
**UNIVERSITI PUTRA MALAYSIA**

# *BIOCHEMICAL AND MOLECULAR CHARACERIZATION OF Pectobacterium carotovorum, THE CAUSAL AGENT OF SOFT ROT IN PENINSULAR MALAYSIA*

**EISA NAZERIAN**

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# BIOCHEMICAL AND MOLECULAR CHARACERIZATION OF *Pectobacterium carotovorum*, THE CAUSAL AGENT OF SOFT ROT IN PENINSULAR MALAYSIA

**By**

**EISA NAZERIAN**



**Thesis Submitted to the School of Graduate Studies, University Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

**April 2012**

## DEDICATION

I would like to dedicate this work to my lovely wife for her patience and understanding throughout my studies. I have no any other way to show my appreciation than to dedicate this entire work to her.

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

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**April 2012**

**Chairman: Associate Professor, Kamaruzaman Sijam, PhD**

**Faculty: Agriculture**

Surveys were conducted between the years 2008 and 2009 in the northern, central and southern regions of Malaysia. The sampling sites included vegetable farms and ornamental greenhouses. A total of 147 samples of chlorotic or necrotic leaves, stems, or fruits with light brown to yellow discoloration and extensive water soaked lesions suspected to be infected by soft rot bacteria of the genus *Pectobacterium* were obtained from 35 sites. Characteristics of 63 bacterial isolates obtained from these samples were studied based on phenotypic observations and molecular methods. All isolates obtained from diseased samples were identified as *P. carotovorum* subsp. *carotovorum* based on phenotypic and molecular features. Biochemical properties clearly showed that *P. carotovorum* subsp. *carotovorum* was the main bacterial pathogen affecting vegetables and

ornamental plants in Malaysia. The species represented 92% of the total isolates sampled.

Twenty one bacterial suspensions at the concentration of $10^6$ CFU/ml derived from the different host plants when inoculated to potato tuber slices, showed clear differences in aggressiveness. The representative isolates elicited HR in tobacco, and produced pectinase caused soft rot symptoms upon inoculation of this bacterial suspension concentration on vegetable and ornamental leaves, roots or stems via pathogenicity tests.

The results revealed that all *P. carotovorum* subsp*. carotovorum*sequences studied here had similarities between 94-100% with gene bank databases using different primers and these were in agreement with the classification based on physiological and biochemical features, and indicated that *P. carotovorum* subsp*. carotovorum* was detected from all the infected plant tissues.

Moreover based on PCR amplification of the pectate lyase-encodinggene (*pel*) and universal rice primer, 16s rRNA analysis, analysis of the intergenic transcribed spacer region (16S-23S rRNA), and ITS-restriction fragment length polymorphism (ITS-RFLP), all isolates were identified as *P. carotovorum* subsp*. carotovorum.*

In spite of the low number of isolates examined, it was shown that BOX-PCR and ERIC-PCR were suitable for characterisation of *P. carotovorum*

subsp*. carotovorum*. Some similarities and differences were indicated by the pattern of DNA fingerprint in BOX-PCR and ERIC-PCR methods during classification of isolates. In both methods, isolates were placed in three main groups. There was no perfect agreement between the ERIC-PCR and BOX-PCR methods in differentiation of isolates. Even isolates that presented similar patterns in BOX-PCR exhibited different patterns in ERIC-PCR.

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

**CHARACERIZATION BIOKIMIA DAN MOLEKUL PECTOBACTERIUM CAROTOVORUM, YANG BERSEBAB AGEN ROT SOFT DI SEMENANJUNG MALAYSIA**

Oleh

**EISA NAZERIAN**

**April 2012**

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Kaji selidik telah dijalankan di kawasan-kawasan utara, tengah dan selatan Malaysia antara tahun 2008 dan 2009. Tapak penyampelan termasuk kebun sayur-sayuran dan rumah hijau hiasan. Sejumlah 147 sampel daun 'chlorotic' atau 'necrotic', batang, atau buah-buahan yang memiliki perubahan warna dari perang muda ke kuning dan luka-luka berair yang telah disyaki bahawa dijangkiti oleh bakteria reput lembut dari genus *Pectobacterium* telah diperolehi dari 35 kawasan. Ciri-ciri 63 'strain' bakteria yang telah diperolehi dari sampel-sampel ini telah dikaji melalui pemerhatian ke atas ciri-ciri fenotipik dan kaedah-kaedah molekul. Berdasarkan ciri-ciri fenotipik dan molekul, kesemua isolat yang diperolehi dari sampel-sampel berpenyakit telah dikenalpasti sebagai *P. carotovorum*

subsp. *carotovorum*. Ciri-ciri biokimia jelas menunjukkan bahawa *P. carotovorum* subsp. *carotovorum* merupakan patogen bakteria utama yang menjejaskan sayur-sayuran dan tanaman hiasan di Malaysia. Spesies tersebut mewakili 92% dari jumlah isolat yang telah dipencilkan.

