UNIVERSITI PUTRA MALAYSIA

CHARACTERISATION AND PATHOGENICITY OF Neofusicoccum ribis AND GENE EXPRESSION STUDY OF PATHOGENESIS –RELATED GENES IN RUBBER (Hevea brasiliensis Muell.Arg)

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NYAKA NGOBISA AURELIE IRENE CLAIRE

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

February 2014

DEDICATION

The work in this thesis is dedicated to the memory of my loving father Nyaka Emmanuel (28/12/1939-19/05/2009) and to the entire Nyaka family. To my husband Mandengue Louis Lucien Heurard for his boundless love, understanding, encouragement and patience thoughout my study

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

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Chairman: Associate Professor Zainal Abidin Mior Ahmad, PhD
Faculty: Agriculture

Rubber tree (*Hevea brasiliensis*) is well known as the tree of life for its by-products such as latex and wood. However, the growth and performances of *H. brasiliensis* are reduced substantially by Fusicoccum Leaf Blight disease, a re- emerging malady first detecte in Malaysia in 1987. This study provides a foundation for understanding the infection of healthy rubber plant tissues by *Fusicoccum* spp. in selected regions in Malaysia, with emphasis on pathogenicity and cross pathogenicity evaluation of associated hosts. The study provides a leaf colonisation pattern since the resistance mechanism provided by pathogenesis-related proteins (PR) and phenylalanine ammonia lyase (PAL) enzymes, both induced in infected rubber, are fairly well studied. Fungal isolates were obtained from leaf samples collected in several rubber plantations in Selangor, Perak and Johor in 2010. All isolates were identified based on cultural characteristics such as conidial production in various cultural media (PDA, MEA, CDA, CMA) and sequence data generated from the internal transcribed spacers (ITS1 and ITS2), the 5.8S rRNA gene and an unknown locus (BotF15) containing microsatellite repeats. Pathogenicity tests were conducted using representative isolates from three locations (SB30, SK10 and SJ20) for confirmation by detached leaf method and seedlings in glasshouse, and cross pathogenicity tests on detached leaves. The roles of β-1, 3-glucanase and chitinase, the two most widely studied groups of pathogenesis-related (PR) proteins (PR3 and PR2) and phenylalanine ammonia lyase (PAL) enzymes present during pathogenesis were investigated.

Thirty three isolates were derived from this study. In different culture media and pH tested, significant differences were observed in spore production, dimensions of the conidia and growth rates among isolates. On PDA, pH 6 supported the best growth compared to others and optimum temperatures of growth were between 25 ^{o}C to 30 ^{o}C. The growth rate was 21.2 mm/day on PDA, 17.9 mm/day on CDA, 17.1 mm / day on MEA and 14.2 mm/day on CMA. The highest spore production of 161.33 x 10 ^{6} spores / ml was obtained on PDA. The optimum temperature for spore germination in distilled water was 25 ^{o}C – 35 ^{o}C within a period of twenty- four hours. The fungus colony color was white to grey; dark greenish grey or greyish.

Conidia were either septate or aseptate, hyaline when young and turning brown with age, oval, elliptic to ovaloid or subglobose in shape. Conidia size ranged 9.0-25.8 µm in length and 1.9-8.5 µm width with the L: B ratio of 3.4.

Molecular characterisation revealed fungal sequences generated from ITS and BotF15 genes, indicating therefore that they formed a supported clade with *Neofusicoccum. ribis*. Tested isolates (SB30, SK10 and SJ20) were pathogenic to *H. brasiliensis* but varied in symptoms. Pathogenicity tests showed high susceptibility of percentage of disease severity on detached rubber leaves from clones RRIM 2024 and PB 350 (39 - 56%) and low susceptibility on clones RRIM 2002 and RRIM 2007 (6.6 - 18.8%). Some alternate hosts such as mango and guava exhibited high susceptibility (50 - 80%) while others (rambutan and papaya) were low (3 - 10%).

Induction of pathogenesis–related genes in rubber leaves in the presence of fungal pathogen showed that patterns of expressions of these enzymes were up-regulated in both tolerant (RRIM 2002) and susceptible (PB 350) rubber clones over time although their expression was much higher in the latter. Generally, pathogenesis-related proteins from *H. brasiliensis* could be used as molecular probes to monitor defence-response activation while the mechanism in *N. ribis* isolate SK10 could serve as an inducer of systemic resistance in rubber.

