UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF Lasiodiplodia theobromae (B4d) ISOLATED FROM KENAF SEEDS AND ITS SENSITIVITY TOWARDS DIAZOTROPHIC BACTERIA

NORHAYATI BINTI MADIHA

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By

NORHAYATI BINTI MADIHA

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

August 2013

With the name of Allah S.W.T.

Specially Dedicated To

My beloved mother and father,

My brothers and family

My best friends and colleagues

Thank you for being with me

through all ups and downs during this long journey

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

CHARACTERIZATION OF *Lasiodiplodia theobromae* (B4d) ISOLATED FROM KENAF SEEDS AND ITS SENSITIVITY TOWARDS DIAZOTROPHIC BACTERIA

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NORHAYATI BINTI MADIHA

August 2013

Chairman: Kamaruzaman Sijam, PhD

Faculty: Agriculture

Hibiscus cannabinus L. is commonly known as kenaf grows natively in east-central Africa and used in production of ropes, canvas, board making, oil absorption and animal feed. Kenaf is not only tolerant to many types of soil and other planting conditions; it is also resistant to pests and diseases. However, several diseases have been reported infecting the kenaf plant. Many studies were conducted on disease infected the leaves and roots but there is insufficient research effort to identify the causal agent of the disease on initial growth of kenaf causing loss of germination in seeds. The study was aimed to isolate and characterize the causal pathogen of seed-borne disease on kenaf seed in Malaysia using morphological and molecular techniques and to confirm the pathogenicity. The second objective was to identify the potential diazotrophic bacteria in order to control the infection of seed-borne fungus. For isolation of the seed-borne fungus, the seed source was obtained from National Tobacco and Kenaf Board (LKTN) in Perlis. Twenty-one of fungal isolated from the

seeds were obtained and tested for their pathogenicity. This study found that the fungus B4d showed highest isolation frequency and pathogenic to the seed. Morphological characterization of fungus B4d indicated that the colony was fast growing fungus. The conidia were dark brown in colour, two-celled, thin cell walled and oval shape with longitudinal striation when mature. In immature stage, the conidia were hyaline, smooth, single-celled and have thick cell walled. The average size of conidia was 24 µm (length) x 15 µm (width) while the average length/width ratio was 1.11 (n=50). The morphological findings described the fungus B4d as *Lasiodiplodia theobromae.* Identification of fungus B4d was also done through molecular characterization using 18S Internal Transcribed Spacer (ITS) regions of ribosomal deoxyribonucleic acid (rDNA). BLAST result indicated that the fungus was 100% similar to that of *L. theobromae* (JQ809341). Phylogenetic tree grouped the fungus in the same group with *L. theobromae* and distinct from other anamorph in the genus *Botryosphaeria*. The dual culture assay was conducted to determine diazotrophic bacteria that can suppress the *L. theobromae* growth. Among 61 bacterial strains isolated from kenaf roots, three strains were exhibited potent antagonistic effect against *L. theobromae*. Percentage inhibition radial growth (PIRG) values showed significant difference ($P \leq 0.05$) on antagonism activity of the fungi. Three bacterial isolates recorded PIRG value more than 60% on *L. theobromae*. The highest PIRG value was shown by IRPP1b followed with RFC1a and RKBP3d, respectively. Selected diazotrophs have been identified. Molecular technique using 16S rDNA markers analysis confirmed the bacterial species were *Burkholderia cepacia* (JF820823), *Burkholderia vietnamiensis* (JF833110) and *Bacillus subtilis* (JF833115). Selected antagonistic bacteria were used in seed treatment in glasshouse. Treatments of seeds with diazotrophic bacterial suspension

significantly reduced the infection of *L. theobromae* ($P \leq 0.05$). This study showed that *B. cepacia* has more potential to reduce the fungal infection was 83% followed by *B. vietnamiensis* (78%) and *B. subtilis* (67%), respectively. The germination rate of kenaf seeds was increased by the time of inoculation with bacterial isolates. Therefore, *B. cepacia, B. vietnamiensis* and *B. subtilis* have a good potential in suppressing the growth of *L. theobromae* and increase the germination rate of kenaf seeds.

