FUNCTIONAL ANALYSIS OF NITRIC OXIDE IN Musa sp. cv. BERANGAN DURING INTERACTION WITH Fusarium oxysporum f. sp. cubense TROPICAL RACE 4

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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September 2018

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Nitric oxide (NO) is one of the important signalling molecules that regulates plant defence against pathogen attack. The mechanism of S-nitrosylation, one of the post-translational modifications involving NO is not known during an interaction between *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc*TR4) and *Musa* spp. (banana). Hence, this study was carried out to investigate the effect of NO on Fusarium wilt progression in Berangan banana plants and to determine the S-nitrosothiol (SNO) content as well as the abundance of S-nitrosylated proteins. The Berangan banana roots that have been treated with NO donor, GSNO and NO scavenger, carboxy-ptio were inoculated with *Foc*TR4 conidial suspension using root dip inoculation method. The disease severity index (DSI) was recorded based on the score obtained for leaf symptom index (LSI) and rhizome discoloration index (RDI). Pre-treatment of infected Berangan banana plants with GSNO resulted in lower DSI with LSI and RDI scores of 2 and 4, respectively in comparison with pre-treatment using carboxy-ptio where the DSI is higher with LSI and DSI scores of 5 and 7, respectively. The pre-treatment with GSNO reduced the disease severity status of the Berangan banana plants from highly susceptible to susceptible. In order to determine the S-nitrosylation of Berangan banana proteome, protein extraction from Berangan banana tissues was first optimized for one-dimensional non-reducing SDS-PAGE analysis. Trichloroacetic acid-acetone (TCA) gave the highest concentration and quality of protein extract compared to phenol and phosphate buffer saline (PBS) extraction protocols. Using Saville-Griess assay, SNO content in infected Berangan banana plants pre-treated with GSNO were recorded at 96.99 µM mg^{-1} while in untreated plants, the SNO was recorded lower at 57.59 µM mg^{-1}. As expected, prior removal of NO in infected Berangan banana plants which were pre-treated with carboxy-ptio resulted in the lowest SNO content at 48.82 µM mg^{-1}. Consistent with the role of NO during nitrosative burst at the early phase of plant defence response, *Foc*TR4 induced higher SNO formation in root protein extract at 2 hours post inoculation compared to

12 hours post inoculation. In relation to that, biotin switch assay showed that more protein were S-nitrosylated in response to *Foc*TR4 compared to non-infected Berangan banana plants. Overall, findings from this study suggest that NO is involved in the basal defence mechanism of Berangan banana during an early interaction with *Foc*TR4 through S-nitrosylation. This study adds value to the existing pool knowledge on the molecular mechanism behind the interaction between *Foc*TR4 and banana.

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISI FUNGSI NITRIK OKSIDA DI DALAM *Musa* sp. cv. BERANGAN KETIKA INTERAKSI DENGAN *Fusarium oxysporum* f. sp. *cubense* TROPICAL RACE 4