Dua puluh satu inokulat bakteria yang diperolehi dari tanaman perumah yang berlainan telah menunjukkan perbezaan yang jelas dari segi keagresifan apabila diinokulat pada kepingan ubi kentang pada kepekatan $10^6$ CFU/ml. Dalam ujian kepatogenan ke atas daun, akar atau batang sayur-sayuran dan tanaman hiasan, wakil-wakil 'strain' bakteria ini telah berupaya untuk menghasilkan gejala-gejala penyakit reput lembut apabila diinokulat pada kepekatan $10^6$ CFU/ml.

Hasil kajian telah menunjukkan bahawa semua urutan *P.carotovorum* subsp. *carotovorum* yang telah dikaji mempunyai persamaan antara 94-100% dengan pangkalan data genbank dengan penggunaan primer yang berbeza dan ia adalah bersamaan dengan klasifikasi berdasarkan ciri-ciri fisiologi dan biokimia, serta menunjukkan bahawa *P. carotovorum* subsp. *carotovorum* telah dikesan pada semua tisu tumbuhan yang dijangkiti.

Melalui amplifikasi PCR gen pengekodan pectate-lyase (*Pel*) dan primer sejagat beras, analisis 16s rDNA, analisis 'intergenic transcribed spacer region' (16-23S rRNA) dan 'ITS-restriction fragment length polymorphism'

(RFLP-ITS), kesemua isolat telah dikenalpasti sebagai *P. carotovorum* subsp. *carotovorum*.

Walaupun bilangan isolat yang dikaji adalah sedikit, hasil kajian telah menunjukkan bahawa kaedah BOX-PCR dan kaedah ERIC-PCR adalah sesuai untuk pencirian *P. carotovorum* subsp. *carotovorum*. Beberapa persamaan dan perbezaan telah diperhatikan daripada pertimbangan keputusan DNA 'fingerprint' dalam kaedah BOX-PCR dan kaedah ERIC-PCR semasa klasifikasi isolat dilakukan. Dalam kedua-dua kaedah, isolat-isolat telah ditempatkan di dalam tiga kumpulan utama. Tiada persamaan yang sempurna diperlihatkan antara kaedah ERIC-PCR dan kaedah BOX-PCR dalam proses pembezaan isolat. Malahan, isolat yang telah mempamerkan corak yang sama dalam kaedah BOX-PCR menunjukkan corak yang berbeza dalam kaedah ERIC-PCR.

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I will always carry with me a good memory of the time spent in the lab together.

This thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctora Phylosophy. The members of the Supervisory Committee were as follows:

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## DECLARATION

I declared that the thesis is my original work except for quotations and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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| | |
|---|---|
| % | Percentage |
| µl | Microliter |
| µM | Micromol |
| $^{o}$C | Degree Celisus |
| bp | Base pairs |
| ca. | Circa |
| CFU | Colony Forming Unit |
| Cm | Centimeter |
| EDTA | Ethylenediaminetetraacetic acid |
| G | Gram |
| h | hours |
| ha | hectare |
| HPLC | High performance liquid chromatography |
| Kg | Kilogram |
| L | Liter |
| Min | minutes |
| Ml | milliliter |
| M | Molar |
| mM | Millimolar |
| OD | Optical density |
| PCR | Polymerase Chain Reaction |
| Pa | *Pectobacterium atrosepticum* |
| Pcc | *P. c*. subsp. *carotovorum* |
| PCWD | Plant Cell Wall Degradation Enzymes |

| | |
|---|---|
| RAPD | Random Amplified Polymorphic DNA |
| REP-PCR | Repetitive-Element Sequence-based PCR |
| Spp | Species (plural) |
| Subsp | subspecies |
| TBE | Tris-Boric-EDTA |
| Uv | Ultraviolet |
| v | Volt |

