UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND TRANSMISSION OF COCONUT CADANGCADANG VIROID VARIANTS IN OIL PALM AND COCONUT

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CHARACTERIZATION AND TRANSMISSION OF COCONUT CADANG-CADANG VIROID VARIANTS IN OIL PALM AND COCONUT

By

RAIMATHY A/P KANAVEDEE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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December 2016

Chairman : Associate Professor Ganesan Vadamalai, PhD
Faculty : Agriculture

Coconut cadang-cadang viroid (CCCVd) is the causal agent of the devastating Coconut cadang-cadang disease in the Philippines. CCCVd variants have been associated with orange spotting (OS) disorder in Malaysian oil palm. The interrelation between characterized CCCVd variants and OS symptom variation as well as transmission of oil palm CCCVd variants into alternate host to observe the expression of symptoms that have not been studied in Malaysia. Characterization of CCCVd variants with OS and pathogenicity trials will enable better understanding of the disease epidemiology, viroid etiology and its transmission to alternate host. CCCVd-like RNAs from OS palms with symptom variation from different locations were extracted and characterized using an optimized and modified conventional Natrium Chloride EDTA Tris-HCL Mercaptoethanol (NETME) extraction method. The modification involved the inclusion of additional ethanol and lithium chloride precipitation stages thereby eliminating further purification through time-consuming non-denaturing Polyacrylamide Gel Electrophoresis (PAGE) process. Transmission study was carried out by inoculating $CCCVd_{OP246}$ clones into 3 months old oil palm and coconut seedlings. The modified total nucleic acid extraction procedure proved to be successful and resulted in high quality RNA with consistent results in detection of CCCVd variants in oil palm. Characterized CCCVd variants from oil palm samples with variation in OS symptom (OPKS, OPBP and OPUP) showed 99% sequence similarity to $CCCVd_{OP246}$ oil palm variant with substitution at positions at positions $C^{104} \rightarrow G$, $G^{105} \rightarrow C$ and $C^{140} \rightarrow A$ (OSKS), $C^{19} \rightarrow G$, and $G^{35} \rightarrow C$ (OSBP), and $C^{24} \rightarrow U$ (OSUP) respectively. Transmission study found that CCCVd variant with 99% sequence similarity to $CCCVd_{OP246}$ was detected in oil palm and coconut seedlings on the 3rd month and 6th month respectively after inoculation. Expression of orange spotting symptoms were observed on oil palm and coconut at the 6th month and 9th month respectively. CCCVd variants from palm with orange spotting symptom variation from different locations were successfully characterized. Variation in orange spotting symptom may be related to nucleotide change in the viroid sequence. Cross-

pathogenicity test of CCCVd variant into coconut were successful with viroid and symptom appearance.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENCIRIAN DAN KEPATOGENAN VARIAN - VARIAN COCONUT CADANG-CADANG VIROID PADA KELAPA SAWIT DAN KELAPA

Oleh

RAIMATHY A/P KANAVEDEE

Disember 2016

Pengerusi : Profesor Madya Ganesan Vadamalai, PhD
Fakulti : Pertanian

Coconut cadang-cadang viroid (CCCVd) merupakan ejen penyebab penyakit Coconut cadang-cadang yang teruk melanda di Filipina. Varian CCCVd sering kali dikaitkan dengan penyakit orange spotting (OS) pada pokok kelapa sawit di Malaysia. Hubung-kait antara varian CCCVd yang telah dicirikan dan variasi simptom OS serta transmisi varian CCCVd kelapa sawit ke perumah alternatif untuk memerhati ekspresi symptom, masih belum dikaji di Malaysia. Pencirian varian CCCVd dengan OS dan ujian kepatogenan membolehkan pemahaman dan pengetahuan yang lebih mendalam tentang epidemiologi penyakit, etiologi viroid dan transmisinya ke perumah alternatif. RNA yang menyerupai CCCVd daripada pokok kelapa sawit OS dengan variasi simptom daripada lokasi yang berlainan telah di ekstrak dan diciri menggunakan kaedah pengekstrakan konvensional Natirum Klorida EDTA Tris-HCl Mercaptoetanol (NETME) yang telah dioptimum dan diubahsuai. Pengubahsuaian yang dilakukan termasuklah penambahan etanol dan peringkat resapan lithium klorida dimana langkah penulenan seterusnya menggunakan gel elektroforesis poliakrilmida (PAGE) yang mengambil masa yang lama tidak digunakan. Kajian transmisi telah dijalankan dengan menginokulasi klon $CCCVd_{OP246}$ pada anak pokok kelapa sawit dan kelapa yang berumur 3 bulan. Pengubahsuaian kaedah pengekstrakan acid nukleik terbukti berjaya dan menghasilkan kualiti RNA yang konsisten dalam pengesanan varian-varian CCCVd dalam kelapa sawit. Varian –varian yang telah dicirikan daripada sampel-sampel kelapa sawit dengan variasi simptom OS (OPKS, OPBP, dan OPUP) menunjukkan 99% persamaan jujukan dengan varian kelapa sawit $CCCVd_{OP246}$dengan penggantian pada posisi- posisi $C^{104} \rightarrow G$, $G^{105} \rightarrow C$ and $C^{140} \rightarrow A$ (OPKS), $C^{19} \rightarrow G$, and $G^{35} \rightarrow C$ (OPBP), and $C^{24} \rightarrow U$(OPUP) masing-masing. Kajian transmisi menunjukkan varian CCCVd mempunyai 99% persamaan jujukan pada $CCCVd_{OP246}$ telah dikesan dalam pokok kelapa sawit dan kelapa pada bulan ke-tiga dan ke-enam masing-masing setelah inokulasi. Ekspresi simptom OS telah diperhatikan pada pokok kelapa sawit dan kelapa pada bulan ke-enam dan ke-sembilan masing-masing setelah penyuntikan. Varian –varian CCCVd daripada pokok kelapa sawit dengan variasi simtom OS daripada lokasi yang berlainan telah berjaya dicirikan. Variasi simptom OS boleh dikaikan dengan perubahan nukleotida jujukan

viroid. Kajian kepatogenan silang varian CCCVd ke dalam pokok kelapa berjaya mengekspreskan viroid and simtom.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

Acryl	Acrylamide
Amp	Ampicillin
Amp	Ampere
AMV-RT	Avian myeloblastosis virus reverse transcription
APS	Ammonium persulphate
Bp	Base pair
Bis	Bisacrylamide
CA	Chloroform: isoamyl alcohol mix
cDNA	Complementary deoxyribonucleic acid
dNTP	Mixture of deoxnucleoside - triposphates in equimolar amounts
DDW	Double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylanediamine tetra acetic acid
EtBr	Ethidium bromide
G	Gram
HCl	Hydrochloric acid
IPTG	Iso-Propyl-β- thiogalactopyranoside
K	Kilo
Kb	Kilo base
L	Litre
LB	Luria - Bertani 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
PAGE	Polyacrylamide gel electrophoresis
PCA	Phenol: chlroform: isoamyl alcohol mix
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDDW	Sterile 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	Ultraviolet
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

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a native plant from West Africa but it has been cultivated in most of the equatorial tropics of South-East Asia and America (Esua *et al*., 2015). Oil palm, with an economic life of around 25 years was first introduced to Malaysia in 1870 for ornamental purposes. The first commercial oil palm plantation in Malaysia was established at Tennamaran estate (Selangor) in 1917 (Sumathi *et al*., 2007; Nadzri and Ahmad, 2016), and thus laid the foundation for the oil palm plantation and the palm oil industry.

According to the Economic Transformation Program report of 2013, as reported by Nadzri and Ahmad (2016), Malaysia is regarded among the largest exporters and producers of palm oil worldwide. With an estimated production of 2.13 tons of palm kernel oil and 17.73 million tons of palm oil from cultivated land area of 4.49 million tons, Malaysia accounts for 27 % oil fats trade export and 11 % world production of fats. The oil palm is the most efficient oil-bearing crop in the world.

Among the limitations of oil palm production is improper soil preparation for growing oil palm, its sustainability and food health concerns. The actual challenge is sustaining the palm economic production, which can be exaggerated either by pest and disease infestations or deprived management practices. *Ganoderma boninense* is the causative organism of Basal Stem Rot (BSR), the major disease of oil palm (Sundram *et al*., 2015), while Orange Spotting (OS) linked with *Coconut cadang-cadang viroid* (CCCVd) is an emerging disease of oil palm. CCCVd is the causative organism of cadang-cadang disease of coconut in the Philippines, serious with death of coconut palms (Randles and Rodriquez, 2003).

Previous studies have displayed 90 % sequence similarity between CCCVd variants present in OS symptomatic and asymptomatic oil palm and CCCVd from coconut (Vadamalai, 2005; Vadamalai *et al*., 2006; Wu *et al*., 2013). In addition, high degree of symptom variation exist in OS palms. This could be attributed to the differences in CCCVd variants present in oil palm. Interrelation of oil palm CCCVd variants to OS variation is important so as to have a proper understanding of the epidemiology of OS since difference in sequence of the CCCVd oil palm variants can result in different severity of OS symptoms and disease.

Studies carried out by Joseph (2012) and Thanarajoo (2014) on transmission of CCCVd-like RNA into oil palm had been established. The cross - transmission between CCCVd-like RNAs from oil palm into alternate host such as coconut is yet to be studied. Significant economic importance is not placed on the OS disorder, and as a result there is absence of major research strength to scrutinise its occurrence within the Malaysia region.

Studies in molecular biology require high quality RNA and polysaccharides, polyphenols and phenol contents of plants especially coconut and oil palm affects PCR sensitivity which may lead to false results (Xiao *et al*., 2012; Ergun *et al*., 2013; Shu *et al*., 2014). Among the available extraction methods, NETME is a suitable choice, as it requires small amount of leaves (Joseph, 2012). A further purification step of Polyacrylamide gel electrophoresis (PAGE) is usually undertaken to achieve high quality RNA. This further step is usually laborious and time consuming as it requires an additional two days experiment. It is therefore of paramount importance to continuously search and develop easier and less time-consuming methods of achieving the required high quality RNA for these studies.

Based on these submissions, the objectives of the study are:

i. To modify and optimize total nucleic acid extraction method from oil palm leaves rich in polysaccharides and polyphenols.
ii. To characterize *Coconut cadang – cadang viroid* (CCCVd) variants in oil palm with orange spotting symptom variation.
iii. To carry out transmission of oil palm CCCVd variant into oil palm and coconut seedlings.

CHAPTER 2

LITERATURE REVIEW

2.1 Oil palm industry

The history of the oil palm dates back to the records of journeys and explorations by the Portuguese, English and Dutch on the coast of West Africa (Corley and Tinker, 2003). It produces the highest yields per hectare when compared to other crops (Corley and Tinker, 2003). Two kinds of oil – palm oil and palm kernel oil is produced from the oil palm. Basiron (2007) reported that production of palm kernel is about 10 % of the palm oil quantity produced. Between 1995 and 2011, worldwide production of palm oil tripled. This resultant demand has contributed significantly to the economies of Indonesia and Malaysia, together which produce about 85 % of world supply of palm oil. Palm oil contributed 28 % to world fats and oils production in 2011, and is currently the most importantly traded in the global market among the major fats and oils. Indications are emerging on the continuous importance and competitiveness of oil palm with other edible oils and fats (CommodityBasis, 2016).

2.2 Oil palm industry in Malaysia

Malaysia contributes about 44 % to world palm oil exports and is currently the world's largest exporter even though it is ranked as the second largest producer after Indonesia. Introduced in 1870 as an ornamental plant with first oil palm plantation in 1917, Malaysia is now home to West African palm which thrives well as a result of 2000 mm annual rainfall that is evenly distributed, ample sunshine, humid tropical climate and temperatures in the range of 24 °C – 34 °C. The expansion of oil palm was rapid in the 1960s and was encouraged by the Malaysian government, which recognized its potentiality as a complementary crop to rubber (May, 2012).

Palm oil production in Malaysia has increased over the years and was expected to contribute 9.87 % to global production of oils and fats in 2015 (Basiron, 2015). Malaysian oil palm accounted for 5.4 million hectares out of global cultivated hectares of 258.9 million for the 10 main oilseed crops. Regarded as the most traded oil crop in the world after soy (Sumathi *et al*., 2007) with yield of about 10 fold higher than that of soybean (Habib *et al*., 2014), it has become the most important commodity crop in Malaysia. Palm oil is acclaimed to be the richest vegetable oil plant and the palm kernel oil is used for making soap, cosmetic, glycerol, margarine, explosives, and refined edible vegetable oil (Basiron, 2015). Tenera variety, a cross between dura and pisifera with a yield of about 5 tons of crude palm oil (CPO) per hectare and 1 ton palm kernels is the most widely cultivated variety in Malaysia (Palm Oil Health, 2016).

2.3 Orange spotting (OS) disease of oil palm

Orange spotting, a disorder of oil palm (Coulter and Rosnquist, 1955; Forde and Leyritz, 1968) was first recognized in West Africa in early nineteenth century. Orange spotting is found in commercially grown plantations in South Pacific and South-East Asia regions and has been associated with losses up to 50 % in a single OS affected palms compared with adjacent healthy palms (Forde and Leyritz, 1968). Randles (1998) and Hanold and Randles (1991) observed that OS has been discerned in oil palm plantations in Indonesia, Malaysia, the Solomon Islands, Thailand, the Philippines, Papua New Guinea, South America and Central America. Possible causes related with this disorder includes genetic origin and nutrient deficiency (Forde and Leyritz, 1968; Coulter and Rosenquist, 1955). Previous studies associated OS with the lethal Coconut cadang-cadang disease in Philippines which is caused by Coconut cadang-cadang viroid (CCCVd). Imperial *et al*. (1985) reported similar disease caused by a viroid exhibiting oil palm OS closely related to Coconut cadang-cadang viroid. The idea that a viroid closely related to CCCVd was the causative organism of OS was birthed with the unpredicted CCCVd-like RNA detection of in 1986, in oil palms with OS symptom in the Solomon Islands (Hanold and Randles, 1991).

Bright orange spotting which appear bronze coloured to necrotic at a distance typically expresses on infected oil palms, although younger fronds are usually spots free, with the number and size of fronds increasing with age (Hanold and Randles, 1991). Young fronds are characterized by 2 to 3 mm long non-necrotic, irregularly shaped spots occurring between leaflet veins. Spots usually coalesce into large irregular circular patches with age, while older fronds expresses distal necrosis of leaflets (Forde and Leyritz, 1968).

Phenotypically, palms with OS are less productive, bears smaller fruit bunches with yields 25 – 50 % lower than healthy plants, and their heights are reduced height (Forde and Leyritz, 1968; Hanold and Randles, 1991). Seed transmission of the disease happen at a rate of 1 in 300 (Bonaobra *et al*., 1998). Orange spotting was first recorded in Malaysia in 1941 (Hartley, 1988) but was revealed as an oil palm disease by Turner (1981). Report by Selvaraja *et al.* (2012) indicated about 74.3 % occurrence of visually-observed incidences of OS disease (CCCVd) from a 15 year-old oil palm stand.

In an earlier study, viroid-like molecules were characterized through isolation and sequencing from an asymptomatic oil palm. Studies showed variants of CCCVd in oil palms in Malaysia had over 90 % sequence similarity in comparison with CCCVd from coconut palm, although they were present at lower concentrations in oil palm compared to CCCVd in coconuts (Vadamalai, 2005). In view of this, these CCCVd variants may pose a risk of unknown epidemic in the development of oil palm industries in Malaysia. Studies by Wu *et al*. (2013) and Joseph (2012) confirmed the existence of variants of CCCVd in OS oil palms with 99 % sequence similarity with CCCVd from coconut. A previous study conducted by Joseph (2012) reported that inoculation of a CCCVd variant containing nucleic acid extract from an OS palm into healthy oil palm seedlings showed 100% similarity to the inoculums after nine months.

Study by Thanarajoo (2014) on the movement of variants of CCCVd in the oil palm seedlings by means of real-time PCR showed that the CCCVd variants concentration in stems and leaves were much higher as compared to its concentration on roots.

2.3.1 Infectivity and spread

The OS disorder and viroid-like molecule associated with this disorder occurs and spread naturally in the field (Hanold and Randles, 1991). In Solomon Island, survey done on 12-year-old plantation showed 85 % of disease incidence while an oil palm plantation in Medan, North Sumatra, showed OS incidence of 1 – 2 % and such an early detection could be due to seed transmission. Orange spotting disorder was reported as seed transmissible (Bonaobra *et al*., 1998). The disease incidence in 10 years old oil palm in Peninsular Malaysia was reported to be at 5 % as compared to 1 – 2 % in east Malaysia (Cheong, 2012). This concurred with the report based on observation on symptom expressions in the oil palms that correlates between disease incidence and palm age (Zelazny, 1980). Survey conducted by Selvaraja *et al*. (2012) in a Malaysian oil palm plantation on spatial variability of the OS incidence through remote sensing showed 74.3 % of the palms had OS symptom and the disease severity studied showed 73 % of the area with low OS disease severity and 2 % with high severity. However the study did not confirm the CCCVd detection on the experimental field.

2.4 Viroids

Diener (1971) introduced the first term of viroid as a virus-like particle to describe the infectious agent. They are industrial, ornamental plants and food pathogens with inconspicuous diseases compared with those caused by bacteria, nematodes and fungi (Randles, 2003). Viroids are small, circular, non-encapsidated, single-stranded pathogenic RNA molecules. Replication and other functions is dependent on plant host enzyme due to their inability to code for proteins. (Keese and Symons, 1985; Flores *et al*., 2005). Plant diseases are induced from host factor interactions but pathogenicity mechanism is rather yet unknown while their effect extends further than direct effects on quality and yield (Randles, 2003).

2.4.1 Structure of viroids

2.4.1.1 Circular nature

The circular nature of viroids was first presented using electron microscopy. The molecules however appear as rods under non-denaturing conditions with average length of 37 mm for PSTVd and axial ratio of 20:1. Sanger *et al*. (1976) and McClements and Kaesberg (1977) showed the molecules as 100 nm contour length of covalently closed circles. As observed in Figure 2.1, viroid preparations typically contain rods and circles with a variable ratio of linear molecules.

