

# **UNIVERSITI PUTRA MALAYSIA**

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

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



SATHIS SRI THANARAJOO

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

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

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

By

#### SATHIS SRI THANARAJOO

September 2014

#### Chairman: Ganesan Vadamalai, PhD Institute: Institute of Tropical Agriculture

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<sub>4</sub>, 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<sub>2460P</sub>. For viroid accumulation study, two variants of CCCVd (CCCVd<sub>246OP</sub> and CCCVd<sub>293OP</sub>) 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<sub>2460P</sub> (Genbank: HQ608513.1) was characterised from seedlings inoculated with CCCVd<sub>293OP</sub> plasmids. In addition, a 246-nt sequence was also recovered from the symptomatic (OS) seedling with 99% sequence similarity to CCCVd<sub>2460P</sub>, which confirmed the pathogenicity of this 246-nt CCCVd variant. In viroid movement study, the CCCVd<sub>2460P</sub> 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

## SATHIS SRI THANARAJOO

#### September 2014

### Pengerusi: Ganesan Vadamalai, PhD Institut: Institut Pertanian Tropika

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<sub>4</sub>, 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<sub>2460P</sub>. 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<sub>2460P</sub> dan CCCVd<sub>2930P</sub>) 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<sub>2460P</sub> (Genbank: HQ608513.1) telah dicirikan dari anak pokok yang disuntik dengan plasmid CCCVd<sub>293OP.</sub> Di samping itu, jujukan 246-nt juga telah diperoleh dari anak pokok yang menunjukkan simtom (OS) dengan 99% persamaan jujukan pada CCCVd<sub>2460P</sub>. Keputusan ini mengesahkan kepatogenan varian CCCVd 246-nt. Bagi kajian translokasi viroid, varian CCCVd<sub>2460P</sub> 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.

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Sequence alignment between CCCVd<sub>2460P</sub> (Genbank accession

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- 6.2d 12 months after inoculation. CCCVd was detected in all the leaves 80 leaves, two to three sections of the stems and; roots of two seedlings (indicated by black dots).

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# LIST OF ABBREVIATIONS

Acryl	Acrylamide
Amp	Ampicilin
Amp	Ampere
AMV-RT	Avian myeloblastosis virus reverse transcriptase
APS	Ammonium persulphate
Вр	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
М	Molar
μ-	Micro- $(10^{-6})$
m-	Milli (10 <sup>-3</sup> )
n-	Nano (10 <sup>-9</sup> )
NaAc	Sodium acetate
NaCl	Sodium chloride
Na <sub>2</sub> EDTA	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

G

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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- $\beta$ -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 19<sup>th</sup> 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 realtime PCR
- iii. To determine the viroid translocation in CCCVd-inoculated oil palm seedlings.

#### REFERENCES

- Ahmad, N., Kuramoto, I.K. and Baroudy, B.M. (1993). A ribonuclease protection assay for the direct detection and quantitation of hepatitis C virus RNA. *Clinical and Diagnostic Virology* 1:233-244.
- Aranda, M.A., Fraile, A. and Carcia-Arenal, F. (1993). Genetic variability and evolution of the satellite RNA of *cucumber mosaic virus* during natural epidemics. *Journal of Virology* 67:5896-5901.
- Aranda, M.A., Fraile, A., Garcia-Arenal, F. and Malpica, J.M. (1995). Experimental evaluation of the ribonuclease protection assay method for assessment of genetic heterogeneity in populations of RNA viruses. *Archives of Virology* 140: 1373-1383.
- Branch, A. D. and Robertson, H. D. (1984). A replication cycle for viroids and other small infectious RNAs. *Science* 233: 450-454.

Bio-Rad Laboratories, 2006. Real-Time PCR Applications Guide.

