TRANSMISSION OF *COCONUT CADANG-CADANG VIROID* OIL PALM VARIANTS TO OIL PALM TISSUE CULTURE PLANTLETS

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ABSTRACT

This study was conducted to transmit *Coconut cadang-cadang viroid* (CCCVd) variants to oil palm tissue culture plantlets. The source of inoculum was extracted by using the polyethylene glycol (PEG) extraction method from the leaflets of an oil palm with the orange spotting (OS) symptom and which has tested positive as a CCCVd oil palm variant. The concentration of leaf extract was adjusted to 20µg/ml and inoculated into 1 to 3-month-old plantlets with a high pressure injector. Six months after inoculation, the orange spotting symptom was observed on the leaflet of the plantlet. When subjected to dot blot assay, the sample hybridised with the $CCCVd_{246}$ full-length complementary probe, indicating the presence of CCCVd-like RNAs in the inoculated plantlet. This suggests that the CCCVd oil palm variants have been successfully transmitted into the host. This study has demonstrated the effectiveness of using a high pressure injector in transmitting CCCVd oil palm variants and showed the potential of using oil palm tissue culture plantlet in pathogenicity studies due to benefits in terms of economies of time, space and labour.

Keywords: CCCVd oil palm variants, oil palm tissue culture plantlet, high pressure injector

ABSTRAK

Satu kajian untuk menjangkitkan plantlet kultur tisu kelapa sawit dengan varian Coconut cadang-cadang viroid (CCCVd) telah dijalankan. Inokulum diekstrak daripada daun kelapa sawit yang telah dikenalpasti positif CCCVd dengan simtom orange spotting (OS) menggunakan kaedah polyethylene glycol (PEG). Kepekatan ekstrak daun disesuaikan pada 20µg/ml dan disuntik ke plantlet yang berusia 1 ke 3 bulan menggunakan injektor bertekanan tinggi. Enam bulan kemudian, plantlet menunjukkan simtom orange spotting. Apabila diuji dengan dot blot assay didapati sampel berhibridisasi dengan prob $CCCVd_{246}$ menunjukkan kehadiran RNA menyerupai CCCVd pada plantlet, seterusnya mencadangkan bahawa CCCVd varian kelapa sawit tersebut telah dipindahkan dengan jayanya di dalam hos. Kajian ini telah membuktikan keberkesanan injektor bertekanan tinggi untuk memindahkan CCCVd varian kelapa sawit dan menunjukkan potensi kultur tisu kelapa sawit dalam kajian patogenisiti kerana kelebihannya yang menjimatkan masa, ruang dan tenaga kerja.

Kata kunci: CCCVd varian kelapa sawit, plantlet kultur tisu kelapa sawit, injektor bertekanan tinggi

1. Introduction

Coconut cadang-cadang disease which is caused by the *Coconut cadang-cadang viroid* (CCCVd) is a lethal coconut disease in the Philippines where it has caused extensive losses to the coconut industry (Zelazny et al., 1982). CCCVd is not controlled because the epidemiology of the disease is poorly understood. Exclusion is the only method considered to be effective in controlling the spread of CCCVd (Hadidi et al., 2003). CCCVd is a single stranded RNA with a basic form of 246 nucleotide molecule, the smallest pathogen known. Initially the disease was thought to be contained in the Philippines. However, Hanold and Randles (1991) reported that CCCVd-like RNAs were present in oil palm plantations in the Solomon Islands. Apparently the palms with CCCVd-like molecules were in oil palms with the orange spotting (OS) disorder.