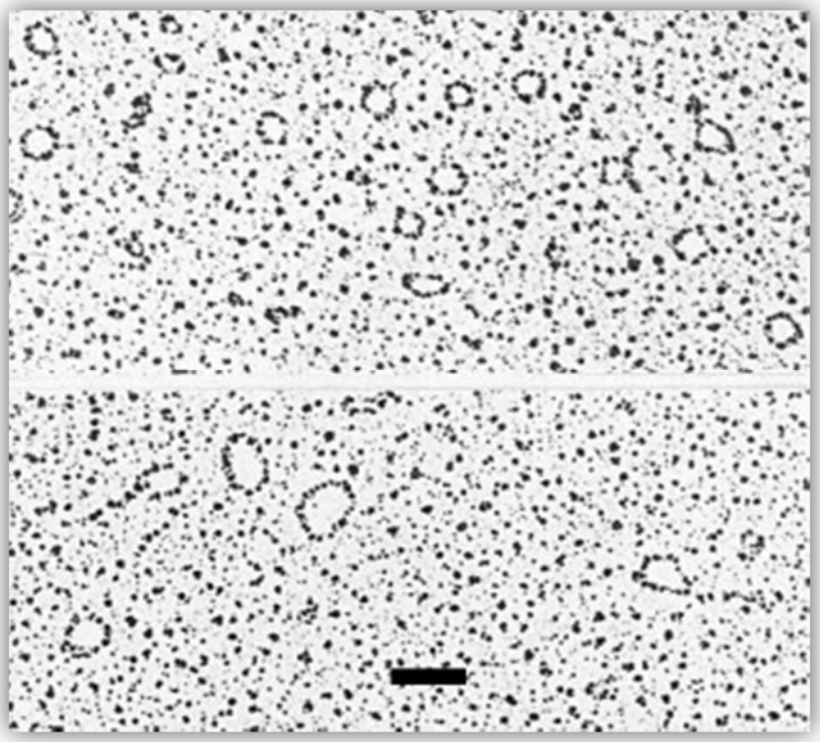


Figure 2.1 : Purified Coconut cadang-cadang RNA under denaturing conditions using electron microscopy showing denatured dimeric circles (below) and monomeric (*above*) forms of the viroid (Randles and Hatta, 1979). Bar represents 300 nm with stained grids of uranyl acetate and rotary shadowed.

2.4.1.2 Sequence of nucleotide

The sequences of nucleotide of about 31 viroids are now acknowledged. The circular structure of the viroid constitutes a range between 246 to 375 nucleotides sequence (Visvader and Symons, 1985). Viroids have certain degree of sequence similarity where 31 different viroids were recognized, and were positioned in seven genera. Viroids have a comparatively high Guanine (G) +Cytosine (C) content (53 - 60 %). The cDNA clones are used for sequencing of viroids from field isolates and have been discovered that a range of closely related sequence variants can be obtained from a single isolate (Visvader and Symons, 1985).

2.4.1.3 Secondary structure

The RNAs of *Avsunviroidae* are less structured and may contain host proteins associated tertiary structure while those of *Pospiviroidae* members form rod-like structures with unpaired loops intermingled base-paired regions (Branch *et al*., 1985; Gast *et al*., 1996). Members of this family owns the capacity to self-cleave with its hammerhead ribozymes through a rolling-circle mechanism (Flores *et al*., 2000) and do not consist a central conserved region (CCR) (Navarro and Flores, 1997) (Figure 2.2). The *Pospiviroidae* family assume rod-like structures and contain five functional

domains: terminal right and left domains (TR and TL respectively), variable (V), central (C) and pathogenicity (P) (Keese and Symons, 1985; Flores *et al*., 2005) (Figure 2.3). Symptom expression is though controlled by bases situated within the TR, TL, V and P domains (Sano *et al*., 1992). The V domain is a variable region and shows highest sequence variability between closely- related viroids (Keese and Symons, 1985; Keese *et al*., 1988). The P domain contains purine nitrogenous bases rich in adenine in one strand and an oligo (U) bases in the opposite strand and both strands have been associated in pathogenesis. The central conserved region (CCR) within the central domain characterizes viroid. Viroid from this family have a nuclear localization whereby the replication occurs through rolling circle mechanism using either a symmetric or asymmetric pathway in three steps, RNA transcription, processing and ligation. The terminal left conserved hairpin (TCH) with sequence (CCCCUCUGGGGAA) are found in viroids about 300 nucleotides unlike TCR (Keese and Symons, 1985).

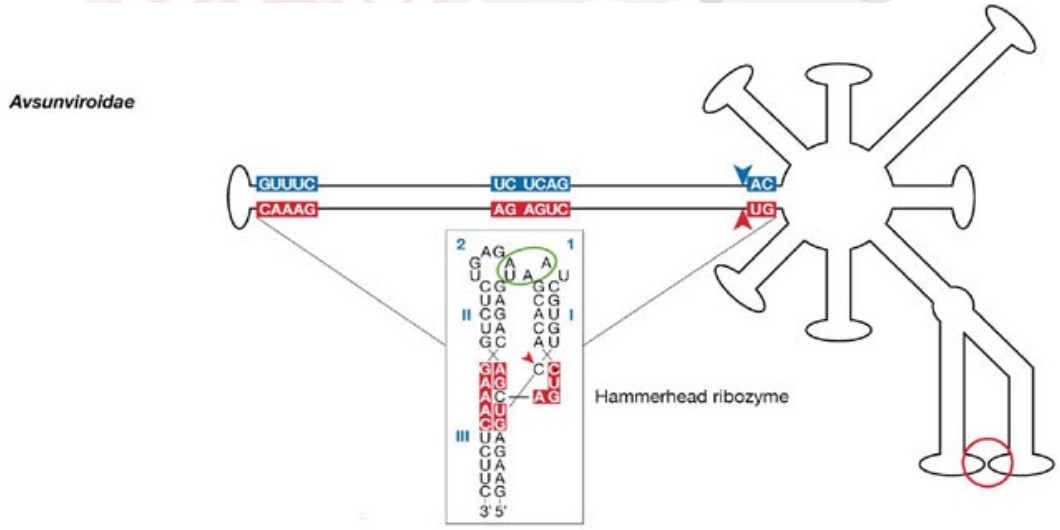


Figure 2.2 : Mechanism of Peach latent mosaic viroid structure (PLMVd; family *Avsunviroidae)*, in which the sequences conserved in most natural hammerhead ribozymes are observed and the self-cleavage sites are directed by arrowheads (Source: Daros *et al*., 2006).

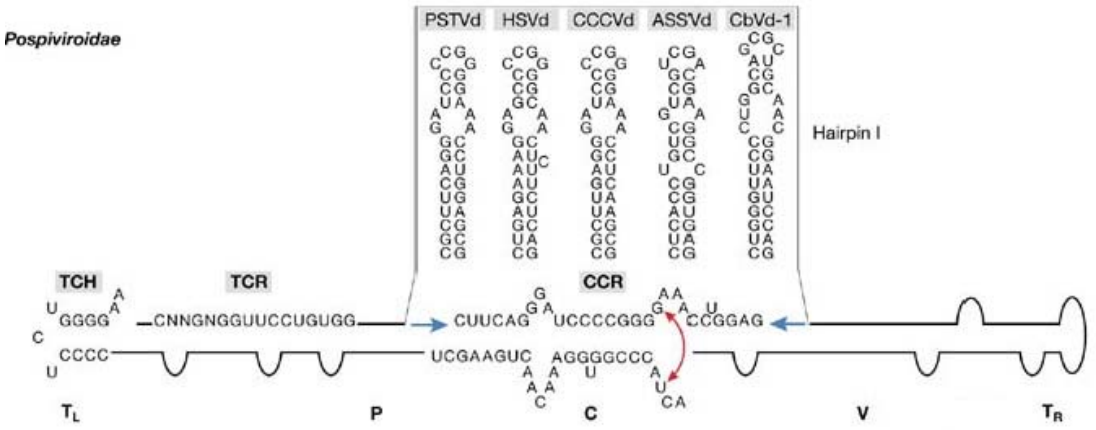


Figure 2.3 : Mechanism of viroid structure of the family *Pospiviroidae* comprised with five structural-functional domains: terminal left and right (TL and TR, respectively) domains, variable (V), pathogenicity (P) and the central conserved region (CCR) within the C domain (Source: Daros *et al*., 2006).

2.4.2 Classification of viroids

Two viroids family classes exist: the *Pospviroidae* and *Avsunviroidae* based on sequence and RNAs structures with each family having several genera (Flores *et al*., 2000). A complete classification according to family and genus is presented in Table 2.1.

Table 2.1 : Classification of viroid according to family and genus

Family *Avsunviroidae*	
Genus *Avsunviroid*	*Avocado sunblotch viroid* (1 species)
Genus *Pelamoviroid*	*Peach latent mosaic viroid, Chrysanthemum chlorotic mottle viroid* (2 species)
Genus Elaviroid	*Eggplant latent viroid* (1 species)
Family Pospiviroidae	
Genus *Pospiviroid*	*Tomato planta macho viroid, Citrus exocortis viroid, Tomato chlorotic dwarf viroid, Columnea latent viroid, Pepper chat fruit viroida, Iresine viroid* 1*, Potato spindle tuber viroid, Mexican papita viroid, Tomato apical stunt viroid, Chrysanthemum stunt viroid* (10 species)
Genus *Hostuviroid*	*Hop stunt viroid* (1 species)
Genus *Cocadviroid*	*Coconut cadang-cadang viroid, Hop latent viroid, Citrus bark cracking viroid, Coconut tinangaja viroid* (4 species)
Genus *Apscaviroid*	*Grapevine yellow speckle viroid* 2*, Citrus viroid Va, Apple dimple fruit viroid, Grapevine yellow speckle viroid* 1*, Apple scar skin viroid, Citrus viroid VIa, Australian grapevine viroid, Citrus dwarfing viroid, Citrus bent leaf viroid, Pear blister canker viroid* (10 species)
Genus *Coleviroid*	*Coleus blumei viroid* 1*, Coleus blumei viroid* 2*, Coleus blumei viroid 3* (3 species)

(Source: Di Serio et al., 2014)

2.4.3 Replication of viroids

Robertson and Branch (1987), Flores *et al*. (1997) and Symons (1997) have reviewed viroid replication in details. Viroids does not have adequate information to code for an RNA replicase instead they could code for a proportionally small polypeptide which can form viroid-specific replicase from proper syndicating with host proteins, but do not translate to give any polypeptides (Sanger, 1987; Symons, 1997). The *Pospiviroidae* RNAs are located in the nucleus while those of the *Avsunviroidae* family accumulates in the chloroplast (Flores *et al*., 2005). Viroids replicate through a mechanism known as RNA rolling – circle (Branch *et al*., 1988). Complexion of circular monomers to long linear multimers provides a rolling circle form of replication which engender to progeny viroid RNA (Owens and Diener, 1982). This mechanism has two variation; symmetric and asymmetric pathways. Members of

family *Avsunviroidae* follows symmetric pathways and members of family *Pospiviroidae* follows the asymmetric pathway (Diener, 2001).

2.4.4 Pathology of viroids

2.4.4.1 Macroscopic disease symptoms

Both monocotyledonous and dicotyledonous plants are infected by viroids. Viroid infection show symptoms such as necrosis, stunting, mottling, chlorosis and leaf epinasty which are escalated by high temperature (Tessitori *et al*., 2007). In the case of CCCVd, symptoms cover an extensive range from the slowly emerging destructive disease in coconut palms (Haseloff *et al*., 1982) to the non-symptomatic viroid infections remain to be discovered (Puchta *et al*., 1988).

2.4.4.2 Subcellular location of viroids

Viroids are located mainly in the nucleus or in the chloroplast. For instance in ASBVd-infected leaves, 80 % of the viroid RNA was detected in the chloroplasts (Lima *et al*., 1994) and thylakoid membranes (Bongfiglioli *et al*., 1994). This was supported by a study done by Marcos and Flores (1990) that the viroid RNA was not found in the nuclei. Viroid RNA of the PLMVd- infected leaves also accumulated in chloroplast (Bussiere *et al*., 1999) and may represent a common subcellular location of all members of the family *Avsunviroidae*, with hammerhead structure. The non-hammerhead structure of family *Pospiviroidae*, were located in the nucleus of cells infected with HSVd (Takahashi *et al*., 1982), TPMVd (Galindo *et al*., 1982), CPFVd (Muhlbach and Sanger, 1979). Within nuclei, CCCVd and PSTVd were located in nucleoli (Harders *et al*., 1989; Bonfiglioli *et al*., 1994; 1996). Few other viroids have been detected in the cytoplasm, membrane fractions but were not associated with organelles (Marcos and Flores, 1990).

2.4.4.3 Movement in the plant

The rapid movement of viroids from one cell to another cell is facilitated by a structural motif through the plasmodesmata (Lazarowitz and Beachy, 1999). This was proven from an experiment whereby RNA transcript of the vector could not move from the injected cell but when the RNA transcript included PSTVd, it moved from one cell to another cell (Zhu *et al*., 2001). Also, the phloem enables observation of long distance movement of the viroid in plants (Zhu *et al*., 2001). The viroid RNA are resistance to nuclease attack and perhaps eases their long-distance movement (Palukaitis and Zaitlin, 1987).

2.4.4.4 Transmission

Viroids transmission in most of their hosts is through a mechanical process. Transmission occurring in the field may be due to contamination by the tools and other similar means (Diener, 1979). The transmission is probably due to the secondary

structure of viroid and viroids complex nature to host constituents during the process of transmission. Also, viroids are seed and pollen transmissible (Wah and Symons, 1999). An example as observed clearly, PSTVd is seed and pollen transmissible in potato plants (Grasmick and Slack, 1986) and can endure in diseased seed for a lengthy period of time. Numerous viroid transmission occurring via pollen and seed have been observed in tomatoes (Kryczynski *et al*., 1988) and grapes (Wah and Symons, 1999).

2.4.4.5 Epidemiology

Viroids are mainly spread through crops by mechanical means, vegetative propagation, pollen, and seed. The different ways of transmission varies with type of viroids and hosts. Many of the viroids were first exist after 1940 (Diener, 1987). Several factors are responsible for the unexpected speedy proliferation of new diseases of viroid origin. Presence of a viroid in a host nature and perhaps causes no disease can jump into nearby crops and rapidly spread in the crops. A study by Barbara *et al*. (1990) observed that recent generality of HLVd in hops is as a result of recognized infection along the propagation management system of hops in the late 1970s.

2.4.5 Molecular basis for biological activity

The fact that many sequence variants of a specific viroid may exist in nature provides a range of viable mutants. The sequence correlation methodology are advantageous as numerous sequence of viroid variants might be present in nature, which gives a reach from claiming feasible mutants. A real problem will be that many infections occurring naturally consist of a mixture of viroid variants. This issue has been overwhelmed by the cDNA clones of isolates preparation each with a solitary characterized nucleotide sequence (Visvader and Symons, 1985). Eleven new variants of CEVd were isolated by cloning of cDNA. Sequence variations situated in the V and P domains (Visvader and Symons, 1986). Practically it is found that numerous nucleotides changes in a sequence of viroid distributed as non-transmittable although changes are expected to have least effects on secondary structure (Owens, 1990). Viroids cause various diseases, ranging from symptomless to destructive.

2.4.6 Diagnostic procedures for viroids

Viroids do not code for protein. Hence, serological methods commonly used in viruses could not be used for diagnostic procedures of viroid diseases. Also, because no particles of characteristic can be ascertained, electron microscopy procedures are irrelevant (Hull, 2002). Molecular diagnosis through biological tests, gel electrophoresis, hybridization experiments and polymerase chain reaction (PCR) have been bound for these reasons.

2.4.6.1 Polyacrylamide gel electrophoresis (PAGE)

Partial purification procedure is required and involves running the nucleic acids in a proper PAGE system. This is due to the low concentration of viroids in the infected

hosts. It has been widely used to detect CCCVd in coconut palms. This gel system is very specific that it separate CCCVd mutants with addition or deletion of single base in secondary structures (Rodriguez and Randles, 1993). However, this system did not perform well and had to undergo additional hybridization procedure in determining CCCVd-like RNAs in oil palms (Vadamalai *et al*., 2006; Joseph, 2012).

2.4.6.2 Reverse transcription –polymerase chain reaction (RT-PCR)

Viroid RNAs are synthesized by converting them to cDNA through the process of reverse transcription and further using the cDNA for RT-PCR amplification. RT- PCR has been widely used in the diagnosis of numerous viroids such as CCCVd, ASBVd, CEVd, ASSVd, and CTiVd (Hadidi and Yang, 1990; Schnell *et al*., 1997). RT-PCR was extensively used in detection of CCCVd variants in oil palm using specific primer sets (Vadamalai, 2005; Joseph, 2012; Wu *et al*., 2013). Also, low concentration of the RNA in oil palm allows re-amplification of RT-PCR products (Vadamalai, 2005; Vadamalai *et al*., 2006). Effective detection of CCCVd was achieved through RT-PCR reducing the need for hybridization probes (Hadidi and Yang, 1990).

2.5 Coconut cadang-cadang viroid (CCCVd)

Coconut cadang-cadang viroid (CCVd) is a species of the genus Cocadviroid in the family *Pospiviroidae* (Flores *et al*., 2000). CCCVd is the causative agent of Coconut cadang-cadang disease in Philippines. Cadang - cadang which originated from 'gadan-gadan' (meaning dying or dead) refers to the premature gradual death or decline of coconut palms infected with the viroid. CCCVd host range comprises the Palmae family members, which includes royal palm (*Oreodoxa regia*), betel nut palm (*Areca catechu*), date palm (*Phoenix dactylifera*), palmera (*Chrysalidocarpus lutescens*), MacArthur palm (*Ptycosperma macarthuri*) and Manila palm (*Adonidia merillii*) (Imperial *et al*., 1985). However, assays using molecular hybridization additional species of palms and other monocotyledons in Oceania and South-East Asia have been shown by molecular hybridization assay to contain CCCVd-related sequences (Hanold and Randles, 1991).

RNAs within the size range anticipated for CCCVd which hybridize with probes and represents a part or all minimal 246 nucleotides are known as CCCVd-related sequences. Sequence information is generally not available for these. Evidence exist of their circularity nature which confirms them to be putative cocadviroids (Hanold and Randles, 1991; Hanold, 1998). Although the means of field natural inoculation is unknown, CCCVd like molecules have been related with OS disorder in plantations of African oil palm across South pacific and South-East Asia (Hanold and Randles, 1991; Randles, 1998). Mother palms assisted pollination using pollen obtained from infected palms have shown encouraging transmission, while positivity for CCCVd have also been established for insignificant percentage of infected seed nuts and produced progenies. Also, successful transmission to palms have been observed from contaminated harvesting scythes (Hanold and Randles, 1991). The basic form of CCCVd is 246 nucleotide molecule.

According to Vadamalai (2005), asymptomatic and symptomatic oil palms have exhibited the presence of CCCVd-like RNAs in their nucleic acid extracts especially in Malaysia. Further studies by Wu *et al*. (2013); Joseph (2012) and Vadamalai, (2005) confirmed the CCCVd variants had more than 90% sequence similarity compared with CCCVd in coconut. This can be considered a threat to oil palm production (Vadamalai, 2005).

2.5.1 CCCVd variants in palms

Small CCCVd forms can be detected at the onset of the development of the disease. Larger viroid forms, typically between 287 and 301 nucleotides are seen as the disease progresses and usually occur as a result of repetition of 41, 50 or 55 nucleotides at the viroid right terminal (Rodriguez and Randles, 1993; Haseloff *et al*., 1982). Variants of coconut CCCVd comprises of 'fast' ($CCCVd_{op246}$ and $CCCVd_{OP247}$) and 'slow' ($CCCVd_{OP296}$ and $CCCVd_{OP297}$) monomeric forms as well as dimeric forms of each 'fast and 'slow' monomers (Randles, 1985). Insertion of cytosine at position 197 resulted in 247 and 297 nucleotides. Variants of CCCVd in coconut in the form of 246, 247, 287, 296, 297 and 301 (Rodriguez and Randles, 1993; Haseloff *et al*., 1982).

