UNIVERSITI PUTRA MALAYSIA

PHENOTYPIC, MOLECULAR AND MATING TYPE ANALYSES OF EXSEROHILUM ROSTRATUM ASSOCIATED WITH LEAF SPOTS OF RICE

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PHENOTYPIC, MOLECULAR AND MATING TYPE ANALYSES OF *Exserohilum rostratum* ASSOCIATED WITH LEAF SPOTS OF RICE

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 Master of Science

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PHENOTYPIC, MOLECULAR AND MATING TYPE ANALYSES OF *Exserohilum rostratum* ASSOCIATED WITH LEAF SPOTS OF RICE

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Chairman : Nur Ain Izzati Mohd Zainudin, PhD
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Exserohilum rostratum is one of the plant pathogens causing leaf spot disease, which affects wide range of plant species, mainly grasses. There has been no report of *E. rostratum* causes the disease on rice plant particularly on leaves, therefore this study was conducted. During a series of sampling in March to August 2013, leaf spot is one of common symptoms found in rice plantations in Malaysia. The objectives of this study were to identify fungi isolated from leaf spot of rice based on phenotypic and molecular approaches, to determine whether or not *E. rostratum* isolates were pathogenic, to analyze the mating type of *E. rostratum* using Polymerase Chain Reaction (PCR) and to examine the sexual development of *E. rostratum* isolates. All isolated fungi were purified and identified based on their phenotypic features for species delimitation and confirmed by sequencing the internal transcribed spacers (ITS) region. Seventy-eight isolates were identified; *E. rostratum* (45 isolates), *Bipolaris sorokiniana* (10 isolates), *Curvularia hawaiiensis* (8 isolates), *C. geniculata* (6 isolates), *C. eragrostidis* (6 isolates), *C. aeria* (2 isolates) and *C. lunata* (1 isolate). A phylogenetic tree was constructed using neighbor-joining method showed the same species grouped in the same clade. Clade I consisted of *E. rostratum* while Clade II were *Bipolaris sorokiniana* and *Curvularia* species. Based on pathogenicity test, some isolates of *E. rostratum* were pathogenic and produced leaf spot symptoms towards rice. In this study, *E. rostratum* is minor or secondary pathogen to rice because it showed smaller lesion compared to infection by *B. oryzae*, the main pathogen of brown spot disease. Mating type (*MAT*) of all isolates of *E. rostratum* were amplified using two sets of primers (NM162MAT1_1F1Seto and NM163MAT1_1R1Seto for *MAT1-1*; NM164MAT1_2F1Seto and NM165MAT1_2R1Seto for *MAT1-2*). Three isolates were confirmed carried *MAT1-1* allele while 42 isolates are *MAT1-2* isolates. The imbalance distribution of both *MAT* may caused by ratio of modes of reproduction and selection pressure in the field. The opposite *MAT* isolates were crossed on Sach's medium for 3 weeks in quadruplicate. Nine isolates were indicated as fertile, which produced mature pseudothecia and ascospores, 12 isolates produced barren pseudothecia (empty asci) and the remaining 24 isolates were non-fertile without formation of pseudothecia. Three isolates of *E. rostratum* from this study; one *MAT1-1* isolate (UPM1109) and two isolates of *MAT1-2* (UPM1247 and UPM1152) are highly fertile and suggested to

be used as tester strains. The developed tester strains may facilitate other researchers to access the fertility on other related study on other *E. rostratum* isolates.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