# CHAPTER 1

# INTRODUCTION

## Introduction

The taxonomic position of soft rot Erwinias (Garrity *et al.*, 2005), was first clarified by Dye (1969) and Graham (1964, 1971). Recently soft rot Erwiniaswere re-classified to *Pectobacterium carotovorum* due to extensive taxonomic and molecular characterization (Kwon *et al.*, 1997; Hauben *et al.*, 1998; Avrova *et al.*, 2002; Gardan *et al.*, 2003; Ma *et al.*, 2007). The genus Pectobacterium includes four species: *P. carotovorum* with two subspecies (subsp. *carotovorum* and subsp. *odoriferum*) *P. atrosepticum*, *P. betavascularum* and *P. wasabiae.*

Pectobacterium is gram-negative, non spore-forming facultative anaerobes producing extracellular polysaccharides. Fimbriae or pili are present on cells in a high proportion of strains of *P. carotovorum*, but are absent from cells of *P. atrosepticum* (Christofi *et al.*, 1979). Membere of this genus are responsible for soft rot, infecting more than 35% of angiosperms and 50% of monocot orders. Major horticultural crop hosts include potato (*Solanum tuberosum*), tomato (*Lycopersicum esculentum*), cabbage (*Brassica* spp), bell pepper (*Capsicum annum*), lettuce (*Lactuca sativa*), and onion (*Allium cepa*). The symptoms of Pectobacterium causing soft rot in most plants are

similar. Disease symptoms start on leaves, stems or plant parts below the surface of the ground. Symptoms begin as small spots, or water soaked lesions, rapidly enlarging with extensive maceration of affected tissues. Several selective media such as crystal violet pectate and modified selective media have been used for detection of soft rot Pectobacterium in different crops (Bdliya, 1995). Biochemical tests have been developed for the identification and characterization of *P. carotovorum*, but these could not discriminate between all Pectobacterium causing soft rot.

The bacterium can affect plants in flowering, fruiting, post harvest, seedling and growing stages. Temperature affects Pectobacterium virulence because of the production of some proteins and phenotypes related to virulence including substances such as pectic lyase, pectin lyase, polygalactronase, and biofilms (Lanham *et al.*, 1991; Hugouvieux-Cotte-Pattat *et al.*, 1996; Smadja *et al.*, 2004; Yap *et al.*, 2005; Hasegawa *et al.*, 2005). Plant cell wall degradation enzymes (PCWDEs) which include cellulases, proteases, pectate lyases, pectin methylesterases, pectin lyases and polygalacturonases, are the major determinants of pathogenicity and virulence (Lund, 1979). Lipopolysaccarides, exopolysaccharides, motility, harpins, other effectors, sidrophores, toxins and some factors, which protect bacteria from oxidative stress, are all contributors to disease development.

More recently, PCR-based methods have been developed and used as sensitive and rapid diagnostic tools. Polymerase chain reaction (PCR)

based techniques provide a comprehensive view of the genetic variability within and among *Pectobacterium carotovorum*. They have been used to characterize many local and international collections of isolates (Bertheau *et al.*, 1998; Hadas *et al.*, 2001). However, no information is available on the distribution of species and genetic diversity in Malaysian populations of soft rot Pectobacterium.

**Problem statement**

In the present work the bacteria under study has been observed to play a very important role in the incidence of soft rot disease in Malaysia. A first step in the management of soft rot is to identify precisely the pathogens present. So far, a number of diseases have been detected in Malaysian vegetable crops and ornamental plants. However, their identification was usually based only on symptoms, and the causal organisms in many cases have not been positively identified. Since several of the aforementioned diseases can be caused by a number of plant pathogen species, which differ from one another, and may show variation in ecological requirements or pathological characteristics between taxonomically related isolates, it is of utmost importance to obtain accurate descriptions and variability of the pathogenic species actually present in Malaysian vegetables and ornamental plants.

The present study was therefore designed to identify the causal agent of soft rot in order to comprehend the etiological and disease management of

*P. carotovorum* subsp. *carotovorum* in Malaysia. The study was initiated with a survey of commercial vegetables farms and ornamental plant production areas. Suspected samples were collected, analyzed and characterized by both biochemical and molecular methods as well as plant responses. It should be noted that the soft rot disease reported for the first time by Nazerian et al. (2011) in cabbage from Malaysia, has shown variations among isolates. It is, therefore hypothesised that *Pectobacterium carotovorum* exists in farms and greenhouses in Malaysia and are genetically divergent.

The objectives of the present study therefore were to: (i) identify the species and subspecies of soft rot Pectobacterium and their distribution in Malaysian vegetable crops and ornamental plants, and (ii) describe the biochemical and molecular variability existing within the Pectobacterium isolates.

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