CCCVd variants have been identified in oil palms, characterization showing over 90 % sequence similarity with coconut CCCVd. Wu *et al*. (2013) observed base changes in pathogenicity domain at $C^{31}\rightarrow U$ and in the central domain at $G^{70}\rightarrow C$ in CCCVd variants of 246 nucleotides in comparison with CCCVd from coconut. Joseph (2012) discovered additional substitution in the terminal right domain at $C^{140}\rightarrow A$ for CCCVd variants from both asymptomatic and symptomatic palms which had 97 % sequence similarity with $CCCVd_{OP246}$. Vadamalai (2005) discovered 3 'slow' forms of CCCVd variant with (270, 293, and 297) nucleotides characterized from asymptomatic palm having over 90 % similarity to the variant from coconut.

2.5.2 Pathogenesis

Pathogenesis by viroids is poorly understood. However, increase in CCCVd size leads to disease progression and development of more severe symptoms (Rodriguez, 1998). The molecular form of host isolated CCCvd is directly co-related with the development stage of the disease. Mohamed *et al*. (1985) states that 'fast' form (246/247) inoculation upon detection gave rise to small length of CCCVd and contributed to the detection of large CCCVd forms known as 'slow' monomeric forms in the infected seedlings.

2.5.3 Pathogenicity study

Whole plant pathogenicity study typically involves viroids introduction through mechanical means like high pressure injection and slashing into specific seedlings. Crude extract inoculums have shown high viroid infectivity rate, but more successful infection rate is typically achieved with the use of cDNA clone viroids (Sanger, 1987). Joseph (2012) observed that inoculation of pre-germinated oil palm seedlings with

extracts of nucleic acid from OS palm expressed symptoms nine months after inoculation, while inoculated tissue culture showed symptoms after 6 month of inoculation. The result obtained was 100 % sequence homology to the inoculum. A more recent study by Thanarajoo (2014) showed no significant correlation between accumulation in inoculated seedlings and viroid variant. The study further observed the random systemic movement of CCCVd variants in oil palm seedlings with CCCVd detected at higher concentrations in leaves and stems as compared to the roots. This is the first study on movement of CCCVd viriods reported.

CHAPTER 3

OPTIMIZATION OF TOTAL NUCLEIC ACID (TNA) EXTRACTION METHOD FOR DETECTION OF COCONUT CADANG-CADANG VIRIOD (CCCVD) VARIANTS IN OIL PALM

3.1 Introduction

Extraction of high quality RNA is necessary for making cDNA libraries, characterization and detection by RT-PCR or investigating gene expression profile (Gehrig *et al*., 2000). However, high phenolic and/or polysaccharides compounds level give rise to poor RNA quality or no absence of RNA. Shu *et al*. (2014) observed that research in molecular biology, especially cDNA synthesis requires high quality RNA, while Ergun *et al*. (2013) pointed out that PCR sensitivity is affected by polysaccharides, polyphenols and phenol contents of plants which may ultimately lead to false results. In the same vein, Xiao *et al*. (2012) reported that high quality and copious RNA is needed for sequencing strategies involving RNA, particularly for coconut and oil palm which are rich in polyphenols and polysaccharides and possess leaves that have high fibre content and waxy cuticle. A study by Loomis (1974) evidenced that readily oxidized compounds of phenolic are linked covalently with quinones, and binds with nucleic acids, while co-precipitation of polysaccharides with RNA can occur in low ionic strength buffers (Ho *et al*., 1996). Based on these submissions, it is therefore paramount that extraction methods produces high concentration of RNA, thus the need for optimization.

There are many extraction methods being modified and optimized for better quality and yield of RNA. A few extraction methods suitable for CCCVd extraction have been tested that includes Polyethylene Glycol (PEG) and Sodium Chloride EDTA Tris-HCl Mercaptoethanol (NETME) extraction methods (Vadamalai, 2005; Joseph, 2012). PEG extraction methods established by Hanolds and Randles (1991) was proven to be successful for detection of CCCVd variants from monocots, especially coconut and oil palm. However, this method is highly labourious as it requires large amount of leaves sample (20-100 g) and time consuming (Hanold and Randles, 1991; Vadamalai, 2005). NETME is a suitable choice for simpler extraction with small amount of leaves sample (2-5 g) used throughout the experiment. NETME extraction method undergoes purification step through Polyacrylamide gel electrophoresis (PAGE). This purification step provides high quality of RNA in terms of its purity and its concentration. However, the purification step is time-consuming and laborious. It requires additional two days experiments and proven to be inconsistent in detection when repeated.

This chapter describes the modification and optimization of NETME extraction method in order to shorten the time for extraction and to obtain a better quality RNA in order for better detection of CCCVd oil palm variants with eliminating the PAGE purification process.

3.2 Materials and Methods

3.2.1 Sample collection

Leaflets from nine years old oil palm with OS symptom and healthy leaflets of oil palm were sampled from commercial oil palm plantations in Sungai Buloh, Selangor, Malaysia. One symptomatic palm with replicates (OSP1, OSP2, and OSP3) and one healthy palm (HP) were collected respectively. The leaf samples were brought to laboratory. The leaves were cut into 15 - 20 cm long after removing the midribs, cleaned, sealed in plastic bag, labelled properly and stored at -80 °C. Brown, wilted or bruised tissues were discarded as this indicated that cell breakdown had occurred with RNA degradation.

3.2.2 Conventional NETME extraction method

Total nucleic acid (TNA) extraction was carried out using the Hodgson *et al*. (1998) method as modified by Vadamalai (2005). Leaf (2 g) was ground in a mortar and pestle with liquid Nitrogen as homogenizer. The slurry obtained from grinding were transferred to 50 mL screw cap tube. Approximately 8 mL of NETME buffer, 4 mL of 1 % SDS and 16 µL 2- Mercaptoethanol were added and undergoes incubation for 30 mins at room temperature at 20 rpm in a mini-rotator (Glas-Col®, Terre Haute USA). The sample was centrifuged for 15 mins at room temperature (25 ºC± 2) at 11, 000 g, (Eppendorf 11K, Germany). The resulting aqueous phase supernatant was mixed with Phenol: Chloroform and Isoamylalcohol (PCA) mix in (1:1) ratio and centrifuged for 15 mins at room temperature at 11, 000 g (Eppendorf 11K, Germany). The supernatant was re-extracted with Cloroform: Isoamylalcohol (CA) mix in (1:1) ratio and centrifugation was ran for 15 mins at 11, 000 g (Eppendorf 11K, Germany) at room temperature. Further the supernatant was transferred to a new screw cap tube and was gently mix-inverted with 0.9 volume (vol) isopropanol. Incubation of the mixture was carried out at -20 °C for 3 hours and centrifugation took place for 10 mins at 11, 000 g (Eppendorf 11K, Germany). After incubation, the supernatant was disposed and the resulting pellet prior to centrifugation was washed with 700 µL of 70 % ethanol by mixing well and was then centrifuged for 10 mins at 11, 000 g (Eppendorf 11K, Germany) at room temperature. The final pellet obtained prior to centrifugation was allowed to air dry for 20 to 30 mins. It was dissolved in 150 mL of sterile double distilled water (SDDW) and stored at -20 °C.

3.2.3 Optimization of NETME extraction method

The optimization of NETME extraction method was carried out by modifying several steps in the conventional method as explained below and summarized in Table 3.1.

Table 3.1 : Summary of optimization steps for conventional NETME extraction method

Conventional NETME Method	Modified NETME Method
Homogenization and Extraction Buffer	
• 2 g of leaf samples were ground with liquid Nitrogen • Incubated in 8 mL NETME buffer • 4 mL 1 % SDS • 12.8 mL 2-Mercaptoethanol	• 10 g of leaf samples were ground with liquid Nitrogen and • 5 mL NETME buffer • 2.4mL 1 % SDS • 12.8 mL 2-Mercaptoethanol
Separation	
• Phenol/Chloroform/isoamyl Alcohol to supernatant ratio (1:1) • Chloroform/isoamyl Alcohol to supernatant (1:1)	• Phenol/Chloroform/isoamyl Alcohol to supernatant ratio (3:2) • Chloroform/isoamyl Alcohol to supernatant ratio (4:3)
Centrifugation	
• 11,000 g, 10 mins, room temperature	• 11,000 g, 15 mins, 4 ºC
Precipitation	
• Isopropanol	• Isopropanol • 1 mL 8 M Lithium Chloride • Supernatant with 1mL 100 % ethanol
Incubation	
• Pellets were incubated at -20 °C (Hodgson *et al*., 1998)	• Pellets were incubated at -20 °C

3.2.3.1 Homogenization and extraction buffer

The amount of leaf samples used for extraction was increased from 2 to 10 g. The leaf samples were ground with liquid Nitrogen to crush and reduced NETME extraction buffer, 1 % SDS (2.4 mL) and 2- Mercaptoethanol (12.8 µL) until fine slurry. Reduction of NETME extraction buffer was from 8 mL to approximately 3 to 5 mL, 1 % SDS from 4 mL to 2.4 mL and 2- Mercaptoethanol from 16 to 12.8 µL as compared to conventional NETME extraction method. Incubation for 30 mins in a mini-rotator described in the conventional NETME extraction method was skipped as a result of this modification.

3.2.3.2 Separation and centrifugation

The separation of supernatant and PCA mix was carried out using a ratio (3:2) rather than (1:1) as observed in conventional NETME extraction method. The following separation of supernatant and CA mix was done using a ratio (4:3) from (1:1) as in conventional method. The centrifugation carried out in this method was modified at 4 ºC for 20 mins at 11, 000 g (Eppendorf 11K, Germany) as compared to centrifugation at room temperature for 10 mins at 11, 000 g (Eppendorf 11K, Germany) as in conventional NETME extraction method.

3.2.3.3 Precipitation and incubation

Modification in this involved additional precipitation stages with isopropanol, lithium chloride and ethanol as compared to only isopropanol precipitation step from conventional NETME extraction method. The supernatant from isopropanol precipitation was re-precipitated with 1 mL of 8.0 M lithium chloride solution and incubation was carried out at -20 °C for 4 hours. Lithium chloride was utilized in the modification process to increase rate of precipitation. Subsequently, the resulting supernatant was further precipitated with 1 mL of 100 % ethanol and was incubated at -80 °C for 30 mins.

3.2.3.4 RNA quality assessment

The RNA quality (A260/280 and A260/230 readings) and concentration was measured using NanoDropTM 1000 spectrophotometer (Thermo SCIENTIFIC, USA). 1 µL of extracted TNA was pipetted onto the end of the fibre optic cable, also known as receiving fibre. A source fibre is introduced and made to have contact with the sample (liquid), thus the liquid bridges the gap between both optic fibre ends controlled to 1 mm and 0.2 mm paths. A PC-based software controls the instrument usually logged in a file on the PC. Light source is typically provided by a pulsed xenon which passes through the sample for easy analysis by a linear Charged-Coupled Device (CCD) array utilizing spectrophotometer.

3.2.4 Polyacrylamide gel electrophoresis (PAGE)

The nucleic acids were fragmented by 5 % non-denaturing polyacrylamide gel [in 50 mL: 5 mL 10 X TBE, 6.25 mL 30 % Acrylamide: Bisacrylamide (39:1), 43.75 µL TEMED, 937.5 µL 10 % ammonium persulphate, 37.78 mL SDDW] in 1 X TBE buffer. Cleaning of the plates made off glass (Biorad®) measured 16 X 20 cm were done with KOH buffer and SDDW (Appendix A). The nucleic acid extracts were assorted with 1:16X Bromophenol loading dye and were loaded on the polyacrylamide gel. Electrophoresis was done using 140 X 120 X 1.5 mm gels (BioRad) or 70 X 100 X 1 mm gels (BioRad) under non-denaturing conditions at 40 mA for 1 hour 30 mins.

3.2.5 Synthesis of cDNA

RNA are synthesized by converting them to cDNA through reverse transcription according to Rodriguez (1993) and Hodgson *et al*. (1998). The RNA for cDNA synthesis was obtained from the modified nucleic acid extraction. First strand cDNA was synthesized with AMV reverse transcriptase from AMV reverse transcription system (PROMEGA) and CCCVd definite reverse primer sets (Table 3.2) in a final vol of 20 µL reaction. According to procedure by Rodriguez (1993), 2 µL of nucleic acid extract and 0.5 of µM primer was added with 7.5 µL of nuclease - free water. Incubation was done at – 80 °C for 12 min was then placed on ice for 5 mins. The reverse transcription buffer requires components such as 24 U of AMV reverse transcriptase, 1 mM each dTTP, dGTP, dCTP, dATP and 20 U RNAsin. The buffer was added to make up a final vol of 20 µL. The process of reverse transcription continued with incubation of the mixture for 20 mins at 50 °C and again for 5 mins at 4 °C.

3.2.6 RT-PCR amplification

RT-PCR amplification of the RNA extracted from oil palm samples were carried out using two CCCVd specific primer sets. Primer set I (GV1 and GVR1) (Hodgson *et al*., 1998) and primer set IV (GV4 and GVR4) (Vadamalai, 2005).

Table 3.2 : CCCVd Specific Primer Set I and Set IV

Set of Primers	Sequence	Primer Type	Reaction	PCR product size
I (GVR1)	5'-d (AGG TTT CCC CGG GGA TCC CTC AAG CGG CCT C)-3'	Reverse	RT and PCR	242 nts
I(GV1)	5'-d(CGA ATC TGG GAA GGG AGC GTA CCT GGG TCG)-3'	Forward	PCR	
IV(GVR4)	5'-d (TGT ATC CAC CGG GTA GTC TC)-3'	Reverse	RT and PCR	246 nts
IV(GV4)	5'-d(ACT CAC GCG GCT CTT ACC)-3'	Forward	PCR	

The PCR was done using Go Taq Green master mix (PROMEGA). The PCR mix contained 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each reverse and forward primers (table 3.2), 25 unit/mL *Taq* polymerase, and nuclease - free water added to make up a total of 25 µL reaction vol. The product from cDNA was aliquoted to 5 µL added was added to 30 µL final vol. Initial denaturation of the mixture stated above was done at 96 °C for 3 min as delineated by Rodriguez (1993). Amplification was done under conditions stated below using T-Personal Thermal Cycler system (Biometra, Germany): 93 °C for 45 sec, 68 °C for 45 sec, and 72 °C for 3 mins, 35 cycles; 72 °C for 15 mins with primer set I and 93 °C for 45 sec, 60 °C for 45 sec, and 72 °C for 3 mins, 35 cycles; 72 °C for 15 mins with primer set IV.

3.2.6.1 Agarose gel electrophoresis

Agarose powder in 1.5 % w/v was weighed and placed into a conical flask. The agarose powder was then dissolved in 1X TBE buffer by heating in a microwave oven for about 2 mins. The agarose was heated until it was dissolved completely and was poured into a gel casting tray and was allowed to cool under room temperature for 10 mins. PCR products were then mix- pipetted with 6X blue/orange loading dye (PROMEGA, Madison, USA). The mixture of PCR product and loading dye was loaded into the gel that was earlier prepared on casting tray and the gel was run for 45 mins at 90 V.

3.2.6.2 Gel staining

The agarose gel run in electrophoresis was then placed into a container with 0.5 µg/mL ethidium bromide for 10 mins in 200 mL of distilled water. The de-staining of the gel with distilled water was done for 5 mins in a clean container. The PCR amplicons were visualized at 254 nm under Molecular Imager Gel Doc XR system (Biorad ®, USA).

3.2.6.3 Gel purification

A MinElute ® Gel Extraction Kit (QIAGEN) was used for excision and purification of the PCR amplicons according to the manufacturer's procedure. The excised PCR amplicons from the gel were transferred into 1.5 mL micro - centrifuge tubes and were weighed. 3 vols of buffer QG was added into the centrifuge tubes and incubation was carried out at 50 °C for 10 mins. Isopropanol of 1 vol was included to one gel ratio and the tube was gently- mixed. This was carried out for the other gel fragments in the centrifuge tube. The gel suspensions with isopropanol were shifted to the QIAprep spin column in collection tube and centrifugation took place at 13, 000 g (Eppendorf Minispin, Germany) for a min. The pass-through collected in the collection tubes were discarded. A total of 500 µL buffer QG measured 500 µL were added to the spin columns and centrifuged for 1 min at 13, 000 g (Eppendorf Minispin, Germany).

The flow-through in the collection tubes were disposed and the additional centrifugation were done at 13, 000 g (Eppendorf Minispin, Germany) for 1 min. The flow-through collected were discarded and the spin columns were added with 750 µL of buffer PE and centrifuged for 1 min at 13, 000 g (Eppendorf Minispin, Germany). The spin columns placed in the collection tubes were re-centrifuged at the same condition after discarding the flow-through. This step is included to wash away remaining ethanol residues from the buffer PE. The spin columns were transferred into a clean 1.5 mL micro-centrifuge tubes. Approximately 30 µL of SDDW were added to the centre of the spin columns and centrifugation occurred at 13, 000 g (Eppendorf Minispin, Germany) for 1 min for elution of DNA.

3.3 Results and Discussion

3.3.1 Assessment of RNA purity and quality using NanoDrop

Intact RNA through assessment of RNA purity and concentration is a crucial step for the successful application of advanced molecular biological methods (Fleige and Pfaffl, 2006). The presence of polyphenols and polysaccharides in the palms usually interfere with viroid appearance (Mohammadi *et al*., 2010). Results expressed in Table 3.3 indicate that the modified and optimized NETME extraction method resulted in higher quality RNA (RNA purity and RNA concentration) when compared with the conventional NETME method based on the A260/280 and A260/230 readings and concentration measured using NanoDrop™ 1000 spectrophotometer (Thermo Scientific, USA). The modified NETME extraction method yielded approximately 0.16 µg per gram of fresh tissue RNA and was at least 4 times more than values obtained using the conventional extraction method. The duration and speed of the centrifugation during lithium chloride precipitation are important to be kept at least at 12, 000 g (Eppendorf 11K, Germany) for more than 15 mins, and at temperature less than 4 °C as stated in the ThermoFisher SCIENTIFIC technical bulletin #160. This method is credited for its time efficiency and simplicity, although the RNA may be more susceptible to degradation during longer period of lithium chloride precipitation.