- Bonfiglioli, R.G., McFadden, G.I. and Symons, R.H. (1994). *In situ* hybridization localises *Avocado sunblotch viroid* on chloroplast thylakoid membranes and *Coconut cadang-cadang viroid* in the nucleolus. *Plant Journal* 6:99-104.
- Bonfiglioli, R.G., Webb, D.R. and Symons, R.H. (1996). Tissue and intra-cellular distribution of *Coconut cadang-cadang viroid* and *Citrus exocortis viroid* determined by *in situ* hybridization and confocal laser scanning and transmission electron microscopy. *Plant Journal* 9:457-465.
- Boonham, N., Pérez, L.G., Mendez, M.S., Peralta, E.L., Blockley, A., Walsh, K.,
   Barker, I. and Mumford, R.A. (2004). Development of a real-time RT-PCR assay for the detection of potato spindle tuber viroid. *Journal of Virological Methods* 116:139–146.
- Boubourakas, I.N., Fukuta, S. and Kyriakopoulou, P.E. (2009). Sensitive and rapid detection of *Peach latent mosaic viroid* by the reverse transcription loop-mediated isothermal amplification. *Journal of Virology* 160:63-68.

- Bussiere, F., Lehoux, J., Thompson, D.A., Skrzeczkowski, L.J. and Perreault, J.P. (1999). Subcellular localization and rolling circle replication of *Peach latent mosaic viroid*: Hallmarks of group A viroids. *Journal of Virology* 73(8): 6353-6360.
- Cabrera, O., Roossinck, M.J. and Scholthof, K.B.G. (2000). Genetic diversity of *Panicum mosaic virus* satellite RNAs in St. Augustinegrass. *Phytopathology* 90:977-980.
- Cooper, R.M., Flood, J., and Rees, R.W. (2011). *Ganoderma boninense* in Oil Palm Plantations: Current Thinking on Epidemiology, Resistance and Pathology\*. *The Planter (Kuala Lumpur)* 87 (1024): 515-526.
- Chen, C. and Cui, S. (2009). Detection of porcine parvovirus by loop-mediated isothermal amplification. *Journal of Virological Methods* 155: 122–125.
- Cheong, L.C. (2012). Incidence of orange spotting and characterization of Coconut *cadang-cadcang viroid* variants in Selangor and Sabah oil palm plantations, Malaysia. MSc. Thesis. Malaysia: Universiti Putra Malaysia.
- Coulter, J.K. and Rosenquist, E.A. (1955). Mineral nutrition of the oil palm. A study of the chemical composition of the frond in relation to chlorosis and yield. *Malayan Agricultural Journal* 38:214-236.
- Daròs, J.A., Elena, S.F. and Flores, R. (2006). Viroids: an Ariadne's thread into the RNA labyrinth. *EMBO reports* 7(6), 593–598.
- Ding, B., Kwon, M.O., Hammond, R. and Owens, R. 1997. Cell-to-cell movement of *Potato spindle tuber viroid. Plant Journal* 12:931-936.
- Ding, B. (2009). The biology of viroid-host interactions. Annual Review of *Phytopathology*. 47:105–31.
- Diener, T.O. (1971). Potato spindle tuber 'virus' IV. A replicating, low molecular weight RNA. *Virology* 45:411-428.

Diener, T.O. (1972). Viroids. Advances in Virus Research 17:295-313.

