A FLUORESCENT qPCR DETECTION OF COCONUT CADANG-CADANG VIROID (CCCVd) VARIANTS IN OIL PALM

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MPOB INFORMATION SERIES • ISSN 1511-7871 • JULY 2018 MPOB TT No. 628

Coconut cadang-cadang viroid (CCCVd) is the smallest pathogen with a size range of 246-401 nt. The viroid is encapsulated, circular and single strand RNA (ribonucleic acid) depending on the host protein for replication (Flores *et al.*, 2005). The lethal coconut cadang-cadang disease caused by CCCVd in Philippines infects the coconut palms and caused a total loss of 40 million coconut palms (Randles and Rodriguez, 2003). A finding in African oil palm (*Elaeis guineensis*) reports the occurrence of CCCVd variants associated with orange spotting (OS) disorder in one commercial oil palm plantation in 2006 (Vadamalai *et al.*, 2006). The occurrence of OS-CCCVd variants in oil palm plantation was found to be isolated cases, with distinct crown discoloration to orange colour in a particular blocks with no incidences on adjacent palms. The symptoms identified in oil palm plantation expressed varied symptoms in which some spots appeared with necrotic lesion in the middle while some orange spots were fused without any necrotic lession (Sundram *et al.*, 2017). CCCVd RNA was found to be present in low concentrations in palm tissue which in turn caused in detection (Vadamalai *et al.*, 2009). Existing molecular detections are reliable for detection of CCCVd in coconut but not in oil palm due to the low concentration (Hanold and Randles, 1991). Even though Ribonuclease Protection Assay (RPA) is sensitive and specific, it has not been widely used due to time-consuming, robust with involvement of radioactive and inconvenient

TABLE 1. SUMMARY OF DIFFERENCES AND NOVELTY OF THE INVENTION TO DETECT CCCVd RNA IN OIL PALM

Characteristic	Detection tools		
	Modified NETME Sundram *et al.*, 2017 (MPOB TT 610)	**RT-LAMP Madihah *et al.*, 2017 (MPOB TT 609)**	**Fluorescent qPCR**
Technology	RT-PCR	RT-LAMP	qPCR
Process	Require post-PCR processing	Naked eye observation	No post-PCR processing
Primers	2 primers	6 primers	2 primers and a probe with two different fluorescent dyes
Sensitivity/specificity	• Sensitive • Low specificity	• Higher specificity • Sensitivity 10 x greater than RT-PCR	• Higher sequence specificity and sensitivity • High-throughput
Measurement method	Qualitative	Qualitative	Quantitative
Limitation	Affected by polyphenol and polysaccharides	Cross contamination	Need for high template quality
Equipment	PCR machine	Specific machine (EIKEN) with specific reagent	PCR machine with fluorescent dyes detector
Days/hr to detect	*RNA extraction:* 1 ½ days *Detection:* 6 hr	*RNA extraction:* 1 day *Detection:* 1 hr	*RNA extraction:* 1 day *Detection:* 2 hr

ISSN 1511-7871
9 771511 787001

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routine analysis. Therefore, quantitative real-time PCR (qPCR) has the potential in detection compared to other available molecular techniques because of its high sensitivity in detecting the lowest range of viroid (1-10 copies) (Lenarcic *et al.*, 2013). Therefore, the current invention is a novel approach for detection of CCCVd variants RNA in oil palm using hydrolysis probe.

PROBLEM STATEMENT

The sensitivity and reliability of viroid detection method depends on the concentration and distribution of the viroid in the host plants. This is because viroid RNA are not evenly distributed and their concentration are considerably very low in oil palm. Research was focused on optimising the fluorescent detection qPCR assay using hydrolysis probe to detect the CCCVd RNA in oil palm which offers a sensitive detection. The optimised method successfully detects the CCCVd variants and also significantly reduces processing cost. The differences and novelty of the invention to detect CCCVd RNA are described in *Table 1*.

