UNIVERSITI PUTRA MALAYSIA

INFECTION PROCESSES AND PATHOGENESIS MECHANISM OF Fusarium proliferatum IN BAKANAE DISEASE

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By

QUAZI SHIREEN AKHTER JAHAN

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

September 2014

DEDICATION

Dedicated to my parents, husband, my daughter Sabera Samin Kafil

and

my son Ayman Kafil

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

INFECTION PROCESSES AND PATHOGENESIS MECHANISM OF *Fusarium proliferatum* IN BAKANAE DISEASE

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Chairperson: Sariah Meon Professor, PhD

Faculty: Institute of Tropical Agriculture

Bakanae disease caused by *Fusarium (F.)* species (spp.) was first reported to have a significant effect on Malaysian rice varieties in 1985 from the rice growing areas of Kedah, Kelantan and Perak. A total of five *Fusarium* spp. i.e. *F. fujikuroi*, *F. proliferatum*, *F. sacchari*, *F. subglutinans* and *F. verticillioides* were isolated and found to be associated with Bakanae disease in Malaysia. It was essential to identify the causal agent using morphological characteristics along with molecular techniques. There was little information on the infection processes and mechanisms of pathogenesis of *Fusarium* species in relation to development and symptoms expression of Bakanae disease in infected plants. Gibberellic acid (GA_3) produced by the causal pathogen has been identified to be responsible for internode elongation, but a number of secondary metabolites produced by the causal pathogen in relation to disease development and symptoms expression were still unknown. Therefore, the aims of this research were to identify the causal *Fusarium* species in Selangor rice growing areas, to establish the role of phytohormonal imbalances and metabolites production by the fungus and pathogenesis-related (PR) protein activities in relation to disease symptoms expression in infected plants and to evaluate the symptoms expression analysis of Bakanae disease by pre-seed treatment with pure (synthetic) phytohormones and metabolites in susceptible rice variety MR 211.
A total of 12 isolates from Tanjung Karang and from Sekinchan areas were obtained. All isolates were identified as *Fusarium proliferatum* by PCR with Pro1/2 primer and further confirmed by sequencing (Acc. JQ807850) in both

directions. Seed inoculation was found to be the most suitable method compared to soil inoculation for pathogenicity test. SEM micrographs showed conidia of *F. proliferatum* germinated on seed surface of susceptible variety MR 211, 24 h after inoculation followed by colonization and symptoms initiation after 5 days after inoculation. Based on the varietal screening, MR 211 was identified as susceptible, whereas G-27 was moderately resistant and BR3 was resistant. In phytohormone analysis, highest up-regulation of IAA (94.1%) and a marginal percentage of GA_3 (9.35%) occurred mostly in leaf tissues, whereas they were down regulated in root tissues (GA_3 = -37.61%; IAA= -16.32%) and in the stem (GA_3 = -29.75%; IAA= -39.21%) of infected susceptible variety MR 211 after 7 days of inoculation at a disease score of 1 (stunted plant with yellowing leaves). This indicates that up- and down regulation of phytohormones was associated with the expressed symptoms in elongation and the stunting effect coincides with chlorosis of leaves. In disease score 3 (abnormal elongated internodes with chlorotic or brownish leaves), the up-regulation of all analyzed phytohormones were observed to be high in stem tissues of susceptible variety MR 211 with GA_3 = 77.46%, IAA = 87.38% and ABA = 98.55% compared to leaf and root tissues. In infected plants with disease score 5 (leaf and stem browning with elongated internodes, fungal mass seen on the infected plants or dead plant), the amounts of GA_3 and IAA were down-regulated in susceptible MR 211 and this down regulation was reflected in the growth and development of leaves and roots and resultant senescence. Among the metabolites evaluated, moniliformin (MON) and fusaric acid (FA) were found to be associated with symptoms development and expression in susceptible variety MR 211. MON was found at disease score 3 (83.67 ng/g) and at disease score 5 (112.81 ng/g) in infected MR 211 plants. FA was found at all three disease score levels in infected susceptible MR 211 plants, but higher amounts were observed at disease score 1 (354.41 µg/g) and at disease score 5 (372.38 µg/g). No MON or FA was detected in resistant BR3 plant samples. The association of phytohormone GA_3 and secondary metabolites FA and MON in relation to Bakanae symptoms expression were further confirmed when seeds of susceptible variety MR 211 were treated with synthetic GA_3 phytohormone and synthetic FA and MON metabolites in the glasshouse. It was observed that GA_3 was responsible for plant height elongation and leaf chlorosis, whereas FA was associated with plant height retardation, root growth retardation and chlorosis of leaves, and moniliformin was found to be associated with leaf and stem browning, crown rot and root necrosis in susceptible variety MR 211. Among the PR-proteins chitinase activity was found to be more prominent in the disease susceptibility/resistance mechanism against *F. proliferatum* than ß-1, 3 glucanase activities. Chitinase activity increased from disease score 1 to disease score 5 in resistant variety BR3, whereas no activity was observed in susceptible variety MR 211. This is the first report that *F. proliferatum* is the pathogen causing Bakanae disease in rice plants and that metabolites FA and MON along with phytohormone GA_3 produced by *F. proliferatum* in diseased plants are associated with a variety of disease symptoms expression.

