UNIVERSITI PUTRA MALAYSIA

PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF Pantoea agglomerans CAUSING CITRUS CANKER OF CALAMANSI (Citrus microcarpa) IN SELANGOR

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FP 2017 63

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SERDANG, SELANGOR DARUL EHSAN

2016/2017

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BY

VINCENT RODERIC JR FUNG

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

Department of Plant Protection

Faculty of Agriculture

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan

2016/2017

This project report entitled "Phenotypic and Molecular Characterization of *Pantoea agglomerans* Causing Citrus Canker of Carambola (*Citrus microcarpa*) in Selangor" is prepared by Vincent Roderic JR Fung and submitted to the Faculty of Agriculture in fulfillment of the requirement of PRT 4999 (Final Year Project) for the award of the degree of Bachelor of Agricultural Science.

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ACKNOWLEDGEMENTS

Thanks to God because of His grace I managed to complete this Final Year Project which I have been struggled throughout a period of time. This report is crucial in order to obtain the degree of Bachelor of Agricultural Science.

My heartfelt appreciation to Dr. Dzarifah Mohamed Zulperi, as my supervisor. Special thanks for the time she spent to teach, for the opportunity to learn and develop starting from the beginning of this project until the end of the thesis writing.

Not to forget, a tremendous thanks to my parent and family members for their support and encouragement throughout my study, which was my biggest motivation to accomplish this project.

Last but not least, thanks to Ms. Junaina Jaafar, from the Bacteriology Laboratory for her assistance and my fellow friends who were also together struggling in order to complete the project given. I really appreciate your help in terms of information and guidance along the way.

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%	percent
°C	degree celcius
cm	centimeter
bp	base pair
DNA	deoxyribonucleic acid
DOA	Department of Agriculture
g	gram
Ha	hectar
L	liter
M	molar
m	meter
mM	millimolar
ml	milliliter
Mt	metric ton
PCR	polymerase chain reaction
RM	Ringgit Malaysia
TAE	tris-acetic EDTA

Taq	*Thermus aquaticus*
µg	microgram
µl	microliter
µg/ml	microgram per mililiter
V	volt

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ABSTRACT

Citrus microcarpa (*C. microcarpa*), commonly known as *limau kasturi* or calamansi fruit has been used widely in food preparation and antibacterial activities due to their strong bioactive components. It belongs to the family *Rutaceae*, and found native to Philippines and widely growing throughout South East Asia region. However, the infestations of canker disease can be induced by the *Pantoea* species (*Pantoea* spp.) as a causal agent, instead of *Xanthomonas axonopodis* pathovar *citri*. The symptoms of this disease include blister-like surrounded by yellow halos on the infected fruits and leaves that will later turn to yellow, and easily fall. This study was conducted to reach these objectives; 1) isolation and identification of *Pantoea* spp. isolated from local calamansi varieties via phenotypic characteristics and 2) molecular identification and phylogenetic analysis of *Pantoea* spp. strains isolated in this study. The samples of *C. microcarpa* with the symptoms of canker disease were obtained from Ladang 10, Universiti Putra Malaysia and were isolated to obtain pure cultures. Then, the characteristics of the causal bacterium were

described through phenotypic characterization. The pure cultures were yellowish in color, Gram-negative with rod-shaped, positive reaction for catalase test and potassium hydroxide (KOH) tests; and negative for oxidase test. The pathogenicity test on four selected strains were showed, leaves started to change in terms of color and leaf-spot also occurred; and volcano-like symptoms, non-uniform lesion occurred on the fruits and started to fall which act as the citrus canker symptoms after seven days of inoculation into healthy calamansi seedlings. Negative control, seedlings remained asymptomatic. Strain cultures were re-isolated and the morphological and biochemical characteristics showed up as mentioned above. For molecular identification, the total genomic DNA of all four strains were extracted as templates for 16S rDNA polymerase chain reaction (PCR) amplification by using primer 8F and 1492R, where they produced ~1400bp amplicon each. Sequencing analyses showed that all strains were 99% identical to *Pantoea agglomerans*, reference gene in GenBank database. Phylogenetic analyses of the 16S rDNA gene sequences clustered all strains into *Pantoea agglomerans*, reference strains with 100% posterior probability. To our knowledge, this is the first report of citrus canker disease caused by *Pantoea agglomerans* of calamansi in Malaysia.

