UNIVERSITI PUTRA MALAYSIA

PHYLOGENETIC, ANTAGONIST POTENTIAL, BIOCHEMICAL PROFILE AND EXPRESSION OF NBS-LRR RESISTANCE GENE CANDIDATES OF Colletotrichum gloeosporioides ON PEANUT SEEDS (Arachcis hypogaea L. cv. Margenta)

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By

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in Fulfillment of the Requirement for the Degree of Doctor of Philosophy

PHYLOGENETIC, ANTAGONIST POTENTIAL, BIOCHEMICAL PROFILE AND EXPRESSION OF NBS-LRR RESISTANCE GENE CANDIDATES OF *Colletotrichum gloeosporioides* ON PEANUT SEEDS (*Arachcis hypogaea* L. cv. Margenta)

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Peanuts (*Arachis hypogaea* L. cv. Margenta) are important as a crop with high content of protein and oil in seeds. At present in Malaysia, the practices for control of pathogenic fungi rely mainly on how the pesticides are applied. This resulted in some of the serious hazards on human health especially the farm workers and consumer. Due to the over use of pesticides to control the growth of pathogenic fungi, some of the strains have developed increased resistance against these chemicals. Various approaches which had been carried out to control plant diseases. Besides, the biological control of plant diseases is feasible and environment-friendly as opposed to chemicals and pesticides which causes environmental pollution. The first objective of the study is to determine the phylogenetic relationship of *Colletotrichum gloeosporioides* from infected peanut seeds. The cultivars of peanuts (Margenta) were found to be susceptible to fungal diseases caused by the pathogens *C. gloeosporioides*. The fungus *Colletotrichum gloeosporioides* was isolated from naturally infected plants and the molecular identification of the species level was done using amplification via ITS1 and 4 primer with sequencing and a phylogenetic tree by MEGA 7. The phylogenetic tree confirmed that the *ITS* region was an isolated from of peanut fungus closely related to *C. gloeosporioides.* The potential biological disease decreasing ability of antagonistic mycorrhizal fungi: *Trichoderma virens, Trichoderma asperellum, Trichoderma harzanium, Hypogea virens* and *Trichoderma begomovirus* to manage seed diseases and root rot of groundnut plants also studied. The antagonistic ability of *T. virens, T. asperellum, T. harzanium, H. virens* and *T. begomovirus* was screened *in vitro* by the dual culture technique. *Trichoderma harzanium* strongly inhibited the fungal growth of *C.*

gloeosporioides by 80.6%, followed by *T. virens* (78.4%), *T. asperellum* (65.3%), *T. begomovirus* (62.2%) and *H. virens* (48.3%). Two fungal biocontrol agents (BCAs), *T. virens* and *T. harzianum* were found to strongly inhibit the growth of *Colletotrichum* sp. through mycoparasitism, competition and antibiosis. The highest chitinase enzyme activity was recorded with *T. harzanium* (5.51 IU/mL), followed by *T. virens* (4.2 IU/mL), *T. asperllum* (2.32 IU/mL), *T. begomovirens* (0.54 IU/mL), and *H. virens* (0.12 IU/mL). The highest β-1,3-glucanase enzyme activity was recorded with *T. virens* (34.4 IU/mL), followed by *H.virens* (34.4 IU/mL), *T. begomovirens* (25.3 IU/mL), *T. harzianum* (7.6 IU/mL), and *T. asperllum* (4.8 IU/mL). The effects of *C. gloeosporioides* on its morphology, germination and fatty acid profile in regards to the quality of peanut seeds aslo conducted. *C. gloeosporioides* infection had a significant effect on the changes of the physical structure of seeds based on seed volume. The 50-seed volume was found to be significantly lower (10.2 mL) in *C. gloeosporioides* infected seeds than that of uninoculated seeds (11.4mL). Germination of inoculated seed significantly reduced (65.0%) compared to un-inoculated seeds (control). The protein content of inoculated seeds was found to be significantly higher (40.6%) compared to un-inoculated seeds (38.5%). However, the oil content is similar between inoculated and un-inoculated seeds. A fatty acid profile determination was done by Gas Chromatography, and the percentage of fatty acids was analysed. The saturated fatty acids were increased while the unsaturated fatty acids decreased due to an oxidation process, and consequently there was a production of toxic metabolites in the seeds which also reduced the germination percentage. In this study, genes related to the plant defense system had been isolated by using PCR, and analyses of resistant gene candidates (RGCs) in specific host-pathogen intractions was carried out. A pair of degenerate oligonucleotide primers designed from conserved motifs of P-loop and GLPL regions, common to many resistance genes, were used to amplify the Nucleotide-Triphosphate Binding Site (NBS) regions of RGAs from the Fabaceae species. A total of three partial RGAs fragments designated as AhRGC 1, AhRGC 2 and AhRGC 3 were amplified from *Arachis hypogaea*. Therefore, the application of biocontrol is alternative to fungicides and environmentally friendly strategy to control seed-borne pathogens. There is a generation of complimentary environment for BCAs as biopriming due to initial moisture content inside the seed, equal chance to obtain nutrient sources from exudates of seed which can contribute to proliferate rapidly at the surface of a seed for their potential of being a biocontrol. As a conclusion, the *T. virens* and *T. harzianum* have the potential to control *C. gloeosporioides* on peanut seeds.