Abstrak tesis yang dikemukakan kepada Senat Umiversiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falasafah

PROSES JANGKITAN DAN MEKANISME PATOGENESIS *Fusarium proliferatum* DALAM PENYAKIT BAKANAE

Oleh

QUAZI SHIREEN AKHTER JAHAN

September 2014

Pengerusi: Sariah Meon Professor, PhD

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Penyakit Bakanae disebabkan oleh *Fusarium* (F.) spesis (spp.) pertama kali dilaporkan mempunyai kesan yang ketara kepada varieti padi Malaysia pada tahun 1985 dari kawasan penanaman padi di Kedah, Kelantan dan Perak. Terdapat lima spesis *Fusarium, F. fujikuroi*, *F. proliferatum*, *F. sacchari*, *F. subglutinans* dan *F. verticillioides* telah disaringkan dan didapati berkaitan dengan penyakit Bakanae di Malaysia. Ia adalah penting untuk mengenalpasti agen penyebab dengan menggunakan ciri morfologi dan teknik molekular. Terdapat sedikit maklumat mengenai proses jangkitan dan mekanisma patogenesis spesies *Fusarium* berhubung dengan perkembangan penyakit Bakanae dalam tumbuhan yang dijangkiti. Asid giberelik (GA_3) yang dihasilkan oleh patogen telah dikenalpasti menyebabkan pemanjangan buku, tetapi beberapa metabolisma sekunder yang dihasilkan oleh patogen berhubung dengan perkembangan penyakit masih tidak diketahui. Oleh itu, tujuan kajian ini adalah untuk mengenalpasti spesis *Fusarium* yang menyebabkan penyakit di kawasan penanaman padi sekitar Selangor, mengenalpasti peranan ketidakseimbangan hormonfito, dan penghasilan metabolit oleh kulat dan hubungan kepatogenan (PR) aktiviti protein patogenesis yang berkaitan dengan gejala penyakit yang ditunjukkan dan untuk menilai analisis gajala penyakit yang ditunjukkan oleh penyakit Bakanae melalui rawatan pra-cambah dengan hormonfito asli (sintetik) dan metabolit dalam biji benih variati yang peka MR 211. Sebanyak 12 saringan; dari kawasan Tanjung Karang dan kawasan Sekinchan telah disaringan. Semua saringan telah dikenalpasti sebagai *F. proliferatum* hasil daripada saringan PCR ini telah diperkuatkan dengan Pro1/2 dan seterusnya disahkan oleh penjujukan (Acc. JQ807850) di kedua-dua hala.

