



UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF NON-RIBOSOMAL PEPTIDE SYNTHETASE IN *Ganoderma boninense* PAT

NEDA SHOKROLLAHI

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By

NEDA SHOKROLLAHI

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

April 2021

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DEDICATION

Specially dedicated to,

My dear supervisor for her ongoing supports, my parents; the reason of what I become today, my husband for his invaluable love, understanding, tolerance, sacrifice, moral support and my twin brothers for their encouragement.



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

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April 2021

Chairman : Professor Wong Mui Yun, PhD
Faculty : Agriculture

Basal stem rot (BSR) is a destructive disease caused by a white-rot fungus, *Ganoderma boninense* Pat. which has threatened the oil palm industry for more than eight decades without any effective treatment available until now. Therefore, it is crucial to understand the role of non-ribosomal peptides (NRPs) implicated as virulence factors during pathogenesis particularly in *Ganoderma*-oil palm pathosystem where this information is lacking. The objectives of this study were i) to isolate NRPS gene of *G. boninense* strain UPMGB001 using PCR-based method, to analyze the sequence with bioinformatics tools, namely antiSMASH and Augustus, also the prediction of the secondary structures and three-dimensional structure modelling of the polypeptides by using SOPMA and the SWISS-MODEL tools, respectively; ii) to analyze the secondary metabolite(s) related to NRPS released by *G. boninense* during its interaction with oil palm *in vitro* by using Liquid Chromatography Triple Quadrupole Mass Spectrometer (LC-MS/MS) and performance liquid chromatography (HPLC) by using analytical standard; and iii) Expression of *G. boninense* NRPS gene and its correlation with basal stem rot disease severity in oil palm seedlings by performing quantitative real-time PCR (qPCR), finally to investigate the correlation between disease severity and NRPS expression within six months. The isolated NRPS fragment of 8,322 bp was used to predict the putative peptide sequence of different domains and showed similarity with NRPS adenylation domain (core motifs of A1 and A10), thiolation and condensation domains. Furthermore, phylogenetic analysis of NRPS sequences demonstrated that NRPS of *G. boninense* belongs to the type VI siderophore family. Ricinoleic acid was detected in the culture filtrate that was produced by *G. boninense* strain UPMGB001 during the pathogenicity in oil palm *in vitro* while this compound was not detected in the control sample. It was the first report on ricinoleic acid synthesis by *G. boninense* that was confirmed by using HPLC and ricinoleic acid analytic standard. The correlation between high disease severity (50%) and high expression

(227 fold change) of *G. boninense* NRPS gene at 4 months after inoculation and above indicated that this gene played a significant role in the advancement of BSR disease. These results showed that the NRPS gene probably plays an imperative role in the development of BSR disease. These findings are new perspectives for the understanding of the functional role of NRPS of *G. boninense*, and they will contribute to the development of an early detection system using the NRPS region as a *Ganoderma*-specific biomarker for more efficient management of BSR disease in oil palm.



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

PENCIRIAN DAN ANALISIS BERFUNGSI PEPTIDA SINTETASE BUKAN RIBOSOMAL DI DALAM *Ganoderma boninense* SEMASA INTERAKSINYA DENGAN KELAPA SAWIT

Oleh

NEDA SHOKROLLAHI

April 2021

Pengerusi : Profesor Wong Mui Yun, PhD
Fakulti : Pertanian

Reput pangkal batang (BSR) merupakan satu penyakit serius yang disebabkan oleh kulat pereputan putih, *Ganoderma boninense* Pat. dan ianya telah mengancam industri kelapa sawit lebih daripada lapan dekad tanpa penyelesaian yang berkesan sehingga kini. Maka, pemahaman fungsi peptide sintetase bukan ribosomal (NRP) sebagai faktor virulen dalam proses patogenesis adalah penting terutamanya dalam pato-sistem *Ganoderma*-kelapa sawit yang mana maklumat ini amat kurang. Objektif kajian ini adalah untuk i) mengasingkan gen NRPS dari *G. boninense* strain UPMGB001 menggunakan kaedah berasaskan PCR, menganalisa turutan tersebut dengan perisian bioinformatik, iaitu antiSMASH dan Augustus, dan juga meramalkan struktur sekunder dan pemodelan struktur tiga dimensi poli peptida masing-masing menggunakan kaedah SOPMA dan SWISS-MODEL; ii) memahami metabolit sekunder yang berkaitan dengan NRPS yang dihasilkan oleh *G. boninense* semasa interaksinya dengan kelapa sawit semasa *in vitro*.; dan iii) mengkaji ekspresi gen NRPS dalam *G. boninense* semasa aktiviti sebagai patogen dalam kelapa sawit sepanjang empat bulan selepas inokulasi tiruan dengan menjalankan proses PCR kuantitatif *real time* (qPCR) dan akhirnya untuk memahami korelasi antara keparahan penyakit dan ekspresi gen NRPS sepanjang 6 bulan. Serpihan NRPS sepanjang 8,322 bp yang diasingkan digunakan untuk meramal urutan peptida untuk domain berbeza yang dijangka dan menunjukkan persamaan dengan domain adenilasi NRPS (motif teras A1 dan A10), domain thiolasi dan kondensasi. Analisa filogenetik ke atas urutan NRPS menunjukkan bahawa NRPS dari *G. boninense* menganggotai keluarga VI siderofor. Menggunakan Spektrometer Massa Kromatografi Cair Triple Quadrupole (LC-MS/MS), didapati asid ricinoleik merupakan metabolit sekunder tunggal yang disintesis oleh NRPS di dalam *G. boninense* strain UPMGB001 semasa interaksinya dengan kelapa sawit *in vitro*, manakala asid ricinoleik tidak dikesan dalam sampel kawalan. Inilah pertama kali asid risinoleik dilaporkan dalam *G. boninense*. Asid ricinoleik dalam *G. boninense*