Table 3.3 : RNA quality from conventional and modified NETME extraction methods

Samples	Methods	A260/280	A260/230	Concentration (ng/ µL)
OSP 1	NETME	1.30	0.74	646.0
	Modified NETME	1.75	1.92	3745.5
OSP 2	NETME	0.99	0.64	1272.1
	Modified NETME	1.80	1.90	3478.7
OSP 3	NETME	1.03	0.66	227.8
	Modified NETME	1.90	1.70	1225.9

Purity ratios are important indicators of sample quality. According to the ThermoFisher SCIENTIFIC technical bulletin T009 and T042, absorbance ratio > 2.0 for 260 nm to 280 nm is generally considered pure for RNA. On the other hand, 260/230 ratio values for pure nucleic acid is often higher than the respective 260/280 values and is typically in the range of 2.0 to 2.2. Although the results from modification method showed higher purity as compared to the conventional method, the 260/230 and 260/280 ratios were still low for all samples and this may be due to the residual phenol from nucleic acid extraction and a very low concentration of nucleic acid respectively (Sambrook *et al*., 1989). Fleige and Pfaffl (2006) also observed that salt concentration and buffer utilized may interfere with optical density measurement under- or over- estimated concentrations of RNA.

3.3.2 Final pellets of TNA with respond to lithium chloride precipitation

Results showed that final pellets of total nucleic acid obtained from the modified extraction method gave rise to colourless and clearer pellet compared to the yellowish orange or brown pellets obtained with the conventional NETME extraction method for all samples (Figure 3.1). Yellowish orange or brown pellets typically indicates unsuccessful removal of contaminating phenols. Shu *et al*. (2014) observed that phenolic compound oxidation and polysaccharides co-precipitation with RNA produces browning effect and causes difficulty in dissolving RNA which lowers yield and hinders RT-PCR amplification because residual polyphenols during the process of extraction. The modified NETME method produced final RNA pellets in one day as compared to the conventional NETME which usually takes three days in addition to the purification PAGE step.

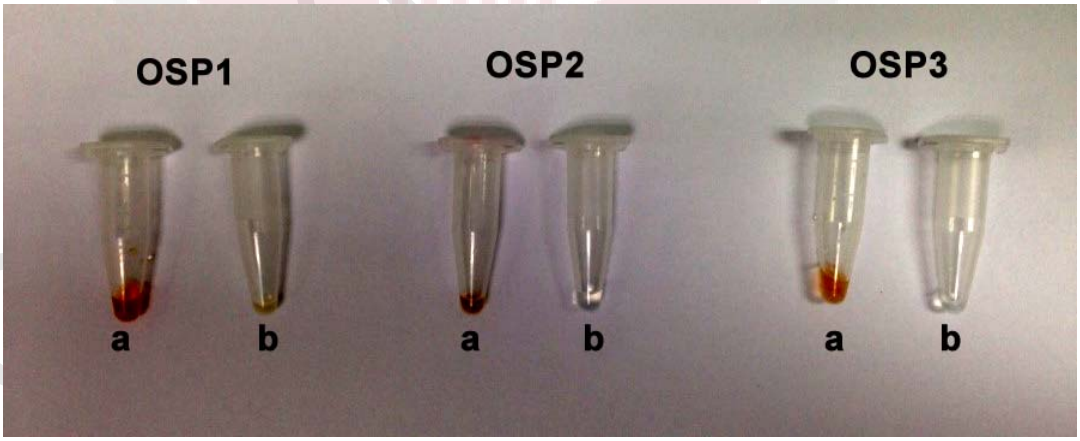


Figure 3.1 : Pellets obtained from (a) conventional NETME Extraction method and (b) modified NETME extraction method for various samples

The clearer and better RNA quality observed could be attributed to the removal of the accompanying DNA and 4S RNAs, recovery of small RNA and low molecular weight viroid necessitated by the additional lithium chloride precipitation (Granell *et al*., 1983), which was a key modification in the extraction protocol for RNA. Lithium chloride precipitation does not efficiently precipitate DNA and carbohydrate, but removes inhibitors of cDNA synthesis (Barlow *et al*., 1963; Cathala *et al*., 1983; Apt *et al*., 1995). The molarity of lithium chloride is normally fixed at 2.0 – 8.0 M for precipitation of RNA (Verwoerd *et al*., 1989; Wilkinson, 2000; ThermoFisher SCIENTIFIC). The task of extracting intact total nucleic acid (TNA) is difficult due to the susceptibility of RNA molecules to the enzymatic degradation by RNase. The modification allowed consistent production of high quality RNA, reduced extraction time and the extracted RNA was suitable for downstream processes like cDNA synthesis and expression pattern analysis as observed by Kundu *et al*. (2011), Pandey *et al*. (1996) and Ghawana *et al*. (2011).

3.3.3 RT-PCR amplification

RT-PCR amplification of samples from the modified NETME method using CCCVd specific primer led to consistency in band retrieval and detection of CCCVd viroid as expressed in Figures 3.2 and 3.3.

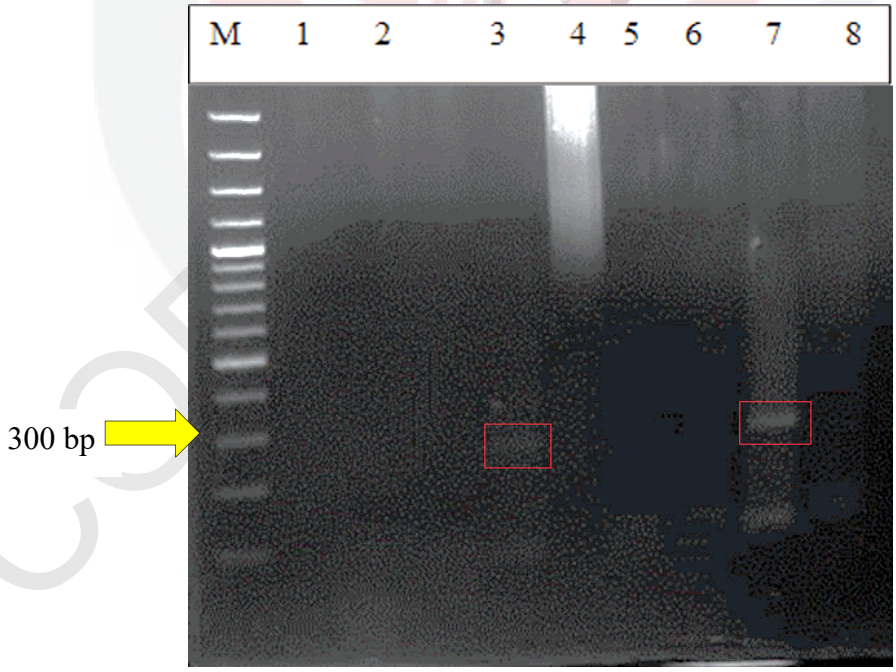


Figure 3.2 : RT – PCR amplification using primers set I of total nucleic acid from modified NETME extraction analysed on 1.5 % agarose gel electrophoresis. M: Marker (100 bp). Lane 1: Negative control (SDW as PCR template), Lane 2: HP, Lane 3: OPS1, Lane 4: OPS1, Lane 5: OPS2, Lane 6: OPS2, Lane 7: OPS3

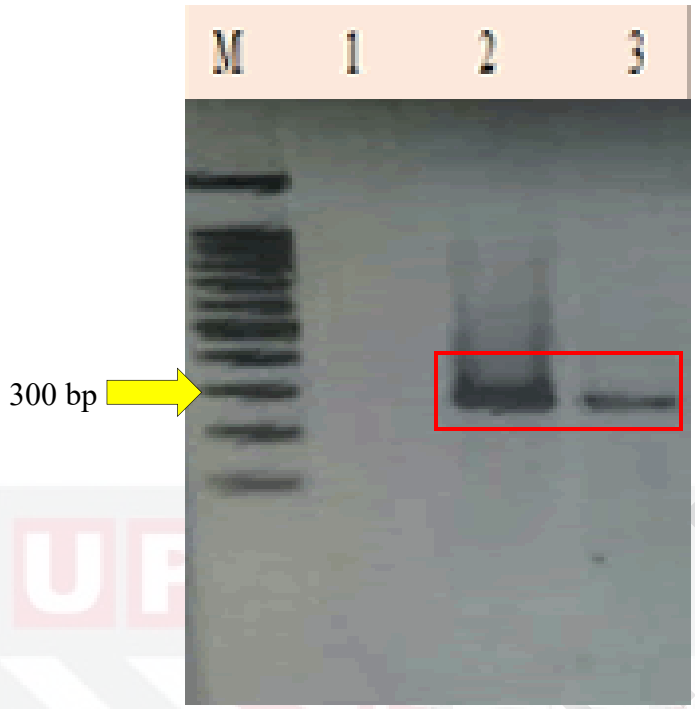


Figure 3.3 : RT – PCR amplification of total nucleic acid from modified NETME extraction method using primers set IV viewed on 1.5 % agarose gel and stained with EtBr. M: Marker (100 bp), Lane 1: HP, Lane 2: OSP1 and Lane 3: OSP2

On the other hand, RT – PCR amplification of samples from the conventional NETME extraction method using primer set IV showed no viroid - like molecules detected, while one sample, OSP1 showed CCCVd- like RNA detected using primer set I as seen in Figure 3.4. Sample OSP1 was able to detect CCCVd- like RNA easily with primer set I as compared to the OSP1 with primer set IV because the primer set I is a partial length primer that only detects the viroid (242 bp). Non detection of viroid may be due to the low yield of RNA from the sample. In comparison, the modified NETME extraction method showed viroid detection on most of the sample with both primers set I and set IV. High quality RNA obtained from modification of extraction procedure enabled the detection of circular form viroid using full length primer set IV that amplified approximately 250 amplicon.

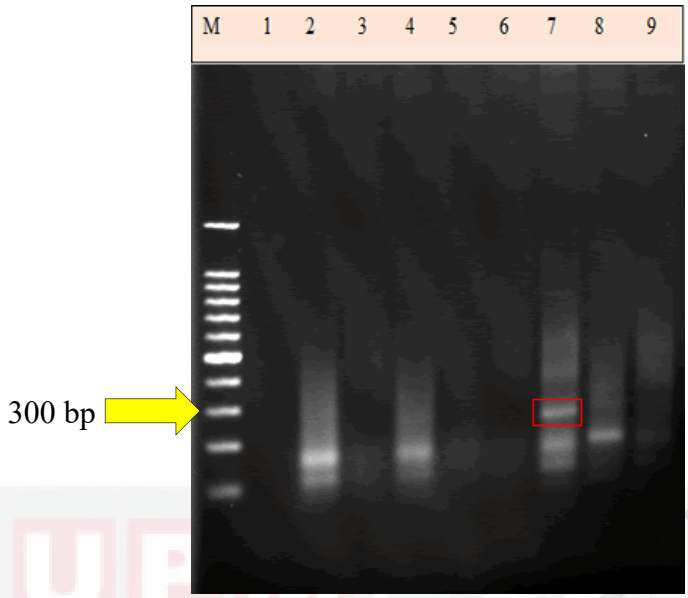


Figure 3.4 : RT – PCR amplification using both primer set I and set IV from conventional NETME extraction method analysed on 1.5 % agarose gel and stained with EtBr. M: Marker (100 bp), Lane 1: negative control, Lane 2: OSP1, Lane 3: OSP2, Lane 4: OSP3, Lane 5: HP with primer set IV and Lane 6: HP, Lane 7: OSP1, Lane 8: OSP2, Lane 9: OSP3 with primer set I.

These results support the fact that the modified NETME extraction method led to high quality RNA which made band retrieval at approximately 250 amplicons using both sets of primer mentioned above possible. Detection of viroid through RT-PCR using RNA extract from conventional NETME method was only feasible after running on 5 % non-denaturing Polyacrylamide Gel Electrophoresis (PAGE) purification step. With this additional step, viroid detection was possible for all three samples OSP1, OSP2 and OSP3 as observed in Figure 3.5. This additional step was eliminated with the modified NETME extraction method which ensured consistency in the detection of CCCVd- like RNAs. These results are further summarized in Table 3.4.

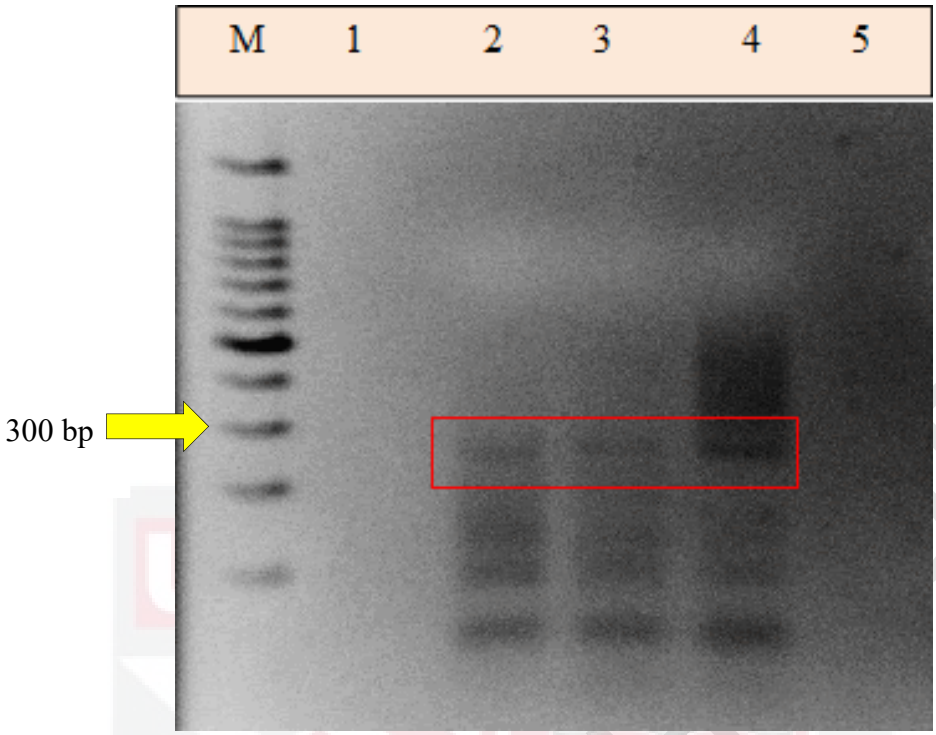


Figure 3.5 : RT - PCR amplification from purification with 5 % non-denaturing PAGE using Primer Set IV generated on 1.5 % agarose gel and stained with EtBr. M: Marker (100 bp), Lane 1: negative control, Lane 2: OSP1, Lane 3: OSP2, Lane 4: OSP3, Lane 5: HP

Table 3.4 : Presence (✓) and absence (x) of CCCVd-like RNA in the leaf samples extracted using conventional and modified NETME extraction method.

Samples	Conventional NETME Extraction Method		Modified NETME Extraction Method	
	Primer Set 1	Primer Set IV	Primer Set 1	Primer Set IV
OSP 1	✓	x	✓	✓
OSP 2	x	x	✓	✓
OSP 3	x	x	x	x
HP	x	x	x	x

* (✓) Presence of CCCVd- like RNA in the leaf sample
* (x) Absence of CCCVd- like RNA in the leaf sample

3.4 Summary

An optimized and modified extraction method from the conventional NETME method resulted in high quality of RNA which includes higher RNA purity and concentration. Modification also ensured the elimination of further purification stage using non-denaturing PAGE, which is laborious and takes between 2 to 3 days to be carried out. Total nucleic acid obtained was suitable for cDNA synthesis, RT-PCR experiments, and provided consistent results in CCCVd variants detection in oil palm.

CHAPTER 4

CHARACTERIZATION OF COCONUT CADANG–CADANG VIROID (CCCVd) VARIANTS IN OIL PALM WITH VARIED ORANGE SPOTTING SYMPTOMS

4.1 Introduction

Viroids do not code for protein nor are they encapsidated by protein thus serological methods applied to viruses are unreliable for the diagnosis of viroid diseases. Similarly, electron microscopy techniques are inappropriate because detection of characteristic particles are not valid. (Hull, 2002). Diagnosis of CCCVd through symptoms observation is unreliable as OS symptom was always associated with potassium deficiency and also found a significant relationship between potassium content and OS in self-progenies of oil palms (Vadamalai, 2005). Thus diagnosis of CCCVd through molecular techniques have been developed.

CCCVd occur in very low concentrations in infected hosts and it is very important to have high concentration of the nucleic acids before molecular detection. Previous study has shown molecular techniques that includes polyacrylamide gel electrophoresis (PAGE) and hybridization assay supported by probe technology used in CCCVd –like RNA detection (Vadamalai, 2005). In a more recent work by Thanarajoo (2014), successfully detected the CCCVd – like RNA using reverse transcription loop - mediated isothermal amplification (RT- LAMP). However, these two techniques do not provide information on the structure of the CCCVd – like RNA. Molecular characterization is required to characterize and identify the sequence variability. To date, there are few CCCVd variants have been characterized in oil palms with more than 90 % sequence similarity with CCCVd in coconut. A 246-nt CCCVd variant has been identified and described from oil palms with OS symptoms in Malaysia. Also, 297, 293 and 270 nucleotides of variants were identified and characterized from asymptomatic palms. There is yet a study that looks into the characterized CCCVd variants from palms with symptom variation.

In a recent survey done by MPOB a high degree of symptom variation and several inconsistent forms of OS was noticed in affected palms (Shamala Sundram, Senior Principal Research officer, Malaysian Palm Oil Board, pers. comm. 24 October 2015). This could be attributed to the differences in CCCVd variants present in oil palm. Interrelation of oil palm CCCVd variants to OS variation is important so as to have a proper understanding of the epidemiology of OS since difference in CCCVd variants can result in different severity of OS symptoms and disease. This can only be confirmed with characterization of CCCVd variants.

Sequence variation in viroid genome is directly responsible for symptom expression (Gora *et al*., 1996). Changes and substitutions of nucleotides that occurs due to mutation were reported to cause symptom severity (Owens, 2007). Viroid sequence

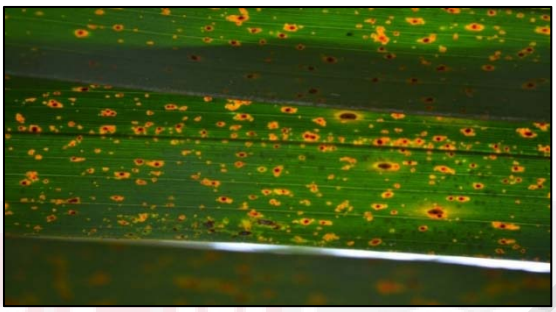
that causes minor symptom varies from one that causes severe symptom by only two bases substitution. (Gross *et al*., 1981). Moreover, several viroid sequences characterized indicated additional nucleotides especially in the TL and P regions (Sano *et al*., 1992). CCCVd mutants that demonstrated CCR and P domain are correlated to symptom severity (Rodriguez and Randles, 1993). This could be the case of OS symptoms in oil palm. The aim of this chapter is therefore to study the interrelation between CCCVd variants in oil palm and OS symptoms variation so as to have a proper understanding of the disease epidemiology and explore the possibility of different CCCVd variants characterized based on the variation in OS symptoms.