Untuk ujian patogenisiti, inokulasi benih dikenalpasti sebagai kaedah yang paling sesuai berbanding dengan inokulasi tanah. Pemerhatian mikroskop SEM menunjukkan konidia *F. proliferatum* bercambah pada permukaan biji benih MR 211 padi yang peka, selepas 24 jam inokulasi diikuti dengan penjajahan dan gejala permulaan selepas 5 hari inokulasi. Berdasarkan pemeriksaan varieti, MR 211 telah dikenalpasti sebagai peka, manakala G-27 adalah sederhana tahan dan BR3 adalah spesis rintang penyakit. Dalam hasil analisis hormonfito menunjukkan, peratusan IAA tertinggi (94.1%) dan peratusan terendah ialah GA_3 (9.35%) kebanyakannya berlaku dalam tisu daun, sedangkan mereka turut dikawal selia dalam tisu akar (GA_3 = -37.61%; IAA= -16.32%) dan dalam batang (GA_3 = -29.75%; IAA = -39.21%) daripada varieti peka penyakit MR 211 selepas 7 hari inokulasi pada skor penyakit 1 (tumbuhan terbantut beserta daun kekuningan). Ini menunjukkan bahawa regulasi hormonfito ke atas dan ke bawah dikaitkan dengan gejala yang dinyatakan di dalam pemanjangan dan kesan pertumbuhan terbantut bertepatan dengan gejala warna klorosis pada daun. Pada penyakit skor 3 (pemanjangan luar biasa antara buku dangan klorosis atau keperangan daun), regulasi meningkat semua hormonfito yang dianalisis diidapati tinggi dalam tisu batang variety peka dengan GA_3 = 77.46%, IAA = 87.38% dan ABA = 98.55% berbanding tisu daun dan akar. Dalam tumbuhan yang dijangkiti dengan skor penyakit 5 (daun dan batang keperangan beserta pemanjangan buku, jisim kulat dilihat pada pokok yang dijangkiti atau pokok mati), jumlah GA_3 dan IAA telah turun dikawal selia dalam varieti peka MR 211 dan regulasi turun ini digambarkan dalam pertumbuhan dan pembangunan daun dan akar dan terhasilnya penuaan. Antara metabolit dinilai, moniliformin (MON) dan asid fusarik (FA) telah didapati berkaitan dengan pembangunan gejala dalam pelbagai varieti peka MR 211. MON ditemui pada penyakit skor 3 (83.67 ng/g) dan pada penyakit skor 5 (112.81 ng/g) dalam tumbuhan MR 211 yang dijangkiti. FA ditemui di semua tiga tahap skor penyakit dalam tumbuhan peka MR 211, tetapi jumlah yang lebih tinggi diperhatikan pada penyakit skor 1 (354.41 εg/g) dan pada penyakit skor 5 (372.38 εg/g). Tiada MON atau FA dikesan pada sampel tumbuhan rintang penyakit BR3. Pertalian hormonfito GA_3 dan metabolit sekunder FA dan MON berhubung dengan gejala Bakanae telah disahkan lagi apabila benih varieti peka MR 211 telah dirawat dengan hormonfito sintetik GA_3 dan sintetik FA dan metabolit MON dalam rumah kaca. Diperhatikan bahawa GA_3 bertanggungjawab untuk pemanjangan tinggi tanaman dan warna kuning pada daun, sedangkan FA dikaitkan dengan perencatan ketinggian tumbuhan, pertumbuhan akar terencat dan warna kuning pada daun, dan moniliformin ini didapati berkaitan dengan daun dan warna coklat pada batang, reput pangkal dan nekrosis pada akar dalam varieti peka MR 211. Antara PR-protein, aktiviti kitinase didapati lebih menonjol dalam mekanisma sesuatu penyakit/rintangan terhadap *F. proliferatum* berbanding aktiviti ɓ -1, 3 glucanase. Aktiviti kitin di dapati pada kadar yang semakin meningkat daripada penyakit skor 1 kepada penyakit skor 5 dalam varieti rintang BR3, manakala tiada aktiviti diperhatikan dalam varieti peka MR 211. Ini adalah laporan pertama *F. proliferatum* adalah patogenik untuk menyebabkan penyakit Bakanae dalam tanaman padi dan metabolit FA dan MON bersama-sama

dengan GA_3 dihasilkan oleh *F. proliferatum* dalam tumbuhan berpenyakit Bakanae dikaitkan dengan pelbagai gejala penyakit.

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I certify that a Thesis Examination Committee has met on 23 September 2014 to conduct the final examination of Quazi Shireen Akhter Jahan on her thesis entitled "Infection Processes and Pathogenesis Mechanism of *Fusarium proliferatum* in ... Bakanae Disease" 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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The 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 Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

Ab	Absorbance
ABA	Abscisic acid
Acc	Accession
AFLP	Amplified Fragment Length Polymorphism
Ŭ	Alpha
ANOVA	Analysis of Variance
BEA	Beauvericin
ɓ	beta
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BR	Brassinosteroid
BRRI	Bangladesh Rice Research Institute
CLA	Carnation leaf agar
cm	Centimeter
CK	Cytokinin
CTAB	Cetytrimethyl ammonium bromide
CRD	Completely randomized design
ºC	Degree Celsius
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DI	Disease incidence
DSI	Disease Severity Index
DSS	Disease Severity Scale