FENOTIP, MOLEKULAR DAN ANALISIS JENIS PENGAWANAN *EXSEROHILUM ROSTRATUM* BERASSOSIASI DENGAN BINTIK DAUN PADA PADI

Oleh

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Exserohilum rostratum merupakan salah satu patogen tumbuhan yang menyebabkan bintik daun yang mana menjejaskan pelbagai jenis spesis tumbuhan terutamanya rumput. Tiada laporan berkenaan *E. rostratum* menyebabkan penyakit pada pokok padi terutamanya pada daun, oleh itu kajian ini dijalankan. Semasa siri pensampelan dijalankan antara Mac hingga Ogos 2013, bintik daun adalah salah satu simptom yang biasa ditemui di sawah padi di Malaysia. Objektif kajian ini adalah untuk memencilkan dan mengecam kulat daripada bintik daun padi berdasarkan pendekatan fenotip dan molekular, untuk mengkaji sama ada pencilan-pencilan *E. rostratum* adalah patogenik, untuk menganalisis jenis pengawanan *E. rostratum* menggunakan *Polymerase Chain Reaction* (*PCR*) dan untuk mengkaji perkembangan seksual pencilan *E. rostratum.* Kesemua pencilan kulat telah dipencilkan dan dicamkan berdasarkan ciri fenotip untuk pengecaman species dan disahkan dengan analisis jujukan kawasan *Internal Transcribed Spacer* (ITS). Tujuh puluh lapan pencilan telah berjaya dicamkan; *E. rostratum* (45 pencilan), *Bipolaris sorokiniana* (10 pencilan), *Curvularia hawaiiensis* (8 pencilan), *C. geniculata* (6 pencilan), *C. eragrostidis* (6 pencilan), *C. aeria* (2 pencilan) and *C. lunata* (1 pencilan). Pohon filogeni telah dibina menggunakan kaedah *neighbor-joining*, menunjukkan bahawa spesies yang sama dikelaskan dalam clad yang sama. Clad 1 terdiri daripada *E. rostratum* manakala Clad II adalah *Bipolaris sorokiniana* dan spesies *Curvularia*. Berdasarkan ujian kepatogenan, beberapa pencilan *E. rostratum* adalah patogenik dan menghasilkan simptom bintik daun terhadap padi. Di dalam kajian ini, *E. rostratum* ialah patogen minor atau sekunder kepada padi kerana menunjukkan lesi bersaiz kecil berbanding jangkitan oleh *B. oryzae*, patogen utama penyakit bintik perang. Jenis pengawanan (*MAT*) untuk semua pencilan *E. rostratum* diamplikasikan menggunakan dua set primer (NM162MAT1_1F1Seto and NM163MAT1_1R1Seto untuk *MAT1-1*; NM164MAT1_2F1Seto and NM165MAT1_2R1Seto untuk *MAT1-2*). Tiga pencilan telah disahkan mempunyai *MAT1-1* manakala 42 pencilan adalah *MAT1-2*. Ketidakseimbangan pengagihan untuk kedua-dua *MAT* mungkin disebabkan nisbah cara pembiakan dan tekanan pemilihan di lapangan. Pencilan yang bertentangan *MAT* telah dikacukkan di atas media Sach selama tiga minggu dengan empat replikasi. Sembilan pencilan adalah subur, di mana menghasilkan pseudotesia dan ascospora

matang, 12 pencilan menghasilkan pseudotesia yang mandul (askus kosong) dan baki 24 pencilan adalah tidak subur tanpa pembentukan pseudotesia. Tiga pencilan *E. rostratum* daripada kajian ini, satu pencilan *MAT1-1* (UPM1109) dan dua pencilan *MAT1-2* (UPM1247 dan UPM1152) adalah paling subur dan dicadangkan untuk digunakan sebagai strain penguji. Strain yang ditemui dapat membantu penyelidik lain untuk memeriksa kesuburan strain *E. rostratum* yang lain di dalam kajian yang berkaitan.

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I certify that a Tesis Examination Committee has met on (10 August 2015) to conduct the final examination of Nor Azizah Kusai on her tesis entitled "Phenotypic, Molecular and Mating Type Analyses of *Exserohilum rostratum* Associated with Leaf Spot of Rice" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putera Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Master of Science.

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LIST OF ABBREVIATIONS

%	Percentage
^{o}C	Degree celcius
µl	Microliter
µm	Micrometer
µM	Micromolar
$\times g$	Centrifugal force
bp	Base pair
$Ca(NO_3)_2$	Calcium nitrate
$CaCO_3$	Calcium carbonate
cm	centimeter
CM	Complete medium
CMX	Complete medium xylose
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dpi	days post-inoculation
EtBr	Ethidium bromide
g	gram
h	hour
ha	hectares
IADA	Kawasan Pembangunan Pertanian Bersepadu
ITS	Internal transcribed spacer
K_2HPO_4	Potassium hydrogen phosphate
KADA	Lembaga Kemajuan Pertanian Kemubu
kb	kilo base
KETARA	Kawasan Pembangunan Pertanian Bersepadu Terengganu Utara
kg	kilogram
L	liter
MADA	Lembaga Kemajuan Pertanian MUDA
MAT	Mating type
mg	miligram
$MgSO_4$	Magnesium sulphate
min	minutes

ml	milliter
ng	nanogram
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
rpm	revolution per minute
s	second
TBE	Tris-Boric acid-EDTA
V	Volt
WA	Water agar