4.2 Materials and Methods

4.2.1 Collection of leaf samples

Leaves were sampled from three different locations namely Sungai Buloh, Selangor, Port Dickson, Negeri Sembilan, and Teluk Intan, Perak. Three symptomatic and one healthy palms were collected from each of the location (Appendix A). The three symptomatic samples from different locations that were collected were observed to have symptom variation as shown on Table 4.1. The orange spots are irregular in shape, scattered and distributed all over the fronds, not concentrated on any of the part of leaf. Three different samples from different locations had difference in orange spotting symptom. From the table shown, sample OSKS from Sungai Buloh shows small orange spots with necrotic lesion or darkening of tissue at the centre and they do not coalesce together. Sample OSBP collected from Port Dickson, Negeri Sembilan reveals absence in necrotic lesion, irregular shape, and smaller size with 2-3 mm long as compared to the other two samples. Sample OSUP from Teluk Intan, Perak shows larger size orange spots as compared to the other two samples. Young orange spots have necrotic lesion at the centre and becomes desiccated as it grows old.

Table 4.1 : Leaf samples from different locations with OS symptom variation namely OSKS (Selangor), OSBP (Port Dickson) and OSUP (Teluk Intan).

Sample Name	Leaf samples	Locations	Symptoms
OSKS		Sungai Buloh, Selangor	Small orange spots with necrotic lesion at the centre
OSBP		Port Dickson, Negeri Sembilan	Small orange spots with no necrotic lesion
OSUP		Teluk Intan, Perak	Large orange spots with necrotic and desiccation

* OSKS: Sample from Sungai Buloh, Selangor, * OSBP: Sample from Port Dickson, Negeri Sembilan, * OSUP: Sample from Teluk Intan, Perak.

4.2.2 Nucleic acid extraction

Leaf samples collected and brought to laboratory for nucleic acid extraction. Total nucleic acid extraction was carried out using the modified NETME extraction method in order to achieve consistent detection of CCCVd with improved RNA quality. Ten g leaf sample was ground with liquid Nitrogen until crush. The crushed sample was further ground with approximately 5 mL of NETME extraction buffer [100 mM NaOAc, 2 M NaCl, 50 mM Tris-HCl (pH 7.5), 50 mM EDTA (pH 8.0) and 20 % ethanol]. Approximately 2.40 mL of 1 % SDS, and 12.8 µL of 2-Mercaptoethanol was added along with the extraction buffer. The ground slurry was transferred into screw cap tube and then centrifugation was carried out for 15 min at 4 °C at 11, 000 g (Eppendorf 11K, Germany). Phenol: Chloroform: Isoamylalcohol (PCA) was transferred into a clean tube containing supernatant from the earlier step. The solutions were well-mixed for approximately 10 min. The mixture was centrifuged at 11, 000 rpm for 10 mins at 4 °C. The supernatant transferred into a new tube was then added with Chloroform: Isoamylalcohol (CA) and mixed and vortexed well again before centrifuging at 11, 000 g (Eppendorf 11K, Germany) for 10 mins at 4 °C.

The supernatant that was transferred into the new tube was precipitated with 1 volume of isopropanol. The mixture was gently invert - mixed thrice and was incubated at -80 °C for 45 mins. The incubated mixture was then centrifuged for 15 mins at 11, 000 g (Eppendorf 11K, Germany) at 4 °C. The supernatant was removed and the resulting pellet was well-rinsed with 1 mL of 70 % ethanol by mixng well for 10 mins and further centrifuged at 11, 000 g (Eppendorf 11K, Germany) for 15 min at 4 °C. The pellet was allowed to dry for approximately 20 mins. The air-dried pellet was re-precipitated with 1 mL of 8 M LiCl solution, vortexed well and was incubated at -20 °C for 4 hours. The mixture was then centrifuged for 15 mins at 11, 000 g (Eppendorf 11K, Germany) at 4 °C and the pellet was removed. The remaining supernatant was precipitated once again with 1 mL of 100 % ethanol and was incubated at -80 °C for 45 mins. The incubated mixture was centrifuged at 11, 000 g (Eppendorf 11K, Germany) for 15 mins at 4 °C. The supernatant was removed and the pellet was re-washed with 1 mL of 70 % ethanol. The mixture was vortexed well and centrifuged at 11, 000 g (Eppendorf 11K, Germany) for 10 mins at 4 °C and the pellet was allowed to air-dry. The pellet was re – suspended with 20 µL SDDW.

4.2.3 cDNA synthesis

The first strand cDNA was synthesized according to Rodriguez (1993) and Hodgson *et al*. (1998) with CCCVd specific antisense primer sets I (GVR 1) and IV (GVR 4) as described in section 3.2.5.

4.2.4 RT-PCR amplification

Amplification of RT-PCR was carried out using specific primer sets I and IV that annealed at 68 ºC and 60 ºC respectively as described in section 3.2.6.

4.2.5 Cloning

PCR product was cloned in the plasmid vector using PCR Cloning Kit (QIAGEN®, Germany) according to manufacturer's protocol. The PCR product was used to ligate PCR product into the plasmid vector. The DNA PCR product was ligated using ligation master mix (QIAGEN®, Germany) into 50 ng/µL pDrive cloning vector in a 10 µL final volume. Storage of ligation mixture was at -20 °C, after incubation at 4 °C for 30 mins.

Plasmids transformation was immediately followed using ice thawed PCR Cloning kit (QIAGEN®, Germany). A tube of competent cell and 2 µL of ligation mixture obtained above was gently mixed, placed on ice for 5 mins, heat-shocked was done for 30 s at 42 °C and allowed to stand on ice for 2 mins.

Approximately 250 µL of SOC medium at room temperature was added to the transformation mixture. Plates of LB agar were prepared by incubating with 80 µg/mL X- Gal and 50 µM IPTG at 37 ºC for an hour for screening of blue/ white recombinants. About 50 to 200 µL of the transformation mixture with SOC medium was aliquoted and gently spread on LB agar plates containing ampicillin as a selection marker. The LB plates were incubated at room temperature until the transformation mixture has absorbed into the agar and were inverted at 37 °C overnight.

Purification of plasmid DNA was carried out using QIAprep Spin Miniprep kit (QIAGEN®, GERMANY) based on manufacturer's procedure. Cultures were obtained from white colonies which grew on the LB agar plates and 10 µg/mL ampicillin containing LB broth and incubated for 16 hours at 37 °C under vigorous shaking. Centrifugation for 10 mins was carried out at 6, 800 g (Eppendorf 11K, Germany) to harvest bacterial cells, after which re-suspension of pellets was done carefully using buffer P1 (250 µL) followed by addition of Buffer P2 (250 µL) in the same tube and centrifugation at 13,000 g (Eppendorf Minispin, Germany) and room temperature for 1 min. The resultant product from centrifugation was placed in a QIAprep Spin Column, washed with 750 µL Buffer PE and centrifuged again at 13, 000 g for 1 min (Eppendorf Minispin, Germany). The flow-through was disposed and 30 µL buffer EB was pipetted through the centre of the spin column in a new centrifuge tube to elute DNA and centrifuged at 13, 000 g (Eppendorf Minispin, Germany) for 1 min. Eluted product obtained was stored at -20 ºC for sequencing.

The presence of target insert (200-300 bp) was analysed by digesting 2 µL of plasmid DNA according to manufacturer's procedures using 1 µL (10U/ µL) EcoR1 FastDigest enzyme (Fermentas, USA) in a 25 µL reaction volume at 37 °C for 5 mins. Further analysis of the mixture was carried out on 1.5 % agarose gel electrophoresis.

4.2.6 Sequencing analysis

Sequencing of purified PCR products was carried out at NHK Bioscience Solutions, Korea. Comparison of results with sequences available in the National Centre for Biotechnology Information (NCBI) database was carried out using BLAST online-software (http://blast.ncbi.nlm.nih.gov).

4.3 Results and Discussion

4.3.1 RT-PCR amplification

Results of this study indicates that all nine OS palms sampled were positive for CCCVd RNA. RT-PCR amplicons from the nine OS symptomatic samples had single amplicon at approximately 250 bp and showed clear band on 1.5 % agarose gel using set IV primers (Figure 4.1). As expected, healthy oil palm samples (HKS, HBP and HUP) did not indicate the presence of CCCVd RNA as shown in Figure 4.2. RT-PCR positive samples were further used for RNA characterization purposes.

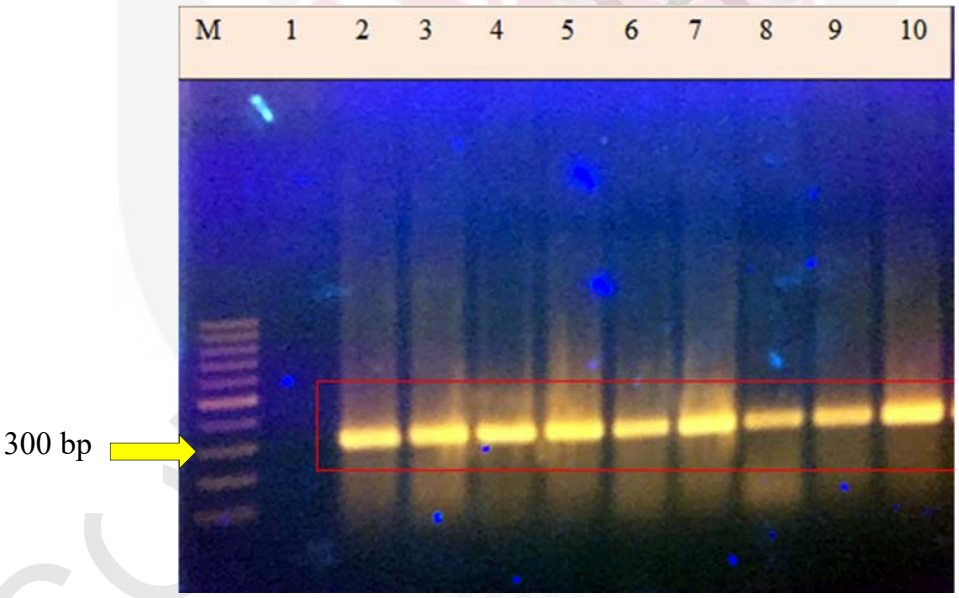


Figure 4.1 : Product of RT - PCR from the nine samples of OS- affected oil palm; OSKS1 (lane 2), OSKS2 (lane 3), OSKS3 (lane 4), OSBP1 (lane 5), OSBP2 (lane 6), OSBP3 (lane 7), OSUP1 (lane 8), OSUP2 (lane 9) and OSUP3 (lane 10), amplified using set IV primers and observed on a 1.5 % agarose gel. Lane 1 is a negative control (SDW as PCR template). A DNA ladder (lane M) sized 100 bp was used as a marker.

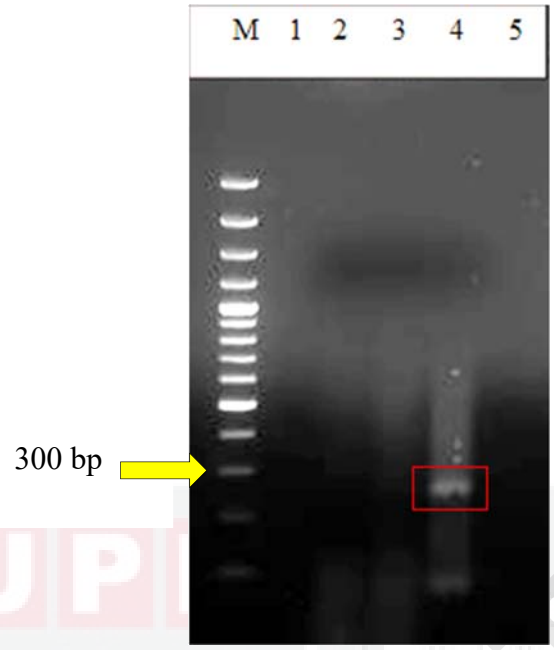


Figure 4.2 : RT-PCR amplification of samples HKS (lane 1), HBP (lane 2), and HUP (lane 3) generated using primers set IV and viewed on 1.5 % agarose gel. Lane 4 is a positive control (oil palm CCCVd $_{OP246}$) and negative control (SDW as PCR template) is as shown I lane 5. 100-bp DNA ladder was used as a size marker.

In addition, CCCVd specific primers set I and IV are expressed at different positions in the viroid molecules (Vadamalai, 2005) and may cause base substitution. Such base substitution could cause mutations which does not necessarily give a true picture of the viroid but may arise due to random errors in RT-PCR amplification (Fernandez *et al*., 2004). RT-PCR amplification was also generated using primer set I (GV1 and GVR1) to check for mutations, using samples with the best quality RNA (OSKS1, OSKS3, OSBP1 and OSUP3) and resulted in partial length amplicons ca. 242 bp in size. These results are presented in Figures 4.1 to 4.6.

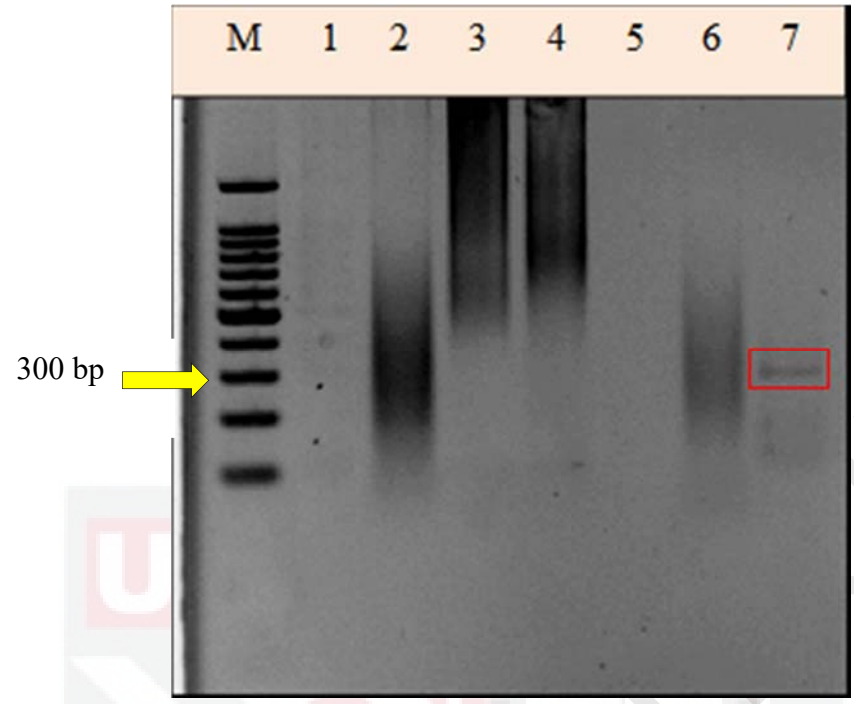


Figure 4.3 : Electrophoresis in an agarose gel (1.5 %) of RT-PCR products amplified from OS symptomatic sample (Lane 7: OSKS1) from Selangor generated using CCCVd specific primer set I. Lane 1 and 5 are negative controls (SDW as PCR templates). Lane M (100 bp DNA ladder) used as a size marker.

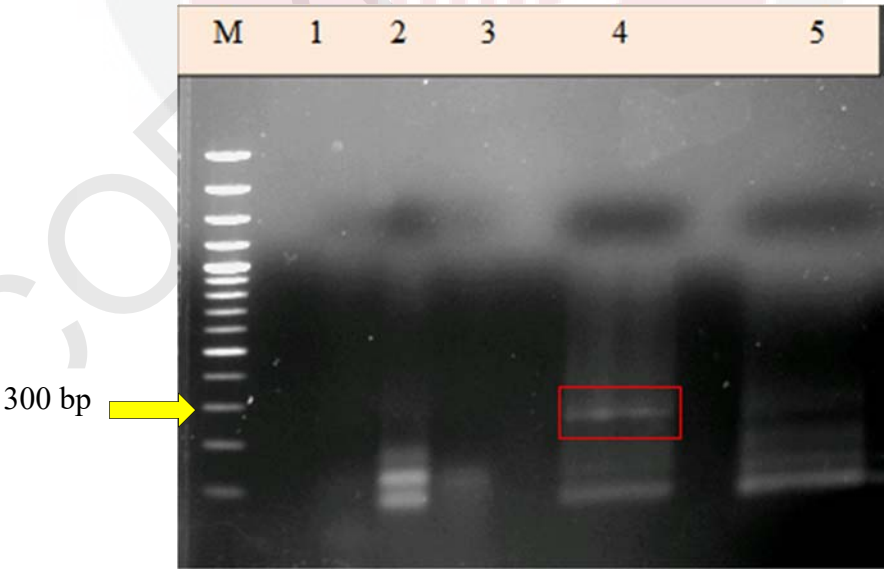


Figure 4.4 : Electrophoresis in an agarose gel (1.5 %) of RT-PCR products amplified from OS symptomatic sample (Lane 4: OSKS3) from Selangor generated using CCCVd specific primer set I. Lane 1 and 3 are negative controls (SDW as PCR templates). Lane M (100 bp DNA ladder) used as a size marker.

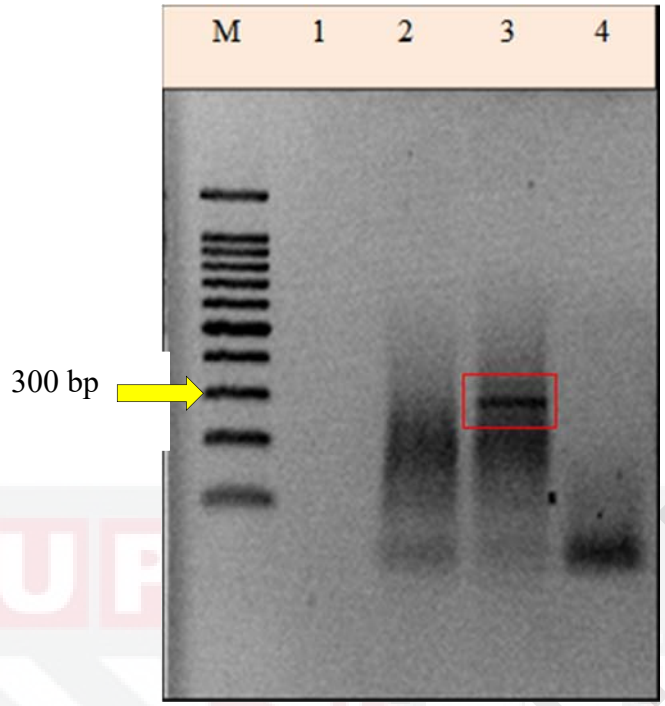


Figure 4.5 : Electrophoresis in an agarose gel (1.5 %) of RT-PCR products amplified from OS symptomatic sample (Lane 3: OSBP1) from N. Sembilan generated using CCCVd specific primer set I. Lane 1 is a negative control (SDW as PCR template). Lane M (100 bp DNA ladder) used as a size marker.