dikenal pasti menggunakan kromatografi cecair berprestasi tinggi (HPLC) dan piawaian analiktikal. Didapati bahawa korelasi keparahan penyakit yang tinggi (50%) mempunyai kaitan dengan kadar ekspresi gen yang tinggi (perubahan 227 kali ganda) dalam gen NRPS *G. boninense* pada bulan keempat selepas inokulasi. Keputusan ini menunjukkan bahawa gen ini memainkan peranan penting dalam penularan penyakit BSR. Pencapaian ini merupakan satu perspektif baru untuk memahami fungsi peranan NRPS *G. boninense*. Penemuan ini akan menyumbang ke arah perkembangan satu sistem pengesanan awal menggunakan kawasan NRPS sebagai biomarker spesifik *Ganoderma* dalam pengurusan penyakit BSR yang lebih berkesan dalam kelapa sawit.



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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Wong Mui Yun, PhD

Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Ho Chai Ling, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Nur Ain Izzati Binti Mohd Zainudin, PhD

Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Mohd As'wad Bin Abdul Wahab, PhD

Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

ZALILAH MOHD SHARIFF, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: _____
Name of Chairman
of Supervisory Committee: Professor
Dr. Wong Mui Yun

Signature: _____
Name of Member
of Supervisory Committee: Professor
Dr. Ho Chai Ling

Signature: _____
Name of Member
of Supervisory Committee: Associate Professor
Dr. Nur Ain Izzati Mohd Zainudin

Signature: _____
Name of Member
of Supervisory Committee: Dr. Mohd As'wad Bin Abdul Wahab

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LIST OF ABBREVIATIONS

°C	Degree Celsius
A domain	Adenylation Domain
aa	Amino Acid
antiSMASH	Antibiotics and Secondary Metabolite Analysis Shell
BGCs	Biosynthetic Gene Clusters
bp	Base Pair
BSR	Basal Stem Rot
C domain	Condensation Domain
CDD	NCBI Conserved Domains
CDs	Coding Sequences
CH ₃ OH	Methanol
CTAB	Cetyl Trimethylammonium Bromide
E domain	Epimerization Domain
EA	Ergot Alkaloid
EtoAC	Ethyl Acetate
ITS	Internal Transcribed Spacer
MDAR	Mono Dehydroascorbate Reductase
min	minute
MS	Murashige and Skoog
MSA	Multiple Sequences Alignment
NCBI	National Center of Biotechnology Information
NRPs	Non-ribosomal Peptides
NRPSs	Non-ribosomal Peptide Synthetases
PDA	Potato Dextrose Agar

PDB	Potato Dextrose Broth
pp-binding	4'-Phosphopantetheine
ROS	Reactive Oxygen Species
RT	Retention Time
SM	Secondary metabolite
T domain (PCP domain)	Thiolation Domain (Peptide Carrier Protein)
TE domain	Termination Domain



CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Oil palm (*Elaeis guineensis* Jacq) is a native tree from West Africa (Kwan et al., 2016). Oil palm, as the most significant plantation crop, plays a crucial role in the national economic development of Malaysia (Tapia et al., 2021). Basal stem rot (BSR) is a destructive disease caused by a white-rot fungus, *Ganoderma boninense* Pat. which has threatened the oil palm industry for more than eight decades without any effective treatment available until now (Alexander et al., 2017; Paterson, 2019). BSR is the most important fungal disease in the oil palm plantations of South East Asia (SEA), particularly, Malaysia and Indonesia that are responsible for up to 85% of the world's palm oil export (Siddiqui et al., 2021).

Ganoderma is a polyporoid fungus, a member of *Ganodermataceae* family and *Basidiomycetes* class which grows on wood (Idris, 2009). These fungi are known as lignolytic fungi that are generally classified as white-rot fungi. They degrade the lignin part of wood whereas subsisting white cellulose is revealed (Paterson, 2007). Thus, *Ganoderma* among other groups of white-rot fungi is more active in degrading lignin. *G. boninense* has been identified as the most virulent species to the oil palm (Moncalvo, 2000). No absolute cure has been identified yet for the BSR disease, and it is a serious concern among investors of oil palm plantations, researchers and others who are working in the oil palm sector (Siddiqui et al., 2021). Therefore, the necessity of an inclusive molecular study on plant pathogenic fungi interaction is more than ever. Furthermore, studies on the biological cycle and virulence factors of fungus (from the molecular approach) and assessment of the interaction with its host, are undeniably significant.