- Diener, T.O. (1979). Viroids: structure and function. *Science* 31:205 (4409):859–866.
- Diener, T.O. (1991). Subviral pathogens of plants: Viroid and virod-like satellite RNAs. FASEB (Federation of American Societies for Experimental Biology) Journal 5:2808-2813.
- Diener, T. O. (2001). The viroid: biological oddity or evolutionary. *Advances in Virus Research* 57: 137-184.
- Eiken Chemical Co. Ltd. Retrieved 10 February 2012 from <u>http://www.eiken.co.jp/en/.</u>
- Enosawa, M., Kageyama, S., Sawai, K., Watanabe, K., Notomi, T., Onoe, S., Mori,
  Y. and Yokomizo, Y. (2003). Use of Loop-Mediated Isothermal Amplification of the IS900 Sequence for Rapid Detection of Cultured Mycobacterium avium subsp. Paratuberculosis. Journal Clinical Microbiology 41:4359–4365.
- Flores, R., Randles, J. W., Bar-Joseph, M., Owens, R. A. and Diener, T. O. (2005a). Viroidae. In Virus Taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses: 1145-1159. Edited by Fauquet, M. A.M. C. M., Maniloff, J., Desselberger, U. and Ball, A. L. London: Elsevier/Academic.
- Flores, R., Hernandez, C., Martinez de Alba, A.E., Daros, J.A. and Di Serio, F. (2005b). Viroids and viroid-host interactions. *Annual Review of Phytopathology* 43:117-139.
- Forde, S.C.M. and Leyritz, M.J.P. (1968). A study of confluent orange spotting of the oil palm in Nigeria. *Journal of the Nigerian Institute for Oil Palm Research (Nigeria)* 4: 371-380.
- Galipienso, L., Janssen, D., Rubio, L., Aramburu, J. and Velasco, L. (2013). Cucumber vein yellowing virus isolate-specific expression of symptoms and viral RNA accumulation in susceptible and resistant cucumber cultivars. *Crop Protection* 43:141-145.
- Góra-Sochacka, A. (2004). Viroids: unusual small pathogenic RNAs. *Acta Biochimica Polonica* 51(3):587-607.

- Hadidi, A. and Yang, X. (1990). Detection of pome fruit viroids by enzymatic cDNA amplification. *Journal of Virological Methods* 30: 261-270.
- Hanold, D. and Randles, J. W. (1991). Detection of Coconut cadang-cadang viroidlike sequences in oil and coconut palm and other monocotyledons in southwest Pacific. *Annals of Applied Biology* 118: 139-151.
- Hanold, D. (1998). Diagnostic methods applicable to viroids. In Report on ACIAR-Funded Research on Viroids and Viruses of Coconut Palm and other Tropical Monocotyledons 1985-1993. 51: 27-39. Edited by Hanold, D. and Randles, J. W. Canberra: Australian Centre for International Agricultural Research.
- Hanold, D. and Randles, J. W. (1998). CCCVd-related sequences in species other than coconut. In Report on ACIAR-Funded Research on Viroids and Viruses of Coconut Palm and other Tropical Monocotyledons 1985-1993. 51, 144-152. Edited by Hanold, D. and Randles, J. W. Canberra: Australia Centre for International Agricultural Research.
- Hajeri, S., Ramadugu, C., Manjunath, K., Ng, J., Lee, R. and Vidalakis, G. (2011). In vivo generated *Citrus exocortis viroid* progeny variants display a range of phenotypes with altered levels of replication, systemic accumulation and pathogenicity. *Journal of Virology* 417(2):400-9.
- Harders, J., Lucas, N., Robert-Nicoud, M., Jovin, T.M. and Riesner, D. (1989). Imaging of viroids in nuclei from tomato leaf tissue by *in situ* hybridization and confocal laser scanning microscopy. *EMBO Journal* 8:3941-3949.
- Haridas, D.V., Pillai, D., Manojkumar, B., Nair, C.M. and Sherief, P.M. (2010). Optimization of reverse transcriptase loop-mediated isothermal amplification assay for rapid detection of *Macrobrachium rosenbergii* noda virus and extra small virus in *Macrobrachium rosenbergii*. Journal of Virological Methods 167: 61-67.
- Hartley, C.W.S. (1988). The Oil Palm (*Elaeis guineensis* Jacq.), 3<sup>rd</sup> edn. UK: Longman Scientific and Technical. 761 pp.
- Haseloff, J., Mohamed, N.A. and Symons, R.H. (1982). Viroid RNAs of cadangcadang disease of coconuts. *Nature* 299, 316 – 321.

