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RESEARCH NOTE

Optimization of total nucleic acid extraction method for detecting Coconut cadang-cadang viroid variants in oil palm

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Abstract Variants of Coconut cadang-cadang viroid (CCCVd) with over 90% sequence similarity to CCCVd in coconut have recently been associated with orange spotting disease in oil palm, especially in low concentrations in infected hosts. Thus, there is a need to extract high-quality nucleic acid for molecular detection of the viroid. Total nucleic acid (TNA) was extracted from oil palm leaf samples with orange spotting symptoms collected from different states in Malaysia using a modified and optimized version of the conventional natrium chloride EDTA Tris-HCL mercaptoethanol extraction method. The modifications involved additional ethanol and lithium chloride precipitation stages, thereby eliminating the need for further purification through non-denaturing polyacrylamide gel electrophoresis (PAGE). The modified procedure yielded a mean volume of 60 μg RNA per 10 g of fresh tissue which is approximately four times the volume produced by the conventional extraction method. Furthermore, the modified method resulted in higher purity than the conventional method according to the ratios of absorbance at wavelengths of 260/280 nm and 260/230 nm, which were 1.838 and 1.883 with the modified method and 1.085 and 0.765 with the conventional method. The modified method enabled extraction of high-quality RNA from all samples investigated. The extracted RNA was suitable for cDNA synthesis and PCR amplification and showed consistent detection of CCCVd-like RNA at approximately 250 amplicons. Using the conventional method, detection of CCCVd-like RNA was inconsistent and only feasible after the PAGE purification step.

Keywords Coconut cadang-cadang viroid · Oil palm · Total nucleic acid · Extraction · NETME

Large amounts of high-quality RNA are needed for sequencing strategies involving RNA, characterization and detection by RT-PCR or investigating gene expression profiles (Gehrig et al. 2000; Shu et al. 2014). However, high phenolic and/or polysaccharide compounds released during cell disruption (particularly for coconut and oil palm, which are rich in polyphenols and polysaccharides and possess leaves with high fiber content and a waxy cuticle [Xiao et al. 2012]), can make it difficult to obtain high-quality RNA. Similarly, the susceptibility of RNA molecules to enzymatic degradation by RNase (Rubio-Pina and Vazquez-Flota 2008) can give rise to poor-quality RNA or an absence of RNA. Ergun et al. (2013) pointed out that PCR sensitivity is affected by polysaccharides, polyphenols and the phenol contents of plants, which may ultimately lead to false results. Kansal et al. (2008) observed that readily oxidized phenolic compounds are linked covalently with quinones and bind with nucleic acids. Furthermore, co-precipitation of polysaccharides with RNA can occur in low ionic strength buffers (Ho et al. 1996). Because of these various challenges involved in extracting high concentrations of usable RNA through conventional methods, it is necessary to optimize RNA extraction techniques.

A few suitable methods for extracting TNA from oil palm have been tested; these include polyethylene glycol and sodium chloride EDTA Tris-HCl mercaptoethanol (NETME) extraction (Hanold and Randles 1991; Vadamalai 2005; Joseph 2012). The polyethylene glycol extraction method, established by Hanold and Randles (1991), has been proven

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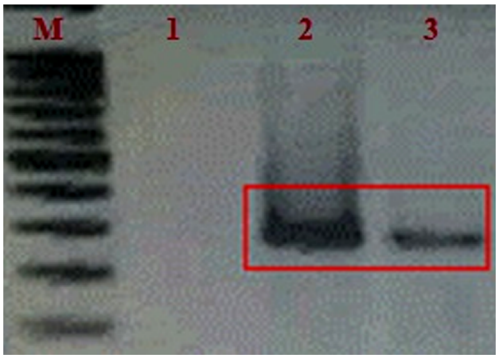

Fig. 1 RT-PCR amplification after the modified NETME extraction method using primer set IV, viewed on 1.5% agarose gel and stained with EtBr. M: Marker (100 bp), Lane 1: HP, Lane 2: OSP1 and Lane 3: OSP2

successful for detecting CCCVd variants in monocots, especially coconut and oil palm. However, this method is time consuming and highly laborious because it requires large volumes of leaf samples (20–100 g). NETME is a suitable alternative because it is a simpler process that requires lower-volume samples, followed by purification step using PAGE. Although this purification step can provide high-quality of RNA, it is also time-consuming and laborious because it requires an additional 48 h of experimental process. Consequently, it is important to develop easier and less time-consuming methods of obtaining high-quality RNA. The current report presents an RNA extraction protocol for oil palm that involves a modified and optimized version of the conventional NETME method. This optimized technique is suitable for recovering high-quality RNA for detection by RT-PCR and further characterization purposes.

