REVERSE TRANSCRIPTION-LOOP MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) KIT FOR DETECTION OF COCONUT CADANG-CADANG VIROID (CCCVd) VARIANTS

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Orange spotting (OS) disease caused by Coconut Cadang-Cadang Viroid (CCCVd) variants in oil palm was discovered by Vadamalai *et al.* (2006). The CCCVd variants did not manifest in any outbreak as observed on coconut in the Philippines. It is only found on isolated palms in the planting blocks indicating no spread of the viroid (Sundram *et al.*, 2017). The viroid is a single-stranded RNA with sizes ranging from 246 to 247 nucleotides in early infection and extended to 296 to 375 nucleotides in the later infection (Haseloff *et al.*, 1982).

Methods for detecting of CCCVd variants such as polyacrylamide gel electrophoresis (PAGE), ribonuclease protection assay (RPA), probe hybridisation and reverse transcription-polymerase chain reaction (RT-PCR) are available but require longer time for analysis and have low consistency. The availability of a rapid, inexpensive and accurate diagnostic technique, which is readily adapted to large-scale screening of CCCVd variants in oil palm, would benefit in decision-making for appropriate control.

Specific detection of PCR method based on loop-mediated isothermal amplification referred as LAMP, offers a simple, robust and rapid screening of the viroid. Sensitivity of the reverse transcription-LAMP (RT-LAMP) assay is higher (up to 10-fold) as compared to the conventional nested PCR (Notomi *et al.*, 2000). RT-LAMP amplification products are detectable using fluorescent dyes, agarose gel electrophoresis and turbidity measurement. RT-LAMP has been used for the detection of human, animal, and plant pathogens such as virus, viroid, bacterial and fungi. The RT-LAMP kit for detection of CCCVd variants in oil palm has been developed and is ready to be used by the oil palm industry.

NOVELTY OF TECHNOLOGY

The RT-LAMP detection is a qualitative *in vitro* test which manipulate colour and turbidity changes using LoopAmp Realtime Turbidimeter machine (Eiken, Japan). The modification and optimisation of RNA extraction method used in this study resulted in high yield, purity and consistent total RNA production both from nursery and field samples. Modified extraction method of cetyltrimethyl ammonium bromide (CTAB) with spermidine as described by Zeng and Yang (2002) promoted total RNA concentration ranging from 1500 ng μl^{-1} to 3000 ng μl^{-1} from 2 g of matured oil palm leaves with absorbance of $A_{260/280}$ and $A_{260/230}$ were more than 1.8. The extraction time was reduced from two days to one day.

The RT-LAMP kit consists of RNA extraction buffers, reaction mix, fluorescent dye, enzyme mix, positive control, specific CCCVd primers and MiliQ water. The RT-LAMP reaction is incubated at 63°C within 60 minutes yet provides high amplification efficiency with DNA amplified at 10^9 - 10^{10} times and promotes high specificity with detection sensitivity up to 2 ng of RNA.

The CCCVd RT-LAMP detection uses a closed system; no gel is needed for detection of the viroid. Hence, reduce diagnostic time, avoid cross contamination between samples and evade false-positive. Since the RT-LAMP is performed at a single temperature with short incubation time, it is practical to be used for screening of large sample size, both in the laboratory and field.

DEVELOPMENT OF RT-LAMP KIT FOR DETECTION OF CCCVd VARIANTS IN OIL PALM

The principle of RT-LAMP method is based on the DNA/cDNA synthesis by the *Bst* DNA polymerase with a high strand displacement activity. It requires a set of four to six specifically designed primers that recognise a total of six different sequences on the target DNA which provides high specificity and sensitivity. Specific CCCVd primers have been designed and developed based on the $CCCVd_{246}$ sequence. The primers consist

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of forward inner primer (CCCVd-FIP), backward inner primer (CCCVd-BIP), forward outer primer (CCCVd-F3) and backward outer primer (CCCVd-B3). Validation of the detection method involved collection of oil palm leaves samples, RNA extraction and RT-LAMP detection.

Oil palm leaf samples were collected and washed under running tap water and rinsed with 10% volume per volume (v/v) sodium hypochlorite and 70% v/v ethanol before the final rinse with sterile distilled water. Samples were dipped into liquid nitrogen and stored in -80°C for further analysis. The extracted RNA was then tested for the presence of CCCVd variants by RT-LAMP. RT-LAMP amplification takes less than 60 minutes and the result is observed based on colorimetric, turbidity or ultraviolet (UV) light. Colorimetric changes can be observed through colour changes from orange to green for positive reaction. Meanwhile, turbidity measurement can be detected using a turbidimeter where the result is expressed as an amplification curve (*Figure 1*).