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

CIRI-CIRI *Lasiodiplodia theobromae* (B4d) DIASINGKAN DARIPADA BIJI BENIH KENAF DAN KESENSITIFANNYA KEPADA BAKTERIA DIAZOTROFIK

Oleh

NORHAYATI BINTI MADIHA

Ogos 2013

Pengerusi: Kamaruzaman Sijam, PhD

Fakulti: Pertanian

Hibiscus cannabinus L. adalah dikenali sebagai kenaf yang tumbuh asli di timur tengah Afrika dan digunakan dalam penghasilan tali, pembuatan papan, penyerap minyak dan makanan ternakan. Kenaf bukan sahaja rentan kepada pelbagai jenis tanah dan keadaan penanaman; ia juga rentan kepada haiwan perosak dan penyakit. Kajian telah dijalankan pada penyakit yang menjangkiti daun dan akar namun masih kurang kajian ke atas pengenalpastian agen penyebab penyakit pada awal pertumbuhan kenaf yang menyebabkan kegagalan percambahan biji benih. Kajian bertujuan untuk mengasingkan dan mengkaji ciri-ciri patogen penyebab penyakit biji benih kenaf di Malaysia menggunakan perincian morfologi dan teknik molekular serta pengesahan kepatogenannya. Objektif kedua adalah mengenalpasti bakteria diazotrofik berpotensi untuk mengawal jangkitan kulat biji benih. Untuk pengasingan kulat, biji benih diperolehi daripada Lembaga Tembakau dan Kenaf Negara (LKTN)

di Perlis. Terdapat 21 isolat kulat diasingkan daripada biji benih dan kepatogenannya diuji. Kajian mendapati kulat B4d menunjukkan frekuensi pengasingan dan kepatogenan paling tinggi ke atas biji benih. Ciri morfologi kulat B4d menunjukkan koloni kulat ini bertumbuh dengan cepat. Koloninya berwarna coklat, sel dubel, dinding sel nipis dan berbentuk oval dengan jalur menegak semasa matang. Pada peringkat pra-matang, konidia adalah lutcahaya, licin, sel tunggal dan dinding sel tebal. Purata saiz konidia adalah 24 µm (panjang) x 15 µm (lebar) manakala purata panjang/lebarnya ialah 1.11 (n=50). Kajian morfologi menerangkan B4d ialah *Lasiodiplodia theobromae.* Pengenalpastian kulat B4d turut dilakukan melalui ciri-ciri molekul menggunakan 18S 'Internal Transcribed Spacer (ITS)' ribosom asid deoksiribonukleik (rDNA). Keputusan BLAST menunjukkan kulat ini 100% sama dengan *L. theobromae* (JQ809341). Hubungan filogeni menghimpunkan kulat ini dalam kumpulan yang sama dengan *L. theobromae* dan berbeza daripada anamof lain di dalam genus *Botryosphaeria*. Kajian kultur dual dijalankan untuk menentukan bakteria diazotrofik yang boleh menghadkan pertumbuhan *L. theobromae.* Di antara 61 isolat bakteria dari akar kenaf, tiga strain menunjukkan kesan antagonis menentang *L. theobromae.* Nilai peratus perencatan pertumbuhan radial (PIRG) menunjukkan perbezaan signifikan ($P \leq 0.05$) pada aktiviti antagonis ke atas kulat. Tiga isolat bakteria merekodkan nilai PIRG lebih daripada 60% ke atas *L. theobromae*. Nilai PIRG tertinggi direkodkan oleh IRPP1b, diikuti dengan RFC1a dan RKBP3d. Bakteria diazotrofik yang terpilih dikenal pasti. Teknik molekular menggunakan analisis 16S rDNA mengesahkan bahawa spesies bakteria tersebut adalah *Bukholderia cepacia* (JF820823), *Bukholderia vietnamiensis* (JF833110) dan *Bacillus subtilis* (JF833115). Bakteria antagonis yang terpilih ini digunakan untuk rawatan biji benih di dalam rumah kaca. Rawatan biji benih dengan bakteria

diazotrofik mengurangkan jangkitan *L. theobromae* ($P \leq 0.05$). Kajian ini menunjukkan *B. cepacia* lebih berpotensi mengurangkan jangkitan kulat sebanyak 83%, diikuti dengan *B. vietnamiensis* (78%) dan *B. subtilis* (67%). Kadar percambahan biji benih kenaf meningkat dengan masa inokulasi isolat bakteria. *B. cepacia*, *B. vietnamiensis* and *B. subtilis* berpotensi baik dalam mengehadkan pertumbuhan *L. theobromae* dan meningkatkan kadar percambahan biji benih kenaf.

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Thank you very much.