- Hodgson, R.A.J. (1998). Molecular tools for plant pathogen diagnosis, pp.135. Glen Osmond, South Australia: Department of Crop Protection, University of Adelaide.
- Hodgson, R. A. J., Wall, G. C. and Randles, J. W. (1998). Specific identification of coconut tinangaja viroid for differential field diagnosis of viroids in coconut palms. *Phytopathology* 88: 774-781.
- Hull, R. (1989). The movement of viruses in plants. *Annual Review of Phytopathology* 27:213-40.
- Imperial, J.S., Bautista, R.M. and Randles, J.W. (1985). Transmission of the *Coconut* cadang-cadang viroid to six species of palm by inoculation with nucleic acid extracts. *Plant Pathology* 34:391-401.
- International Committee on Taxonomy of Viruses (ICTV), updated in 2013. Retrieved 12 January 2014 from (http://www.ictvonline.org).
- Itaya, A., Foliomonov, A., Matsuda, Y., Nelson, R. S. and Ding, B. (2001). Potato spindle tuber viroid as inducer of RNA silencing in infected tomato. Molecular Plant-Microbe Interactions 14: 1332-1334.
- Jiang, T., Liu, J., Deng, Y.Q., Su, J.L., Xu, L.J., Liu, Z.H., Li, XF., Yu, X.D., Zhu, S.Y., Gao, G.F., Qin, E.D. and Qin, C.F. (2012). Development of RT-LAMP and real-time RT-PCR assays for the rapid detection of the new duck Tembusu-like BYD virus. Archives of Virology 157:2273-2280.
- Joseph, H. (2012). Characterization and pathogenicity of *Coconut cadang-cadang viroid* variants in oil palm (*Elaeis guineensis* Jacq.) seedlings. Ph. D Thesis. Malaysia: Universiti Putra Malaysia.
- Jun, P., Zaifeng, F. and Junsheng, H., (2012). Rapid Detection of Banana Streak Virus by Loop-mediated Isothermal Amplification Assay in South China. *Journal of Phytopathology* 160:248-250.
- Kurath, G. and Palukaitis, P. (1989). RNA sequence heterogeneity in natural populations of three satellite RNAs of cucumber mosaic virus. *Virology* 173(1):231–240.

- Le, D.T., Netsu, O., Uehara-Ichiki, T., Shimizu, T., Choi, II-Ryong., Omura, T. and Sasaya, T. (2010). Molecular detection of nine rice viruses by a reverse-transcription loop-mediated isothermal amplification assay. *Journal of Virological Methods* 170(1-2):90-3.
- Lek-Uthai, U. (2009). Loop-Mediated Isothermal Amplification Method (LAMP): Low and Effective cost Novel Tool for Molecular Public Health. Retrieved 24 January 2012 from <u>http://www.malariaworld.org/blog/loop-mediated-isothermal-amplification-method-lamp-low-and-effective-cost-novel-tool-molecular</u>.
- Lenarcic, R., Morisset, D., Mehle, N. and Ravnikar, M. (2012). Fast real-time detection of Potato spindle tuber viroid by RT-LAMP. *Plant Pathology* 62(5): 1147-1156.
- Lima, M.I., Fonseca, M.E.N., Flores, R. and Kitajima, E.W. (1994). Detection of *Avocado sunblotch viroid* in chloroplasts of avocado leaves by *in situ* hybridization. *Archives of Virology* 138:385-390.
- Lopez-Galindez, C., Lopez, J.A., Melero, J.A., De La Fuente, L., Martinez, C. Ortin, J. and Perucho, M. (1988). Analysis of genetic variability and mapping of point mutations in influenza virus by the RNase mismatch cleavage method. *Proceedings of the National Academy of Sciences USA* 85:3522-3526.
- Luigi, M. and Faggioli, F. (2011). Development of quantitative real-time RT-PCR for the detection and quantification of Peach latent mosaic viroid. *European Journal of Plant Pathology* 130: 109–116.
- Malaysian palm oil council (MPOC, 2012). Retrieved 10 January 2014 from <u>http://www.mpoc.org.my/Industry\_Overview.aspx</u>.
- Martinez-Soriano, J.P., Galindo-Alonso, J., Maroon, J.M., Yucel, I., Smith, D.R. and Diener, T.O. (1996). *Mexican papita viroid*: putative ancestor of crop viroids. *Proceedings of the National Academy of Sciences USA* 93:9397-9401.
- Mohammadi, M. R., Vadamalai, G. and Joseph, H. (2010). An optimized method for extraction and detection of Coconut cadang-cadang viroid (CCCVd) from oil palm. *Communications in agricultural and applied biological sciences* 75(4):777-81.