In Malaysia, Vadamalai (2005) detected the presence of CCCVd-like RNAs in both OS symptomatic and asymptomatic oil palms in commercial oil palm plantations. The CCCVd-like RNAs showed over 90% similarity with CCCVd from coconuts in the Philippines but none were identical. The oil palm CCCVd variants (comprising 246, 270, 293 or 297 nt) were classified as CCCVd because they show greater than 90% sequence similarity with the coconut viroid (Vadamalai et al., 2006; Wu et al., 2013). The nucleotide substitution of the oil palm variants were at position 31 (C to U) and position 70 (G to C) compared to the CCCVd from coconuts. To relate the veracity of a CCCVd lethal infection on coconuts and CCCVd oil palm variants infectivity on oil palm, a pathogenicity study has to be conducted. Joseph (2012) established the pathogenicity of CCCVd oil palm variants by infecting pre-germinated oil palm seedlings using partially purified oil palm leaf extracts infected with CCCVd variants as a source of inoculum. DNA sequencing showed that the 246 nt variant was found only in oil palms expressing OS symptoms and had 99% sequence similarity with $CCCVd_{246}$ from coconut palm (Joseph, 2012; Wu et al., 2013). The three larger variants were found in an asymptomatic oil palm (Vadamalai, 2005). This showed that a range of sequence variants of CCCVd can coexist in one oil palm (Vadamalai et al., 2006). Recently, Thanarajoo (2014) successfully transmitted the plasmid clone of the $CCCVd_{246}$ oil palm variant to oil palm seedlings. Both techniques observed OS symptoms six months after inoculation. Their studies have proven that CCCVd not only infects coconut but also infects and replicates autonomously in oil palm. In relation to these findings, an effective and economically sound research is required for further investigation on CCCVd epidemiology and its control measures.

Viroids can be mechanically transmitted (Diener, 1979) and this usually involves the introduction of plant extracts that contain viroids into particular plants through mechanical inoculation such as through slashing and using high pressure injection. In the case of the *cadang-cadang* coconut disease, the viroid was first successfully transmitted to 4 to 18-month-old coconut seedlings using plant extracts by high pressure injection combined with either rubbing with carborundum or slashing of petioles with a razor (Randles et al., 1977). A later study showed that transmission could be achieved by high pressure injection of nucleic acid extracts alone into younger seedlings (Randles et al., 1980). Therefore, in our study, an attempt was made to transmit CCCVd variants to oil palm tissue culture plantlets using partially purified oil palm leaf extracts with CCCVd oil palm variants. Oil palm tissue culture plantlets were used because the *in-vitro* system allows inoculation in controlled conditions.

2. Materials and Methods

2.1. *Source of oil palm tissue culture plantlets*

The oil palm tissue culture plantlets (Figure 1) were obtained from Associate Professor Dr. Uma Rani Sinniah (Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia) with an age range of between 1 to 3 months old, and these were randomly selected to contain a mixture of rooted and non-rooted plantlets.

Figure 1: The oil palm tissue culture plantlets

2.2 *Source of CCCVd variant inoculums*

The leaves (Figure 2) from oil palms reported with CCCVd oil palm variants and showing the OS symptom (Vadamalai, 2005) were extracted using the polyethylene glycol (PEG) extraction method (Hanold & Randles, 1991). Ten to 20g of leaves was cut into small pieces and blended in 120ml of 100mM Na_2 SO_3. The slurry was then strained through cotton muslin and shaken vigorously for 30 minutes at 40^0C with 20g/L polyvinylpolypyrrolidone (PVPP). Fifty mililitres of chloroform was added and mixed vigorously for 5 minutes. The mixture was centrifuged at 10,000g for 10 minutes. The aqueous supernatant was collected and PEG 6000 was added at 80g/L. After stirring to dissolve the PEG, and incubating it for 2 hours at 4^0C, the resulting precipitate was collected by centrifugation at 10,000g for 10 minutes. Nucleic acids were extracted from the precipitate by dissolving it in 2ml of 10g/L sodium dodecyl sulphate (SDS) and then adding 2ml of aqueous phenol (900g/L) containing 1g/L 8-hydroxy quinoline and shaking it vigorously for 1 hour. The aqueous supernatant was collected by centrifugation at 10,000g for 10 minutes and re-extracted with 1ml of phenol and 1ml of chloroform for 5 minutes. NaCl was added to 0.1M, followed by cetyl trimethylammonium bromide (CTAB) to 3.3g/L and incubating it for 30 minutes at 0^0C. The precipitate was collected by centrifugation for 30 minutes at 10,000g. The pellet was washed two times with 0.1M Na-acetate in 75% ethanol and once with 100% ethanol. The pellet was air-dried and re-suspended with 500µl of Milli-Q and stored in -20^0C. For inoculation purpose, the inoculum concentrations were adjusted to 20µg/ml and measured using a ND-1000 spectrophotometer (NanoDrop Technologies Inc. USA).