One of the impressive achievements is the discovery of secondary metabolites (SMs) during the fungal pathogenicity process. SMs, unlike primary metabolites, are compounds that are not involved in essential life processes, however, often have potential physiological roles (Keller, 2019). Research on non-ribosomal peptides (NRPs), as one of SMs, has been noticed because of their role in biotic stress responses, including pathogenicity (Sayari et al., 2019). NRPs are fungal secondary metabolites whose biosynthesis is independent of ribosomes and mRNA but synthesized by non ribosomal peptide synthetases (NRPSs) (Eisfeld, 2009). NRPS was detected in *G. boninense* and demonstrated to be associated with BSR disease development (Wong et al., 2011). So, it was hypothesized that there is/are NRPS(s) in *G. boninense* with the conserved core motifs in A, T and C-domain, and NRPS gene in *G. boninense* may be involved in the pathogenesis of basal stem rot in oil palm. Most studies about NRPS have been on filamentous ascomycete fungi such as *Aspergillus* sp and *Cochliobolus heterostrophus*.

A canonical module of NRPS enzyme is composed of three different standard domains: adenylation (A), thiolation (T) or peptidyl carrier protein (PCP), and condensation/peptide-bond formation (C) which are recognized as one NRPS module (Süssmuth and Mainz, 2017). NRPS-derived metabolites show a wide chemical variety such as the incorporation of proteinogenic (the 22 amino acids that encoded by genes for the synthesis of proteins (Ambrogelly et al., 2007)) and non-proteinogenic amino acids (non-coded amino acids containing those not typically encoded or detected in the genetic code. In addition to the 22 standard amino acids, over 140 amino acids are recognized normally in proteins, and more than a thousand may be synthesized *in vivo* or *in vitro* (Lobanov et al., 2010)) in their D - and L - configurations by multi-domain, multi-modular enzymes (Mukherjee et al., 2012). ACV synthetase (ACVS), enniatin synthetase, cyclosporine synthetase, HC-toxin synthetase (HTS1), AM-toxin synthetase, and bassianolide synthetase are some examples of NRPSs found in plant pathogenic fungi (Soukup et al., 2016).

Prediction on secondary metabolite biosynthesis based on microbial genome sequences requires bioinformatic analyses. Some tools such as, ClustScan (Starcevic et al., 2008), CLUSEAN (Weber et al., 2009), SBSPKS (Anand et al., 2010) and SMURF (Khaldi et al., 2010) have been developed to identify and analyze the enzymatic domains in multi-modular polyketide synthases (PKSs) and/or NRPSs, which are the essential enzymes for the production of clinically crucial secondary metabolites. 'Antibiotics and secondary metabolite analysis shell' (antiSMASH) was launched in 2011 as a tool with a broad group of compound-specific analysis algorithms for the automated identification of the gene cluster for secondary metabolites (Blin et al., 2017).

Therefore, this study included three specific objectives:

1. To isolate and characterize the nonribosomal peptide synthetase gene in *Ganoderma boninense*.
2. To identify the secondary metabolites produced by *G. boninense* during interaction with oil palm *in vitro*.
3. To determine the expression of *G. boninense* NRPS gene and its correlation with basal stem rot disease severity in oil palm seedlings.

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BIODATA OF STUDENT

Neda Shokrollahi was born in Qom, Iran on June. 19, 1984. She finished Pre-University on June 2002. She graduated in B.A. Plant Pathology from Zanjan University on September 13, 2006. She graduated with her Master degree in the field of Agriculture Engineering, Plant Pathology from Damghan Branch, Islamic Azad University on November 01, 2011. She has enrolled as a PhD candidate in the Faculty of Agriculture, Universiti Putra Malaysia since February 2016.



LIST OF PUBLICATIONS

Journal

Neda Shokrollahi, Chai-Ling Ho, Nur Ain Izzati Mohd Zainudin, Mohd As'wad Bin Abul Wahab, Mui-Yun Wong. Identification of non-ribosomal peptide synthetase in *Ganoderma boninense* Pat. that was expressed during the interaction with oil palm. *Sci Rep* 11, 16330 (2021). <https://doi.org/10.1038/s41598-021-95549-8>.

Neda Shokrollahi, Chai-Ling Ho, Nur Ain Izzati Mohd Zainudin, Mohd As'wad Bin Abul Wahab, Mui-Yun Wong. Ricinoleic acid biosynthesis detected in *Ganoderma boninense* strain UPMGB001 during interaction with oil palm *in vitro*

Poster Presentation

Shokrollahi, N., Ling, H. C., Zainudin, N. A. I. B. M., Bin, M. A. W., Wahab, A., & Yun, W. M. Molecular Analysis of Condensation Domain in Non-Ribosomal Peptide Synthetase (NRPS) GENE of *Ganoderma boninense*. 13–15 November 2018 TNCPI Building, Universiti Putra Malaysia Serdang, Selangor Malaysia, 294.



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