THE TECHNOLOGY

The invention describes a new hydrolysis probe and primers for the detection of CCCVd variants in oil palm and was adapted from Taqman real-time PCR principle whereby two fluorescent dyes were added at 5′ end as reporter (FAM) and 3′ end as quencher (BHQ) of the hydrolysis probe (*Figure 1*). Together with the new specific primer target, the probe will specifically bind to target and the *Taq polymerase* will extend the primer until probe

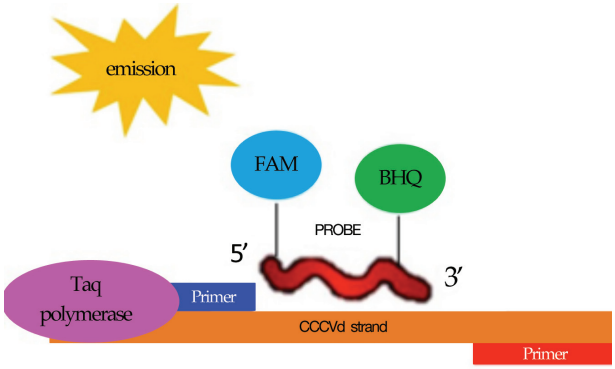

Figure 1. Principle of hydrolysis probe targeting CCCVd RNA.

cleaves the 3′ reporter and emits the fluorescent. The optimised probe, primers and PCR condition gave an effective and reliable technique in detection of CCCVd variants in oil palm and does not involve the time consuming post-PCR processing. Validation was carried out using healthy and OS leaves from field (*Figure 2*) with the range of viroid RNA copy number detected (*Table 2*). The cycle in which fluorescence can be detected is termed as quantitation cycle (*Cq*) and is the basic result of qPCR: lower *Cq* values mean higher initial copy numbers of the target. For detection purpose, *Cq* value of 35 cycles was established as the cut-off to discriminate positive from negative samples.

NOVELTY OF TECHNOLOGY

The optimised qPCR hydrolysis probe assay method provide sensitive detection of CCCVd RNA in oil palm. The assay can be carried out on

TABLE 2. THE MEAN OF QUANTITATION CYCLE (*Cq*) VALUE, DETECTION AND COPY NUMBER OF VIROID RNA

Sample	Mean Cq value (SD ±)*	Detection of CCCVd RNA	Viroid RNA copy numbers
Healthy leaves	nil	×	nil
Healthy leaves + Spiked positive control	15.14 ± 0.499	✔	5 313
Positive control (plasmid containing CCCVd sequence)	14.95 ± 0.483	✔	6 009
Orange spotting (OS) samples	26.57 ± 1.348	✔	3
Orange spotting (OS) samples	26.18 ± 1.827	✔	4

Note: * Standard deviation (SD ± are based on triplet measurement).
nil – Not detected.
× - Negative without CCCVd RNA.
✔ - Positive without CCCVd RNA.

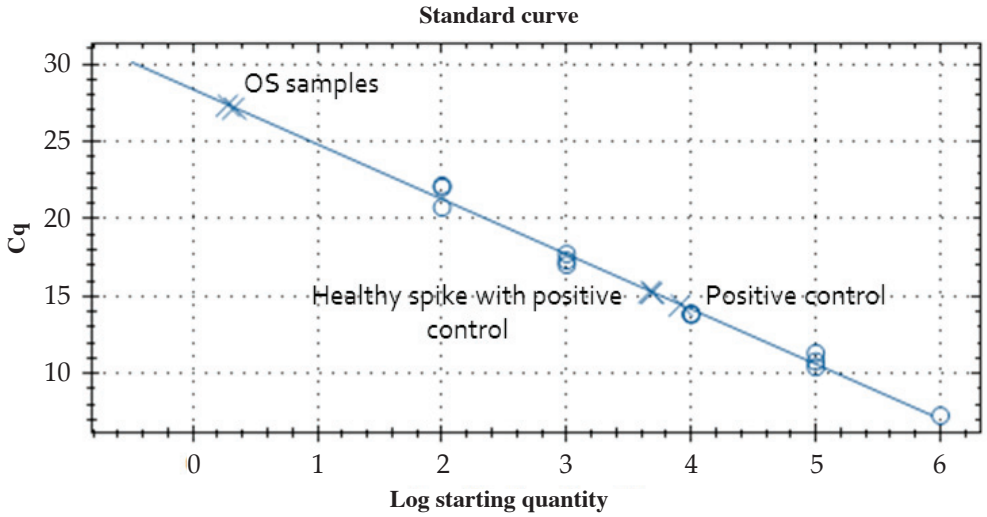

Figure 2. Standard curve obtained for CCCVd RNA quantification. The horizontal axis is logarithm of the log starting quantity while vertical axis is the quantification value (Cq). Empty circle (o) represent five dilution point and the cross (X) represent quantified samples. The OS sample (symptomatic); Healthy spiked with positive control; Positive control (plasmid containing CCCVd sequence).

oil palm seedling to avoid future introduction of CCCVd variants in field. The novel hydrolysis probe and primer would provide a sensitive and reliable detection of CCCVd variants for plantations to screen their oil palm materials and is suitable to be adopted by quarantine agencies and oil palm seedling producer with molecular laboratory facility. This approach resulted in the detection of very low copy number of viroid present in field samples in approximately 2 hr of reaction time and the presence detection was evaluated from the amplification graph. Post-PCR processing was not required thus the assay was less time consuming and more sensitive.

