UNIVERSITI PUTRA MALAYSIA

ANTIOXIDANT-MEDIATED DEFENSE RESPONSE OF Carica papaya L. VAR. EKSOTIKA AGAINST A COMPATIBLE Erwinia mallotivora STRAIN BT-MARDI

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By

SUHAINA SUPIAN

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Chairman : Associate Professor Mohd Puad Abdullah, PhD

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Papaya dieback disease, caused by the bacterium *Erwinia mallotivora*, is the most devastating papaya disease in Malaysia. Most papaya cultivars in Malaysia are susceptible to this disease which suggests that the molecular basis of the interaction between these cultivars and the pathogen is based on a compatible interaction, leading to disease development. However, in certain pathosystems, such interaction could also develop moderate defense response mediated by antioxidant system. Hence, it was hypothesized that early defense response in compatible interaction of papaya and *E. mallotivora* is mediated by antioxidant system. Therefore, this study was aimed to determine the expression of antioxidant-related genes and proteins and activity of antioxidant-related enzymes in the early response of Eksotika papaya to *E. mallotivora* infection. In addition, this study also optimized the inoculation procedures for artificial infection of Eksotika papaya with this pathogen. Two important parameters in the artificial infection of *E. mallotivora*, inoculum concentration and leaf position, affected significantly the disease development in papaya. The probability of the infection depends on the number of bacteria inoculated where greater number of bacteria is required to defeat host defenses as well as the position of leaf where lower or older leaf is less metabolically active thus, lead to higher susceptibility to this infection. Therefore, for efficient artificial infection of papaya with this pathogen, the use of second leaf from the top and inoculum concentration of 10^7 CFU/inoculation site was recommended. Using these optimized parameters, the inoculation of papaya with this pathogen was carried out and the inoculated leaf samples were collected at 0, 2, 4, 8, 12 and 24 h of post-infection (hpi). Following the bacterial challenge, through semi-quantitative reverse-transcription-PCR (RT-PCR) method, the expression of two selected antioxidant marker genes [superoxide dismutase (*SOD*) and peroxidase] was analyzed. *SOD* gene was found to be transiently up-regulated at 2 hpi whereas, peroxidase gene was up-regulated throughout the experimental

period. Through enzyme assays, enzymatic activity of SOD and peroxidase also showed a significant progressive increase occurred after 8 hpi (p-value<0.05). These observations were further confirmed through protein profiling of the infected leaf proteome at 4 and 8 hpi using two dimensional-polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry analysis where a significant up-regulation of SOD protein at 4 hpi was observed (p-value<0.10). Overall, the findings in this study proved that antioxidant system is involved in early defense response of papaya to *E. mallotivora* infection which may suggest that this defense pathway is also activated in this compatible interaction. This study provides new insights into molecular mechanisms underlying this interaction.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains (*Bioteknologi Tumbuhan*)

TINDAKBALAS PERTAHANAN AWAL *Carica papaya* L. VAR. EKSOTIKA MELAWAN STRAIN BT-MARDI *Erwinia mallotivora* YANG SERASI YANG DIPERANTARAI OLEH SISTEM ANTIOKSIDAN

Oleh

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Penyakit mati rosot betik, yang disebabkan oleh bakteria *Erwinia mallotivora*, adalah penyakit betik yang paling dahsyat di Malaysia. Kebanyakan kultivar betik yang ditanam di Malaysia adalah rentan kepada penyakit ini di mana ini menunjukkan bahawa asas molekul interaksi antara kultivar dan patogen ini adalah berasaskan interaksi yang serasi, yang membawa kepada pembangunan penyakit. Walau bagaimanapun, dalam sistem-sistem tumbuhan-patogen yang tertentu, interaksi seperti itu boleh juga membangunkan tindak balas pertahanan yang sederhana yang diperantarai oleh sistem antioksidan. Oleh itu, dihipotesiskan bahawa tindak balas pertahanan awal dalam interaksi serasi antara betik dan *E. mallotivora* diperantarai oleh sistem antioksidan. Oleh itu, kajian ini bertujuan untuk menentukan ekspresi gen dan protin yang berkaitan antioksidan dan aktiviti enzim yang berkaitan antioksidan dalam tindak balas awal betik Eksotika terhadap jangkitan *E. mallotivora*. Selain itu, kajian ini juga mengoptimumkan prosedur inokulasi untuk jangkitan buatan betik Eksotika dengan patogen ini. Dua parameter yang penting dalam jangkitan buatan *E. mallotivora*, kepekatan inokulum dan kedudukan daun, mempengaruhi pembangunan penyakit pada betik secara signifikan. Kebarangkalian jangkitan bergantung kepada jumlah bakteria yang diinokulasi di mana bilangan bakteria yang lebih besar diperlukan untuk mengalahkan pertahanan perumah dan kedudukan daun di mana daun yang lebih rendah atau lebih tua adalah kurang aktif secara metabolisma yang membawa kepada kerentanan yang lebih tinggi terhadap jangkitan ini. Oleh yang demikian, untuk jangkitan buatan betik dengan patogen ini dengan efisien, penggunaan daun kedua dari atas dan kepekatan inokulum pada 10^7 CFU/tapak inokulasi adalah diusulkan. Menggunakan parameter-parameter yang dioptimumkan ini, inokulasi betik dengan patogen ini telah dijalankan dan sampel daun yang diinokulasi telah dikumpulkan pada

