



UNIVERSITI PUTRA MALAYSIA

***DETECTION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF
NANO SILVER AGAINST COCONUT YELLOW DECLINE PHYTOPLASMAS
IN COCONUT AND ORNAMENTAL PALMS IN MALAYSIA***

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ITA 2015 7



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PALMS IN MALAYSIA**

**By
NEDA NADERALI**

**Thesis Submitted to the School of Graduated Studies, Universiti Putra Malaysia
In Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

August 2015

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Associate Professor Ganesan Vadamalai, PhD
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Phytoplasmas are prokaryotes within the class Mollicutes and have been associated with over 700 diseases in different plant species including agricultural crops and ornamentals universally. Detection and characterization of phytoplasmas are very difficult because of the inability to culture them in vitro. They inhabit the phloem tissue of plants at a very low concentration especially in woody monocotyledonous plant hosts. Symptoms such as yellowing of fronds particularly older leaves which eventually turned brown, the crown inflorescence yellowing, stunting and decline in growth. Popular evergreen ornamental palms which are infected by phytoplasma not only lose their green and vivid appearance but they harbour this pathogen as a source for next infection. Yellowing symptoms similar to coconut yellow decline phytoplasma disease have been observed on ornamental palms in Selangor, Malaysia. Extracted DNA was amplified from 15 symptomatic lipstick palms (*Cyrtostachys renda*), 15 foxtail palms (*Wodyetia bifurcate*), 15 royal palms (*Roystonea regia*) and 13 Red Coconut (MRD) palms (*Cocos nucifera*) by PCR using phytoplasma universal primer pair P1/P7 followed by R16F2n/ R16R2. Phytoplasma presence was confirmed and a 1250 bp product of 34 positive samples (12 of 15 symptomatic foxtail palms, 10 of 15 symptomatic lipstick palms and 12 of 15 symptomatic royal palms) was cloned and sequenced. R16F2n/ R16R2 is a specific primer for phytoplasma detection and a PCR product of approximately 1250 bp should be amplified using this primer. Sequence analysis indicated that the phytoplasmas associated with yellow disease on ornamental palms were belonging to 16SrI '*Candidatus* Phytoplasma asteris' and 16SrXIV '*Candidatus* Phytoplasma cynodontis' groups with accession numbers: KC924727 & KC924728 for lipstick phytoplasma, KF803561 for royal phytoplasma, KC751560 & KC751561 for foxtail phytoplasma). Virtual RFLP analysis of the resulting profiles revealed that these palm-infecting phytoplasmas belonged to a subgroup most similar to 16SrI-B subgroup and a possibly new 16SrI-subgroup. In addition, because of low concentration of phytoplasma in coconut palm a real-time PCR assay with 16SCYD primer (specific primer for 16SrXIV group) was applied for sensitive detection of yellow decline phytoplasma in Malayan red coconut palms (MRD). Periwinkle phytoplasma was used as the reference gene for real-time PCR. The results revealed that this method is useful for sensitive detection of low titer phytoplasma in woody

plants such as MRD coconut palms in comparison with nested-PCR. An antimicrobial activity of nano silver was tested against phytoplasma in yellow coconut palms and resulted in a significant reduction of phytoplasma concentration within five months of nano silver injection. Nano silver antimicrobial activity analysis with real-time PCR yielded a significant fluorescence signal ($P < 0.05$). The results of SQ values were consistent with Ct values and concentration of CYD phytoplasma was decreased after applying nano silver (from 11.00 to 30.00 ng/ μ l). The values showed that, after applying nano silver, the concentration of the pathogen was decreased. This reduction was significant.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk mendapat Ijazah Doktor Falsafah

**PENGESANAN, PENCIRIAN DAN AKTIVITI MIKROB NANO SILVER
TERHADAP COCONUT YELLOW DECLINE PHYTOPLASMA
DALAM KELAPA DAN POKOK KELAPA HIASAN
DI MALAYSIA**