BENEFITS AND ADVANTAGES

The assay for the detection and quantification of CCCVd RNA has the potential to be used in disease management and control, both of which having direct effects in sanitary and certification programs of quarantines agencies. The developed assay is also environmentally friendly whereby the invention delivers a less hazardous and free from radioactive probes. This is because the conventional assay uses radioactive probes for higher sensitivity. In this invention, two types of fluorescent dyes were attached to the probe combined with two primers increases the sensitivity of detection in a single tube reaction.

MARKET AND COMMERCIAL POTENTIAL

The estimated expenditure and other economic parameters are as shown in *Table 3*.

TABLE 3. ESTIMATED EXPENDITURE AND ECONOMIC VALUES

Item	Value
Net present value @ 10% (NPV)	RM 2 078 299
Internal rate of return (IRR)	44.31%
Discounted payback period (in years)	1 yr
Discounted BC ratio	1.11

The technology can be adopted by quarantine agencies and all seedling producers with molecular laboratory facilities.

IP STATUS

This technology has been submitted for patent filling (Application No.: PI 2018701227)

IMPACT

Detection methods should be given greater consideration, because reliable results are crucial for CCCVd RNA detection and identification. This is important for disease prevention and quarantine purposes.

CONCLUSION

Detection methods should be specific, sensitive, reliable and precise but at the same time also rapid, simple, cost-effective and appropriate for routine analysis. The current qPCR technology offers faster detection with an increase in sensitivity and detection limits.

REFERENCES

Flores, R; Hernandez, C; Martinez De Alba, A E; Daros, J A and Di Serio, F (2005). Viroids and viroid-host interactions. *Annual Review Phytopathology, 43*: 117-139.

Hanold, D (1998). Diagnostic methods applicable to viroids is subtopic. In report on ACIAR-funded research on viruses on viroids and viruses of coconut palm and other tropical monocotyledons. Australian Government (Australian Centre for International Agricultural Research). The monograph can be accessed at: http://aciar.gov.au/publication/mn045.

Hanold, D and Randles, J W (1991). Detection of coconut cadang-cadang viroid-like sequences in oil and coconut palm and other monocotyledons in the South-west Pacific. *Annals Applied Biology. 118(1)*: 139-151.

Lenarcic, R; Morisset, D; Mehle N and Ravnikar, M (2013). Fast real-time detection of potato spindle tuber viroid by RT-LAMP. *Plant Pathology, 62*: 1147-56.

Madihah, A Z; Idris, A S and Maizatul, S M (2017). Reverse transcription loop-mediated isothermal amplification (RT-LAMP) detection kit for orange spotting disease caused by coconut cadang-cadang viroid (CCCVd) variants in oil palm. *MPOB Information Series No. 609.*

Randles, J W and Rodrigues, M J B (2003). Coconut cadang-cadang viroid. *Viroids* (Hadidi, A; Flores, R; Randles, J W; Semanchik, J S eds.). CSIRO Publishing, Collingwood, VIC. p. 233-241.

Sundram, S; Kamarudin, N; Roslan, N D; Ramachandran, V; Idris, A S and Kushairi, A (2017). *Orange Spotting on Oil Palm in Malaysia – A Pictorial Guide to Identify Symptoms of Orange Spotting in Oil Palms*. MPOB, Bangi, Selangor. 15 pp.

Sundram, S; Kanavedee R; Idris; A S and Nur Diyana, R (2017). Modified extraction method for detection of coconut cadang-cadang viroid (CCCVd) variants. *MPOB Information Series No. 610.*

Vadamalai, G (2005). An investigation of oil palm orange spotting disorder. Ph.D thesis, University of Adelaide, Australia.

Vadamalai, G; Hanold, D; Rezaian, M A and Randles, J W (2006). Variants of coconut cadang-cadang viroid isolated from an African oil palm (*Elaeis guineensis* Jacq.) in Malaysia. *Archive Virology, 151*: 1447-1456.

Vadamalai, G; Perera, A A F L K; Hanold, D; Rezaian, M A and Randles, J W (2009). Detection of coconut cadang-cadang viroid sequences in oil and coconut palm by ribonuclease protection assay. *Annals Applies Biology, 154*: 117-125.

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