UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF STEMPHYLIUM SPECIES ASSOCIATED WITH DISEASE OF VEGETABLE CROPS IN MALAYSIA

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FP 2014 42

CHARACTERIZATION OF *STEMPHYLIUM* SPECIES ASSOCIATED WITH DISEASE OF VEGETABLE CROPS IN MALAYSIA

By

ABBAS NASEHI

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

June 2014

DEDICATION

This thesis is dedicated to:

All I love,

My beloved mother,

My beloved father,

Specially my beloved wife "Hajar" who have been a constant source of encouragement to continue my study and for their boundless love, understanding, patience and support throughout my study in Malaysia.

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

CHARACTERIZATION OF *STEMPHYLIUM* SPECIES ASSOCIATED WITH DISEASE OF VEGETABLE CROPS IN MALAYSIA

By

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June 2014

Chairman: Associate Professor Jugah B Kadir, PhD
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Leaf spot caused by *Stemphylium* spp. is one of the important diseases of vegetable crops worldwide. Forty-six isolates of *Stemphylium* were collected from diseased tomato (*Lycopersicon esculentum* L.), eggplant (*Solanum melongena* L.), pepper (*Capsicum annuum* L.) and lettuce (*Lactuca sativa* L.) from major vegetable production regions of Malaysia, including the states of Pahang, Johor and Selangor during 2011 - 2012. The isolates were examined based on morphological, cultural and molecular characterizations and pathogenicity. Morphological characterization revealed that the isolates were variable in color, texture, shape and pigmentation of the colonies on potato dextrose agar (PDA) and divided into 5 groups. The isolates were also variable in terms of colony size, growth rate, and size of conidia and conidiophores. Little variations were observed in the number of transvers and longitudinal septa of conidia in the isolates. The variations observed among the isolates were not correlated either to host or geographical origins of the isolates. Conidia of the isolates were similar in length, width and length/width ratio to *Stemphylium solani* G. F. Weber, but the isolates were also similar to *S. lycopersici* (Enjoji) W. Yamam. with distinct constrictions at the main transverse septa of conidia and in cultural features, particularly secretion of a yellow to dark red pigment of majority of the isolates on PDA.

Various DNA regions of the isolates, including the internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*gpd*), elongation factor 1-alpha protein (*EF-1 alpha*) and the noncoding region between the vacuolar membrane ATPase catalytic subunit A gene (*vmaA*) and a gene involved in vacuolar biogenesis (*vpsA*) regions, were compared to epitype species of the genus *Stemphylium* existing in the GenBank. No variation was observed among the isolates based on the four DNA regions examined and the isolates showed 100 % similarity to each other. Based on molecular analyses, the isolates associated with vegetable crops were confirmed to be *S. lycopersici*,

and were distinguished clearly from *S. solani* and other *Stemphylium* species available in the GenBank.

Pathogenicity tests revealed that all *S. lycopersici* isolates were pathogenic on the original hosts, namely tomato, eggplant, pepper and lettuce. However, virulence variability was observed among the isolates. This study confirmed that *S. lycopersici* isolates were the causal agent of leaf spot on the vegetable crops examined. In cross-inoculation assay, the representative *S. lycopersici* isolates selected from each crop were able to cause leaf spot on the inoculated leaves of host plants found in this study, but not on cabbage as a non-host plant. This indicated an apparent lack of host-specificity in *S. lycopersici* isolates on the four host crops.

Genetic diversity was also assessed among 46 *S. lycopersici* isolates using RAPD and ISSR markers. Both RAPD and ISSR markers used to investigate the genetic variability among the isolates from the vegetable crops were able to differentiate the isolates examined. The results between the markers were congruent, however the effective multilocus marker ratio and subsequent to that the number of private alleles was greater with ISSR markers. Analysis of the genetic variation among the isolates showed significant concordance with the host plants, and no apparent geographic effect was observed. The results of the present study will provide better insight into the biology and etiology of *S. lycopersici* and these achievements will be helpful in the development of better disease management strategies and in the breeding of resistant varieties for vegetable production in Malaysia.