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Nitrik oksida (NO) adalah salah satu molekul isyarat penting yang mengawal pertumbuhan tumbuhan dan pertahanan terhadap serangan patogen.Mekanisme S-nitrosilasi, salah satu pengubahsuaian pasca translasi yang melibatkan NO tidak diketahui semasa interaksi antara *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc*TR4) dan *Musa* spp. (banana). Oleh itu, kajian ini dijalankan untuk mengkaji kesan NO terhadap perkembangan perkembangan penyakit "*Fusarium* wilt" pada pisang Berangan serta menentukan kandungan S-nitrosothiol (SNO) dan kesan terhadap protein yang menjalani proses S-nitrosilasi. Akar pisang Berangan telah diinokulasi dengan cecair spora *Foc*TR4 menggunakan kaedah inokulasi rendaman akar setelah dirawat dengan GSNO dan carboxy-ptio. Indeks keparahan penyakit (DSI) direkodkan berdasarkan skor yang diperolehi untuk indeks gejala daun (LSI) dan indeks perubahan warna rhizome (RDI). Pra-rawatan menggunakan penderma NO (GSNO) ke atas pisang Berangan yang dijangkiti dengan *Foc*TR4 memberikan skor LSI 2 dan skor RDI 4 yang lebih rendah dibandingkan dengan pra-rawatan menggunakan pengusir NO (carboxy-ptio) yang menyumbang kepada DSI yang lebih tinggi yang mengakibatkan LSI mencapai skor 5 dan RDI mencapai skor 7. Oleh itu, status ekspresi penyakit untuk tumbuhan pisang yang dijangkiti *Foc*TR4 sebelum dirawat dengan GSNO adalah mudah diserang penyakit manakala tanaman pisang yang dijangkiti yang dijangkiti dengan carboxy-ptio sangat mudah diserang penyakit. Untuk mengesan protein yang menjalani proses S-nitrosilasi, pengekstrakan protein dari tisu pisang Berangan dioptimumkan terlebih dahulu untuk analisis SDS-PAGE satu dimensi. Protokol pengekstrakan asid-aseton memberikan kepekatan protein yang tertinggi dan kualiti ekstrak protein yang bagus berbanding dengan protokol pengekstrakan fenol dan fosfat saline. Pengesanan kandungan S-nitrosotiol (SNO) menggunakan teknik Saville menunjukkan bahawa pisang Berangan yang dijangkiti *Foc*TR4 selepas dirawat dengan GSNO memberi kandungan SNO tertinggi dengan nilai 96.99 μM mg^{-1} manakala pisang Berangan yang tidak dirawat menunjukkan

kandungan SNO yang lebih rendah iaitu 57.59 μM mg^{-1}. Seperti yang telah diramalkan, penyingkiran NO terlebih dahulu di dalam pisang Berangan yang dirawat dengan carboxy-ptio sebelum dijangkiti *Foc*TR4 menunjukkan kandungan SNO terendah dengan nilai 48.82 μM mg-1. Selari dengan peranan penting NO semasa pecah oksidatif yang berlaku pada fasa awal respon pertahanan tumbuhan, *Foc*TR4 mendorong pembentukan SNO yang lebih tinggi dalam ekstrak protein daripada akar pada 2 jam selepas inokulasi berbanding 12 jam selepas inokulasi. Sehubungan dengan perkara tersebut, teknik "biotin switch" menunjukkan bahawa terdapat lebih banyak protein yang menjalani proses S-nitrosilasi berikutan tindak balas terhadap inokulasi dengan *Foc*TR4 berbanding dengan pisang Berangan yang tidak dijangkiti. Secara keseluruhan, penemuan daripada kajian ini menunjukkan bahawa NO terlibat dalam mekanisme pertahanan tumbuhan semasa interaksi awal dengan *Foc*TR4 melalui proses S-nitrosilasi. Kajian ini boleh meningkatkan lagi pengetahuan tentang mekanisme molekul di sebalik interaksi antara *Foc*TR4 dan pisang.

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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. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

APX	ascorbate peroxidase
Biotin-HPDP	N-[6-(biotinamido)hexyl]-3'-(2'pyridyldithio)propionamide
bp	base pair
BSA	bovine serum albumin
BST	biotin switch technique
carboxy-ptio	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CO_2	carbon dioxide
Cys	cysteine
DAMP	damage associated molecular pattern
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Foc	*Fusarium oxysporum* f.sp. *cubense*
GSH	glutathione
GSNO	nitrosoglutathione
GSNOR	niitrosoglutathione reductase
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	hypersensitive response
ITIS	Integrated Taxonomic Information System
MMTS	methyl methanethiosulfonate
NO	nitric oxide
NOS	nitric oxide synthase
NPR1	non-expressor of pathogenesis-related gene 1
O^2	oxygen
$ONOO^-$	peroxynitrite

PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PR	pathogenesis-related
Prx II E	peroxiredoxin II E
PTM	post-translational modification
PVDF	polyvinylidene difluoride
PVPP	polyvinylpolypyrrolidone
ROS	reactive oxygen species
RBOHD	respiratory burst oxidase homolog D
SA	salicylic acid
SABP3	salicylic acid-binding protein 3
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
SNA	Spezieller nahrstoffarmer agar
SNO	s-nitrosothiol
STR4	subtropical race 4
TCA	trichloroacetic acid
TR4	tropical race 4
Tyr	tyrosine

CHAPTER 1

INTRODUCTION

1.1 Introduction

Musa spp., or known as banana is one of the most important crops in the world. It serves as staple food in some regions and also an addition to diet as it contains high nutritional value and minerals (Bjarnadottir, 2017). In 2011, the global gross production value of banana worth US$44 billion with combined global production estimated at 145 million tons. Banana is a major contributor to the economy of the leading producers such as Ecuador and Philippines (Ploetz, 2015). In Malaysia, the increasing demand for banana supply causes an expansion of the harvested area from 27,084.61 hectares in the year 2013 to 34,894.06 hectares in year 2017 (DOA, 2017). Despite the expansion in the harvested area, the banana production has declined from 325,499.60 tonnes in year 2015 to 309,507.65 tonnes in year 2016 (DOA, 2017) which caused severe economic loss in banana plantation industry. The declined in banana production is mainly due to the outbreak of diseases and one of the most lethal disease is known as *Fusarium* wilt disease which is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*). The infection occurs as the *Foc* infects the roots of a banana and progress to the pseudostem (Ploetz, 2015). Eventually, the leaves of the banana plant wilt and the whole plant collapses. Until now, there is no effective methods to control this disease and it is still spreading in the world. One of the main reasons is the lack of understanding on the molecular basis of plant defence mechanism during host-pathogen interaction.

In response to microbe-associated molecular pattern (MAMPs) or damage-associated molecular pattern (DAMPs) of a pathogen, plant activates a signalling cascade that involves many interacting components such as production of reactive oxygen species (ROS), nitric oxide (NO), Ca^{2+} flux and various hormones (Li et al., 2013). Thereby, understanding the molecular mechanism of each component in the signalling cascade could lead to better strategies in combating a pathogen attack.

Nitric oxide (NO) has been widely studied in plant as it is involved in many physiological processes such as plant development, hormone signalling and response to biotic and abiotic stresses (Trapet et al., 2015). Most importantly, NO plays a significant role in the regulation of defence-related genes, activation of secondary metabolite production as well as hypersensitive response (HR) in plant under pathogen attack (Romero-Puertas et al., 2008; Trapet et al., 2015; Yun et al., 2016). The predominant mode of NO bioactivity is through a post-translational modification (PTM) known as S-nitrosylation, in which NO moiety is attached to a reactive cysteine residue via a covalent bond (Kovacs et al., 2015). S-nitrosylation of several key genes in plant immunity such as salicylic acid-binding protein 3 (SABP3) and non-expressor of pathogenesis-related gene 1 (NPR1) has been shown to modulate plant responses to pathogen. Although NO plays an important role in plant immunity, its involvement during banana-*Foc* interaction is yet to be elucidated. Thus, this study aimed to

investigate the potential role of NO during defence mechanism in banana plants upon *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc*TR4) attack to better understand banana-*Foc*TR4 interaction. It is hypothesized that NO is involved in disease resistance aspect during banana-*Foc* interaction and the infected sample of banana plants has a higher level of S-nitrosothiol (SNO) and S-nitrosylated proteins compared to non-infected sample.

1.2 Objectives of the Study

The objectives of this study are as stated below:

1. To investigate the effect of NO *Fusarium* wilt progression in Berangan banana plants;
2. To optimize protein extraction protocol from banana tissues for the determination of S-nitrosothiol content and abundance of S-nitrosylated proteins in protein extract from healthy and *Foc*TR4-infected Berangan banana.

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