I certify that a Thesis Examination Committee has met on 28 August 2013 to conduct the final examination of Norhayati binti Madiha on her thesis entitled "Characterization of *Lasiodiplodia theobromae* (B4d) isolated from kenaf seeds and its sensitivity towards diazotrophic bacteria" in accordance with the Universities and University College 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

Lau Wei Hong, PhD
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Shuhaimi Mustafa, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner 1)

Jugah bin Kadir, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner 2)

Latiffah binti Zakaria, PhD
Associate Professor
School of Biological Science
Universiti Sains Malaysia
(External Examiner)

_____

NORITAH OMAR, Phd
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 20 November 2013

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:

Kamaruzaman Sijam, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Radziah Othman, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

_____

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotation and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any degree at Universiti Putra Malaysia or at any other institution.

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NORHAYATI BINTI MADIHA

Date: 28 August 2013

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LIST OF ABBREVIATIONS

µM	micromolar
g	Gram
µL	microlitre
mL	millilitre
min	minute
^{o}C	degree celcius
%	percent
rpm	rotation per minute
UV	ultraviolet
CLA	carnation leaf agar
WA	water agar
CRD	complete randomized design
CTAB	N-Cetyl-N,N,N-trimethyl-ammonium bromide
DNA	deoxyribonucleic acid
kb	kilo base
PDA	potato dextrose agar
h	hour
PCR	Polymerase chain reaction
DMRT	Duncan's Multple Range Test
µm	Micrometre
L/W ratio	Length/width ratio
mm	millimetre
M	Molar
EDTA	Ethylenediamine tetra acetic acid
bp	base pair

NFA	Nitrogen free agar
ITS	internal transcribe spacer
V	Volt
TAE	Tris-acetate EDTA
NA	nutrient agar
cfu	colony forming unit
µmol	micromolar
kg	kilogram
cmol	centimol
ppm	part per million
N	nitrogen
ha	hectares
mg	milligram

CHAPTER 1

INTRODUCTION

Kenaf is a fibrous plant which belongs to the *Malvaceae* family. The plant is generally grouped together with genus *Hibiscus* with more than 400 species grown worldwide. Kenaf is known to be similar to roselle (*Hibiscus sabdariffa* L.) has been recognized as kenaf in several places. Kenaf has more than 129 common names (Dempsey, 1975) such as mustard (India), Stokroos (South, Africa), Java jute (Indonesia), ambary (Taiwan) (Li, 1980).

Kenaf was cultivated around the world to made variety of products such as ropes and oil absorbent. Kenaf produce natural fibres which have a good quality and suitable as a board raw material. Kenaf has been used as well as oil absorbents, filtration medium, ropes and carpets. Kenaf seeds are suitable for biomass production such as animal feed.

In Malaysia, kenaf is cultivated in several states including Kelantan, Terengganu, Perlis and Pahang. The current policy introduced by the Government is to plant kenaf as a replacement crop of tobacco nationwide. Like any other fibre crops, kenaf is also affected by biotic and abiotic stresses that can limit their productivity. Kenaf was previously considered as resistant crop because they require minimum pesticide application during their cultivation period. Recent years, kenaf has been reported to be infested by a few pests and diseases.

Seed-borne disease affects a wide range of plant host and several pathogens including *Rhizoctonia solani*, *Fusarium monoliforme*, *Ustilago maydis*, *Aspergillus flavus*, *Alternaria* sp. (Gwary et al., 2006) and *Botryosphaeria* sp. These pathogens can cause many types of symptoms on seeds such as blacken of seeds, discoloration, shrivelling and un-germinated. This will reduce seed germination and affects the seedling growth (Mamatha et al. 2000). Seed-borne pathogens infect externally and internally from the cracks or wounds present on the seed (Singh, 1996). Severe infection reduces the emergence of radical and later infection causing rotten of seed and cotyledon. Chemical treatment is common method used by seed production companies in order to control the infection of seed-borne pathogens. However, these treatments sometimes affect the seeds quality. Hence, a new approach using beneficial microorganisms has been implemented to control the pathogens for example diazotrophic bacteria such as *Bacillus* spp., and *Pseudomonas* spp (Anjaiah et al., 2003; Ongena et al., 2005)

Seed-borne pathogens have recently been reported in kenaf seeds in Malaysia. However, there are no official reports or research efforts been taken to identify the causal agent and the effect of seed-borne fungal pathogen on seed germination. Therefore, the objectives of this research were:

1. To isolate, identify and characterize the seed-borne fungal of kenaf seeds in Malaysia using morphological and molecular characterization.
2. To characterize diazotrophic bacteria against seed-borne fungal pathogen.

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