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

FILOGENETIK, KEKUATAN ANTAGONIS , BIOKIMIA PROFIL Dan PENYATAAN HASRAT NBS-LRR KETAHANAN GEN DARI *Colletotrichum gloeosporioides* PADA BIJI BENIH KACANG TANAH (*Arachcis hypogaea* L. cv. Margenta)

Oleh

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Kacang tanah (*Arachis hypogaea* L. cv. Margenta) merupakan tanaman yang penting di Malaysia. Pereputan biji benih, batang dan akar merupakan ancaman utama kepada tanaman. Kacang tanah (Margenta) didapati terdedah kepada penyakit kulat yang disebabkan oleh patogen *Colletotrichum gloeosporioides* dan menyebabkan kerugian yang tinggi. Kawalan biologi merupakan kaedah strategik untuk mengawal penyakit kulat ini berbanding dengan pengunaan bahan kimia dan racun perosak di mana menyebabkan pencemaran alam sekitar. Kajian ini dilakukan bertujuan mengkaji hubungan filogenetik *Colletotrichum* yang dipencilkan daripada biji benih kacang tanah yang dijangkiti oleh kulat tersebut. Primer ITS 1 dan 4 digunakan untuk ‘PCR’ dan jujukan gen ITS1 dan 4 dianalisis. MEGA 7 digunakan untuk menghasilkan pokok filogenetik. Kajian filogenetik membuktikan *Colletotrichum* yang dipencilkan biji benih kacang adalah *Colletotrichum gloeosporioides.* Potensi *Trichoderma virens, Trichoderma asperellum, Trichoderma harzanium, Hypogea virens dan Trichoderma begomovirus* untuk mengawal penyakit biji benih dan akar reput tumbuhan kacang tanah dijalan secara *in vitro* dengan menggunakan teknik ‘dual culture’. Keputusan mendapati keupayaan *T. harzanium* menghalang pertumbuhan kulat *C. gloeosporioides* adalah paling tinggi dengan nilai PIRG 80.6%, dan diikuti oleh *T. virens* adalah nilai PIRG 78.4% , *T. asperellum* adalah 65.3%, *T. begomovirus* dan *H. virens* adalah 62.2% dan 48.3%. *Trichoderma. harzanium* menunjukkan Aktiviti enzim chitinase tertinggi (5.51 IU / mL), diikuti oleh *T. virens* (4.2 IU / mL), *T. asperllum* (2.32 IU / mL), *T. begomovirens* (0.54 IU / mL), dan *H. virens* (0.12 IU / mL). Manakala, aktiviti enzim β-1,3-glucanase tertinggi ditunjukkan oleh *T. virens* (34.4 IU / mL), diikuti oleh *H. virens* (34.4 IU / mL), *T. begomovirens* (25.3 IU