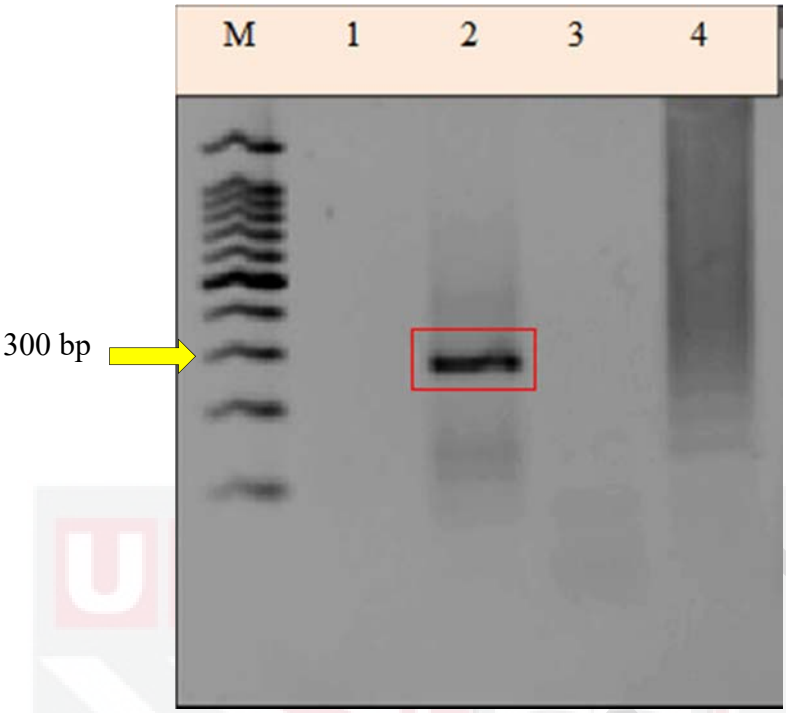


Figure 4.6 : Electrophoresis in an agarose gel (1.5 %) of RT-PCR products amplified from OS symptomatic sample (Lane 2: OSUP3) from Perak generated using CCCVd specific primer set I. Lane 1 and 3 are negative controls (SDW as PCR templates). Lane M (100 bp DNA ladder) used as a size marker.

4.3.2 Cloning and analysis of insert with EcoRI

Altogether, 15 recombinant bacterial colonies were successfully cloned and sequenced from the three sample sources. Inserts size were approximately 250 bp in size (Figure 4.7). Among the 15 clones that were sequenced, 8 contained insert of 246 nucleotides (1 OSKS1, 1 OSKS3, 1 OSBP1 and 3 OSUP3), and another 7 contained inserts of 242 nucleotides (two of OSKS1, one of OSKS3, two of OSBP1, and two of OSUP3). All the clones showed more than 90 % sequence similarity with 246 nucleotide variant of CCCVd from oil palm ($CCCVd_{OP246}$) with accession number HQ 608513.1 from GenBank.

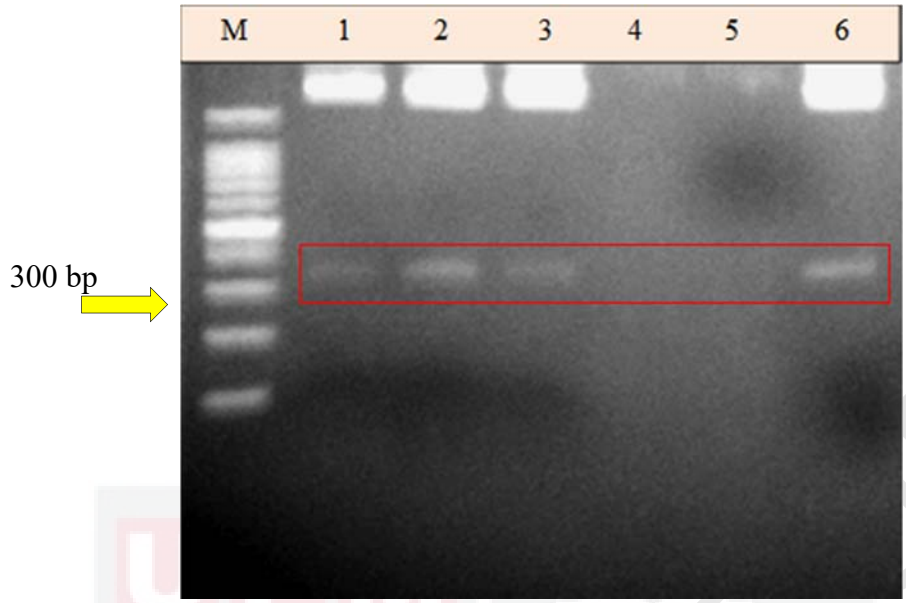


Figure 4.7 : Clones with inserts were identified by digestion of plasmid with EcoRI. Lane 1: OSKS1; lane 2: OSKS3; lane 3: OSBP1 and lane 6: OSUP3. Negative control (SDW) was used in lane 4 and 5. A DNA ladder (lane M) sized 100 bp was used as a size marker.

4.3.3 Sequence analysis

Sequences of OSKS1 and OSKS3 of primer set I with CCCVd$_{OP246}$ showed that they had 97 % sequence similarity while sequences of OSKS 1 and OSKS3 of primer set IV showed 99 % sequence similarity (Figure 4.8). Samples OPKS1 and OPKS3 with both sets of primers had three single base substitutions compared to CCCVd$_{OP246}$ at positions 104 (C → G), 105 (G → C) and 140 (C →A) of the terminal right (TR) domain. The sequences of OSBP 1 with two different primer sets had two base substitutions at positions 19 (C → G) and 35 (G → C) which occurred in the pathogenicity (P) domain of the molecule (Figure 4.9). It therefore had 98 % sequence similarity from samples with primer set I and 99 % sequence similarity from samples with primer set IV. Alignment of the sequences of samples OSUP 1 and OSUP3 using primer set I with CCCVd $_{OP246}$ showed 98 % sequence identity while the alignment of the sequences of the samples using primer set IV showed 99 % sequence identity (Figure 4.10). Substitution took place at position 24 (C →U) of the P domain.

The base substitutions of OPKS, OPBP and OPUP took place at different positions. Samples OSBP and OSUP showed nucleotide substitution at pathogenicity domain while samples OSKS1 and OSKS3 showed substitution at terminal right domain. The substitutions were consistently present in all the samples as compared to the CCCVd$_{OP246}$, suggesting that the substitutions were not due to random errors by PCR amplification of CCCVd variant (Wu *et al*., 2013). The clones of samples OSKS, OSBP and OSUP can be considered as variants of CCCVd as the arbitrary level of 90 % sequence similarity was achieved and their difference in biological properties separates viroid species from viroid variants (Flores *et al*., 2005; Vadamalai *et al*., 2006).

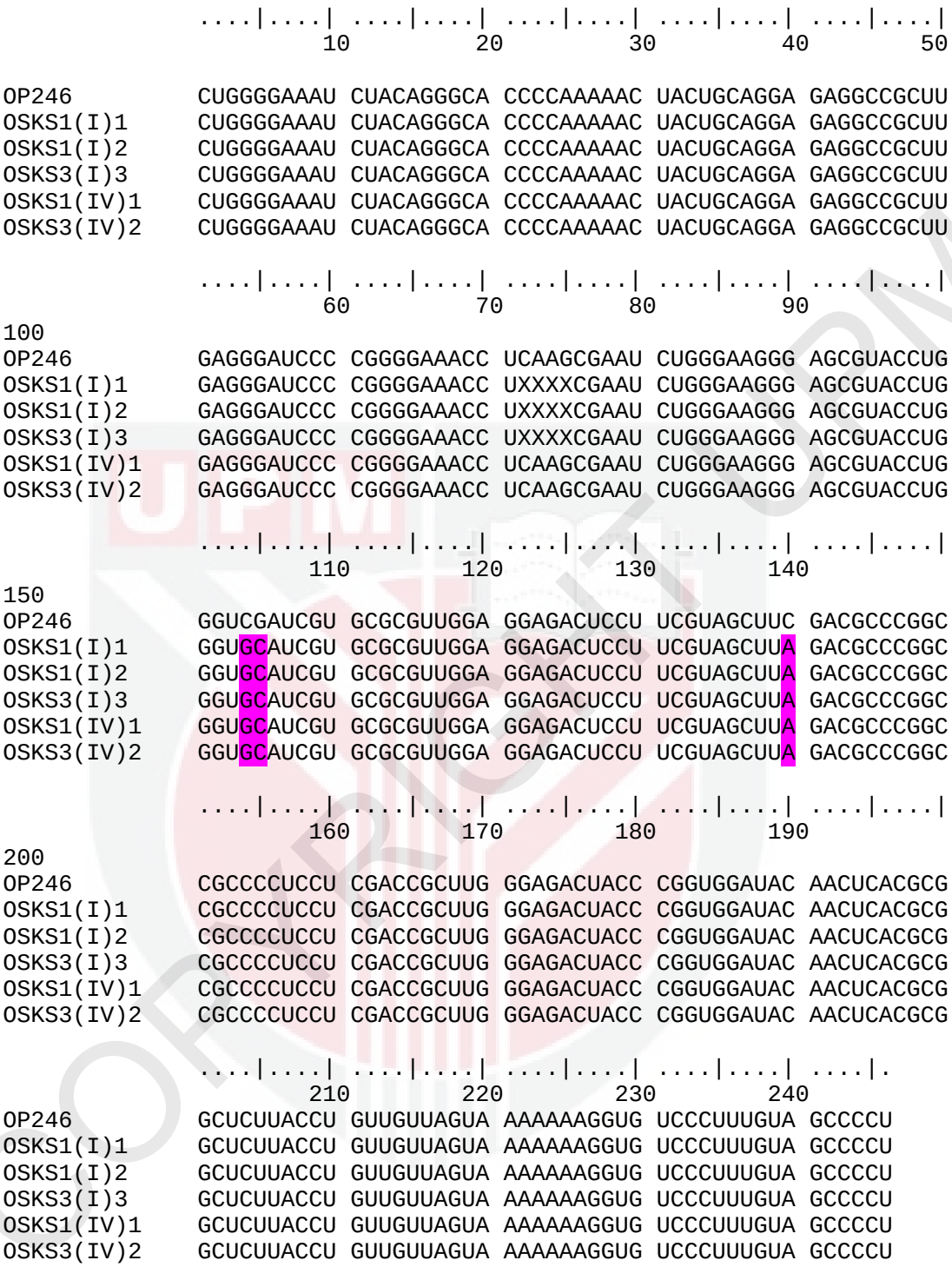


Figure 4.8 : Alignment of sequence between CCCVd $_{OP246}$ from GenBank with accession number; HQ608513.1 and clones resulted from characterization of variant of CCCVd from oil palm with orange spotting symptom variation with Set I and Set IV primer (sample OSKS1 and OSKS3). Clone OSKS1 (I)1, OSKS1(I)2, and OSKS3(I)3 had 97 % while OSKS1(IV)1 and OSKS3(IV)2 had 99 % sequence similarity with CCCVd$_{OP246}$. All the clones of OSKS (I and IV) showed nucleotide changes at positions $C^{104} \rightarrow G$, $G^{105} \rightarrow C$ and $C^{140} \rightarrow A$ (terminal right domain). 'X' indicate nucleotides not read with primer Set I.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50

OP246           CUGGGGAAAU CUACAGGGCA CCCCAAAAAC UACUGCAGGA GAGGCCGCUU
OSBP1(I)1       CUGGGGAAAU CUACAGGGGA CCCCAAAAAC UACUCCAGGA GAGGCCGCUU
OSBP1(I)2       CUGGGGAAAU CUACAGGGGA CCCCAAAAAC UACUCCAGGA GAGGCCGCUU
OSBP1(IV)1      CUGGGGAAAU CUACAGGGGA CCCCAAAAAC UACUCCAGGA GAGGCCGCUU
OSBP1(IV)2      CUGGGGAAAU CUACAGGGGA CCCCAAAAAC UACUCCAGGA GAGGCCGCUU

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90
100
OP246           GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG
OSBP1(I)1       GAGGGAUCCC CGGGGAAACC UXXXXCGAAU CUGGGAAGGG AGCGUACCUG
OSBP1(I)2       GAGGGAUCCC CGGGGAAACC UXXXXCGAAU CUGGGAAGGG AGCGUACCUG
OSBP1(IV)1      GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG
OSBP1(IV)2      GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140
150
OP246           GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
OSBP1(I)1       GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
OSBP1(I)2       GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
OSBP1(IV)1      GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
OSBP1(IV)2      GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190
200
OP246           CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
OSBP1(I)1       CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
OSBP1(I)2       CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
OSBP1(IV)1      CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
OSBP1(IV)2      CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG

                ....|....| ....|....| ....|....| ....|....| ....|.
                        210        220        230        240
OP246           GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
OSBP1(I)1       GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
OSBP1(I)2       GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
```

Figure 4.9 : Alignment of sequence between CCCVd $_{OP246}$ (GenBank: HQ608513.1) and clones obtained from characterization of CCCVd variant from oil palm with orange spotting symptom variation with Set I and Set IV primer (sample OSBP1). Clone OSBP1(I)1 and OSBP1(I)2 had 98 % while OSBP(IV)1 and OSBP(IV)2 had 99 % sequence similarity with CCCVd $_{OP246}$. All the clones of OSBP (I and IV) showed nucleotide changes at position C^{19} → G and G^{35} → C (pathogenicity domain). 'X' indicate nucleotides not read with primer Set I.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50

OP246           CUGGGGAAAU CUACAGGGCA CCCCAAAAAC UACUGCAGGA GAGGCCGCUU
OSUP3(I)1       CUGGGGAAAU CUACAGGGCA CCCUAAAAAC UACUGCAGGA GAGGCCGCUU
OSUP3(I)2       CUGGGGAAAU CUACAGGGCA CCCUAAAAAC UACUGCAGGA GAGGCCGCUU
OSUP3(IV)1      CUGGGGAAAU CUACAGGGCA CCCUAAAAAC UACUGCAGGA GAGGCCGCUU
OSUP3(IV)2      CUGGGGAAAU CUACAGGGCA CCCUAAAAAC UACUGCAGGA GAGGCCGCUU
OSUP3(IV)3      CUGGGGAAAU CUACAGGGCA CCCUAAAAAC UACUGCAGGA GAGGCCGCUU

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90
100
OP246           GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG
OSUP3(I)1       GAGGGAUCCC CGGGGAAACC UXXXXCGAAU CUGGGAAGGG AGCGUACCUG
OSUP3(I)2       GAGGGAUCCC CGGGGAAACC UXXXXCGAAU CUGGGAAGGG AGCGUACCUG
OSUP3(IV)1      GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG
OSUP3(IV)2      GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG
OSUP3(IV)3      GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140
150
OP246           GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
OSUP3(I)1       GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
OSUP3(I)2       GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
OSUP3(IV)1      GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
OSUP3(IV)2      GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
OSUP3(IV)3      GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC

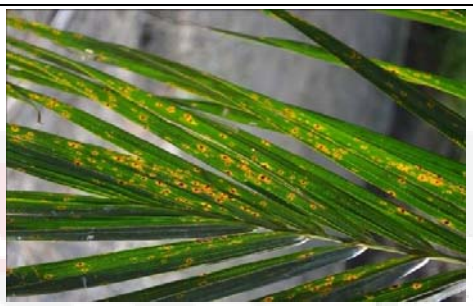
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190
200
OP246           CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
OSUP3(I)1       CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
OSUP3(I)2       CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
OSUP3(IV)1      CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
OSUP3(IV)2      CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
OSUP3(IV)3      CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG

                ....|....| ....|....| ....|....| ....|....| ....|.
                       210        220        230        240
OP246           GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
OSUP3(I)1       GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
OSUP3(I)2       GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
OSUP3(IV)1      GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
OSUP3(IV)2      GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
OSUP3(IV)3      GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
```

Figure 4.10 : Sequence alignment between CCCVd $_{OP246}$ (GenBank: HQ608513.1) and clones obtained from characterization of CCCVd variant from oil palm with orange spotting symptom variation with Set I and Set IV primer (sample OSUP3). Clone OSUP3(I)1 and OSUP3(I)2 had 98 % while OSUP3(IV)1, OSUP3(IV)2 and OSUP3(IV)3 had 99 % sequence similarity with CCCVd $_{OP246}$. All the clones of OSBP3 (I and IV) showed nucleotide change at position C^{24} → U (pathogenicity domain). 'X' indicate nucleotides not read with primer Set I.

Leaf samples from three different locations with orange spotting symptom variation showed difference in characterization of CCCVd variants as summarized in Table 4.2.

Table 4.2 : Symptomatic samples and respective nucleotide substitution

Sample Name and Source	Leaf Samples	% Sequence Similarity to $CCCVd_{OP246}$	Substitution in CCCVd Position
OSKS, Selangor		97 % (primer set I) 99 % (primer set IV)	104, 105, 140 C → G G → C C → A
OSBP, Negeri Sembilan		98 % (primer set I) 99 % (primer set IV)	19, 35 C → G G → C
OSUP, Perak		98 % (primer set I) 99 % (primer set IV)	24 C → U

Nucleotide substitution in CCCVd variants may play a role on symptom variation. However, this requires further investigation but this findings definitely suggest the possibility. CCCVd variants characterized and nucleotide substitution occurred at positions 19, 24, and 35 (pathogenicity domain), 104, 105 and 140 (terminal right) domain of the viroid may have interrelation with the OS symptom variation. Variation in sequence of viroid can give rise to a wide range of phenotypes of disease especially sequence with changes in P and C domain (Rodriguez and Randles, 1993). Mutations due to nucleotide alternation in P domain of the viroid are known to affect the severity of symptom expression and 1 to 2 changes in nucleotides can result in different symptom and disease severity (Wassengger *et al*., 1996; Joseph, 2012). Quasispecies effects (Itaya *et al*., 2002; Codoner *et al*., 2006) may play a role in disease severity and symptom variation (Visvader and Symons, 1985; Wassenger *et al*., 1996).

4.4 Summary

Variants of CCCVd from oil palm with variation in OS symptom from different locations were successfully characterized and gave rise to three different CCCVd variants. Nucleotide substitution in the viroids characterized occurred at positions 19, 24, and 35 (pathogenicity domain), 104, 105 and 140 (terminal right) domain of the viroid, may have interrelation with the OS symptom variation and affect the severity of symptom expression.

CHAPTER 5

TRANSMISSION OF CCCVd VARIANT ISOLATED FROM OIL PALM INTO OIL PALM AND COCONUT

5.1 Introduction

There are four ways viroid transmission can occur that includes seed and pollen (Singh and Finnie, 1973), infected sources causing vegetative transmission, mechanical transmission through systematic inoculation (Wallace and Drake, 1962; Fernow *et al*., 1970; Desjardins, 1987; Kryczynski *et al*., 1988; Bonaobra *et al*., 1998) and insect transmission (Orense *et al*., 1998). Among all the four transmission methods, the most commonly cited method is the mechanical transmission as attempts to transmit viroid agent by rubbing sap extract was unsuccessful with CCCVd (Randles *et al*., 1977). The purposes of inoculation includes testing infectivity, resistance screening and observation of disease development. Studies by Vadamalai (2005) and Vadamalai *et al*. (2006) showed that CCCVd variants were detected in OS palm and in symptomless palms with more than 90 % sequence homology with CCCVd from coconut. One of the fulfilment of Koch's postulate is the study of transmission whereby the ability of a specific pathogen transmission is confirmed when results from inoculation of purified preparations into host plants and show similar symptom to the infected plant that is used as source of inoculums and the same pathogen recovered (Agrios, 2005). Transmission of CCVd into 4 – 18 month old through inoculation using high-pressure injector with either carborandum or using razor for slashing petioles was successful according to Randles *et al*. (1977). However, Randles *et al.* (1980) showed that transmission could be attained by injection using high pressure injector without including other additional methods of inoculation such as using carborandum or slashing. CCCVd can be detected as early as six months and symptom expression would appear after four years and above (Imperial *et al*., 1985).