FENOTIP DAN PENCIRIAN MOLEKUL KE ATAS *Pantoea agglomerans* YANG MENYEBABKAN KANKER SITRUS PADA CALAMANSI (*Citrus micocarpa*) DI SELANGOR

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ABSTRAK

Citrus microcarpa (*C. microcarpa*), biasanya dikenali sebagai limau kasturi atau buah calamansi telah digunakan secara meluas dalam penyediaan makanan dan aktiviti anti-bakteria kerana komponen bioaktif yang kuat. Ia tergolong dalam keluarga *Rutaceae*, dan didapati berasal dari Filipina dan berkembang secara meluas di seluruh rantau Asia Tenggara. Walau bagaimanapun, serangan penyakit kanker boleh disebabkan oleh *Pantoea* spesies (*Pantoea* spp.) sebagai agen penyebab, selain daripada *Xanthomonas axonopodis* pathovar *Citri*. Tanda-tanda penyakit ini termasuk lepuh dikelilingi oleh lingkaran kuning pada buah-buahan dan daun yang dijangkiti pada kemudiannya akan bertukar menjadi kuning, serta mudah gugur. Kajian ini dijalankan untuk mencapai objektif ini; 1) pengasingan dan pengenalpastian strain *Pantoea* spp. daripada calamansi jenis tempatan melalui ciri-ciri fenotip; dan 2) mengenal pasti molekul dan analisis filogenetik strain *Pantoea* spp. yang diasingkan dalam kajian ini. Sampel *C. microcarpa* dengan simptom penyakit kanker diperolehi dari Ladang 10, Universiti Putra Malaysia dan

telah dipilih untuk mendapatkan pencirian lanjut. Kemudian, ciri-ciri bakteria penyebab telah diklasifikasikan melalui pencirian fenotip. Di mana, koloni dari pengasingan adalah warna kekuningan, gram-negatif dengan berbentuk rod pendek, tindak balas positif bagi ujian catalase dan ujian kalium hidroksida (KOH); dan tindak balas negatif bagi ujian oxidase. Untuk ujian kepatogenan, empat strains yang dipilih dan menunjukkan simptom sitrus kanker, daun mula bertukar warna dan bintik daun juga kelihatan; dan pada buah, luka seperti gunung berapi dan luka yang tidak sekata serta keguguran buah terjadi selepas tujuh hari disuntik keatas anak benih limau kasturi yang sihat. Manakala, kawalan negatif disuntik dengan air suling. Kultur strain telah diasingkan semula dan ciri-ciri morfologi dan biokimia muncul seperti dinyatakan di atas. Untuk pengenalpastian molekul, jumlah DNA untuk empat strains diekstrak sebagai template untuk 16S rDNA tindak balas rantai polymerase (PCR) amplikasi dengan menggunakan primer 8F dan 1492R; dan mempamerkan ~1400 bp amplikon untuk setiap strain. Analisis penjujukan menunjukkan bahawa semua strain adalah 99% sama dengan *Pantoea agglomerans*, rujukan gen pada GenBank database. Analisis filogenetik dengan urutan gen 16S rDNA berkelompok semua strain kepada *Pantoea agglomerans*, rujukan strains dengan 100% kebarangkalian posterior. Untuk pengetahuan kita, ini adalah laporan pertama penyakit kanker sitrus disebabkan oleh *Pantoea agglomerans* ke atas calamansi di Malaysia.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Calamansi or Calamondin Lime (*Citrus microcarpa*), a species of citrus which are widely distributed throughout Asia and Worldwide. In Malaysia, it is known as 'Limau Kasturi' that mainly being used as herbs and in food preparation (Othman *et al.*, 2016). In addition, it is a popular fruit because of the taste and fragrance that produces by the fruit itself, which have a medicinal value that essential in the human diet.

Calamansi belongs to the family of *Rutaceae*, is 3-5 m tall abundant of long spine on the stem, branches and twigs. The leaves are 2-7 cm long and 2-3 cm thick with dark green in color (Othman *et al.*, 2016). The fruit is round or oblate in shape with 4.5 cm wide that produced aromatic scent when peeled (Morton, 1987).

This research was carried out in a mature calamansi orchard, which might be new niche for plant pathogenic bacteria that can be the causal agent of citrus canker disease.

1.2 Problem statement

Citrus bacterial canker disease is a serious matter towards the citrus cultivated area. This type of disease is a major problem in terms of production, which can reduce the market value of the fruits and increase the management costs of the disease. Once the plant was infected with the bacterial canker disease, the quality and quantity of the production also affected. These may reduce the economic value of the yield when it comes to marketing.

1.3 Significance of the study

Currently, no study has been conducted on *Pantoea* spp. causing bacterial canker disease of *C. microcarpa* in Malaysia. Therefore, this study was conducted to determine the presence of *Pantoea* spp. and its pathogenicity towards citrus mainly in *C. microcarpa* tree. Results obtained from the study will be an update documentation of bacterial canker disease of citrus in Malaysia.

1.4 Objectives of the study

This study will be conducted to reach these objectives; 1) isolation and identification of *Pantoea* spp. isolated from local calamansi varieties via phenotypic characteristics and 2) molecular identification and phylogenetic analysis of *Pantoea* spp. strains isolated in this study.

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