/ mL), *T. harzianum* (7.6 IU / mL), dan *T. asperllum* (4.8 IU / mL). Oleh yang demikian, *Trichoderma virens* dan *T. harzianum* menghalang pertumbuhan *Colletotrichum* sp. melalui mycoparasitism, persaingan dan antibiosis. Kesan *C. gloeosporioides* ke atas morfologi, percambahan dan profil asid lemak biji benih kacang tanag yang dijangikiti oleh *C. gloeosporioides* juga dijalankan. Kasil kajian mendapati *C. gloeosporioides* menyebabkan perubahan struktur fizikal biji. Isipadu 50 biji benih yang diinokulat dengan *C. gloeosporioides* didapati jauh lebih rendah (10.2 mL) berbanding dengan yang tidak diinokulat dengan *C. gloeosporioides* (11.4 mL). Percambahan biji benih yang diinokulat dengan *C. gloeosporioides* adalah rendah (65.0%) berbanding benih yang tidak diinokulat dengan *C. gloeosporioides*. Walaubagaimanapun, kandungan protein biji benih yang diinokulat dengan *C. gloeosporioides* jauh lebih tinggi (40.6%) berbanding dengan benih yang tidak diinokulat dengan fungi *C. gloeosporioides* (38.5%). Kandungan minyak untuk kedua dua rawatan adalah sama. Analisis profil asid lemak profil menggunakan GC (Gas Kromatografi) mendapati asid lemak tepu meningkat dan asid lemak tak tepu menurun disebabkan oleh proses pengoksidaan dan pengeluaran metabolit toksik. Isolasi “Resistance Gene” (RGC) dan interasi ‘host-patogen’ juga dilakukan dalam kajian ini. Sebanyak tiga gen kode RGAs pada tumbuhan kacang tanah telah diasingkan dari genome iaitu AhRGC 1, AhRGC 2 dan AhRGC 3. Dengan itu, biokontrol adalah alternatif kepada racun kulat dan strategi mesra alam untuk mengawal pathogen biji benih. ‘Biocontrol’ sebagai bioprim memberi kandungan lembapan awal di dalam biji benih, peluang yang sama untuk mendapatkan nutrien dari benih benih yang dapat menyumbang untuk berkembang dengan cepat di permukaan benih. Dan, kaedah bioprim merupakan strategi yang bekesan untuk mengawal kulat, *C. gloeosporioides*. Sebagai kesimpulan, *T. virens* dan *T. harzianum* berpotensi untuk mengawal jangkitan *C. gloeosporioides* pada kacang tanah.

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I certify that a Thesis Examination Committee has met on 24 August 2017 to conduct the final examination of Allegendran a/l Rajeendran on his thesis entitled "Phylogenetic, Antagonist Potential, Biochemical Profile and Expression of NBS-LRR Resistance Gene Candidates of *Colletotrichum gloeosporioides* on Peanut Seeds (*Arachcis hypogaea* L. cv. Margenta)" in accordance with the Universities and University Colleges 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 Doctor of Philosophy.

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%	Percentage
°C	Degree Celsius
µl	Micro Litter
Avr	Avirulence
BCAs	Biocontrol agents
BLAST	Basic local alignment serach tool
CBD	Carbohydrate binding domain
CITRX	Cf-9-interacting thioredoxin
CMC	Carboxy methyl cellulose
CPD	Critical drying point
CTAB	Cetyl-trimethylammonium bromide
ddH_2O	Distilled water
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
FAO	Food and agriculture organization
FFAs	Free fatty acids
Fkr 2	Frucktokinase
FW	Fresh weight
GMO	Genetic modified organisms
H^+	Hydrogen ion
HR	Hypersensitive response
ITS	Inner transcribed spacer
DNA	Deoxyribonucleic acid
IU/mL	A unit of enzyme activity per microliter
LAR	Localized acquired resistance

LRR	Leu-rich repeat resistance
MAMPK	Mitogen-activated protein kinase
MARDI	Malaysian agriculture research & Development institute
MEGA	Multiple sequence genetic analysis
mg	Milligram
mg/g	Milligram per gram
mg/L	Milligram per litre
mins	Minutes
MKT-1	Accession number of peanut from MARDI
mL	Millilitres
mM	Mili molar
Na_2SO_4	Sodium sulphate
NAG	N-acetyleglucosamine
NBS	Nucleotide binding site
NJ	Neighbour joining
NO	Nitric acid
NRPS	Non-ribosomal peptide synthetase
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
P-loop	Phosphorylating-loop
PR	Pathogenesis-related gene
PRR	Proline-rich region
R	Resistance
RGCs	Resistant gene candidate
RNBS-p	Fuctionally unidentified conserved motif

ROI	Reactive oxygen intermediates
SA	Salicyclic acid
JA	Jasmonic acid
SAR	Systemic acquired resistance
sec	Seconds
TcMPK3	Mitogen-activated protein kinase 3
Tcrbcs	Rubisco-small-unit
TcTpp	Trehalose-6-phosphate
TIPK	Trichoderma induced protein kinase
TIR	Toll/interleukin receptor
TMS	Transmembrane
UPM	Universiti Putra Malaysia
UV	Ultraviolet
V	Voltage
VIGS	Virus-induced gene silencing