EC	Emulsifiable concentrate
EDTA	Ethylene diamine tetra acetic acid
EF	Elongation factor
ELISA	Enzyme - linked immunosorbent assays
ET	Ethylene
F	*Fusarium*
FA	Fusaric acid
FB1	Fumonisin
G	Germplasm
g	Gram
GRSD	Genetic Resource Division
GA	Gibberellic acid
HPLC	High Performance Liquid Chromatography
h	Hour
ha	Hectare
HCL	Hydrochloric acid
Id	Internal diameter
IAA	Indole Acetic Acid
Iso	Isolate
ITS	Internal transcribed spacer
JA	Jasmonic acid
KOH	Potassium hydroxide
ş	Lambda
Kg	Kilogram
L	Liter

LC	Liquid Chromatography
LSD	Least Significant Difference
$MgCl_2$	Magnesium chloride
$MgS0_4$	Magnesium sulphate
MARDI	Malaysian Agricultural Research and Development Institute
MP	Mating population
m	meter
MeOH	Methanol
min	Minute
µL	Microliter
µg	Microgram
εm	Micrometer
mg	Milligram
mm	Millimeter
mM	Millimolar
M	Molar
mL	Mililiter
MQ	Milli - Q
MON	Moniliformin
MR	Malaysian rice
Myc	Mycellium
ND	Nano Drop
N	Normality
N	Nitrogen
NaCl	Sodium chloride

NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
OD	Optical density
%	Percent
P	Phosphorus
pmol	Picomole
PCR	Polymerase chain reaction
P	Potassium
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
KNO_3	Potassium nitrate
PDA	Potato Dextrose Agar
PDA	Photo diod array
PDB	Potato Dextrose Broth
PR	Pathogenesis-related
pv	Plant variety
RNA	Ribonucleic acid
rDNA	Ribosomal DNA
R^2	Correlation co-efficient
rpm	Revolution per minute
S1	Score 1
S3	Score 3
S5	Score 5

SA	Salicylic acid
SAR	Systemic acquired resistance
SAS	Statistical Analysis Software
SEM	Scanning Electron Microscopy
SNA	Spezieller Nährstoffarmer Agar
SPE	Solid Phase Extraction
SAX	Strong anion Exchange
TBAHS	Terbutylammnium hydrogen sulfate
TBE	Tris-borate-EDTA
Tris-HCl	Tris-Hydrochloric acid
Spp	Species
U	Unit
UV	Ultra violet
U.S.A.	United States of America
VCG	Vegetative compatibility grouping
v/v	Volume per volume
WE	Weighable emulsion
WG	Weighable granules.
wp	Weighable powder
WP	Whole plant
w/v	Weight per volume
X	*Xanthomonas*

CHAPTER 1

INTRODUCTION

Rice, the staple and important food crop in Asia is being threatened by a number of serious diseases with estimated pre and post harvest yield losses amounting to 1-10% (Savary *et al*., 2000). Among the main rice diseases, Bakanae is common in Asia and causes more damage in the summer than in the spring season (Graves, 2009). Bakanae is alarming in the affected rice growing areas as it is difficult to develop Bakanae resistant varieties due to high genetic variation of the pathogen (Serafica and Cruz, 2009). Moreover, Bakanae disease is becoming a threat for sustainable rice production as the disease is not solely influenced by environment (Cother, 2002)**.** Thus, Bakanae is drawing the most concern in the affected rice growing areas of Asia and is also becoming a threat to sustainable rice production in other parts of the rice growing world.

The disease has worldwide distribution. It has been reported in Bangladesh (Haq *et al.,* 2011), China and India (IRRI, 2009a), Pakistan, Thailand, Nepal, Japan, Philippines, and Vietnam (Desjardins *et al*., 2000). Bakanae disease is becoming increasing important as yield loss due to this disease has been recorded to be in the range of. 3.7- 50.0% (Misra *et al.,* 1994). Both local and improved varieties of rice have been identified as susceptible to Bakanae in Nepal (Desjardins *et al*., 2000). Moreover, in recent years Bakanae disease is spreading and has been reported from new parts of Asia where previously this disease was not recorded as a greater concern. For instance, Haq *et al.* (2011) reported that the incidence of Bakanae is increasing in Bangladesh and it was presumed that it might be due to an increase in the minimum temperature in that rice growing region. In Pakistan, Bakanae has been considered as a major disease since the last five years (Bhalli *et al*., 2001), and in Malaysia the disease has been detected as serious since 1985 as it was found to severely affect rice in Perak, Kedah and Kelantan (Zainudin *et al*., 2008a). Subsequently, in Malaysia and in three provinces of Indonesia the disease was reported to be in the range of 1 to 5 on the disease severity scale with a disease incidence of 0.5 - 12.5% during 2004 – 2005 (Zainudin *et al.,* 2008b).