- Mohamed, N. A., Bautista, R. M., Buenaflor, G. G. and Imperial, J. S. (1985). Purification and infectivity of the coconut cadang-cadang viroid. *Phytopathology* 75, 79-84.
- Mori, Y., Kitao, M., Tomita, N. and Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods* 59:145-157.
- Nagamine, K., Kuzihara, Y. and Notomi, T. (2002). Isolation of single-stranded DNA from loop-mediated isothermal amplification products. *Biochemical Biophysical Research Communications* 290, 1195–1198.
- Navarro, B., Daros, J.A. and Flores, R. (1999). Complexes containing both polarity strands of avocado sunblotch viroid: identification in chloroplast and characterization. *Virology* 253: 77-85.
- Nie, X. (2005). Reverse transcription loop-mediated isothermal amplification of DNA for detection of Potato virus Y. *Plant Disease* 89:605-610.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* 28: E63.
- Olioso, D., Boaretti, M., Ligozzi, M., Lo Cascio, G. and Fontana, R. (2007). Detection and quantification of hepatitis B virus DNA by SYBR green realtime polymerase chain reaction. *European Journal of Clinical Microbiology* and Infectious Diseases 26:43–50.
- Owens, R.A., Blackburn, M. and Ding, B. (2001). Possible involvement of the phloem lectin in long-distance viroid movement. *Molecular Plant Microbe Interaction* 14(7): 905-909.
- Palukaitis, P. (1987) Potato spindle tuber viroid: investigation of the long-distance, intra-plant transport route. *Virology* 158, 239-241.
- Parida, M., Posadas, G., Inoue, S., Hasebe, F. and Morita, K. (2004). Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. *Journal of Clinical Microbiology* 42:257-263.

- Papaefthimiou, I., Hamilton, A., Denti, M., Baulcombe, D., Tsagris, M. and Tabler, M. (2001). Replicating Potato spindle tuber viroid RNA is accompanied by short RNA fragments that the characteristics of posttranscriptional gene silencing. Nucleic Acids Research 29: 2395-2400.
- Parisi, O., Lepoivre, P. and Jijakli, M.H. (2010). Plant-RNA viroid relationship: a complex host pathogen interaction. *Biotechnology, Agronomy, Society and Environment* 14(3), 461-470
- Putcha, H., Ramm, K. and Sanger, H. L. (1989). The molecular structure of hop latent viroid (HLV). A new viroid occurring worldwide in hops. *Nucleic Acids Research* 16: 4197-4126.
- Randles, J.W., Boccardo, G., Retuerma, M.L. and Rillo, E.P. (1977). Transmission of the RNA species associated with cadang-cadang of coconut palm, and the insensitivity of the disease to antibiotics. *Phytopathology* 67: 1211-1216.
- Randles, J. W. (1985). Coconut cadang-cadang viroid. In subviral Pathogens of Plants and Animals: Viroids and Prions 39-74. Edited by Maramorosch, K. and McKelvey Jr, J. J. Orlando, Florida. Academic Press Inc.
- Randles, J. W. (1987). Coconut cadang-cadang. In *The Viroids* 265-277. Edited by Diener, T. O. New York : Plenum Press.
- Randles, J. W. (1998). CCCVd-related sequences in species other than coconut. In Report on ACIAR-Funded Research on Viroids and Viruses of Coconut Palm and other Tropical Monocotyledons 1985-1993. 51, 144-152. Edited by Hanold, D. and Randles, J. W. Canberra: Australia Centre for International Agricultural Research.
- Randles, J.W. and Rodriguez, M.J.B. (2003). *Coconut cadang-cadang viroid*. In: *Viroids* 233-241. Edited by Hadidi, A., Flores, R., Randles, J. W. and Semancik, J. S. Collingwood, Australia: CSIRO Publishing.
- Rodriguez, M. J. B. and Randles, J. W. (1993). *Coconut cadang-cadang viroid* (CCCVd) mutants associated with severe disease vary in both the pathogenicity domain and the central conserved region. *Nucleic Acids Research* 21: 2771.