Imperial *et al*. (1985) stated that high rates of infection in coconut is achieved with injection very near to the meristem of young seedlings. According to a study by (Hanolds and Randles (1998), Malayan Yellow Dwarf (MYD) cultivar of coconut had high rate of CCCVd infection. Possible routes of spread of the CCCVd were also detected in husks of young nuts, anther extract, peduncles and the anther component movement by insects. Although pathogenicity has been established in oil palm (Joseph, 2012; Thanarajoo, 2014), the same cannot be said for coconut. It is important to study the cross-transmission between oil palm and coconut in order to detect the CCCVd variants replication and to relate the sequence variation and symptom expression as there is no study done on cross –transmission between oil palm and alternate host in Malaysia. It is possible for CCCVd variant from oil palm to transmit into coconut due to more than 90 % sequence similarity between oil palm and coconut in previous study. This possibility can be referred to coconut that it might be the reservoir for CCCVd and that it had jumped host into oil palm. The CCCVd detected in coconut in Philippines are the pathogenic ones while the ones in oil palm are variants of CCCVd.

OS disorder is not regarded as economically important in Malaysia, and as such limited research efforts exist to investigate its occurrence. There is no studies done on CCCVd variant into coconut seedlings and no studies showed its route of spread. This study therefore seeks to study the correlation that exists (if any) between variants of CCCVd and OS symptom expression on coconut and oil palm and also to observe if CCCVd variant replication causes nucleotide changes in the molecule of the viroid prior to transmission.

5.2 Materials and Methods

5.2.1 Source of oil palm and coconut seedlings

20 Dura X Pisifera 3-month oil palms seedlings were bought from Sime Darby Plantation, Banting. Twenty 3-month old coconut seedlings of Malayan yellow dwarf cultivar were bought from United Plantation, Teluk Intan. The seedlings were brought to Malaysian Palm Oil Board/Universiti Kebangsaan Malaysia (MPOB/UKM) nursery and were transferred into 20 X 20 cm polybags. The polybags were filled with mixture of soil and sand with ratio 3:1.

5.2.2 Inoculation procedure

CCCVd $_{OP246}$ plasmid was obtained from Assoc. Prof. Dr. Ganesan Vadamalai from Department of Plant Protection, Faculty of Agriculture, UPM. The plasmid was sent for sequencing at NHK Sequencing Services, Korea and was aligned with NCBI sequences from GenBank with accession numbers HQ608513.1. The plasmid concentration was assured to reach 0.2 mg/mL when measured using a NanoDropTM 1000 spectrophotometer (Thermo SCIENTIFIC, USA).

The oil palm and coconut seedlings that were placed at MPOB/UKM nursery were kept for three days in dark condition as described by Joseph (2012) and Thanarajoo (2014). The inoculation of CCCVd$_{OP246}$ into oil palm and coconut seedlings were carried out using a hand-primed, Panjet injector with high pressure (Schuco International London, Ltd) as described by Randles *et al*. (1977) and Imperial *et al*. (1985). Inoculation was done into 10 oil palm and 10 coconut seedlings. Ten other seedlings were kept as control with and without injection of Milli-Q water. Each seedling was inoculated, two doses at the lower base and one dose at the leaflet with approximately 0.6 mg/mL of the CCCVd inocula as described by Thanarajoo (2014).

5.2.3 Seedlings arrangement

A row of infected oil palm and coconut seedlings were arranged side by side with a distance of 1 m from each other as shown in Figure 5.1. The control seedlings were also kept at the same distance from the infected seedlings. Labelling was done for all the seedlings (COCO1, OP1, COCO1 C, and OP1 C). The seedlings at the nursery were watered trice a day and application of basal fertilizer (NPK yellow) at 10 gm/seedling were done every month.

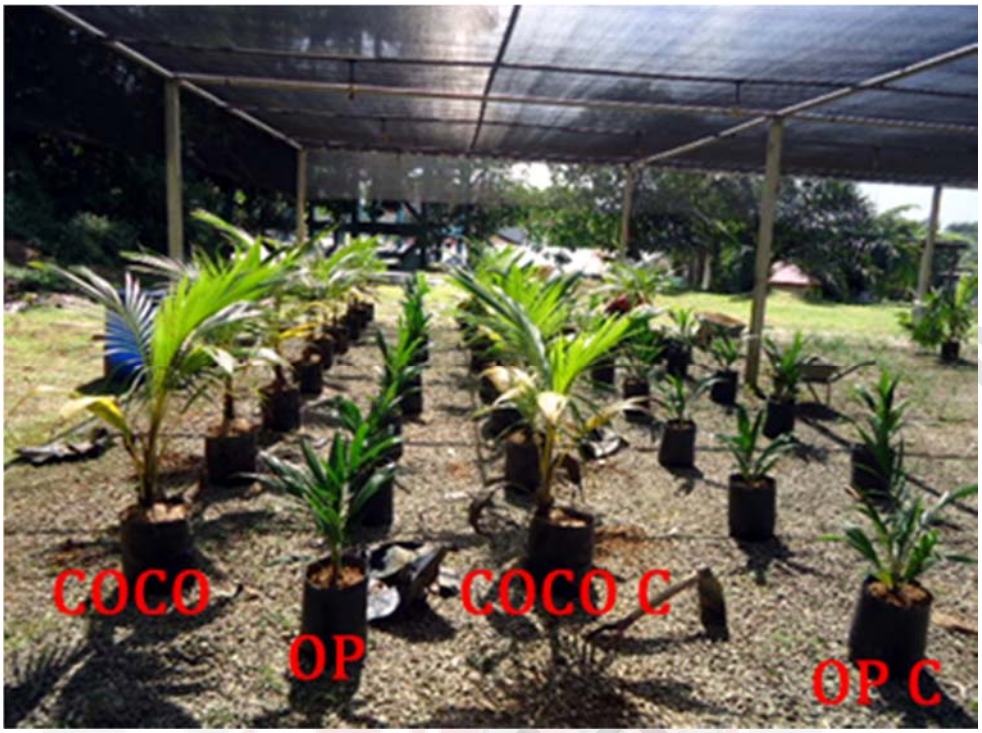


Figure 5.1 : Seedlings arrangement in nursery

5.2.4 Viroid detection

Two to three mature leaflets from the oil palm and coconut seedlings were sampled every three months after inoculation. The samples were then extracted using modified NETME extraction method as described in Section 4.2.2, its cDNA synthesis and further RT-PCR amplification were carried out using CCCVd specific primer set IV as mentioned in section 3.2.5 and 3.2.6 respectively.

5.2.5 Symptom expression

OS symptom expression observation was done on both oil palm and coconut seedlings inoculated with CCCVd$_{OP246}$ and observation was recorded.

5.3 Results and Discussion

5.3.1 RT-PCR amplification for oil palm seedlings

The results of CCCVd$_{OP246}$ transmission into oil palm seedlings are presented in table 5.1. Results showed viroid detection through RT-PCR amplification using CCCVd specific primer set and analysed on 1.5 % agarose gel and stained in EtBr solution from the 3rd month of observation for all oil palm seedlings and indicate symptom expression from the 6th month after inoculation for only seedling samples OP4 and OP5. A possible explanation for this may be the heterogeneity nature of oil palm. High infectivity of viroid usually occurs at the meristematic tissue of the cell located at the

base of the seedlings (Hanolds and Randles, 1991). Moreover, monocotyledonous seedlings might have different tolerance level to pathogens (Rusli,). Thus, even though all seedlings underwent similar stress pattern to weaken the entire plant cell, especially the meristematic tissue.

Table 5.1 : Transmission of CCCVd$_{OP246}$ into oil palm seedlings showed presence (✓) and absence (×) of viroid detection and symptom expression.

Samples	Viroid Detection				Symptom Expression			
	Observation Period (Months)							
	0	3	6	9	0	3	6	9
OP1	×	✓	✓	✓	×	×	×	×
OP2	×	✓	✓	✓	×	×	×	×
OP3	×	✓	✓	✓	×	×	×	×
OP4	×	✓	✓	✓	×	×	✓	✓
OP5	×	✓	✓	✓	×	×	✓	✓
OP6	×	✓	✓	✓	×	×	×	×
OP7	×	✓	✓	✓	×	×	×	×
OP8	×	✓	✓	✓	×	×	×	×
OP9	×	✓	✓	✓	×	×	×	×
OP10	×	✓	✓	✓	×	×	×	×

* ✓ (Presence of viroid and symptom appearance)
* × (Absence of viroid and symptom appearance)

The expression of the viroid on the leaflet of oil palm was very strong for sample OP5 on the 6th month of observation and a close-up leaflet image showing symptom expression is presented in Figure 5.2.

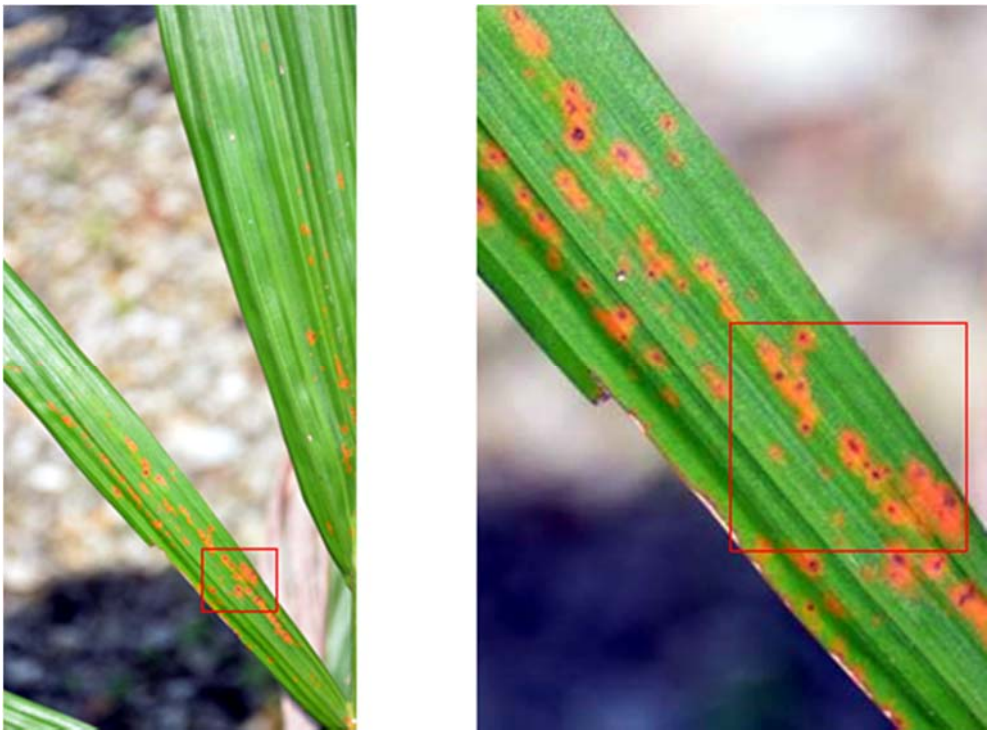


Figure 5.2 : Orange spotting symptom expression 6 months after inoculation in oil palm seedling (OP5)

Similar study carried out by Imperial *et al.* (1985) indicated symptom was expressed on leaves 2 years after inoculation. Joseph (2012) observed OS symptom in oil palm 9 months after inoculation using sap extracted form OS palm as inoculum. The plasmid clone used for this study typically have high concentration and could hasten replication and symptom expression. In addition, temperature could also be responsible for OS symptom expression recorded earlier than reported studies. During the month of July 2015 to April 2016, it was very hot as the temperature rose. Efficiency of viroid transmission is influenced by temperature (Singh, 1983). This was proven in a study by Verhoeven *et al*. (2010) indicated that increase of temperature at a range of 5 ºC to 10 ºC had intensified the transmission of *Potato spindle tuber viroid* (PSTVd) to *Solanum jasminoides,* potato, *Brugmansia suaveolens*, and tomato in a very high rate.

RT-PCR assay amplification results of all samples showed clear band at 250 bp on primer set IV. The bands were clearer for samples OP4 and OP5 as shown in Figure 5.3. Meanwhile, there was no amplification of PCR for seedlings samples inoculated with Milli-Q water.

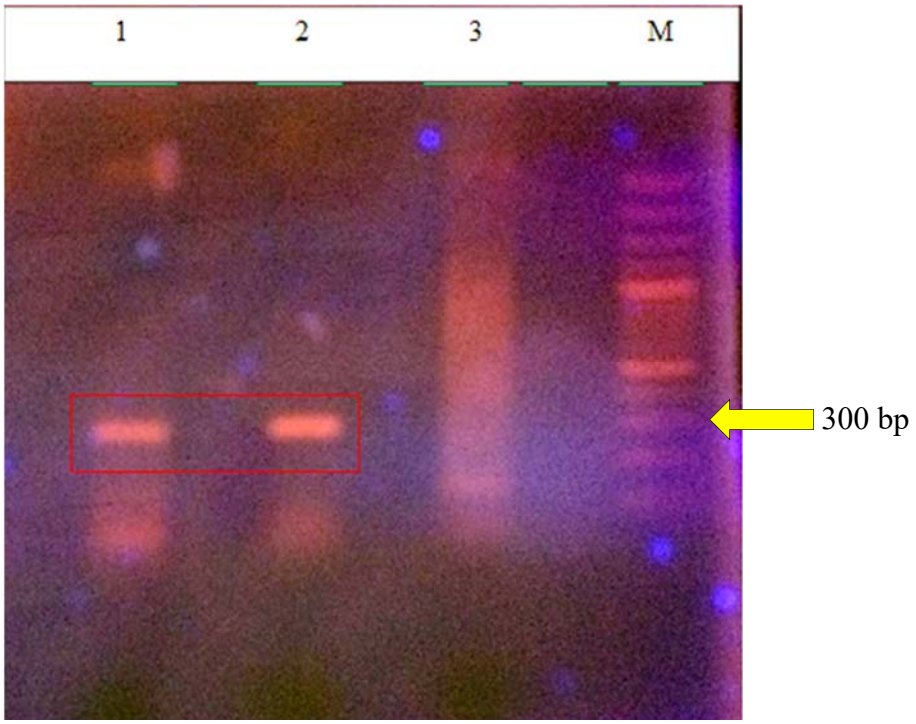


Figure 5.3 : RT-PCR amplification of samples OP4 (Lane 1) and OP5 (Lane 2) generated using set IV primers and fractioned on 1.5 % agarose gel. Lane 3 is a negative control (SDW as PCR template). 100 bp DNA ladder was used as a marker (Lane M).

5.3.2 Cloning of positive sample of oil palm seedlings

Two recombinant bacterial colonies were successfully cloned and sequenced from leaflet of OP5 seedling. As observed in Figure 5.4, analysis of inserts showed 2 clones approximately 250 bp in length and 100% sequence similarity with 246 nucleotide variant of the plasmid clone CCCVd_{OP246} used in the study as observed in Figure 5.5.

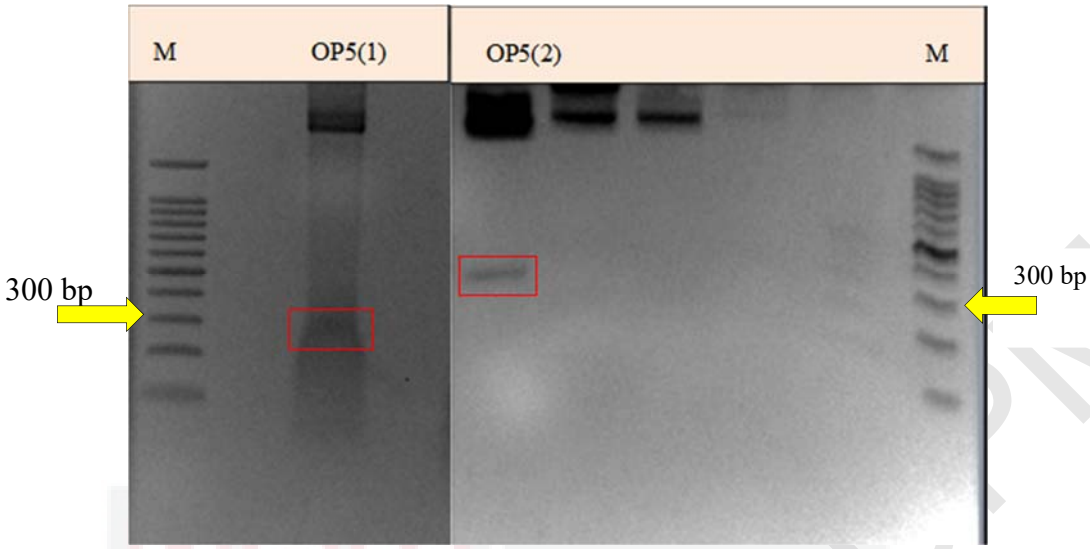


Figure 5.4 : Identification of clones with inserts (250 bp in size) for samples OP5 (1) and OP5 (2).

5.3.3 Sequencing analysis of oil palm seedlings

Figure 5.5 : Alignment of sequence between CCCVd$_{OP246}$ (GenBank: HQ608513.1) and clones from characterization of variant of CCCVd variant from oil palm seedling (OP5) with orange spotting symptom with Set IV had 100 % sequence similarity with CCCVd$_{OP246}$.

5.3.4 RT-PCR amplification of coconut seedlings

Viroid detection was only successful with two coconut seedling samples, COCO5 and COCO7. Disease development stages usually vary with variation in geographical isolate (Imperial *et al*., 1981). Detection and symptom expression of inoculated CCCVd$_{OP246}$ was observed on the two seedlings from the 9th month. Results are presented in Table 5.2, while a close-up image of a leaflet from the sample showing viroid expression is presented in Figure 5.6.