Figure 2: The leaves of oil palms reported with CCCVd oil palm variants and showing the OS symptom (Vadamalai, 2005)

2.3. *Transmission of partially purified leaf extracts containing CCCVd oil palm variants with a Panjet high pressure injector*

Ten plantlets were inoculated with using a Panjet high pressure injector (Schuco International, UK) (Figure 3) at three shots per plantlet: one shot on the leaflet and two shots at the lower base that gave 100µl per shot. Five plantlets were inoculated with sterilised distilled water as control. After 1 week, all plantlets were taken out from the tubes, their roots were washed with sterile distilled water and then transplanted in vermiculite. The plantlets were covered with clear plastic bags and kept in the tissue culture room with temperatures of 26^0C to 28^0C and 12 hours of photoperiod at approximately 3000-lux intensity (normal fluorescent light at180 $\mu molm^{-2}s^{-1}$). The plastic bags were taken off gradually within 4 weeks until the plantlets were hardened and then they were transplanted in soil and kept in a glasshouse. Symptom development was observed every month and dot blot assay was conducted at 6 months after inoculation.

Figure 3: Transmission of partially purified leaf extracts containing CCCVd oil palm variants with a Panjet high pressure injector (Schuco International, UK) into oil palm tissue culture plantlets

2.4. *Nucleic acid extraction for CCCVd oil palm variant detection*

This method was modified from Hodgson (1998). One gram of leaf sample was reduced to powder in liquid nitrogen and homogenised with 2ml NETME extraction buffer (2M NaCl, 100mM sodium acetate, 50mM Tris-HCl pH 7.5, 20mM ethylenediamine tetra acetic acid (EDTA) pH 8.0, 20% ethanol, 0.25% (v/v) 2-mercaptoethanol) and ground with a mortar and pestle until it became fine. SDS was added to 1% and the mixture was incubated for 30 minutes at room temperature. From the resulting slurry, 900µl was transferred to a 1.5ml centrifuge tube, mixed with 600µl phenol:chloroform:iso-amyl alcohol (PCA) mix and centrifuged at 10,000 g for 15 minutes at room temperature. Approximately 800µl of the supernatant was transferred to a new tube, mixed with 600µl chloroform:iso-amyl alcohol (CA) and centrifuged at 10,000g for 15 minutes. The supernatant was then transferred to a new tube and mixed gently with 0.9 vol isopropanol and incubated at -20^0C for 3 hours and centrifuged at 10,000g for 10 minutes. The supernatant was discarded and the pellet was washed with 700µl 70% ethanol by gentle mixing for about 2 hours. The pellet was then collected by centrifugation at 10,000g for 10 minutes and air-dried, and dissolved in 50ml of Milli-Q overnight incubation at 4^0C and stored at -20^0C.

2.5. *Preparation of Digoxygenin (DIG) labeled full-length cRNA probe*

A plasmid vector containing a monomeric insert of the 246 nucleotide form of CCCVd was obtained from Prof. J.W. Randles of the School of Agriculture, Food and Wine, University of Adelaide and used for cRNA probe preparation. A 20µl reaction was prepared for transcription of the probe using the Roche Applied Science DIG nucleic acid labelling kit. According to the manufacturer's recommendation, approximately 1µg linearised plasmid was mixed with a transcription reaction containing 10x NTP labelling mixture, 10x transcription buffer, protector RNase inhibitor and RNA polymerase T7 and incubated for 2 hours at 37^0C. The reaction was stopped by adding 2ul 0.2 M EDTA (pH 8.0) and analysed on 1.5% agarose gel.

2.6. *Dot blot hybridisation*

Four microlitres each of undiluted nucleic acid extract from the inoculated oil palm plantlet, control oil palm plantlet, SRH2 and SRD6 extracts of oil palm samples reported positive with CCCVd oil palm variants (Vadamalai, 2005) were spotted on nylon membrane (Zeta Probe® BioRad), air-dried at room temperature and UV irradiated for 1 to 5 minutes.