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

PENCIRIAN SPESIS *STEMPHYLUM* YANG TERLIBAT DALAM PENYAKIT TANAMAN SAYUR DI MALAYSIA

Oleh

ABBAS NASEHI
Jun 2014

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Kesan bintik pada daun yang disebabkan oleh *Stemphylium* spp. merupakan salah satu penyakit yang utama dalam tanaman sayur-sayuran di seluruh dunia. Empat puluh enam isolat Stemphylium telah dikumpulkan daripada tomato berpenyakit (*Lycopersicon esculentum* L.), terung (*Solanum melongena* L.), lada (*Capsicum annuum* L.) dan salad (*Lactuca sativa* L.) dari kawasan pengeluaran sayur-sayuran utama di Malaysia termasuklah negeri Pahang, Johor dan Selangor pada 2011 - 2012. Isolat telah diperiksa berdasarkan ciri morfologi , kultur, ciri-ciri molekular dan patogenisiti. Pencirian morfologi mendedahkan bahawa isolat-isolat mempunyai kepelbagaian koloni dari segi warna, tekstur, bentuk dan pigmentasi di atas agar potato dextrose (PDA) dan telah dibahagikan kepada 5 kumpulan. Isolat juga berubah dari segi saiz koloni, kadar pertumbuhan, dan saiz konidia dan konidiofor. Sedikit variasi diperhatikan pada beberapa septa konidia menegak dan melintang dalam isolat tersebut. Perbezaan antara isolat-isolat tersebut tidak mempunyai korelasi, sama ada kepada perumah atau asal-usul geografi isolat tersebut. Konidia daripada isolat-isolat tersebut mempunyai persamaan dari segi panjang, lebar dan nisbah panjang/lebar *Stemphylium solani* GF Weber, tetapi isolat tersebut juga sama dengan *S. lycopersici* (Enjoji) W. Yamam dengan keadaan jelas lebih sempit pada septa utama melintang di konidia dan juga pada ciri-ciri kultur; terutamanya terdapat rembesan pigmen kuning sehingga warna merah gelap pada kebanyakan isolat atas PDA.

Pelbagai kawasan DNA pada isolat-isolat tersebut telah dikenalpasti termasuklah, spacer dalaman disalin (ITS), gliseraldehid-3-fosfat dehidrogenase (*gpd*), faktor pemanjangan 1 -alfa protein (*EF- 1 alfa*) dan kawasan tidak berkod antara membran vakuolar ATPase sub unit pemangkin gen A (*vmaA*) dan satu gen yang terlibat dalam kawasan vakuolar biogenesis (*vpsA*). Kesemua kawasan yang telah dikenal pasti itu telah dibandingkan dengan spesies epitype daripada genus *Stemphylium* yang sedia ada dalam

GenBank. Tiada perubahan yang diperhatikan di kalangan isolat-isolat berdasarkan empat kawasan DNA dikaji dan isolat tersebut menunjukkan 100% persamaan antara satu sama lain. Berdasarkan analisis molekular, isolat-isolat yang diperoleh dari tanaman sayur-sayuran telah disahkan merupakan *S. lycopersici*, dan telah dibezakan dengan jelas dari *S. solani* dan spesies *Stemphylium* lain yang terdapat di GenBank.

Ujian pathogenisiti telah menunjukkan bahawa semua isolat-isolat *S. lycopersici* adalah bersifat patogenik pada perumah asal, iaitu tomato, terung, lada dan salad. Walau bagaimanapun, tahap kepelbagaian yang agak tinggi dalam sifat patogenisiti telah diperhatikan antara isolat-isolat tersebut terutamanya perbezaan dari segi keagresifannya. Kajian ini telah mengesahkan bahawa isolat-isolat *S. lycopersici* merupakan ejen penyebab penyakit daun berbintik pada tanaman sayur-sayuran yang telah diperiksa. Dalam kajian silang inokulasi, isolat *S. lycopersici* yang dipilih dari setiap tanaman telah menunjukkan simptom berbintik pada daun tumbuh-tumbuhan perumah yang telah diinokulasi dalam kajian ini, tetapi tidak pada kobis sebagai tumbuhan bukan perumah. Ini menunjukkan bahawa, kekurangan kekhususan isolat *S. lycopersici* terhadap perumah di dalam empat tanaman tersebut.

