IDENTIFICATION, MOLECULAR CHARACTERIZATION AND SEED TRANSMISSION OF PHYTOPLASMA IN VEGETABLE AND FRUIT CROPS IN MALAYSIA

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements of Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman : Associated Professor Ganesan a/l Vadamalai, PhD
Faculty : Agriculture

Phytoplasmas are prokaryotes belong to class Mollicutes that caues diseases in more than 700 plant species including vegetables, fruits, ornamentals, cereals and forest plants worldwide. In Malaysia, phytoplasma diseases have been reported in periwinkle, coconut and ornamental palms but not in vegetable and fruit crops due lack of knowledge and research on the presence and transmission of phytoplasma in these crops. In this study, phytoplasmas were characterised and classified based on the 16SrRNA gene sequence and the seed transmission of this pathogen was tested. Samples exhibiting phytoplasma disease symptoms (34) were collected from farms in Selangor, Malaysia including pumpkin (*Cucurbita moschata*), bitter gourd (*Momordica charantia),* papaya (*Carica papaya*), key lime (*Citrus aurantiifolia*), chili (*Capsicum annuum*) and cucumber (*Cucumis sativus*). Non-symptomatic samples (20) were also collected. DNA extraction was done using CTAB method and Nested PCR was performed using universal primers, P1/ P7 and R16F2n/ R16R2 to amplify 16SrRNA gene. The amplicons generated from primer pairs R16F2n / R16 R2, were cloned and sequenced in both directions. The nucleotide sequences were assembled, edited and compared with available sequences in GenBank using blast alignment. Phylogenetic analysis by MEGA7 software using neighbour joining method done in 1,000 replicates. Virtual RFLP analysis of the F2nR2 fragments of the 16SrRNA gene was accomplished with 17 restriction enzymes; *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI(*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I, using *i*PhyClassifier software. Transmission electron microscopy on phytoplasma infected chilli leaf petiole samples were conducted to observe the presence of elliptical bodies of the phytoplasma. Seeds were collected from phytoplasma infected chili plants and were allowed to germinate under aseptic condition to test for seed transmission of phytoplasma in chili. The presence of Phytoplasma DNA in all the symptomatic samples of pumpkin, bitter gourd, chili, key lime, cucumber and papaya was confirmed by nested PCR of 16SrRNA gene. Sequence analysis indicated that the phytoplasma association with vine malformation of pumpkin (MN585898, MT742801), Chili little leaf phytoplasma

(MT192345, MT780128), bitter gourd little leaf phytoplasma (MT422719) and papaya phytoplasma (MT764331) belong to *Candidatus* phytoplasma asteris (16SrI), while cucumber phytoplasma (MT764158) and key lime phytoplasma (MT764157) belong to *Candidatus* phytoplasma trifoli (16SrVI) and *Candidatus* phytoplasma aurantifolia (16SrII) respectively. Virtual RFLP analysis of the 16SrRNA gene fragment profiles revealed that pumpkin phytoplasma and chili little leaf phytoplasma belong to the new subgroup of 16SrI. It has been reported to infect phytoplasma in several plant species in Malaysia but this is the first report of pumpkin, chilli, bitter gourd, cucumber, key lime and papaya infected with *Candidatus* phytoplasma related strain in Malaysia. In addition, transmission electron microscopy showed the presence elliptical bodies in the sieve elements of phytoplasma infected chilli plant samples. Seed transmission of the phyoplasma in infected chilli plants was established. Phytoplasma DNA was detected by Nested PCR assay in 15 days old germinated chilli seedlings. Infected seedling percentage reached up to 95%. This is the first record of phytoplasma transmission in chilli through seeds.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGENALPASTIAN, PENCIRIAN MOLEKUL DAN PENULARAN PHYTOPLASMA MELALUI BENIH DALAM TANAMAN SAYUR- SAYURAN DAN BUAH-BUAHAN DI MALAYSIA