0, 2, 4, 8, 12 dan 24 jam selepas jangkitan (hpi). Berikutan cabaran bacteria tersebut, melalui kaedah kuantitatif separa transkripsi terbalik-PCR (RT-PCR), pengekspresan dua gen penanda antioksida yang dipilih [superoxide dismutase (*SOD*) dan peroxidase] telah dianalisa. Didapati gen *SOD* telah diregulasi tinggi secara sementara pada 2 hpi manakala, gen peroxidase telah diregulasi tinggi sepanjang tempoh eksperimen. Melalui asai-asai enzim, aktiviti enzim SOD dan peroxidase juga menunjukkan peningkatan progresif yang ketara berlaku selepas 8 hpi (nilai-p<0.05). Pemerhatian ini telah disahkan lagi melalui profil protin pada proteom daun yang dijangkiti pada 4 dan 8 hpi menggunakan dua dimensi- elektroforesis gel poliakrilamida (2D-PAGE) dan analisis spektrometri jisim di mana regulasi tinggi SOD protin yang ketara pada 4 hpi telah diperhatikan (nilai-p<0.10). Secara keseluruhannya, penemuan-penemuan dalam kajian ini membuktikan bahawa sistem antioksidan terlibat dalam tindak balas pertahanan awal betik terhadap jangkitan *E. mallotivora* yang boleh mencadangkan bahawa laluan pertahanan ini juga diaktifkan dalam interaksi serasi ini. Kajian ini memberi pemahaman baru ke dalam mekanisma molekul yang mendasari interaksi ini.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science (Plant Biotechnology). The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

2D-PAGE	Two Dimensional Polyacrylamide Gel Electrophoresis
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	Bovine serum albumin
cDNA	complementary DNA
CFU	Colony-forming unit
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EIF	Eukaryotic initiation factor 4A
g	gram
ha	hectar
HCl	Hydrogen chloride
HR	Hypersensitive response
IEF	Isoelectric focusing
IPG	immobilized pH gradient
LB	Luria-Bertani
MALDI-TOF/TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
MARDI	Malaysian Agricultural and Research Development Institute
min	minute
mRNA	messenger RNA

NBT	Nitroblue tetrazolium
OD	Optical density
OEE1	Oxygen evolving enhancer protein 1
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase Chain Reaction
pI	Isoelectric point
PR protein	Pathogenesis-related protein
rDNA	ribosomal DNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
sec	second
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
µg	microgram
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Papaya dieback, caused by *Erwinia mallotivora* (*E. mallotivora*) bacterium, is one of the most destructive diseases to *Carica papaya L.* (*C. papaya*) in Malaysia. This disease has severely affected most papaya fields in Malaysia. First incidence of papaya dieback disease was reported in Batu Pahat, Johor in late 2003. Since then, this disease was spread aggressively throughout Malaysia including Sabah and Sarawak. In 2006, this disease had affected approximately 800 ha of papaya fields and caused the destruction of 1 million trees with total yield losses of 200,000 metric tons, which equivalent to US$ 58 million (Maktar *et al*., 2008). Due to major losses of papaya yield, total national export volume in Peninsular Malaysia plunged from 57,113 tons in 2003 to 10,323 tons in 2008 (Eng, 2011). This disease is difficult to control due to ineffective chemical sprays and none resistant variety to this disease identified through breeding and genetic engineering. In addition, there is limited information underlying the interaction of papaya and *E. mallotivora* at molecular level.