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

PENCIRIAN DAN PATOGENISITI *Neofusicoccum ribis* DAN KAJIAN EKSPRESI GEN PERKAITAN-PATOGENESIS DALAM GETAH (*Hevea brasiliensis* Muell.Arg)

Oleh

NYAKA NGOBISA AURELIE IRENE CLAIRE

Februari 2014

Pengerusi: Profesor Madya Zainal Abidin Mior Ahmad, PhD
Fakulti: Pertanian

Pokok getah (*Hevea brasiliensis)* lebih dikenali sebagai pokok hayat bagi produk sampingan seperti getah dan kayu. Walau bagaimanapun, pertumbuhan dan perkembangan *H. brasiliensis* terjejas dengan ketara disebabkan oleh penyakit daun hawar *Fusicoccum*, penyakit yang dikesan muncul semula di Malaysia pada tahun 1987. Kajian ini memberi asas pemhaman jangkitan *Fusicoccum* spp. terhadap tisu tumbuhan getah yang sihat di kawasan terpilih di Malaysia, dengan penekanan kepada patogenisiti dan penilaian patogenisiti silang pada hos yang berkaitan. Kajian ini adalah wajar dalam menerangkan corak kolonisasi daun apabila mekanisme rintangan terhasil dari protein yang berkaitan dengan patogenesis (PR) dan enzim ammonia phenylalanine lyase (PAL) yang mana kedua-duanya disebabkan oleh getah yang telah dijangkiti.

Pengasingan kulat diperolehi daripada sampel daun yang dikutip di beberapa ladang getah di Selangor, Perak dan Johor pada tahun 2010. Semua pengasingan telah dikenal pasti berdasarkan ciri-ciri kultur seperti pengeluaran conidial dalam pelbagai medium kultur (PDA, MEA, CDA, CMA) dan urutan data yang terhasil daripada ruang salinan dalaman (ITS1 dan ITS2), gen rRNA 5.8S dan lokus yang tidak diketahui (BotF15) yang mengandungi mengulangi mikrosatelit. Ujian patogenisiti telah dijalankan menggunakan pengasingan mewakili tiga lokasi (SB30, SK10 dan SJ20) untuk pengesahan dengan kaedah helaian daun dan benih di rumah kaca, dan ujian silang patogenisiti pada daun berkembar. Fungsi β-1, 3-glucanase dan chitinase, dua kumpulan ezmin yang dikaji paling meluas yang berkaitan dengan patogenesis (PR) protein (PR3 dan PR2) dan penyediaan enzim lyase ammonia phenylalanine (PAL) dalam patogenesis telah dikaji.

Tiga puluh tiga pengasingan telah diperoleh daripada kajian ini. Dalam medium kultur berbeza dan pengujian pH, perbezaan ketara telah diperhatikan dalam pengeluaran spora, dimensi konidia dan kadar pertumbuhan di kalangan pengasingan. Pada PDA, pH 6 menyokong pertumbuhan yang terbaik berbanding

dengan yang lain dan suhu optimum pertumbuhan adalah di antara 25 ^{o}C hingga 30 ^{o}C. Kadar pertumbuhan adalah 21.2 mm / hari untuk PDA, 17.9 mm / hari untuk CDA, 17.1 mm / hari MEA dan 14.2 mm / hari untuk AKM. Pengeluaran tertinggi spora 161.33 x 10^{6} spora / ml telah diperolehi untuk PDA. Suhu optimum untuk percambahan spora di dalam air suling adalah antara 25 ^{o}C - 35 ^{o}C dalam tempoh dua puluh empat jam. Warna koloni kulat adalah putih kelabu, kelabu kehijauan gelap atau kekelabuan. Konidia sama ada septate atau aseptate, hialin ketika muda dan bertukar perang pada usia, bujur, eliptik ke ovaloid atau berbentuk subglobose. Saiz panjang konidia antara 9.0-25.8 μm dan lebar antara 1.9-8.5 μm dengan L: B nisbah 3.4.

