MODIFIED EXTRACTION METHOD FOR DETECTION OF COCONUT CADANG-CADANG VIROID (CCCVd) VARIANTS

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The oil palm is an important perennial oil crop for a number of countries in the tropics. A recent finding reported the presence of *Coconut cadang-cadang viroid* (CCCVd) variant in oil palm. The viroid as reported by Vadamalai (2005) implicated the movement of oil palm planting materials in Malaysia although there was no evidence of the viroid causing devastating disease loss compared to the loss recorded in the Philippines. In reality, the infected oil palm is only found isolated within a planting block (Sundram *et al.*, 2017). The viroid sequence in oil palm was also found with a few base changes when compared to the viroid sequence in coconut of the Philippines. The viroid present in the form of free RNA strands replicating with protein of the host, was difficult to be detected and the available techniques were unable to give reliable and consistent detection. Therefore this technology improved the existing Natrium Chloride EDTA Tris-HCL Mercaptoethanol (NETME) extraction developed originally for coconut, to detect CCCVd variants consistently in oil palm. Extraction of high quality RNA is necessary in generating cDNA libraries, characterisation and detection by Reverse Transcriptase – Polymerase Chain Reaction (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. Based on these submissions, it is therefore paramount that extraction methods produces high concentration of RNA, thus the absolute need of optimisation. NETME is a suitable choice for simpler extraction with small amount of leaves sample (2-5 g) used (Vadamalai, 2005; Joseph, 2012). NETME extraction method undergoes purification step through Polyacrylamide gel electrophoresis (PAGE) that is time consuming and laborious (Hanold and Randles, 1991; Vadamalai, 2005). It requires additional two to three days procedure and is proven to be inconsistent in detection when repeated. Modification and optimisation of the existing NETME extraction method gave rise to shortened extraction time and provide a higher quality RNA for better detection of CCCVd variants in oil palm with the complete elimination of PAGE purification process.

NOVELTY OF TECHNOLOGY

The approach for modification and optimisation of total nucleic acid (TNA) extraction method resulted in consistent detection of CCCVd variants in oil palm both in nursery and field samples. The highlighted modification with increasing the precipitation stages with the addition of lithium chloride resulted in the consistent detection of CCCVd variants. Unlike conventional method, this approach provides usage of reduced volume of buffer, reduction in experimental period from 4 days to 1 ½ day, and elimination of laborious PAGE purification step. The optimised protocol gave a more cost-effective, less time consuming and a more reliable technique in detecting the viroid variant in oil palm.

IMPROVEMENT IN MODIFIED NETME RNA EXTRACTION ASSAY

The conventional NETME protocol only managed to detect partial length sequence of CCCVd variants. Therefore research was focused on modifying and optimising the existing technique to detect the full length sequence of CCCVd variants. The modified method successfully detects the full length sequence and further eliminates the laborious non-denaturing PAGE. The elimination of PAGE reduces the time taken to complete the assay from 4 days to 1 ½ days. The method also significantly reduces processing cost to approximately RM 172 per sample. The modifications and cost reductions on the conventional and modified NETME method are described in *Table 1*.

VERIFICATION OF MODIFIED NETME ASSAY FOR ACCURACY IN DETECTION

The modified and optimised NETME extraction method resulted in higher quality RNA (purity and concentration) when compared with

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TABLE 1. SUMMARY OF MODIFICATIONS AND COST REDUCTIONS ON THE CONVENTIONAL NETME EXTRACTION METHOD

Conventional NETME Method	Modified NETME Method	Cost difference by using Modified NETME Method
Homogenisation and Extraction Buffer		
5g of leaf samples were ground with liquid Nitrogen Incubated in NETME buffer 1% SDS 2-Mercaptoethanol	5g of leaf samples were ground with liquid Nitrogen NETME buffer 1% SDS 2-Mercaptoethanol	– RM 4
Separation		
Phenol/Chloroform/isoamyl Alcohol to supernatant ratio Chloroform/isoamyl Alcohol to supernatant	Phenol/Chloroform/isoamyl Alcohol to supernatant ratio Chloroform/isoamyl Alcohol to supernatant ratio	– RM 23
Centrifugation		
11 000 g, 10 mins, room temperature	11 000 g, 15 mins, 4°C	
Precipitation		
Isopropanol	Isopropanol Lithium Chloride Supernatant with ethanol	+ RM 5
Incubation		
Pellets were incubated at -20°C (Hodgson *et al*., 1998)	Pellets were incubated at -20°C	
Total Nucleic Acid Purification		
PAGE PURIFICATION Acrylamide Bisacrylamide Tris Borate EDTA (TBE) Ammonium Persulfate (APS) Potassium Hydroxide Tetramethylethylenediamine (TEMED) Acetic Acid Ethanol Silver Nitrate Sodium Bohydrate Sodium Hyroxide Formaldehyde Ammonium Acetate 0.1% SDS EDTA	**ELIMINATION OF PAGE**	– RM 150
Total cost reduction for one sample processing		**– RM 172**

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 RNA per gram of fresh tissue and was at least 4 times more than values obtained using the conventional extraction method. *Table 2* details the 4 samples of OS samples subjected to RNA extraction with modified method giving higher quality of RNA with significant difference.