CHAPTER 1

INTRODUCTION

1.1 Background Study

In terms of food, energy, nutrients, one of the most important sources of food supply are legumes especially in the developing countries of the world. Not only proteins, it has been proven scientifically that legumes are rich in some other nutrients like minerals, vitamins and carbohydrates etc. Pathogenic fungus act as causal agents as they result in certain serious infections and cause damage to the crop leading to considerable loss in terms of yield as well. Wide range of plant species are attacked by pathogenic fungi while sometimes they are limited to one or few of the specific hosts. Peanut (*Arachis hypogaea* L. cv. Margenta) is regarded as highly valuable legume in terms of economy and widely consumed crops in the world. It is grown for a good and cheaper source of good quality protein and vegetable oil for human and livestock nutrition (Kumar *et al.*, 2014). Fungi causing seed-borne diseases such as anthracnose, phomopsis seed decay, frogeye leaf spot and purple seed stain consequently drop the production.

Nowadays, chemical fungicides such as chlorothalonil or tebuconazole had been used to control seed-borne diseases. The usage of these has been debated due to its adverse effects on the environment. These chemicals have been observed to cause environment pollution including leaching of nitrates into the ground water system under agriculture lands, accumulation of heavy metals in soils and presence of excessive pesticides in water and aquatic organisms (Zaller *et al*., 2016). One of the alternatives to complement the role of chemical fungicides is by using beneficial microorganisms, such as bacteria (*Pseudomonas fluorescens*) and fungi (*Trichoderma harzianum*) (Wang *et al.,* 2013).

1.2 Problem Statements, Justification and Objectives of Study

Peanut is one of the most commonly eaten fruits nut and its yields in smallholder cropping system in Malaysia. Pathogenic fungi are considered as key constraints as far as production of peanut is concerned. Several pathogens can attack the crop during the course of its development. At present in Malaysia, the practices for control of pathogenic fungi rely mainly on how the pesticides are applied. The condition has resulted in some of the serious hazards on human health especially the farm workers and those who consume the crop (Rao *et al*., 2016). Due to the over use of pesticides to control the growth of pathogenic fungi, some of the strains have developed increased resistance against these chemicals (Saha *et al.,* 2017). Therefore a alternative ways of controlling fungal pathogens such as *Colletotrichum gloeosporioides* of peanuts is the use of biocontrol application.

Smallholder farmers in the main peanut growing regions of the country grow peanuts in open fields. They face challenges of fungal pathogens, seasonality of the crop production as well as lower yields (Smith *et al.,* 2015). There is a need to reduce pesticide use, increase yields as well as year round production of peanut in the country. Biocontrol application in legumes production has been imposed in several countries has shown that the reduction in the pathogens incidences (Dignam *et al.,* 2016). Biocontrol application such as *Trichoderma* sp. solubilized either macronutrients or micronutrients in the soil to be utilized by plants, resulting in enhanced plant growth (Avis *et al*., 2008) and protection against biotic and abiotic stresses (Benítez *et al*., 2004). There are no specific data about the prospective biocontrol agents (BCAs) against anthracnose of peanut caused by *C. gloeosporioides*. The success of BCAs depends on its ability to survive, grow and proliferate across the environment where the seed is exposed. Thus, seed treatment systems that will enhance the efficacy of biological agents are needed.

Therefore, the application of biocontrol is an alternative to fungicides and environmentally friendly strategy to control seed-borne pathogens. There is a generation of complimentary environment for BCAs as biopriming due to initial moisture content inside the seed, equal chance to obtain nutrient sources from exudates of seed which can contribute to proliferate rapidly at the surface of a seed for their potential of being a biocontrol (Mangmang *et al.,* 2016). When BCAs are inoculated along with priming, there are more efficacies of BCAs against pathogen. On the other hand, there are several approaches for the pathogenic control especially for those pathogens which are soil borne. Therefore, this current work was undertaken with the following objectives:

1. To construct the phylogenetic relationship of *Colletotrichum* sp. from infected
   peanut seeds (*Arachis hypogaea L. cv Margenta*)

2. To study the antagonist potential of *Trichoderma* sp. as biocontrol against *C. gloeosporioides.*

3. To evaluate the effect of *C. gloeosporioides* on morphology, germination and fatty acid profile on the quality of peanut seed (*Arachis hypogaea L. cv Margenta*)

4. To determine the gene expression of putative Resistance Gene Candidates (RGCs) of peanut resistance to *C. gloeosporioides* with inoculant *Trichoderma* sp.

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