CHAPTER 1

INTRODUCTION

Kingdom of Fungi is composed of most diverse species on Earth consisting of pathogens, saprobes and endophytes. According to The Dictionary of Fungi by Kirk *et al.* (2008), there are about 99000 known species recorded. The ascomycetes fungus, *Exserohilum* is an important phytopathogens that cause high yield losses and pathogenic towards human and animals. *Exserohilum rostratum* is a plant pathogen that affects more than 30 plants species comprises of 28 genera in 11 families (Lin *et al.*, 2011) including the important crops such as sugarcane, tomato, sweet sorghum and wheat which leads severe yield losses (Ahmadpour *et al.*, 2013; Cardona and González, 2007; Luan *et al*., 2004). *Exserohilum rostratum* has been reported to cause leaf spot and blight in many plant species particularly grasses (Sharma *et al.*, 2014). However, there have been no reports found on disease of rice plant caused by *E. rostratum.*

Rice (*Oryza sativa*); classified under family Poaceae is an important cereal plants cultivated across the world. Rice supplied with variety of nutrient and consumed as staple food for humans and animals in many parts of the world. Rice consumption was projected to increase by 2030 with 873 million tonnes compared to year 2000 (Kubo and Purevdorj, 2004). People in Southeast Asia; consume rice as more than 50% of their daily diet composition (Fitzgerald *et al.*, 2009). A high demand of rice gives impact on rice industry over the world and gets a lot of attention by researchers. Rice production has contributed in Malaysia's agricultural sector development. Up to 2013, Malaysia has 688207 hectares of rice cultivation, which is the third largest crop cultivated (Department of Agriculture, 2014a). In Malaysia, wetland rice is grown in Peninsular Malaysia as the main rice cultivation compared to upland rice, which can only be found in Borneo Island. Rice production plays important roles in Malaysia as national's food security, self-sufficiency and source of country's income (Fahmi *et al.*, 2013). With that, the studies on etiology and disease distribution of rice are important to help in disease management.

Abiotic and biotic pressure are the limiting factors in rice cultivation and always give significant problems to rice growing in field especially in large cultivation areas. Biotic pressures such as pests and pathogens caused severe damage and losses of rice yield (Oerke *et al*., 2012). Fungi are major pathogens infecting rice during seedling up to maturing stage occurring on all parts of plants. Leaf spot, blast, blight and smut are the common diseases found in field caused by infestation of several fungal species. Leaf spots such as narrow brown spot, brown spot and blast disease frequently found in all rice growing areas. However, in Asia, especially Malaysia, limited number of references was available on the disease. Therefore, etiology of ascomycete fungi including *Exserohilum* species was studied.

Phenotypic characteristics are the primary step in identifying plant pathogens. Characterization based on phenotype of *E. rostratum* was identified through macroscopic and microscopic level. Both levels provide information to determine and limit the species identification. However, this step always leads to misidentification because of lack of standardized terminology and high subjectivity depends on environmental condition (Guarro *et al.*, 1999). Therefore, phytopathology researchers have extensively used molecular method to confirm the species identification. In recent years, characterization of DNA sequences have been used to determine fungal species based on phylogenetic species concept (Taylor *et al.*, 2000). Advances of molecular studies have enabled to distinguish small DNA sequences between species. Target genes such as rRNA, translation elongation factors 1-alpha (*tef1-α*), β-tubulin, glyceraldehyde-3-phosphate dehydrogenase (GPDH) are generally used to provide sufficient information to determine species (Manamgoda *et al*., 2012; Geiser *et al*., 2004). Internal transcribed spacer of rRNA was widely used for species differentiation between *Exserohilum* since it clearly defined interspecific variation (Sharma *et al.*, 2014; Wu and Turgeon, 2013; Lin *et al.*, 2011).