Pencirian molekul mendedahkan urutan kulat terhasil daripada ITS dan gen BotF15, ini menunjukkan bahawa kulat tersebut membentuk klad yang baik yang dibantu oleh *Neofusicoccum*. *ribis.* Hasil ujian penasingan (SB30, SK10 dan SJ20) adalah patogenik pada *H. brasiliensis* tetapi berbeza pada simptom. Ujian Patogenisiti menunjukkan kecenderungan yang tinggi pada daun getah berkembar dari klon RRIM 2024 dan PB 350 (39 - 56%) dan kecenderungan rendah pada klon RRIM 2002 dan RRIM 2007 (6.6-18.8%). Sesetengah alternatif hos rumah seperti mangga dan jambu menunjukkan kecenderungan yang tinggi (50 - 80%) manakala yang lain (rambutan dan betik) adalah rendah (3 - 10%). Induksi gen yang berkaitan dengan patogenesis dalam getah daun tanpa kulat patogen menunjukkan bahawa corak ungkapan enzim ini adalah up-selia kedua-dua toleran (RRIM 2002) dan mudah (PB 350) klon getah dari semasa ke semasa walaupun ungkapan mereka adalah jauh lebih tinggi dalam kedua. Secara umumnya, protein yang berkaitan dengan patogenesis daripada *H. brasiliensis* boleh digunakan sebagai probe molekul untuk mengawal pengaktifan tindak balas pertahanan manakala mekanisme untuk ketegangan *N. ribis* SK10 yang mampu bertindak sebagai pencetus penentangan sistemik dalam getah.

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

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DECLARATION

Declaration by the student

I hereby confirm that:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as slated in Rule 41 in Rules 2003 (Revision 2012-2013) were adhered to.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABCT	Gene encoding ATP-binding cassette transporters
ACO2	Aminocyclopropanecarboxylate oxidase 2
ANPC	Association of Natural Rubber Producing Countries
ANOVA	Analysis of variance
Avr gene	Avirulence gene
BLAST	Basic Local Alignment Search Tool
Bot F15	Microsatellites
Bp	Base pair
^{0}C	Degree Celsius
cDNA	Complementary dexoxyribonucleic acid
CRD	Completed randomised design
CTAB	Hexadecyltrimethyl-Ammonium Bromide
Chit	Chitinase
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
DAI	Day after inoculation
DEPC	Diethylprocarbonate
DMS	Degrees Minutes Seconds
DNase	Deoxyribonuclease
DI	Disease incidence
DSI	Disease severity index
EDTA	Ethylenediaminetetraacetic acid
ETR 1	Ethylene receptor protein 1
FLB	Fusicoccum Leaf Blight
GAPDH	Glyceraldehyde phosphate dehydrogenase

Glu	Glucanase
GPX	Gluthation peroxidase
ITS	Internal Transcribed Spacer
IRSG	International Rubber Study Group
ISR	Induction of systemic resistance
Kb	Kilo base –pair
LiCl	Lithium Chloride
mL	Millilitre
Mm	Millimetre
mM	Milimolar
MW	Molecular weight
Ng	Nanogram
Nm	Nanometre
NCBI	National Centre for Biotechnology Information
OD	Optical density
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PDB	Potato dextrose broth
PDI	Percent disease intensity
pH	Potential of hydrogen
PR	Pathogenesis related protein
PVP	Polyvinyl pyrrolidone
RNA	Ribonucleic Acid
RNase	Ribonuclease
rRNA	Ribosomal Ribonucleic Acid
Rpm	Revolution Per Minute
RRIM	Rubber Research Institute Malaysia
SDS	Sodium dodecyl sulphate

SA	Salicylic acid
SAR	Systemic acquiered resistance
SE	Standard error
SSR	Simple Sequence repeats
TAE	Tris acetate EDTA
µg	Microgram
µM	Micromole
µl	Microlitre
U	Unit
UV	Ultra violet
V	Volts
V/V	Volume per volume
W/V	Weight per volume

CHAPTER 1

INTRODUCTION

The para rubber tree (*Hevea brasiliensis*) is well known as the tree of life for its latex and wood (Hayashi, 2009). It belongs to the genus *Hevea* (Euphorbiaceae, Malpighiales) which accommodates three species that yield usable rubber: *H. brasiliensis*, *H. guianensis* and *H. benthamiana.* Although the *H. brasiliensis* is native to the South American Amazonian rainforest, the plant gained popularity in Asia and became increasingly important in some African and then Latin American countries where it was commercially planted as the primary source of natural rubber, the main structural component of which is the poly (*cis* -1,4 isoprene) (Thomas et al., 2011).