Oleh

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Phytoplasma adalah organisma prokaryot dalam kelas Mollicutes yang menyebabkan penyakit pada lebih 700 spesies tumbuhan termasuk sayur-sayuran, buah-buahan, tanaman hiasan, bijirin dan tumbuhan hutan di seluruh dunia. Di Malaysia, penyakit phytoplasma telah dilaporkan pada periwinkle, kelapa dan palma hiasan tetapi tidak pada tanaman sayur dan buah disebakan kekurangan pengetahuan dan penyelidikan mengenai kehadiran dan penularan phytoplasma dalam tanaman ini. Dalam kajian ini, phytoplama telah dicirikan dan dikelaskan berdasarkan urutan gen 16SrRNA dan transmisi patogen ini melalui biji benih juga telah diuji. Sampel yang menunjukkan simptom penyakit phytoplasma (34) dikutip dari ladang di Selangor, Malaysia termasuk labu (*Cucurbita moschata*), peria (*Momordica charantia*), betik (*Carica papaya*), limau nipis (*Citrus aurantiifolia*), cili (*Capsicum annuum*) dan timun (*Cucumis sativus*). Sampel bukan simptomatik (20) juga dikutip. Pengekstrakan DNA dilakukan menggunakan kaedah CTAB dan Nested PCR dilakukan menggunakan primer sejagat, P1/P7 dan R16F2n/R16R2 untuk mengamplifikasi gen 16SrRNA. Amplikon yang dijana daripada pasangan primer R16F2n/R16 R2 telah diklon dan jujukan nukleotida dibuat dalam kedua-dua arah. Urutan nukleotida digabungkan, disunting dan dibandingkan dengan jujukan yang ada di GenBank menggunakan penjajaran Blast. Analisis filogenetik dengan perisian MEGA7 menggunakan kaedah *'neighbour joinning'* dilakukan dalam 1,000 replikasi. Analisis RFLP maya terhadap serpihan F2nR2 gen 16SrRNA dicapai dengan 17 enzim sekatan; *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI(*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I dan *Taq*I menggunakan perisian iPhyClassifier. Mikroskopi transmisi elektron pada sampel petiole daun cili yang dijangkiti phytoplasma dilakukan untuk memerhatikan kehadiran badan eliptikal phytoplasma. Benih dikumpulkan dari tanaman cili yang dijangkiti phytoplasma dan dibiarkan bercambah dalam keadaan aseptik untuk menguji penyebaran phytoplasma melalui biji benih. Kehadiran DNA Phytoplasma dalam semua sampel simptomatik labu, peria, cili, limau nipis, timun dan betik telah disahkan melalui kaedah Nested PCR gen 16SrRNA. Analisis urutan menunjukkan bahawa phytoplasma yang berhubungkait

dengan malformasi labu (MN585898, MT742801), phytoplasma daun kecil cili (MT192345, MT780128), phytoplasma daun kecil peria (MT422719) dan phytoplasma betik (MT764331) tergolong dalam tergolong dalam *Candidatus* phytoplasma Asteris (16SrI), sementara phytoplasma timun (MT764158) dan phytoplasma limau nipis (MT764157) masing-masing tergolong dalam *Candidatus* phytoplasma trifoli (16SrVI) dan *Candidatus* phytoplasma aurantifolia (16SrII). Analisis RFLP maya dari profil fragmen gen 16SrRNA menunjukkan bahawa phytoplasma labu dan phytoplasma daun kecil cili tergolong dalam subkumpulan baru 16SrI. Ia telah dilaporkan menjangkiti phytoplasma pada beberapa spesies tumbuhan di Malaysia tetapi ini adalah laporan pertama jangkitan strain berhubungkait dengan *Candidatus* phytoplasma dalam labu, cili, peria, timun, limau nipis dan betik di Malaysia. Selain itu, mikroskopi elektron transmisi menunjukkan adanya badan eliptikal pada elemen penyaring dari sampel tanaman cili yang dijangkiti phytoplasma. Penyebaran phytoplasma melalui biji benih dalam tanaman cili yang dijangkiti telah dilakukan. DNA phytoplasma dikesan dengan ujian Nested PCR pada anak benih cili berumur 15 hari. Peratusan anak benih yang dijangkiti mencapai 95%. Ini adalah catatan pertama penularan phytoplasma dalam cili melalui biji benih.