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Phytoplasmas adalah prokariot dalam kelas Mollicutes dan telah dikaitkan dengan lebih 700 penyakit dalam spesis tumbuhan yang berbeza termasuk tanaman pertanian dan hiasan secara universal. Pengesanan dan pencirian phytoplasmas sangat sukar kerana tidak boleh di kultur dalam vitro. Phytoplasma mendiami tisu floem tumbuhan pada kepekatan yang sangat rendah terutama di perumah tumbuhan monokot berkayu. Simptom seperti kekuningan daun terutamanya daun tua yang akhirnya bertukar coklat, crown yang menguning dan berkembang, terbantut dan penurunan dalam pertumbuhan. Pokok kelapa hiasan malar hijau popular yang dijangkiti phytoplasma bukan sahaja kehilangan penampilan hijau yang jelas tetapi mereka menyimpan patogen ini sebagai sumber untuk jangkitan seterusnya. Simptom kuning serupa dengan kelapa kuning penyakit kemerosotan phytoplasma telah diperhatikan pada pokok kelapa hiasan di Selangor, Malaysia. DNA yang telah diekstrak daripada 15 pokok kelapa lipstick (*Cyrtostachys renda*), 15 pokok kelapa foxtail (*Wodyetia bifurcate*), 15 pokok kelapa royal (*Roystonea regia*) dan 13 pokok kelapa (*Cocos nucifera*) Red Coconut (MRD) telah diamplifikasi oleh PCR menggunakan phytoplasma pasangan primer universal P1/P7 diikuti oleh R16F2n/R16R2. Kehadiran Phytoplasma telah disahkan dan produk 1250 bp daripada 34 sampel positif (12 daripada 15 pokok kelapa foxtail dengan simptom, 10 daripada 15 pokok kelapa lipstick dengan simptom dan 12 daripada 15 pokok kelapa royal dengan simptom) telah diklon dan disusun. R16F2n/R16R2 adalah spesifik primer untuk mengesan phytoplasma dan produk PCR kira-kira 1250 bp harus diamplifikasi menggunakan primer spesifik ini. Analisis jujukan menunjukkan bahawa phytoplasmas dikaitkan dengan penyakit kuning pada pokok kelapa hiasan adalah dimiliki oleh 16SrI '*Candidatus* Phytoplasma Asteris' dan kumpulan 16SrXIV '*Candidatus* Phytoplasma cynodontis' dengan nombor kesertaan: KC924727 & KC924728 untuk lipstick phytoplasma, KF803561 untuk royal phytoplasma, KC751560 & KC751561 untuk foxtail phytoplasma). Analisis RFLP maya profil mendedahkan bahawa phytoplasmas yang menjangkiti pokok kelapa tersebut dipunyai oleh kumpulan kecil yang paling serupa dengan 16SrI-B kumpulan kecil dan mungkin 16SrI kumpulan kecil baru. Di samping itu, kerana kepekatan phytoplasma yang rendah di dalam pokok kelapa, 'real-time' PCR dengan 16SCYD primer (primer khusus untuk kumpulan 16SrXIV) telah digunakan untuk pengesanan

sensitif simptom kuning penurunan phytoplasma di pokok kelapa merah Malaya (MRD). Phytoplasma periwinkle telah digunakan sebagai gen rujukan untuk 'real-time' PCR. Hasil kajian menunjukkan bahawa kaedah ini amat berguna untuk pengesanan sensitif phytoplasma titer rendah dalam tumbuhan berkayu seperti pokok kelapa MRD bandingan dengan 'nested'-PCR. Aktiviti antimikrob nanosilver telah diuji terhadap phytoplasma di pokok kelapa kuning dan mengakibatkan pengurangan ketara kepekatan phytoplasma dalam tempoh lima bulan suntikan nano silver. Nano silver analisis aktiviti antimikrob dengan 'real-time' PCR menghasilkan isyarat pendarfluor ketara ($P < 0.05$). Keputusan nilai SQ adalah selaras dengan nilai-nilai Ct dan kepekatan CYD phytoplasma telah menurun selepas menggunakan nano silver (dari 11.00-30.00 ng/ul). Nilai ini menunjukkan bahawa, selepas menggunakan nano silver, kepekatan patogen telah berkurangan. Pengurangan ini adalah penting.



