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

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By

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A project report submitted of Faculty of Agriculture, Universiti Putra Malaysia in fulfillment of the requirement of PRT 4999 (Final Year Project) for award of the Degree of Bachelor of Agricultural Science

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CERTIFICATION FORM

This project entitled 'Detection of Coconut Cadang-Cadang Viroid variants (CCCVd) in Coconut by Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP)' was prepared by Siti Rohani Binti Mat Husain and submitted to the Faculty of Agriculture in fulfillment of the requirement of PRT 4999 for the award of the Degree of Bachelor of Agricultural Science.

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LIST OF ABBREVIATIONS

µL	microliter
mL	Milliliter
L	Liter
g	Gram
min	minute
V	Voltage
rpm	Rotation per minute
ºC	Degree Celsius
%	Percentage
RNA	Ribonucleic acid
mRNA	Messenger RNA
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
RNAse	Ribonuclease
UV	Ultraviolet light
NaCl	Sodium chloride
Tris-HCl	(hydroxymethyl)aminomethane hydrochloride
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulphate
RT buffer	Reverse transcription buffer

LiCl	Lithium chloride
NaOAc	Sodium acetate
AMV	Avian Myleloblastosis Virus
dNTP	Deoxyribonucleic triphosphate
$MgSO_4$	Magnesium sulfate
SDW	Sterile Distilled Water
CTAB	Cetyltrimethylammonium bromide
Tris	(hydroxymethyl)aminomethane
PCA	Phenol: Cholofoam: IsoAmyl
CA	Chlorofoam: IsoAmyl
PVP	Polyvinylpolypyrrolidone

ABSTRACT

Coconut cadang-cadang disease is a major destructive disease in coconut palms in the Philippines caused by *Coconut cadang-cadang viroid* (CCCVd). CCCVd variants have been characterized in Malaysia's oil palms associated with orange spotting disease. Recently, CCCVd variant was also reported in Malaysian coconut. Detection and characterization of the variants was reported to be difficult due low concentration in infected hosts. Molecular techniques like polyacrylamide gel electrophoresis (PAGE), hybridization assay and conventional RT-PCR have been widely used for detection of CCCVd in coconut but they were not reliable due to their low concentrations. RT-LAMP is a novel method which depends on auto-cycling strand displacement DNA synthesis performed by a *Bst* DNA polymerase and under isothermal conditions (60-65ºC). The assay is high specificity with use of four target specific primers which recognizes six distinct regions of the sequence. This study was conducted to detect CCCVd variants in coconut using the RT-LAMP and validated by RT-PCR using CCCVd specific and LAMP primers. Total nucleic acid was extracted using N-STEP extraction from nine coconut samples that showed CCCVd infection-like symptoms. The nucleic acid was amplified by RT-LAMP with CCCVd specific primers. The amplicons analysed by 2% agarose gel electrophoresis. The results of this study show that CCCVd variants were detected in coconut samples using RT-LAMP assay. Two (AL2 and AL3) out of nine coconut samples were positive for the presence of CCCVd by RT-LAMP assay. The optimized condition for RT-LAMP assay for coconut samples were at 65°C

for 60 min. The N-STEP extraction procedure used in this study may not be suitable for RT-PCR analysis as no amplification was observed in RT-PCR using CCCVd specific and LAMP primers.

ABSTRAK

Penyakit cadang-cadang kelapa merupakan penyakit utama yang merosakkan tanaman kelapa di Filipina disebabkan oleh *Coconut cadang-cadang viroid* (CCCVd). Varian CCCVd telah dikenalpasti pada pokok kelapa sawit di Malaysia yang mempunyai kaitan dengan penyakit bintik oren. Kini, varian CCCVd juga telah dilaporkan dalam spesies pokok kelapa di Malaysia. Pengesanan dan pencirian varian dilaporkan menjadi sukar disebabkan kepekatan yang rendah pada pokok yang dijangkiti. Teknik molekul seperti polyacrylamide gel elektroforesis (PAGE), asai penghibridan dan RT-PCR konvensional telah digunakan secara meluas untuk mengesan CCCVd dalam kelapa tetapi mereka tidak boleh dipercayai disebabkan oleh kepekatan yang rendah. RT-LAMP adalah satu kaedah baru yang bergantung kepada auto-kitaran sintesis untai DNA yang dilakukan oleh polimerase DNA *Bst* dan di jalankan di bawah keadaan isoterma (60-65ºC). Asai ini mempunyai pengkhususan yang tinggi dengan penggunaan empat primer khusus yang menyasarkan kepada enam kawasan berbeza daripada urutan itu. Kajian ini dijalankan untuk mengesan variasi CCCVd dalam kelapa menggunakan RT-LAMP dan disahkan oleh RT-PCR menggunakan primer CCCVd tertentu dan primer LAMP. Jumlah asid nukleik telah diekstrak menggunakan pengekstrakan N-STEP daripada sampel kelapa yang mempunyai gejala-gejala CCCVd. Asid nukleik dikuatkan lagi dengan menggunakan RT-LAMP dengan primer CCCVd tertentu. Amplikon akan dianalisis melalui 2% agarose gel elektroforesis. Keputusan kajian ini menunjukkan bahawa CCCVd varian dikesan dalam sampel kelapa menggunakan asai RT-LAMP. Dua (AL2 dan AL3) daripada sembilan sampel kelapa

