas 2 % gel agarose menunjukkan *band* yang berbentuk tetangga dengan saiz yang berbeza. Kondisi optima RT-LAMP untuk mengesan varian CCCVd dari kelapa sawit adalah 6.0 mM $MgSO_4$, 0.8 M betaine, 2.4 mM dNTPs, 1.6 µM primer FIP dan BIP, 0.4 µM primer B3 dan F3 dan 8U *Bst* DNA polymerase. Tahap sensitiviti bagi RT-LAMP dan RT-PCR adalah sama. Primer RT-LAMP adalah spesifik dalam mengesan CCCVd dimana ia tidak mengesan viroid lain yang digunakan di dalam ujian spesifikasi. CCCVd juga dikesan dalam sampel daun kelapa sawit yang dikumpul secara rawak dari ladang. Program BLAST menujukkan bahawa produk RT-LAMP adalah varian $CCCVd_{246OP}$. Sementara itu, dalam *real-time PCR* kaedah kuantifikasi mutlak menggunakan SYBR-green I dilakukan untuk mengesan varian CCCVd di kelapa sawit. Bagi kajian pengumpulan viroid, dua varian CCCVd

(CCCVd$_{246OP}$ dan CCCVd$_{293OP}$) disuntik ke dalam 10 anak kelapa sawit berusia tiga bulan. RT-PCR dan penjujukan DNA dijalankan bagi tujuan pencirian varian CCCVd yang didapati dalam anak pokok yang disuntik pada selang masa yang berbeza. Dalam kajian pengumpulan, CCCVd dikesan tiga bulan selepas disuntik ke dalam anak pokok dengan kepekatan viroid yang sangat rendah dan tiada corak tertentu untuk menunjukkan ianya meningkat atau menurun sepanjang 12 bulan. Hasil penjujukan menunjukkan bahawa CCCVd$_{246OP}$ (Genbank: HQ608513.1) telah dicirikan dari anak pokok yang disuntik dengan plasmid CCCVd$_{293OP.}$ Di samping itu, jujukan 246-nt juga telah diperoleh dari anak pokok yang menunjukkan simtom (OS) dengan 99% persamaan jujukan pada CCCVd$_{246OP}$. Keputusan ini mengesahkan kepatogenan varian CCCVd 246-nt. Bagi kajian translokasi viroid, varian CCCVd$_{246OP}$ disuntik ke dalam 12 anak pokok. Anak pokok disampel selepas tiga bulan dengan tiga replikasi; memisahkan bahagian daun, batang dan akar. CCCVd dikesan di bahagian daun, batang dan akar setelah tiga, enam, sembilan dan dua belas bulan disuntik. Kepekatan CCCVd didapati berbeza di setiap bahagian anak pokok, di mana kepekatan di batang dan daun adalah lebih tinggi berbanding akar.

ACKNOWLEDGEMENTS

I am grateful to God that finally I have managed to complete this dissertation. The completion of this thesis would have been impossible without the assistance of so many kind-hearted individuals.

I would like to extend my deepest appreciation to my supervisor Assoc. Prof. Dr. Ganesan Vadamalai for his knowledge, guidance, advice, and support throughout my research and completion of this dissertation. I appreciate his willingness to allocate time to guide me in my research whenever necessary.

I would also like to thank my supervisory committee members, Assoc. Prof. Dr. Jugah Kadir and Dr. Lau Wei Hong for their advice and intellectual contribution towards my research plan.

My special thanks to Dr. Kong Lih Ling for her endless guidance and opinions. I also wish to express my sincere thanks to all of my lab mates; Ying Hooi, Li Chu, Dr. Hendry, Reza, Roslina, Neda and Rabiatul for their knowledge and experience sharing. Also, my heartfelt thanks to my good friends; Faizah, Kumar and Puvanes who have given me moral support from the beginning of my research journey and spent their quality time to assist me.

I extend my appreciation to the Ministry of Education for the scholarship (MyPhD). My sincere gratitude to the oil palm plantations for the permission to collect samples for my research work. I would also like to thank Institute of Tropical Agriculture for providing the research facilities and Institute of Bio-science for allowing me to use their real-time PCR machine.

Finally, I owe a considerable debt of gratitude to my parents, sister and brother-in-law for the encouragement, love and support given through all the years of my study in Universiti Putra Malaysia.

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 follow:

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LIST OF ABBREVIATIONS

Acryl	Acrylamide
Amp	Ampicilin
Amp	Ampere
AMV-RT	Avian myeloblastosis virus reverse transcriptase
APS	Ammonium persulphate
Bp	Base pair
Bis	Bisacrylamide
CA	Chloroform:iso-amyl alcohol mix
cDNA	Complementary deoxyribonucleic acid
dNTP	Mixture of deoxynucleoside-triposphates in equimolar amounts
DDW	Double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylanediamine tetra acetic acid
EtBr	Ethidium bromide
g	Gram
g	Centrifugal force
HCl	Hydrochloride acid
IPTG	Iso-Propyl-β-D-thiogalactopyranoside
k	Kilo
Kb	Kilo base
L	Litre
LB	Lysogeny broth
M	Molar
µ-	Micro- (10^{-6})
m-	Milli (10^{-3})
n-	Nano (10^{-9})
NaAc	Sodium acetate
NaCl	Sodium chloride
Na_2EDTA	di-sodium ethylenediamine tetra acetic acid
nt	Nucleotides
O/N	Overnight
PAGE	Poyacrylamide gel elecphoresis
PCA	Phenol:chloroform:iso-amyl alcohol mix
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDDW	Steriled double distilled water
SDS	Sodium dodecyl sulphate
SOC	Super Optimal Broth
TBE	Tris-borate EDTA
TEMED	N,N,N'-N'-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
u	Unit