Five *Fusarium* species belonging to section *Liseola* have been isolated and associated with Bakanae disease, but *F. fujikuroi* was found to be highly virulent (Nur Izzati and Salleh, 2009; Zainudin *et al*., 2008b). However, the association of other *Fusarium* spp. to Bakanae disease development has not been clearly proven yet. The causal pathogen has been associated as seed borne or soil borne, but the infection process is not well understood. Moreover, it has been reported that abnormal internode elongation of infected plants was due to the

high amounts of gibberellins (GA_3) production by the causal agent. The role of increased GA_3 in Bakanae diseased plants, its association with other phytohormones such as indole acetic acid (IAA) and abscisic acid (ABA) and subsequent functions of these phytohormones in plant defense mechanisms have not been investigated.

Moreover, different *Fusarium* spp. produces different types of secondary metabolites and can be distinguished as a separate species according to the metabolite profile pattern of individual species. For example, a wide range of metabolites including fumonisin (FB1), moniliformin (MON), beauvericin (BEA), fusaric acid (FA) have been isolated and reported from *Fusarium fujikuroi* and *F. proliferatum,* but *F. proliferatum* was found to produce higher amounts of FB1 compared to *F. fujikuroi* (Cruz *et al*., 2013; Zainuddin *et al.,* 2008a; Desjerdins *et al*., 2000). The metabolites produced by *F. fujikuroi* and *F. proliferatum* have been identified as toxic to humans and animals and have been shown to have some phytotoxic effects, but their production in Bakanae diseased plants in association with Bakanae symptoms expression have not been reported (Li *et al*., 2012; Parmar *et al*., 2010; *Š r o b á et al.,* 2009a; Liu *et al*., 2005; Jestoi *et al*., 2004; Desjerdins *et al*., 2000; Kostecki *et al.,* 1999; Desjerdins *et al.,* 1997).

Resistance responses in plants are mediated by resistance genes that influence induction of plant defense proteins or pathogenesis-related (PR) proteins. Among the PR proteins *β*-1,3 glucanase and endochitinase are found to have influence on the degradation □R□-1,3 glu□□ans or chitin of fungal cell walls, repectively (Muthukrishnan *et al*., 2001). The *β*-1,3 glucanase and chitinase activities have also been observed and reported for other plant disease development, but it is still unknown about the roles of these two PR-proteins in relation to Bakanae disease development and symptoms expression.

In view of the potential importance of this emerging disease and considering the immense yield losses, the present research was carried out to identify the main *Fusarium spp*. or group of species that were responsible for multiple or complex disease symptoms. It was also necessary to identify the infection process that might be helpful for understanding the spread in infection by the causal pathogen in the rice growing world. The role of gibberellin (GA_3) production in relation to regulation of other phytohormones, fungal metabolites and PR-proteins in the development of Bakanae disease could be a basis for the formulation of new control strategies. Thus, understanding the relationship between phytohormones, metabolites and PR-proteins for Bakanae disese development through symptoms expression will be helpful for breeders in developing tolerant/resistant varieties.

Therefore, justification of doing research on this new emerging but destructive disease were that identification of the causal pathogen and infection process of the causal pathogen in the development of Bakanae disease had not been extensively studied before. Moreover, mechanism of pathogenesis with respect to the role of phytohormones, fungal metabolites, pathogenesis-related (PR) proteins and their interactions in Bakanae disease symptoms expression had not been established.

Hence, the objectives of this research were to: (1) identify the causal *Fusarium* species in Selangor rice growing areas (2) establish the role of phytohormonal imbalances and metabolites production by the fungus and pathogenesis-related (PR) protein activities in relation to disease symptoms expression in infected plants and (3) evaluate the symptoms expression analysis of Bakanae disease by pre-seed treatment with pure (synthetic) phytohormones and metabolites in susceptible rice variety MR 211.

The hypothesis of this research project was that the mechanism of Bakanae disease development was reliant on phytohormones imbalance, as well as on phytohormones, metabolites and PR-proteins interactions.

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