adalah positif kehadiran CCCVd oleh asai RT-LAMP. Keadaan yang optimum untuk asai RT-LAMP untuk sampel kelapa adalah 65ºC selama 60 min. Prosedur pengekstrakan N-STEP yang digunakan dalam kajian ini mungkin tidak sesuai untuk analisis oleh RT-PCR kerana amplifikasi tidak dapat dilihat dalam RT-PCR menggunakan primer CCCVd tertentu dan primer LAMP.

CHAPTER 1

INTRODUCTION

Coconut palm (*Cocos nucifera* L.) is an important plantation crop in Malaysia. In the past, coconut oil was the second highest production of oil after soybean oil in the world. Recently, palm oil became the top oil production followed with soybean oil, palm kernel oil, coconut oil and lastly rapeseed oil (Sivaprasagam, 2008). The coconut is under threat due to factors such as conversion of farmers to other oil crops (especially oil palm), urbanization and vagaries of the market particularly the volatility of its various products in global trade (Sivaprasagam, 2008). Besides that, coconut industry also declined due to pest and disease. Some examples of coconut disease are coconut cadang-cadang disease, bud rot and nutfall, ganoderma butt rot and coconut foliar decay virus (PlantVillage.com).

Coconut cadang-cadang disease is one the most economically important disease of coconut. It was first reported in the San Miguel Island (Philippines) and since then around 40 million palms have died due to this disease (Zelazny *et al*., 1982). The name of 'cadang-cadang' disease came from the local dialect called gadan-gadan means dead or dying-dying (Hanold and Randles, 1991a). The disease is caused by *Coconut cadang-cadang viroid* (CCCVd).

Coconut cadang-cadang viroid (CCCVd) is the smallest viroid from the family Pospiviroidae. Their basic form is 246 nucleotide. In addition, CCCVd has a number of molecule forms depending in stages of the disease (Haseloff *et al*., 1982; Keese *et al*., 1988). CCCVd variants had been identified in oil palm in Malaysia. According to the Vadamalai *et al.*, 2006, the CCCVd in oil palm was the first sequences reported for variants of *Coconut cadang-cadang viroid* in a species other than coconut palm, and this was the first evidence that variants closely related to CCCVd occur outside the Philippines. Recently, CCCVd variants have also been reported in coconut palms in Malaysia (Reza *et al.*, 2013). Coconut palms showing severe yellow spotting symptom were collected and tested through RT-PCR using CCCVd specific and LAMP primers. However, detection using RT-PCR was not consistent due to the low concentration of the viroid (Reza *et al.*, 2013).

Molecular techniques like polyacrylamide gel electrophoresis (PAGE), hybridization assay and conventional RT-PCR have been widely used for detection of CCCVd in coconut but they were not reliable for the oil palm CCCVd variants due to their low concentrations (Hanold and Randles, 1991; Vadamalai, 2005; Vadamalai *et al.*, 2006; Reza *et al.*, 2013). Recently, Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) assay was reported to be sensitive to detect CCCVd variants in oil palm (Thanarajoo *et al.*, 2014).

RT-LAMP is a novel method which depends on auto-cycling strand displacement DNA synthesis performed by a *Bst* DNA polymerase; under isothermal

conditions (60-65 ºC). The assay is of high specificity with the use of four target specific primers (inner and outer primers) which recognizes six distinct regions of the sequence (Notomi *et al.*, 2000). This assay has been rapid and sensitive in detecting *Potato spindle tuber viroid* and *Peach latent mosaic viroid* (Boubourakas *et al.*, 2009; Tsutsumi *et al.*, 2010).

In this study, RT-LAMP will be used for detection of CCCVd from coconut leaf samples and validated by RT-PCR with CCCVd specific and LAMP primers.

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