Table 5.2 : Transmission of CCCVd$_{OP246}$ into coconut seedlings showed presence (✓) and absence (×) of viroid detection and symptom expression. Transmission of CCCVd$_{OP246}$ into coconut seedlings

Samples	Viroid Detection				Symptom Expression			
	Observation Period (Months)							
	0	3	6	9	0	3	6	9
COCO1	×	×	×	×	×	×	×	×
COCO2	×	×	×	×	×	×	×	×
COCO3	×	×	×	×	×	×	×	×
COCO4	×	×	×	×	×	×	×	×
COCO5	×	×	×	✓	×	×	×	✓
COCO6	×	×	×	×	×	×	×	×
COCO7	×	×	×	✓	×	×	×	✓
COCO8	×	×	×	×	×	×	×	×
COCO9	×	×	×	×	×	×	×	×
COCO10	×	×	×	×	×	×	×	×

* ✓ (Presence of viroid and symptom appearance)
* × (Absence of viroid and symptom appearance)

Results for RT-PCR assay showed RNA from coconut seedlings at 250 bp, with clear bands for both samples (COCO5 and COCO7) as shown in Figure 5.7. Susceptibility and tolerance level of seedlings are likely causes. Sequencing analysis also revealed 100 % similarity to the plasmid clone CCCVd$_{OP246}$ used in the transmission study as observed in Figure 5.9. Transmission of CCCVd$_{OP246}$ was therefore successful for both oil palm and coconutt seedlings, with the transmiting ability confirmed when oil palm and coconut seedlings inoculated with inoculum CCCVd$_{OP246}$ expressed symptom similar to infected plant and CCCVd variant was recovered. No PCR product was observed with coconut seedling inoculated with Milli-Q water.

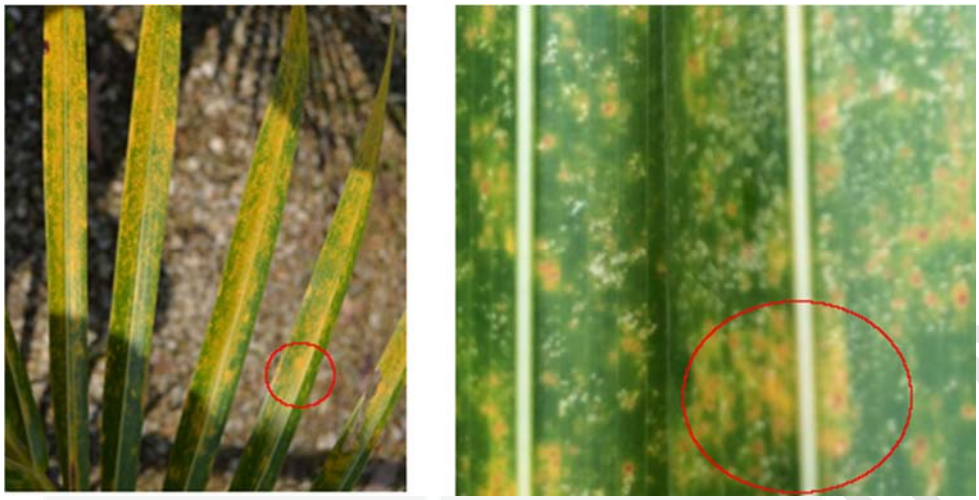


Figure 5.6 : Orange spotting symptom expression 9 months after inoculation in coconut seedling (COCO7)

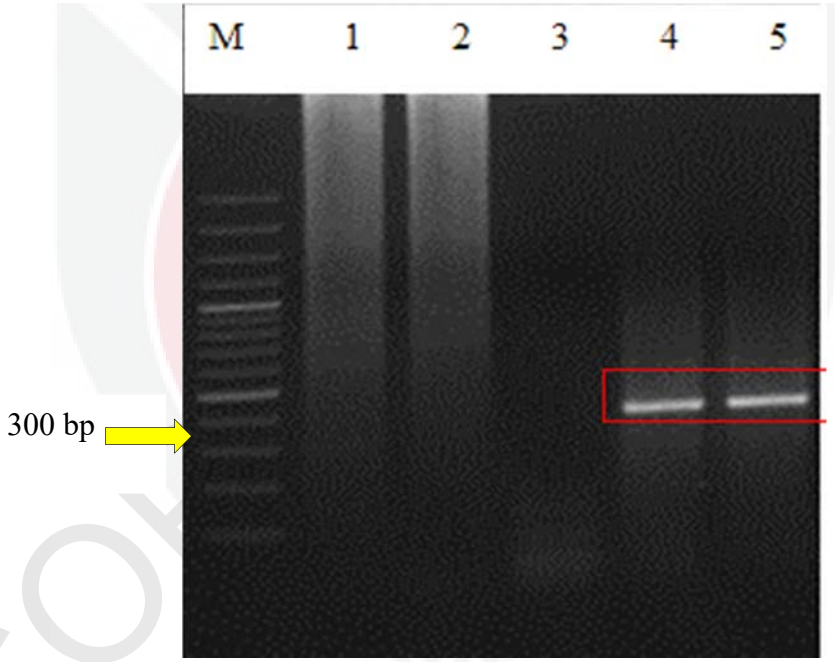


Figure 5.7 : RT-PCR amplification of samples COCO5 (Lane 4) and COCO7 (Lane 5) generated using set IV primers and fractioned on 1.5 % agarose gel. Lane 3 is a negative control (SDW as PCR template). DNA ladder with 100 bp was used as a size marker (Lane M).

5.3.5 Cloning of positive sample of coconut seedlings

Two recombinant bacterial colonies were successfully cloned and sequenced from leaflet of COCO7 seedling. As observed in Figure 5.8, analysis of inserts showed 2 clones approximately 300 bp in length and 100 % sequence similarity with 246 nucleotide variant of the plasmid clone CCCVd$_{OP246}$ used in the study as observed in Figure 5.9.

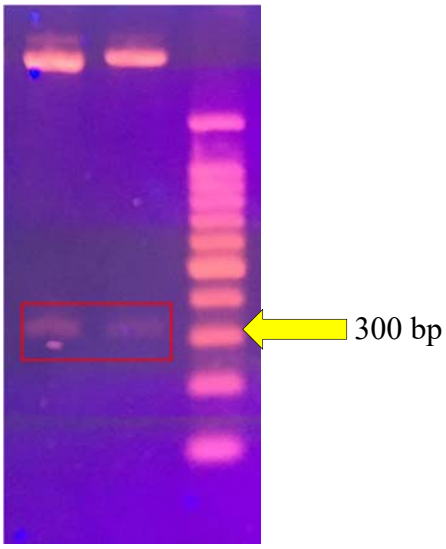


Figure 5.8 : Identification of clones with inserts (250 bp in size) for samples COCO7 (1) and COCO7 (2).

5.3.6 Sequence analysis of coconut seedlings

The CCCVd variant appears to be stable and no nucleotide alteration was observed during replication and transmission. According to Martinez-Soriano *et al.* (1996), most of the transmission from viroid-infected *Solanum cardiophyllum* (papita plants), to potato late blight fungus (*Phytophthora infestans*) involved either minor or major substitution in viroid sequence indicated viroid with high genetic variability. Absence of nucleotide changes in the sequence results from transmission of $CCCVd_{OP246}$ into oil palm seedlings indicates that the CCCVd variant with a solid structure might have low genetic variability and viroid population with low genetic diversity required to maintain secondary structure was limited (Aranda *et al*., 1997). In addition, viroid requires longer incubation period for it to generate mutants (Ellenuch *et al*., 2003), while the ability for autonomous viroid replication and symptoms expression is dependent upon host-encoded function for replication (Owens, 1990).

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50

OP246           CUGGGGAAAU CUACAGGGCA CCCCAAAAAC UACUGCAGGA GAGGCCGCUU
COCO7(1)        CUGGGGAAAU CUACAGGGCA CCCCAAAAAC UACUGCAGGA GAGGCCGCUU
COCO7(2)        CUGGGGAAAU CUACAGGGCA CCCCAAAAAC UACUGCAGGA GAGGCCGCUU


                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
OP246           GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG
COCO7(1)        GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG
COCO7(2)        GAGGGAUCCC CGGGGAAACC UCAAGCGAAU CUGGGAAGGG AGCGUACCUG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
OP246           GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
COCO7(1)        GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC
COCO7(2)        GGUCGAUCGU GCGCGUUGGA GGAGACUCCU UCGUAGCUUC GACGCCCGGC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
OP246           CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
COCO7(1)        CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG
COCO7(2)        CGCCCCUCCU CGACCGCUUG GGAGACUACC CGGUGGAUAC AACUCACGCG


                ....|....| ....|....| ....|....| ....|....| ....|.
                        210        220        230        240
OP246           GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
COCO7(1)        GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
COCO7(2)        GCUCUUACCU GUUGUUAGUA AAAAAAGGUG UCCCUUUGUA GCCCCU
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Figure 5.9 : Sequence alignment between CCCVd$_{OP246}$ (GenBank: HQ608513.1) and clones obtained from characterization of CCCVd variant from coconut seedling (COCO7) with OS symptom with Set IV had 100 % sequence similarity with CCCVd$_{OP246}$.

5.4 Summary

CCCVd RNA detected after 3 – months in the inoculated oil palm seedlings while symptom expression was observed from the 6th month after inoculation. Cross-pathogenicity or transmission of CCCVd$_{OP246}$ variant from oil palm into coconut showed CCCVd detection and symptom expression on the 9th month of observation. Transmission of oil palm CCCVd variant into coconut confirms the pathogenic or transmitting ability of CCCVd variant from oil palm into alternate host.

CHAPTER 6

SUMMARY, GENERAL CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

Consistent detection method was crucial for any characterization study. Although a few methods were available, the methods were either time consuming, laborious or inconsistent. High quality RNA usually results in good band retrieval. The method was subjected for improvement and was successfully modified and optimized as it played a crucial role in characterization of CCCVd variants through consistent detection of viroid. Inclusion of LiCl removes large ribosomal RNA and viroid RNA was recovered. These benefits of LiCl give rise to high quality RNA, whereby purification of time-consuming and laborious 5 % non – denaturing PAGE was eliminated.

The symptom variation in OS palms was one of the striking phenotypic characteristics that were observed in a survey carried out by MPOB. This gave rise to the question on whether there might be the involvement of different viroid variants in the manifestation of these symptoms. Therefore the second objective looked into the characterization of the viroids from different leaflet samples showing OS symptoms. Characterization of CCCVd variants from OS palms with symptom variation was successful. CCCVd variants characterized showed nucleotide substitution occurred at positions 19, 24, and 35 (pathogenic domain) and 104, 105 and 140 (terminal right domain) in the viroid. This finding showed that nucleotide substitution may have interrelation with the OS symptom variation. Changes in 1 to 2 nucleotides in the pathogenicity domain can result in different severity of the symptom and disease.

Symptom expression of severity and the rate of disease spreading are affected by the mutation in viroids in which alteration in nucleotide would influence viroid transmission. These corresponding substitutions in nucleotides in oil palm may be responsive to the self-defence mechanism of the host against pathogens but not aggressive as Coconut cadang-cadang disease in coconut host. Mutations occurring in CCCVd variants in oil palm that causes nucleotide substitution in its sequence can lead to similar CCCVd sequence of coconut over time, hence characterization work should be continuous as it will serve as an eye opener, especially as it concerns symptom expression by the palms to the industry and the country in general.

One of the most important epidemiology question was the confirmation of viroids isolated from oil palm through Koch's postulate experiment. Koch's postulate study was fulfilled confirming the oil palm CCCVd variant OP246 was pathogenic and replicate autonomously in its host with symptom expression. Transmission of $CCCVd_{OP246}$ into oil palm and coconut seedlings were successful for both viroid with symptom appearance. The transmitting ability of viroid was confirmed when oil palm and coconut inoculated with inoculum OP246 expressing OS symptoms and similar CCCVd variant was recovered. However, the transmission of the new variants found

on oil palm and into alternate host have not been studied, thus require further research to observe actual symptom expression on adult palms for better understanding of disease epidemiology and viroid etiology. In addition, transmission of oil palm CCCVd variants to coconut showed 100 % sequence similarity to oil palm CCCVd variant. Host factors and favourable environmental conditions may trigger mutations which could produce a more virulent variant. Thus, proper monitoring is necessary.

Efficiency of strategies of disease management is developed with the use of information regarding epidemiology. It is advisable to implement CCCVd free oil palm planting materials usage in accordance to ensure no further spread of oil palm CCCVd variants in Malaysian oil palms. Further work need to be carried out on epigenetic and other environmental factors related to the mutations in viroid position eventually causing disease symptom. It is important to study on the transmission of CCCVd variants characterized from oil palm into oil palm in order to observe for similar viroid sequence and symptoms expression that may answer to the arising question on the epidemiology of OS disease.

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APPENDICES

APPENDIX A

MATERIALS USED IN STUDY

Samples of Oil Palm Leaf from Malaysia

Samples	Characteristic	Seed Source	Site
OSKS 1 OSKS 2 OSKS 3	Symptomatic, 9 years old, tree bears small fruit bunch	Dura x pisifera (Golden Hope commercial)	Sungai Buloh, Selangor, Sime Darby Plantations
HOS 1	Asymptomatic, 9 years old, tree bears large fruit bunches	Dura x pisifera (Golden Hope commercial)	Sungai Buloh, Selangor, Sime Darby Plantations
OSBP 1 OSBP 2 OSBP 3	Symptomatic, 9 years old, tree bears small fruit bunches	Dura x pisifera (Golden Hope commercial)	Bukit Pelandok, Negeri Sembilan, Sime Darby Plantations
HBP 1	Asymptomatic, 9 years old, tree bears large fruit bunches	Dura x pisifera (Golden Hope commercial)	Bukit Pelandok, Negeri Sembilan, Sime Darby Plantations
OSUP 1 OSUP 2 OSUP 3	Symptomatic, 9 years old, tree bears small fruit bunches	Dura x pisifera (Golden Hope commercial)	Teluk Intan, Perak, United Plantations
HUP 1	Asymptomatic, 9 years old, tree bears large fruit bunches	Dura x pisifera (Golden Hope commercial)	Teluk Intan, Perak, United Plantations

APPENDIX B

BUFFERS, GELS, BACTERIAL MEDIA AND SOLVENTS

Acrylamide solutions

39 g acrylamide and 1 g bisacrylamide (40 % Acryl: Bis 39:1) dissolved in distilled sterile water. The vol was adjusted to 100 mL and stored at room temperature in dark condition.

Polyacrylamide gel

Preparation of 5 % non –denaturing PAGE by mixing 5 mL 10 X TBE, 38 mL sterile water and 6.25 mL of 40 % Acryl: Bis (39:1). 60 µL TEMED and 625 µL 10 % APS was added before casting into glass plates.

Analytical agarose gel

Heating of dissolved 1.5 % agarose in 1 X TBE in microwave oven.

Bacterial media

LB medium (1 L)

Luria broth measured 25 g was dissolved in 1 L SDW and autoclave at 121 ºC/15 psi for 20 mins.

Bacterial agar

LB agar (1 L)

40 g of Luria agar was dissolved in 1 L of SDW and autoclave at 121 ºC/15 psi for 20 mins. Solution poured into petri dishes (20 mL per plate) after cooling at 50 ºC and left to solidify in a laminar air flow before storage at 4 ºC.

Plates were spread with 25µL of 1 M IPTG (4µL per plate) and 80 mg/ml X-gal (20 µg/plate) mixed was spread over LB ampicillin plates surfaces and allowed to absorb at 37ºC for 30 min for cloning purposes.

Buffers

10X TBE

20 mM Na_2EDTA, 0.8 M HBO_3, 0.9 M Tris, adjusted using with acetic acid to pH 7.2.

KOH buffer

10 mL SDDW, 10 g KOH and 90 mL absolute ethanol.

Elution buffer

0.5 M NH_4Ac, 1 mM EDTA, 0.1 % SDS, pH 8.0

Gel loading buffers

10X Glycerol

60 % Glycerol, 0.25 % bromophenol blue, 0.25 % xylene cyanol (stored at 4 ºC).

Organic reagents

CA buffer

1 vol isoamyl alcohol mixed with 24 vol chloroform, stored at room temperature in the dark.

Ethanol

75 % (v/v), absolut and 70 % (v/v) ethanol stored at 4 ºC and room temperature respectively.

PCA solution

One vol of CA solution was mixed with 1 vol of 90 % phenol mixed with 1 vol CA solution stored at room temperature in the dark.

90 % Phenol

500 g phenol melted in a water bath set at 65 ºC, 8-hydroxyquiniline added to 0.1 % (w/w), 55 mL of SDW added and mixed. Saturated phenol stored in the dark.

APPENDIX C

KITS COMPONENT

Reverse Transcription System (Promega, USA)

Components	Final concentration (20µL Vol.)
25 mM $MgCl_2$	5 mM
10 X Reverse Transcription buffer -500 mM KCl -1 % Triton®X-100 -100 mM Tris-HCl (pH 9.0 at 25 ºC)	1X Reverse Transcription buffer -50 mM KCl -0.1 % Triton®X-10010 M -10 mM Tris-HCl (pH 9.0 at 25 ºC)
10 mM dNTP mixture	1mM each dNTP
Recombinant RNasin® Ribonuclease Inhibitor (40U/µL)	20 U
AMV Reverse Transcriptase (24 U/µL)	24 U
10 µM	0.5 µM
(~ 200 ng) RNA template	5 µL
Nuclease - free water	To a final volume of 20 µL

PCR Master Mix (Promega, USA)

2X, Master Mix

50 units/ml *taq* NDA polymerase, 400µM each dCTP, dATP, dTTP, dGTP, 3 mM $MgCl_2$, 1 X master mix utilized for PCR reaction.

PCR Cloning Kit (QIAGEN, Australia)

Components	Final concentration (10 µL reaction)
pDrive cloning vector (50 ng/ µL)	1 µL(50 ng)
2X ligation master mix	5 µL(1 X)
PCR product (50g/ µL)	4 µL(200 ng)

BIODATA OF STUDENT

Raimathy Kanavedee was born in Ipoh, Perak. She had her kindergarten education at St. Marks, Taiping from 1994 – 1996, primary education at girls school S. R. K. Convent Kota, Taiping from 1997-2002 and continued her secondary education at girl school S.M.K. Convent, Taiping from 2003 – 2007. She then enrolled in S. M. J. K. (CINA) Hua Lian, Taiping for her STPM for two years from 2008- 2009. She furthered her undergraduate level studies at Universiti Malaysia Sabah (UMS), Kota Kinabalu, Sabah in Bachelor in Agriculture Science majoring in Crop Production from 2010 – 2014. Her final year project title was comparison of invertebrate abundance in soil amended with bio-organic and chemical fertilizers in Sekong oil palm estate, Kinabatangan district under the supervision of Assoc. Prof. Dr. Suzan Benedick from School of Sustainable Agriculture, UMS. She experience industrial training at Malaysian Palm Oil Board (MPOB) under the supervision of Dr. Shamala Sundram, Senior Principal and Group Leader of Emerging and Exotic Disease (EEDG). She was given an opportunity to then pursue her Master programme at Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia (UPM) in September 2014. She is a recipient of Graduate Student Assistantship Scheme (GSAS) scholarship provided by MPOB. She attended two international conferences and presented posters.

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LIST OF PUBLICATIONS

Raimathy Kanavedee, Ganesan Vadamalai, Nur Diyana Roslan, and Shamala Sundram (2015). Optimization of total nucleic acid extraction from oil palm leaves rich in polyphenol and polysaccharides. Proceedings of the International Congress of the Malaysian Society for Microbiology 2015 (ICMSM 2015), 7th – 10th December 2015, Bayview Beach Resort, Penang, Malaysia.

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