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

Bp	basepair
CTAB	Cetyltrimethyl-ammonium bromide
EDAT	Ethylenediaminetetraacetic acid disodium salt
g	Gram
h	Hour
Kb	kilobase
Min	Minute
MLO	Mycoplasmalike organism
µl	Microliter
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
rDNA	Ribosomal DNA gene
rRNA	Ribosomal RNA
Sec	seconds
UV	Ultraviolet
V	Voltage
X-Gal	5-bromo-4-chloro-3- indolyl β-D- galactopyranoside

CHAPTER 1

INTRODUCTION

Vegetables and fruits are rich in nutrients, vitamins, dietary fibres and phytochemicals. They are grown in different seasons of the year which fetch high economic returns. Biotic and abiotic stresses causes significant yield losses in vegetable and fruit production worldwide. The presence of phytoplasmas and their association with vegetable and fruit crops is an emerging threat for production, which can lead to severe yield losses (Kumari et al., 2019).

Phytoplasmas are phloem-limited cell wall-less pathogens, associated with numerous plant diseases causing symptoms like virescence, phyllody, stunting, witches' broom, yellowing, stunting, die back and leaf and flower malformations. These symptoms depend on the host, environmental factors and the phytoplasma strain infecting the host (Lee et, al. 2000; Dickinson & Hodgetts, 2013).

Phytoplasma can be visualized by microscope, but is not ideal as a routine and rapid diagnostic method. Few attempts have been made to identify the pathogen using antibody–based detection systems but it would be specific for a particular phytoplasma rather than generic. Nucleic acid based detection and diagnostic systems are widely used for the detection of pathogens including phytoplasma since it is rapid and can be used as a generic tool (Dickinson & Hodgetts, 2013). The most common and simplest diagnostic method for the detection of phytoplasma is PCR using specific primers to amplify various regions of rRNA operon. Diagnostic, phylogenetic analysis and classification of phytoplasmas are mostly based on the 16SrRNA gene due to the availability of universal primers to detect this region (Lee et, al. 2000).

Phytoplasmas are mainly transmitted by insect vectors, dodder plants and infected planting materials. But recent studies have been identified that the pathogen can be transmitted by seeds too (Calari et al., 2011;Satta et al., 2020). Vascular colonization pattern indicated that the phytoplasma can move photosynthate flows in the phloem sieve elements. It flows from source to sinks such as expanding roots, shoots and shoot apex. (Kuske and Kirkpatrick, 1992). The phytoplasma affects the seed production, reduce the vigour and finally cause death (Pilkington et al., 2003).

In Malaysia, phytoplasma have been reported in periwinkle, coconut and ornamental palms but not in vegetable and fruit crops (Nejat et al., 2009; Naghmeh et al., 2010; Naderali et al., 2015). Although the phytoplasma associated symptoms were observed in vegetable and fruit crops in Malaysia but there is lack of knowledge and research on the presence and trasnmission of phytoplasma in these crops. In view of this, the present study was undertaken with the below objectives:

1.1 Objectives

1. To isolate and detect phytoplasma DNA from vegetable and fruit crops by nested PCR using universal phytoplasma primers.
2. Classification of phytoplasma associated with diseases in vegetable and fruit crops based on analysis of 16SrRNA gene operon sequences and *in silico* restriction enzyme digestion and virtual gel plotting will be done for 16SrRNA sequence.
3. To detect phytoplasma transmission through seeds in chili.

1.2 The Hypothesis

1. Little leaf, stunting, yellowing and phyllody, vine and floral malformations, die back symptoms are associated with phytoplasma diseases in vegetable and fruit crops.
2. Based on the symptoms and the host plant they can be in different groups and sub groups.
3. Phytoplasma DNA can be transmitted from mother plant to seeds.

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