The modified NETME method also produced final RNA of colourless and clearer pellets in one day as compared to the conventional NETME with yellowish orange or brown pellets typically indicating unsuccessful removal of contaminating phenols (Shu *et al*., 2014) which usually takes three days in addition to the purification PAGE step (*Figure 1*).

RT-PCR amplification of samples from the modified NETME method using CCCVd specific primer; full length base pairs (246 bp) and partial length (242 bp) primers led to consistency in band retrieval and detection of CCCVd variants as expressed in *Figures 2* and *3*.

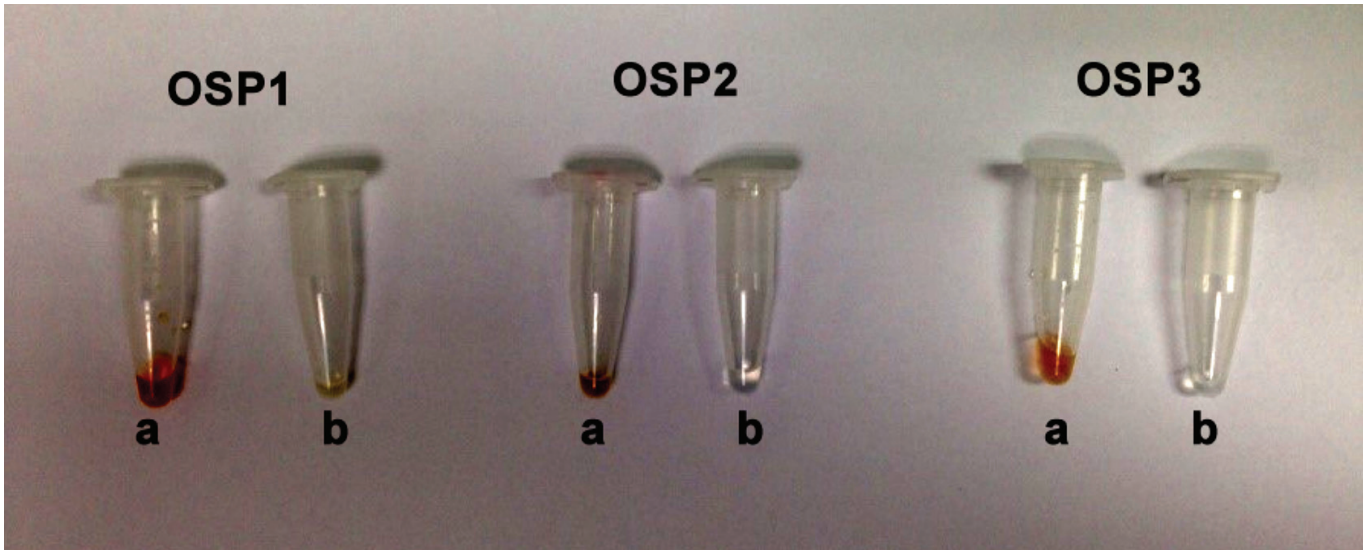

Figure 1. Pellets obtained from (a) conventional NETME Extraction method and (b) modified NETME extraction method for various samples.

TABLE 2. RNA QUALITY FROM CONVENTIONAL AND MODIFIED NETME EXTRACTION METHODS

Sample	Methods	A260/280	A260/230	Concentration (ng μlitre^{-1})
OSP	NETME	1.085[a]	0.765[a]	649.625[a]
	Modified NETME	**1.8375[b]**	**1.8825[b]**	**3004.2[b]**

Note: Mean is representative of n = 4. Means with different superscript letters are significantly different at P ≤ 0.05 with t- test. OSP: orange spotting palms.