- Rosenau, C., Kaboord, B. and Qoronfleh, M.W. (2002). Development of a chemiluminescence-based ribonuclease protection assay. *BioTechniques* 33: 1354-1358.
- Sambrook, J., Fritsch, E. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> edn, Cold Spring Harbor, New York: CSH Laboratory Press.
- Sambrook, J. and Russell, D. W. (2001). Molecular cloning: A laboratory manual, 3<sup>rd</sup> edn, Cold Spring Harbor, New York: CSH Laboratory Press.
- Sanger, H. (1987). Viroid function in viroid replication. In: *The Viroids* 117-166. Edited by Diener, T. O. New York: Plenum Press.
- Selvaraja, S., Balasundram, S.K., Vadamalai, G. and Husni M.H.A. (2012). Spatial variability of Orange Spotting disease in oil palm. *Journal of Biological Sciences* 12(4): 232-238.
- Sanger, H.L., Klotz, G., Riesner, D., Gross H.J. and Kleinschmidt, A. (1976). Viroids are single stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. *Proceedings of the National Academy* of Sciences USA 73(11):3852–56.
- Saunders, N.A. (2004). An introduction to real-time PCR. In Edwards, K., Logan, J. and Saunders, N. *Real-time PCR: An essential guide* (pp. 1-11). Norfolk: Horizon Bioscience Publishers.
- Schnell, R. J., Kuhn, D. N., Ronning, C. M. and Harkins, D. (1997). Application of RT-PCR for indexing avocado sunblotch viroid. *Plant Disease* 81:1023-1026.
- Spiesmacher, E., Muhlbach, H.P., Schnolzer, M., Haas, B. and Sanger, H. L. (1983). Oligomeric forms of *Potato spindle tuber viroid* (PSTVd) and its complementary RNA present in nuclei isolated from viroid-infected potato cells. *Bioscience Reports* 3:767-774.
- Steger, G. and Riesner, D. (2003). Properties of viroids: molecular characteristics. Edited by Hadidi, A., Flores, R., Randles, J. and Semancik, J. Viroids, CSIRO Publishing, Australia, pp. 15–29.

- Symons, R.H. (1991). The intriguing viroids and virusoids: What is their information content and how did they evolve? *Molecular Plant-Microbe Interactions* 4:111-121.
- Tao, Z.Y., Zhou, H.Y., Xia, H., Xu, S., Zhu, H.W., Culleton, R.L., Han, E.T., Lu, F., Fang, Q., Gu, Y.P., Liu, Y.B., Zhu, G.D., Wang, W.M., Li, J.L., Cao, J. and Gao, Q. (2011). Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of Plasmodium vivax infection. *Parasites and Vectors* 4:115.
- Tessitori, M., Rizza, S., Reina, A. and Catara, A. Real-Time RT-PCR Based on SYBR-Green I for the detection of Citrus Exocortis and Citrus Cachexia Diseases. In *Plant Pathology*. Proceedings of the Sixteenth Conference of the International Organization of Citrus Virologists, Montery, Mexico, Nov 7-12, 2004. Hilf, M.E., Duran-Vila, N. and Rocha-Peña, M.A. (Eds.); E. O. Painter Printing Co.: Florida, 2005.
- Tessitori, M., Maria, G., Capasso, C., Catara, G., Rizza, S., De Luca, V., Catara, A., Capasso, A. and Carginale, V. (2007). Differential display analysis of gene expression in Ertog citron leaves infected by Citrus Viroid III. *Biochimica et Biophysica Acta* 1769, 228-235.