Rubber plantation has a critical social implication worldwide as over 20 million people are dependent on the rubber industry. Owing to demand following the phenomenal growth of the tyre and automobile industries (IRSG, 2013), natural rubber production is expected to increase considerably in the years ahead and to even double by the year 2020 (Burger and Smit, 2004; IRGS, 2007), Indeed, the Association of Natural Rubber Producing Countries (ANRPC, 2011) estimated land use for rubber production in Malaysia at 1.25 million hectares in 2007 with a total production of 1.20 million tonnes contributing to about a third of the world's rubber production (MRB, 2009) next to Thailand and Indonesia (ANRPC, 2010; 2011). However, the planted area has been continuously declining since 1982, a situation that is worsened by the concurrent drop in rubber cultivated in all West Malaysia owing to some constraints: aging of trees, less and less availability of suitable land, abiotic and biotic stresses (Jayasinghe, 2009; Thomas et al., 2011).

That notwithstanding, biotic stress, climate change and introduction of new clones in Malaysian rubber plantations could contribute to a change in the diseases scenario. A new disease dubbed Fusicoccum Leaf Blight (FLB) was detected in Johore, Malaysia (Radziah and Chee, 1989) that was caused by a fungal pathogen that infected leaves of mature and immature rubber and inflicted defoliation and premature leaf fall (Hashim et al., 2010) resulting in significant reduction in growth performance, yield and survival of the four –year-old trees in a mixed planting of rubber clones: RRIM 600 (55%), PB260 (15%), PB255 (15%), PB261 (15%), and PB217 (15%), with GT1 as supplies (Radziah and Chee, 1989).

The causal agent was first identified based on the morphology of the vegetative and reproductive structures on infected tissues (Radziah and Chee, 1989). However, although many identification methods for pathogenic fungi associated with leaf blight disease have been developed, the clear taxonomic position of this fungus remains uncertain (Phillips et al., 2008). Hence, the use of a combination of morphological and molecular techniques for the identification of FLB disease would be the choice to implement the appropriate control strategies in the future.

Current knowledge of the taxonomy of *Fusicoccum* through morphological characterisation is unclear. Crous et al. (2006) hence introduced a single generic name for the anamorph which occurs with a Dichomera-like synanamorph, which is the more informative morphological state: *Neofusicoccum* (Crous, Slippers and A.J.L. Phillips, gen. nov. MycoBank MB500870). Interest in this fungal group emerged from the fact that it can lead an endophytic life style within plant organs (Slippers and Wingfield, 2007), and ability to become pathogenic under favourable conditions.

Despite its seriousness to the rubber industry, the mechanism(s) by which the disease develops on rubber tree, the identity of the causal fungus based on morphology and molecular characteristic; and pathogenicity in Malaysia are not known. Indeed, a better understanding of the plant's response to fungal infections could lead to improvement in identification of disease and determination of resistant genes involved as the induction of systemic resistance (ISR) in host plants results in unremitting protection against many pathogens (Kloepper et al., 1992). The ISR is phenotypically similar to systemic acquired resistance (SAR) that develops when plants successfully activate their defence mechanism in response to primary infection by a pathogen (Compant et al., 2005). Pathogen infections can trigger the expression of several classes of pathogenesis –related (PR) genes in a wide range of plant species (Shaji, 2006). However, very little is known about pathogen-induced defence responses in rubber. To gain more insight into the molecular mechanisms of attack by *Neofusicoccum* spp, the role of chitinase, β-1, 3-glucanase; the most widely studied groups of pathogenesis-related (PR) proteins (PR3, PR2), that have been previously demonstrated as reliable markers for induced defense responses in other plant species (Dixon and Harrison, 1990: Van Loon et al., 2006) and phenylalanine ammonia lyase (PAL), a key enzyme in the phenylpropanoid pathway that could perform defence-related functions (Wen et al., 2005), during pathogenesis would be useful.
This research was therefore initiated with the following objectives:

1) To isolate fungus from FLB infected leaves from rubber plantations in West Malaysia.
2) To determine the characteristics of the *Neofusicoccum* spp. based on morphology and molecular characterisations.
3) To conduct pathogenicity tests of *Neofusicoccum* spp. on rubber clones and cross inoculations on alternative host plants (guava, mango, papaya and rambutan).
4) To study gene expression of pathogenesis –related (PR) genes during plant – pathogen interaction.

The hypotheses of the study were as follows:

1) There is diversity among the fungal isolates from FLB infected rubber in terms of morphological and molecular characteristics.

2) Resistant and susceptible rubber clones and other alternative host plants will express different symptoms of disease when inoculated with different isolates of the fungus.

3) The fungus induces pathogenesis –related (PR) genes in rubber.

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