To date, there is no report yet on the molecular basis of papaya early response towards *E. mallotivora* infection. Basic information on molecular mechanisms involved during the compatible interaction of papaya and *E. mallotivora* at the early stage of interaction is still unknown. Lack of the information underlying this pathosystem limits the options to find an effective solution to overcome and control this disease. Thus, investigating the interaction at the molecular level would be helpful to identify key enzymes or genes that play defensive roles in papaya challenged with the pathogen. This will eventually provide better understanding of the defense mechanisms involved in this pathosystem.

To understand the defense mechanisms in plants upon pathogen infection, research should be carried out at the very early stage of the infection process. This is because plant defense system is triggered as soon as the pathogen is in contact with the host plant. During the early stage of plant and pathogen interaction, plants activate their first line of defenses including oxidative burst. Oxidative burst constitutes plant's early defense against pathogen infection by producing a rapid, transient and high amount of reactive oxygen species (ROS) in the cell. Over accumulation of ROS is, however, capable of causing oxidative damage to plant cell (Sharma *et al*., 2012; Mendoza, 2011).

To prevent cell damage caused by oxidation, antioxidant system is induced to control and equilibrate ROS to acceptable levels (Mendoza, 2011). SOD and peroxidase are two of the major enzymes involved in this system. The involvement of these two enzymes in plant defense mechanism has been reported in many plant-pathogen systems (Viljevac *et al*., 2009; Mandal *et al*., 2008; Venisse *et al*., 2001). These enzymes have been proven to be involved

in defense mechanism during early interaction of plant and pathogen even in the compatible interaction (Sgherri *et al*., 2013; Mandal *et al*., 2011). However, in the case of papaya and *E. mallotivora* interaction, the involvement and role of these enzymes are unknown.

Therefore, in the present study, it was hypothesized that the antioxidant system, particularly SOD and peroxidase, are involved in the early defense response of papaya to *E. mallotivora* infection. To prove this, changes of gene expression and enzymatic activity of both SOD and peroxidase in papaya plants in response to this infection were analyzed through semi-quantitative RT-PCR and enzyme assays, respectively. To further confirm the involvement of SOD and peroxidase proteins during the early interaction of papaya and this pathogen, protein expression analysis and identification were carried out through 2D-PAGE and mass spectrometry analysis. Hence, the present study was carried out with the following objectives:

1) to optimize the inoculation procedure for artificial infection of *Carica papaya* L. var. Eksotika with *Erwinia mallotivora,*
2) to determine the expression patterns and activities of the antioxidant genes and enzymes in early response of *Carica papaya* L. var. Eksotika to *Erwinia mallotivora* infection, and
3) to profile and identify antioxidant-related proteins in early response of *Carica papaya* L. var. Eksotika to *Erwinia mallotivora* infection.

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LIST OF PUBLICATIONS

Poster and Proceeding

Suhaina Supian, Noor Baiti Saidi, Wee Chien Yeong & Mohd Puad Abdullah (2014). Potential antioxidant enzymes to combat papaya dieback disease. Proceeding of the BioJohor Biotechnology Conference, 25-27 August 2014, Puteri Pacific Hotel, Johor Bahru, Johor.

Suhaina Supian, Noor Baiti Saidi, Wee Chien Yeong & Mohd Puad Abdullah (2014). Deciphering the defense mechanism in papaya for combating dieback disease. Pameran Sains dan Teknologi MARDI Edisi ke-9, 4-5 November 2014. Silver medal.

Suhaina Supian, Noor Baiti Saidi, Wee Chien Yeong & Mohd Puad Abdullah (2014). Deciphering the defense mechanism in papaya for combating dieback disease. Malaysian Agricultural Innovation Challenge 2014. 6-8 November 2014.

Biotech Colloquium

Suhaina Supian, Noor Baiti Saidi, Wee Chien Yeong & Mohd Puad Abdullah (2012). Identification and characterization of proteins related to papaya dieback disease. 18th Biotech Colloquium, 7 & 21 December 2012, Universiti Putra Malaysia. Pp 32.

Suhaina Supian, Noor Baiti Saidi, Wee Chien Yeong & Mohd Puad Abdullah (2013). Early molecular responses of papaya plants to *Erwinia mallotivora* infection. 20th Biotech Colloquium, 6-7 November 2013, Universiti Putra Malaysia. Pp 3.