To make the study more comprehensible, biological species concept are also applied in characterization of *E. rostratum* based on sexual development. Development of fungus progeny sexually requires nuclear fusion and meiosis processes. This process leads to exchange of genetic materials between two organisms and form variation and recombination of progeny. The progeny produced through sexual stage is more vigor and have better chances to survive in harsh environment. Sexually produced generations will display variation in their traits such as virulence phenotype (Alters and Alters, 2006). Sexual reproduction can be applied in investigation of species invasion, conservation biology, pathogen evolution and control method of pathogen (Billiard *et al*., 2012). Billiard *et al.* (2012) also state that sexual reproduction important which can affect the ecological process of the species through adaptation or colonization.

The study of mating type of organism has increased throughout the years since a century ago. Mate selection and mating process is significant event in the life of a fungus. Mating type (*MAT*) is used as a term in filamentous fungi and non-filamentous fungi to indicate genetic regulation of mating specificity (Kronstad and Staben, 1997). *MAT* consists of two functional alleles, *MAT1-1* and *MAT1-2* that occupy in single locus chromosome (Debuchy and Turgeon, 2006). Sexual reproduction process was initiated when there is interaction between opposite *MAT* alleles under favorable condition. The sexual development of fungus will form fruiting body known as ascocarps and ascospores as the progeny. Traditionally, *MAT allele* was determined using sexual crosses that required tester strains. However, this approach is time-consuming method. Mating type analyses using molecular technique is new insight and recently used in mating type studies because of rapid, effective, and accurate routine technique (Steenkamp *et al*., 2000; Gafur *et al.*, 1997).

Exserohilum rostratum is heterothallic fungus and the teleomorph stage can only be obtained by crossing between opposite mating type. The teleomorph stage of *E.rostratum* known as *Setosphaeria rostrata* and the fruiting body formed is called pseudothecia with bitunicate ascus. Leonard (1976) firstly described teleomorph stage

of *E. rostratum* under laboratory condition using in-vivo culture. As *E. rostratum* becoming important plant pathogens and found in broad range host, the studies on their sexual development are crucial. The tester strains for *E. rostratum* are still undeveloped, to study the specific aspect of *E. rostratum* such as genetic, physiological and plant-pathogen interaction. Therefore, this study was conducted to develop the standard tester strain for *E. rostratum* studies.

The result will provide the information on diversity of *E. rostratum* associated with leaf spots of rice in Peninsular Malaysia, as references for related species and facilitate other researchers on *E. rostratum* studies.

Therefore, the objectives of this study were;

1) To isolate and identify *Exserohilum rostratum* and other associated fungi based on morphological and molecular characterization
2) To determine whether or not the *E. rostratum* isolates isolated from leaf spot of rice were pathogenic
3) To determine the mating type and to examine the sexual development of *E.rostratum* isolates

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LIST OF PUBLICATIONS

Journal:

Nor Azizah Kusai, Madihah Mior Zakuan Azmi, Shahrizim Zulkifly, Mohd Termizi Yusof, and Nur Ain Izzati Mohd Zainudin. (2015). Morphological and molecular characterization of *Curvularia* and related species associated with leaf spot disease of rice in Peninsular Malaysia. Published online in *Rendiconti Lincei*. DOI 10.1007/s12210-015-0458-6.

Nor Azizah Kusai, Madihah Mior Zakuan Azmi, Mohd Termizi Yusof, Azmi Abd Razak and Nur Ain Izzati Mohd Zainudin (2015). Morphological, molecular, virulence and development of *Setosphaeria rostrata* associated with leaf spot of rice. Accepted with major correction in *Mycologia*.

Proceedings:

Nor Azizah Kusai, Mohd Termizi Yusof, Shahrizim, Zulkifly, Azmi Abd Razak, and Nur Ain Izzati Mohd Zainudin (2014). *Morphological Characterization of Setosphaeria rostrata and Cochliobolus species Isolated from Diseased-Rice Leaves Samples in Selangor*. Paper presented at the Malaysia International Biological Symposium (i-SIMBIOMAS 2014), Palm Garden Hotel, Putrajaya.

Nor Azizah Kusai, Mohd Termizi Yusof and Nur Ain Izzati Mohd Zainudin (2014). *Mating Type Analyses of Setosphaeria rostrata Isolated From Brown Spot Disease of Rice in Peninsular Malaysia.* Paper presented at the Monash Science Symposium 2014, Monash University Campus Bandar Sunway.

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