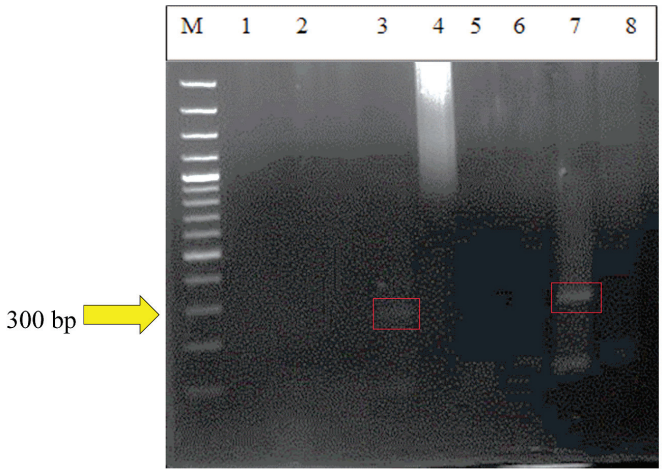

Figure 2. RT – PCR amplification using set I primers of total nucleic acid from modified NETME extraction analysed on 1.5% agarose gel electrophoresis. M: Marker (100 bp). Lane 1: Negative control (sterilised distilled water - SDW as PCR template), lane 2: HP, lane 3: OPS1, lane 4: OPS1, lane 5: OPS2,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

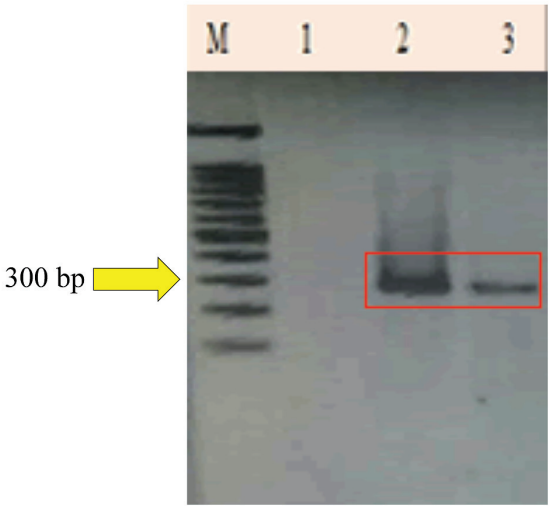

Figure 3. RT – PCR amplification of total nucleic acid from modified NETME extraction method using primers set IV viewed on 1.5% agarose gel and stained with EtBr. M: Marker (100 bp), Lane 1: HP, lane 2: OSP1 and lane 3: OSP2; OPS – oil palm samples.

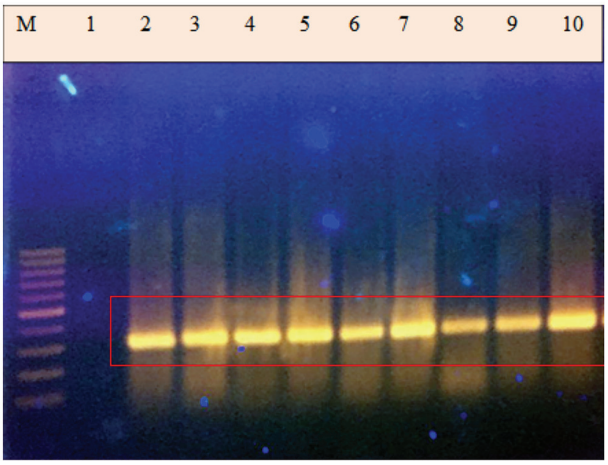

Figure 4. Product of RT - PCR from the nine samples of OS-affected oil palm; OS1 (lane 2), OS2 (lane 3), OS3 (lane 4), OS4 (lane 5), OS5 (lane 6), OS6 (lane 7), OS7 (lane 8), OS8 (lane 9) and OS9 (lane 10), amplified using set IV primers and observed on a 1.5% agarose gel. Lane 1 is a negative control (sterilised distilled water (SDW) as PCR template). A DNA ladder (lane M) sized 100 bp was used as a marker.

VERIFICATION WITH FIELD AND NURSERY SAMPLES

The NETME assay was tested with a number of field and nursery samples (Koch's Postulate). The extracted field samples (from different geographical locations) were run on RT-PCR resulted with amplification at 250 bp on the agarose gel.

POTENTIAL USERS

The technology is suitable to be adopted by quarantine departments and all seed producers with molecular laboratory facilities.

BENEFITS TO THE INDUSTRY

The assay can be carried out on mother palms selected for seed production and breeding trials to avoid future introduction of CCCVd variants in field. The quarantine issue requires reliable detection of CCCVd variants in oil palm. An optimised and modified NETME extraction method results in high quality of RNA which includes higher RNA purity and concentration. The optimised method uses lesser buffer volume which contributes significantly to the economics of the CCCVd variants detection. The modified technique would now provide a faster and more reliable detection of CCCVd variants for plantations to screen their oil palms materials.

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