Kepelbagaian genetik turut dinilai di antara 46 isolat *S. lycopersici* dengan menggunakan penanda RAPD dan ISSR. Kedua-dua penanda RAPD dan ISSR digunakan untuk mengkaji kepelbagaian genetik di kalangan isolat-isolat yang diambil daripada tanaman sayur-sayuran dan ia dapat membuat perbezaan antara isolat yang diperiksa. Keputusan antara penanda adalah kongruen, tetapi nisbah penanda berkesan dan seterusnya bilangan alel yang lebih tinggi diperolehi dengan menggunakan petanda ISSR. Analisis perubahan genetik antara isolat menunjukkan konkordans yang signifikan dengan tumbuhan perumah dan tiada kesan geografi yang jelas diperhatikan. Hasil kajian ini akan memberikan gambaran yang lebih baik ke dalam biologi dan etiologi *S. lycopersici* dan pencapaian ini akan membantu dalam pembangunan strategi pengurusan penyakit yang lebih baik dan di dalam pembiakbakaan varieti yang lebih resistan terutama bagi pengeluaran sayur-sayuran di Malaysia.

ACKNOWLEDGEMENTS

My special thanks go to my supervisor Assoc. Prof. Dr. Jugah Kadir for his guidance, patience and support throughout this study. This dissertation would not have been possible without his support and guidance. Much thanks to all of my committee members; Assoc. Prof. Dr. Zainal Abidin b Mior Ahmad and Assoc. Prof. Dr. Wong Mui Yun. They have been an excellent source of advice throughout the source of my study. My gratitude also goes to former and current lab assistants. Department of plant protection and agriculture program faculty and staff members were also very helpful. I want to express deepest gratitude to the people I have had the pleasure to work within the Department of Plant Protection, UPM. I would like to acknowledge the support and encouragement of my family. Thank you especially to my wife, Hajar, for her love, endless support and being there for me throughout it all. I just want to say thank you. Last but not least, I want to express my gratitude to my family members especially my mom, my father, my brothers and sisters, who have always been encouraging me, giving me hope for my life from beginning until now. Above all, I am grateful to God for health and the many blessings in my life.

I certify that a Thesis Examination Committee has met on 6 June 2014 to conduct the final examination of Abbas Nasehi on his thesis entitled "Characterization of *Stemphylium* Species Associated with Disease of Vegetable Crops in Malaysia" in accordance with the Universities and University College 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 degree of Doctor of Philosophy.

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LIST OF ABBREVIATIONS

CRD	Complete Randomized Design
LSD	Least Significant difference
PDA	Potato Dextrose Agar
PCA	Potato Carrot Agar
WA	Water Agar
MEA	Malt Extract Agar
PSA	Potato Sugar Agar
V-8	V-8 juice agar
SDS	Sodium Dodecyl Sulfate
ddH2O	Double Distilled Water
NUV	Near Ultraviolet
EB	Ethidium Bromide
v	Volt
EDTA	Ethylene Diamine Tetraacetic Acid
TAE	Tris Acetic Acid EDTA
TE	Tris EDTA
ng	Nanogram
pg	Picogram
fg	Festogram
rpm	Revolutions Per Minute
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
NCBI	National Center Biotechnology Information
ISSR	Inter Simple Sequence Repeat
RAPD	Random amplification of polymorphic DNA
ERIC	Enterobacterial Repetitive Intergenic Consensus
REP-PCR	Repetitive Extragenic Palindromes
ITS	Internal Transcribed Spacer
gpd	Glyceraldehyde-3-Phosphate Dehydrogenase
EF-1 alpha	Elongation Factor 1-Alpha Protein
BLASTn	Basic Local Alignment Search Tool Nucleotide