NURSERY AND FIELD VERIFICATION

Leaves samples collected from the nursery and field were tested with the RT-LAMP kit. Results of the nursery and field samples are presented in *Table 1*. For field evaluation, RT-LAMP has shown positive detection of CCCVd variants in 100% of oil palm leaves collected in Kuala Selangor, Selangor, 40% in oil palm samples from Teluk Intan and Seberang Perak in Perak and none in Keratong, Pahang. No detection was found in oil palm leaves seedling samples in Bangi, Selangor and Keratong, Pahang.

CONCLUSION

RT-LAMP detection provides a new simplified method for diagnostic and screening of CCCVd variants in oil palm which will benefits quarantine agencies (*e.g.* DOA and MAQIS) and oil palm industries (*e.g.* seed producers and plantations) to safeguard the oil palm industry.

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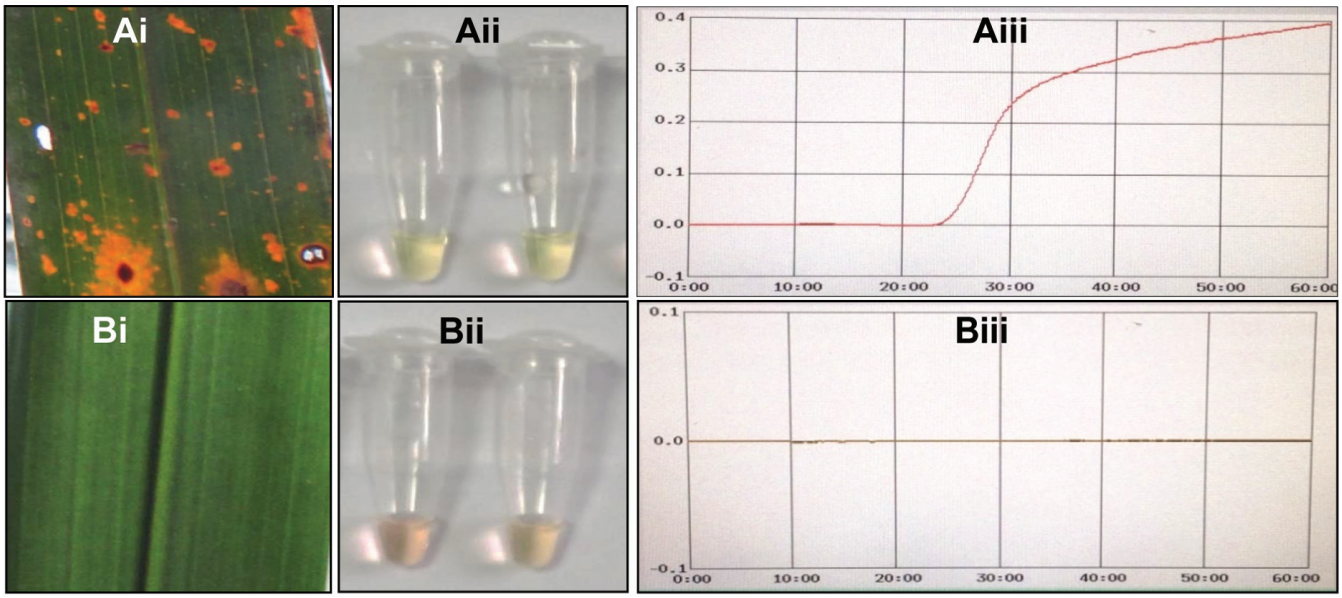

Figure 1. RT-LAMP detection showing positive detection of CCCVd variants of OS symptom-leaf (Ai) based on colour changes from orange to green (Aii) and amplification curve by turbidimeter (Aiii) and negative detection of asymptom-leaf (Bi) showing no colour changes (orange) (Bii) with no amplification curve (Biii).

TABLE 1. NURSERY AND FIELD EVALUATION USING RT-LAMP KIT FOR DETECTION OF CCCVd VARIANTS IN OIL PALM

	Sampling sites	Number of samples	Results using RT-LAMP Kit with positive detection (%)	Remarks
Field oil palms	Kuala Selangor	15	100	Palms with orange spotting
	Seberang Perak	15	40	Palms with orange spotting, leaf spot disease and nutrient deficiencies
	Teluk Intan	15	40	Palms with orange spotting, leaf spot disease and nutrient deficiencies
	Keratong	14	0	Palms with leaf spot disease and nutrient deficiencies
Oil palm seedlings in nursery	Bangi	15	0	Seedlings with leaf spot disease and nutrient deficiencies
	Keratong	14	0	Seedlings with leaf spot disease and nutrient deficiencies

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