Leaflets from nine-year old healthy oil palm (HP) and orange spotted palm (OSP) were sampled from a commercial oil palm plantation in Malaysia. The leaves were cut into 15–20-cm-long samples after removing the midribs. Then, samples were cleaned, sealed in plastic bags, labeled and stored at −80 °C. TNA extraction was carried out using conventional NETME extraction according to the methods of Hodgson et al. (1998) as modified by Vadamalai (2005). Optimization was achieved by grinding 5–10 g leaf samples in liquid nitrogen and grinding them further using reduced NETME extraction buffer (5 mL) with continuous addition of 2.40 mL of 1% SDS, and 12.8 μL of 2-Mercaptoethanol. The ground slurry was centrifuged for 15 min at 4 °C × 11,000 g (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany). The resulting supernatant and phenol-chloroform-isoamylalcohol were mixed to a ratio of 4:3, centrifuged and subsequently mixed with chloroform-isoamylalcohol (CA) to a ratio of 3:2 and centrifuged again. The supernatant obtained was precipitated with 1 volume of isopropanol and the mixture was gently inverted, mixed three times and incubated at −80 °C for 45 min, followed immediately after by centrifuging for 15 min. The pellet was rinsed with 1 mL of 70% ethanol and further centrifuged for 5 min at 4 °C. The pellet was allowed to air dry for 10 min, re-precipitated with 1 mL of 8 M LiCl solution, vortexed, incubated at −20 °C for 4 h and centrifuged for 10 min. The supernatant was precipitated with 1 mL of 100% ethanol, incubated at −80 °C for 45 min and centrifuged for 15 mins at 4 °C. The pellet was re-washed with 1 mL of 70% ethanol, vortexed, centrifuged for 10 min, allowed to air dry, re-suspended with 20 μL sterile of distilled water and stored at −20 °C. RNA quality testing, cDNA synthesis, and RT-PCR amplification were achieved according to the methods of Rodriguez (1993), Hodgson et al. (1998) and Vadamalai (2005) using primer set I (GV1 and GVR1) and primer set IV (GV4 and GVR4). Agarose gel electrophoresis and gel staining were carried out according to the methods of Vadamalai (2005).

The modified extraction method resulted in relatively clear and colorless pellets. This was in contrast with the yellowish-orange or brown pellets obtained with the conventional NETME extraction method for all samples, which typically indicates unsuccessful removal of contaminating phenols (Shu et al. 2014). The modified NETME method produced clear RNA pellets in 1 day in contrast to the conventional NETME method, which took 4 days (in addition to the PAGE purification step) to obtain clear pellets.

The modified method yielded approximately 60 μg per 10 g of fresh tissue RNA, which was at least four times as high as the values obtained using the conventional extraction method. The modified NETME method also resulted in higher-quality RNA according to the ratios of absorbance at wavelengths of 260/280 nm and 260/230 nm, which were 1.838 and 1.883 with the modified method and 1.085 and

Table 1 Presence (✓) and absence (x) of CCCVd-like RNA in RNA extracted from oil palm leaf samples through the conventional and modified NETME extraction methods

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	✓	✓
HP	x	x	x	x

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

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

0.765 with the conventional method. Absorbance ratio ~ 2.0 for 260/280 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 RNA extracted from the modified method had higher purity than that from the conventional method, the 260/230 and 260/280 ratios were still low for all samples. These relatively low values for the two ratios may have resulted from residual phenol from nucleic acid extraction and a very low concentration of nucleic acid, respectively (Sambrook et al. 1989). Fleige and Pfaffl (2006) observed that the salt concentration and buffers used may interfere with optical density measurements and lead to under- or over-estimates of RNA concentrations.

The clearer and better-quality RNA observed through the modified extraction method may be attributable to the removal of the accompanying DNA and 4S RNAs, recovery of small RNA and low-molecular weight viroids as a result of the additional lithium chloride precipitation (Granell et al. 1983), which is a key modification in the extraction protocol. Lithium chloride precipitation does not efficiently precipitate DNA and carbohydrate, but removes inhibitors of cDNA synthesis (Cathala et al. 1983; Apt et al. 1995). High-quality RNA obtained from the modified procedure enabled consistent detection of CCCVd-like RNA for most samples using a full-length primer that amplified approximately 250 amplicons (Fig. 1). In contrast, the conventional method only enabled detection for one sample (OSP1), using primer set I (Table 1).

Detection of CCCVd-like RNA through the conventional NETME method was only feasible after the PAGE purification step. With this additional step, viroid detection was possible for all three samples (OSP1, OSP2 and OSP3.) The modified NETME extraction method allowed us to eliminate this purification step. The modification enabled consistent production of high-quality RNA and reduced extraction time. Furthermore, the extracted RNA using the modified method was suitable for downstream processes such as cDNA synthesis and expression pattern analysis. Thus, we consider the modified NETME to be more efficient, consistent and easier to carry out than the conventional method plus PAGE purification.

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