UV	Ultra Violet
V	Voltage
vol	Volume
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactosidase

CHAPTER 1

GENERAL INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is Malaysia's most important agricultural crop. The first commercial plantation in Malaysia was established in 1917 and since then, it became a high revenue commodity which accounted for 39 % of world palm oil production (MPOC, 2012). Numerous scientific studies were conducted to increase its productivity including breeding, genetic engineering and tissue culture. However, the oil palm production is affected by pest and diseases. Basal stem rot disease caused by the fungus, *Ganoderma boninense* is a major problem in the Malaysian oil palm plantations. This incidence caused the trees to die off with unopened spears and retarded growth (Cooper *et al*., 2011). Another emerging disease is the orange spotting disorder (Turner, 1981).

Orange spotting (OS) is a disorder recognised in oil palm in the early 19th century in Africa (Forde and Leyritz, 1968; Coulter and Rosenquist, 1955) and since then it has been observed in commercial oil palm plantations in Indonesia, Malaysia, Thailand, Papua New Guinea, the Philippines, the Solomon Islands and Central America (Randles, 1998). It has also been observed in oil palms in South America (Hanold and Randles, 1991). OS has been observed in plantations at an incidence between 0.1% and 10% (Hanold and Randles, 1991). A number of possible causes were associated with this disorder including nutrient deficiency and genetic origin (Forde and Leyritz, 1968; Coulter and Rosenquist, 1955). The unexpected detection of *Coconut cadang-cadang viroid* (CCCVd)-like molecules in OS oil palms in the Solomon Islands in 1986 led to suggestion that OS was caused by a viroid closely allied to CCCVd (Hanold and Randles, 1991).

Recently CCCVd variants were sequenced from oil palm in Malaysia with sequence similarity greater than 90% compared with CCCVd in coconut (Wu *et al.,* 2013; Vadamalai *et al.*, 2006). Joseph (2012) observed that nucleic acid extract from the OS palms containing a CCCVd variant reproduced OS symptom when inoculated into healthy seedlings suggesting the role of the CCCVd variant in OS symptom expression. The incidence rate of this disorder was estimated at 5% in Malaysian oil palm plantation (Cheong, 2012).

The CCCVd variants in oil palms were reported to be present at very low concentrations compared to CCCVd in coconut (Joseph, 2012; Vadamalai, 2005). Lower concentration of CCCVd RNAs in the oil palm made detection difficult and inconsistent. This disorder is a potential threat to the oil palm industry since CCCVd has caused death of more than 40 million coconut palms in the Philippines. Hence, it is crucial that a rapid and reliable detection method is available to assist in the management of OS in oil palm.

At present, the molecular methods used for the detection of CCCVd variants in oil palms were not reliable due to their low concentrations (Vadamalai *et al.*, 2009, 2006; Vadamalai 2005; Hanold and Randles, 1991). For example, hybridization assay was widely used to detect CCCVd variants; however it was time-consuming and laborious. Reverse-transcription Polymerase Chain reaction (RT-PCR) was later developed for the detection of CCCVd variants; it worked well despite taking three to four hours to complete and the results needed to be sent for DNA sequencing for validation (Wu *et al.*, 2013; Joseph, 2012; Vadamalai *et al.*, 2006; Vadamalai, 2005). These methods were good in detecting CCCVd in the oil palm but were not rapid and sensitive due to the low concentrations of the viroid. Recently, reverse transcription loop-mediated isothermal amplification (RT-LAMP), a rapid diagnostic assay has been used to detect viriods (Tsutsumi *et al.*, 2010; Boubourakas *et al.*, 2009).

Low concentration of CCCVd variants in oil palm affected the research efforts to understand the aetiology of this viroid in the oil palm. Routine diagnostic methods such as RT-PCR needed re-amplification step to be able to detect and characterise the viroid. Despite this, no research has been conducted to analyse the accumulation of CCCVd variants in oil palm and its progress by time. Viroid concentration or accumulation within the host plant has not been much studied. Real-time PCR has been used in many studies to detect and quantify viroid load (Hajeri *et al.*, 2011; Tessitori *et al.*, 2005).

A general theory on the movement of viroids upon invasion into the host has been discussed in the organelle level (Ding, 2009; Daròs *et al.*, 2006; Flores *et al.*, 2005b). The movement of viroids through the plasmodesmata to other cells helps in replication and establishment of infection. Nevertheless the specific movement pattern of viroids within the infected host is less studied. The distribution of CCCVd within the oil palm has not been studied yet, and information on translocation of CCCVd is not available.

In view of this, the objectives of this study are:

i. To develop a rapid detection method for oil palm CCCVd variants using Reverse Transcription Loop-mediated isothermal amplification (RT-LAMP) method.

ii. To examine the CCCVd accumulation in the oil palm seedlings using real-time PCR

iii. To determine the viroid translocation in CCCVd-inoculated oil palm seedlings.

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