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I certify that a Thesis Examination Committee has met on 4 August 2015 to conduct the final examination of Neda Naderali on her thesis entitled "Detection, Characterization and Antimicrobial Activity of Nano Silver against Coconut Yellow Decline Phytoplasmas in Coconut and Ornamental Palms in Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

| | |
|-------|---|
| Bp | basepair |
| CTAB | Cetyltrimethyl-ammonium bromide |
| CYD | C oconut yellow decline |
| dNTP | Deoxyribonucleotides (Datp, DCTP, DGTP, DTPP) |
| EDTA | Ethylene diaminetetraacetic acid, disodium salt |
| gr | Gram |
| h | Hour |
| Kb | kilobase |
| Min | Minute |
| MLO | mycoplasmalike organism |
| MRD | Malayan red dwarf |
| MT | Malayan tall |
| MYD | Malayan yellow dwarf |
| μl | Microliter |
| PCR | polymerase chain reaction |
| PVP | Polyvinylpyrrolidone |
| RFLP | Restriction fragment length polymorphism |
| rDNA | Ribosomal DNA gene |
| rRNA | Ribosomal RNA |
| Sec | Second |
| UV | Ultraviolet |
| V | Voltage |
| W/V | Weight/volume |
| X-Gal | 5-bromo-4-chloro-3-indolyl β-D- galactopyranoside |

CHAPTER I

INTRODUCTION

Phytoplasmas are prokaryotes from the class *Mollicutes*, which have branched off from gram-positive bacteria (Hogenhout *et al.*, 2008). Phytoplasmas have been associated with many diseases in different plant species containing agricultural crops and ornamentals universally (Lee and Davis, 2000). In Malaysia, phytoplasma have been reported to cause coconut yellow decline disease (CYD) in coconut palms. Symptoms of CYD include yellowing of fronds particularly older leaves which eventually turned brown, gradual collapse of older fronds, the crown inflorescence yellowing, stunting and decline in growth. CYD is important disease of coconut in Malaysia. Destruction of a population of susceptible coconut palms is important over the world therefore economic loss is very important and valuable. In Malaysia there were no reports of phytoplasma host range except coconut palm and periwinkle. Similar symptoms of CYD have been observed on ornamental palms in Malaysia. Popular evergreen ornamental palms which are infected by phytoplasma not only lose their green and vivid appearance as decorative and landscape used trees, but they also can harbor this pathogen as a source for next infection.

Therefore, detecting and characterization of phytoplasma associated with yellow disease on ornamental palms is important to determine the host range and its role as an alternate host for the CYD phytoplasma. Phytoplasmas detection and characterization is a troublesome procedure because of the inability to culture them *in vitro*. They reside in the phloem tissue of host plants at a very low concentration especially in woody plant hosts (Weintraub and Beanland, 2006). Conventional polymerase chain reaction (PCR), sequence analysis of PCR-amplified products, restriction fragment length polymorphism (RFLP) and virtual RFLP analysis of 16S rDNA sequences are useful methods for detection, identification and classification of groups and subgroups of phytoplasmas (Zhao *et al.*, 2009; Nejat and Vadamalai, 2013). At present, control of phytoplasma is carried out by applying compounds and antibiotics in several ways (spraying, root absorption and scion dipping), and at different concentrations, number and timing of treatments. Four antibiotics that have been tested (tetracycline, oxytetracycline, streptomycin, erythromycin A) were all capable of delaying the symptom appearance and phytoplasma multiplication although not active in blocking phytoplasma infection (Mcmanus *et al.*, 2002).

However, the lack of finding of a totally active antibiotic together with the difficulty of culturing these pathogens outside their host still remains a major concern to formulate a disease management strategy of phytoplasma specially in plants. Recently, Nanoparticles have been applied as a special group of materials with unique features and extensive in diverse fields (Matei *et al.*, 2008). Also, there is no report of applying silver nanoparticles against phytoplasmas which could be an alternative control method against plant pathogenic phytoplasmas. The yellowing symptoms on fronds and inflorescence have been reduced by the application of nanosilver. In addition, nanosize materials are used in small amounts in compare to other antimicrobial agents which could be economically beneficial.

OBJECTIVES

1. To detect phytoplasma from coconut and ornamental palms showing yellowing symptoms by standard and nested PCR.
2. To classify and identify of the phytoplasmas associated with disease of ornamental palm in Malaysia based on analysis of 16S rRNA sequence and RFLP.
3. To apply real-time PCR assay for sensitive detection of yellow decline phytoplasma in red coconut palms (MRD).
4. To study the nano silver antimicrobial activity on phytoplasmas associated with disease of coconut palm in Malaysia using real-time PCR.

THE HYPOTHESIS

1. Phytoplasmas are associated with yellow decline disease of ornamental and coconut palms in Malaysia
2. Silver nanoparticles are able to control plant pathogenic phytoplasmas.

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