CHAPTER 1

INTRODUCTION

1.1 Importance and constraints of vegetable production

More than 10,000 vegetable species have been reported around the world (Decoteau, 2000). Vegetable crops have been always considered an important part of a healthy diet. Vegetable crops contain rich sources of vitamins and minerals (Prior and Guohua, 2000), and also significant amounts of bioactive compounds such as polyphenols, which provide a wide range of health benefits beyond basic nutrition. An association between high consumption of vegetable crops and a lower risk of chronic diseases such as certain types of cancer (Kwon *et al.,* 2006; Reddivari *et al.,* 2007; Rose *et al.,* 2005; Smiechowska *et al.,* 2008), type II diabetes (Liu *et al.,* 2004; Villegas *et al.,* 2008), and cardiovascular diseases (Radhika *et al.,* 2008; Terao *et al.,* 2008; Dauchet *et al.,* 2009), have consistently been indicated by epidemiological studies. Additionally, low consumption of vegetables and fruits were identified as an important risk factor for chronic diseases as reported by the World Health Organization World Health Report 2005 (Lock *et al.,* 2005). In Malaysia, a wide range of more than 50 types of vegetables grown by smallholders in both Highland and Lowland areas, have formed the important daily diet for the nation (Ding *et al.,* 1981). In 2006, the total area under vegetable cultivation in Malaysia was reported to be 39,660 ha, of which 11,415 and 10,498 ha was located in Johor (28.8 %) and Pahang (26.8 %), respectively (Anonymous, 2008).

Leaf spot caused by *Stemphylium* spp. is one of the important diseases of vegetable crops that attack the fruit, stem and foliage (Ellis, 1971). This disease is most common and severe on vegetables such as tomato, pepper, eggplant, lettuce, onion, garlic, spinach and asparagus (Farr and Rossman, 2014). Yield loss due to Stemphylium leaf spot can be high and in some cases is 100 % when proper disease control measures are not adopted (Cedeño and Carrero, 1997). The genus *Stemphylium*, anamorphic *Pleospora* (Dothideomycetes), was proposed by Wallroth (1833) with *S. botryosum* Wallr. as the type species. More than 33 species have been recognized in this genus (Câmara *et al.,* 2002). Most of *Stemphylium* species are saprophytes growing on dead plants and cellulose materials (Ellis, 1971, Simmons, 1969), but various *Stemphylium* species such as *S. botryosum*, *S. solani*, *S. lycopersici* and *S. vesicarium* have been reported as common pathogens on vegetables causing severe disease in almost all regions where these crops are grown (Ellis, 1971).

Unfortunately to date, there is no study on the causal agent of Stemphylium leaf spot of vegetable crops in Malaysia. Therefore, studies on *Stemphylium* species associated with leaf spot of vegetable crops in Malaysia are addressed in the present study. Additionally, genetic variability of *Stemphylium* populations obtained from various vegetables and different geographical regions was investigated. The results of this study will provide better insight into the biology and etiology of the pathogen and these achievements will be helpful in the development of better disease management strategies and in the breeding of resistant varieties for vegetable production in Malaysia.

1.2 Objectives of study

The present study was aimed at identifying and characterizing the biology and diversity of *Stemphylium* isolates from various vegetable crops collected from different geographical regions of Malaysia. The objectives of this study were to: (1) identify the causal agent of leaf spot of vegetable crops, and assess phenotype variability among monoconidial isolates using morphological and cultural characteristics; (2) evaluate the pathogenicity and cross-inoculation of *Stemphylium* isolates on host plants; (3) confirm the morphological studies, and provide a taxonomic position of *Stemphylium* isolates at the species level using the ITS, *gpd*, *EF-1 alpha* and *vmaA-vpsA* sequence comparisons; and (4) estimate the genetic variability among *Stemphylium* isolates associated with the various vegetable crops using randomly amplified polymorphic DNA (RAPD) and Internal simple sequence repeats (ISSR) molecular markers.

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