2.7. *Hybridisation and immunological detection*

The membrane was pre-hybridised for 90 minutes at 45^0C in pre-hybridisation buffer. The full-length cRNA probe was mixed with 100µl pre-hybridisation buffer and added to the hybridisation solution. The membranes were incubated in a hybridisation oven at 45^0C for 15-24 hours and then washed twice in 0.5X SSC, 0.1% SDS for 5 minutes at 25^0C followed by high stringency washes in 0.1X SSC, 0.1 % SDS for 60 minutes at 60^0C. The membrane was blocked with blocking solution and the DIG labelled cRNA probe was detected with commercial monoclonal phosphate-conjugated antibody and substrate (Roche Applied Science).

3. Result and Discussion

3.1. *Transmission of partially purified leaf extracts containing CCCVd oil palm variants with a Panjet high pressure injector*

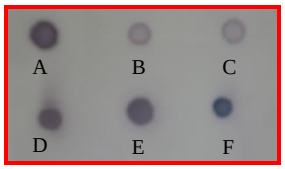
The results of this sudy showed that only one out of 10 oil palm plantlet that were inoculated survived. Two plantlets survived for control treatment. All perished plantlets were non-rooted plantlets which explained its inability to survive. Six months after inoculation, the OS symptom was observed on the sole surviving plantlet leaf (Figure 4). Meanwhile, no OS symptom was observed in the control plantlets (Figure 5). This transmission study using tissue culture plantlets produced the symptom much earlier compared to the pathogenicity study carried out by Imperial et al. (1985) using oil palm seedlings, where they observed yellowing symptoms only from the fourth year onwards after inoculation. It was reported that some of the advantages of using tissue culture plantlets for transmission studies are that they are easy to maintain, large quantities with uniform shape can be obtained fast and they require less time for symptom development (Rey & Hong, 1990).

Figure 4: The orange spotting (OS) symptom was observed on the sole surviving plantlet 6 months after inoculation

Figure 5: No orange spotting (OS) symptom was observed on the control plantlets

3.2. *Dot blot hybridisation*

Dot blot hybridisation with DIG-labeled full-length $CCCVd_{246}$ cRNA probe detected the presence of CCCVd molecules from the inoculated plantlet and oil palm samples reported positive with CCCVd oil palm variants (SRH2 and SRD6) showed by the intensified spot signal as compared to the control (Figure 6). A good spotting signal can be observed within 20 to 60 minutes of the colour development period. Prolonged incubation may result in darker background making it difficult to distinguish between the infected and healthy samples. A weak non-specific signal in the control leaf extract was observed which was also reported by other researchers (Kanematsu et al., 1991). This is a known disadvantage of using dot blot as a colourometric based detection method. To reduce the background problem, Welnicki and Hiruki (1992) suggested the blocking period of the membrane to be prolonged to overnight.



Denotation:
A: Extract of plantlet inoculated with partially purified extract inoculums
B and C: Extract of plantlet inoculated with sterilized distilled water (control)
D and E: Extract of oil palm sample reported positive with CCCVd oil palm variants
F: Unlabeled CCCVd SP6 transcript (viroid sense)

Figure 6: Dot blot hybridisation with DIG-labeled full-length $CCCVd_{246}$ cRNA probe detected the presence of CCCVd molecules from the inoculated plantlet and oil palm samples reported positive with CCCVd oil palm variants (SRH2 and SRD6)

4. Conclusion

Results from this study showed that partially purified CCCVd oil palm variants from leaf extracts were sucessfully transmitted to the tissue culture plantlet. Partially purified inoculums were used because the preparation is economical compared to plasmid clone. The presence of CCCVd molecules were detected by dot blot hybridisation using DIG-labeled full-length $CCCVd_{246}$ cRNA probe. Detection with DIG labelling is a fast, effective and cheaper alternative to DNA sequencing. The use of oil palm tissue culture plantlet is useful in pathogenicity studies. However the technique needs to be improved because of the high mortality of the inoculated plantlets.

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