Thermo scientific (2009). qPCR Optimization and Troubleshooting Guide.

- Tomita, N., Mori, Y., Kanda, H. and Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature Protocols* 3, 877 882.
- Tomlinson, J.A., Boonham, N. and Dickinson, M. (2010). Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. *Plant Pathology* 59(3): 465-471.
- Tsai, S.M., Chan, K.W., Hsu, W.L., Chang, T.J., Wong, M.L. and Wang, C.Y. (2009). Development of a loop-mediated isothermal amplification for rapid detection of orf virus. *Journal of Virological Methods* 157: 200–204.
- Tsutsumi, N., Yanagisawa, H., Fujiwara, Y. and Ohara, T. (2010). Detection of Potato Spindle Tuber Viroid by Reverse Transcription Loop-mediated Isothermal Amplification. *Research Bulletin of the Plant Protection Service*, Japan 46:61-67.

- Turner, P.D. (1981). *Oil Palm Diseases and Disorders*. Kuala Lumpur: Oxford University Press. 280 pp.
- Vadamalai, G. (2005). An investigation of oil palm orange spotting disorder. Ph.D Thesis. Adelaide: The University of Adelaide.
- Vadamalai, G., Hanold, D., Rezaian, M. A. and Randles, J. W. (2006). Variants of Coconut cadang-cadang viroid isolated from an African oil palm (Elaies guineensis Jacq.) in Malaysia. Archives of Virology 151(7): 1447-1456.
- Vadamalai, G., Perera, A.A.F.L.K., Hanold, D., Rezaian, M.A. and Randles, J.W. (2009). Detection of *Coconut cadang-cadang viroid* sequences in oil and coconut palm by ribonuclease protection assay. *Annals of Applied Biology* 154(1): 117-125.
- Wang, M. B., Bian, X. Y., Wu, L. M., Liu, L. X., Smith, N. A., Isenegger, D., Wu, R. M., Masuta, C., Vance, V. B., Watson, J. M., Rezaian, A., Dennis, E. S. and Waterhouse, P. M. (2004). On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. *Proceedings of the National Academy of Sciences USA* 101. 9: 3275-3280.
- Wang, Y., Shibuya, M., Taneda, A., Senda, M., Owens, R.A. and Sano, T. (2011). Accumulation of Potato spindle tuber viroid-specific small RNAs is accompanied by specific changes in gene expression in two tomato cultivars. *Journal of Virology* 413(1):72-83.
- Wu, Y.H. (2012). Post-transcriptional gene silencing (PTGS) in oil palms infected with *Coconut cadang-cadang viroid* (CCCVd) variants. . MSc. Thesis. Malaysia: Universiti Putra Malaysia.
- Wu, Y. H, Cheong, L. C., Meon, S., Lau, W. H., Kong, L.L., Joseph, H. and Vadamalai, G. (2013).Characterization of Coconut cadang-cadang viroid variants from oil palm affected by orange spotting disease in Malaysia. *Archives of Virology* 158:1407-1410.
- Yang, X., Hadidi. A. and Garnsey, S. M. (1992). Enymatic cDNA amplification of citrus exorcortis and cachexia viroids from infected citrus hosts. *Phytopathology* 82: 279-285.
- Zelazny, B. (1980). Ecology of cadang-cadang disease of coconut palm in the Philippines. *Phytopathology* 70, 700-703.

- Zhu, Y., Green, L., Woo, Y.M., Owens, S. and Ding, B. (2001). Cellular basis of Potato spindle tuber viroid systemic movement. *Virology* 279:69-77.
- Zhu, Y., Qi, Y., Xun, Y., Owens, R. and Ding, B. (2002). Movement of *Potato* Spindle Tuber Viroid Reveals Regulatory Points of Phloem-Mediated RNA Traffic. *Plant Physiology* 130(1):138-146.

