UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF Colletotrichum truncatum CP2 AND ITS INTERACTION WITH CHILLIES (Capsicum annuum L.) DURING PATHOGENESIS

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FBSB 2018 44

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By

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

January 2018

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

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January 2018

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Anthracnose caused by Colletotrichum species is the most destructive disease of chilli worldwide. It is responsible for worldwide yield losses and could be even more severe without a successful control that still relies on the use of fungicides. Due to the growing concern about environmental and health damages caused by this control, an understanding of the mechanisms leading to the fungal pathogenicity in a particular host is essential for the implementation of effective disease control. This study aimed to investigate the mechanism leading to pathogenesis of Colletotrichum species in chilli fruit as little is known about the pathogenicity factor involved in this interaction. Thirty five fungal isolates were isolated from chilli lesions of anthracnose from different geographic locations in Malaysia. The ability of fungal isolates to produce cell wall-degrading enzymes was screened and the best cell wall-degrading enzymes producer was selected for further study. Based on its morphological, biochemical and molecular identification, fungal isolate CP2 was identified as Colletotrichum truncatum. Successful inoculation of the C. truncatum CP2 on detached chilli fruits proved its pathogenicity and was confirmed to be a primary pathogen of chilli when it successfully infected the chilli fruits. In order to illustrate the infection strategy adopted by C. truncatum CP2, the infection process of this fungus in the chilli fruit was characterized using light, scanning and transmission microscope. C. truncatum CP2 exhibited a prolonged biotrophic phase of about 48 hour, before switched to necrotrophic phase at approximately 72 hour after inoculation. The first phase of necrotrophy in C. truncatum CP2 was characterized by formation of germ tube, appresorium and infectious hyphae. The destructive necrotrophic phase was characterized by formation of sunken lesions and production of numerous acervuli. The role of cell wall-degrading enzymes in facilitating the C. truncatum CP2 to colonize the host cell was investigated taking into consideration changes in the morphological and chemical compositions of the chilli fruits. The results of enzymatic activity experiment indicated that polygalacturonase (PG) was

the first cell wall-degrading enzymes detected and the activities obtained were higher (0.24±0.10 U/mL) than other enzymes, which appeared later and in lower amount. Significant changes in the pectin (total uronide content increased up to 50.33% - 71.85%) and cellulose contents (decreased to 11.45% - 12.32%) in chilli treated with PG and combination of PG and cellulases showed the main role of these enzymes in facilitating the C. truncatum CP2 during pathogenesis in chilli fruits. According to Fourier transform infrared analysis, there were remarkable changes in the vibration side of cellulose (3290 cm-1 and 2924 cm-1) and ring and vibration side of pectin (1581, 1337 and 1029 cm-1) in the cell wall of chilli treated with PG and mixture of both enzymes. In order to understand the exact role of PG enzymes in pathogenesis, PG enzymes from C. truncatum CP2 was purified using aqueous two phase system. The optimum purification condition of PG was achieved using 22% (w/w) polyethylene glycol and 15% (w/w) sodium citrate comprising crude load of 16% (w/w) at pH 7.0 with addition of 1.0% (w/w) sodium chloride. The necrotizing activity of the crude and purified PG from C. truncatum CP2 was then tested on detached chilli fruits. The faster lesion formation on the chilli treated with purified PG had confirmed the involvement of this enzyme in anthracnose of chilli. In conclusion, C. truncatum CP2 possess all the features to be termed as a serious anthracnose pathogen with the presence of pathogenicity factors such as PG enzymes. The results from this study provide a better insight into the interaction of C. truncatum CP2 and chilli fruits and these findings may be used in the development of efficient disease management strategies in Malaysia.

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

PENCIRIAN *Colletotrichum truncatum* CP2 DAN INTERAKSINYA DENGAN BUAH CILI (*Capsicum annuum* L.) SEMASA PATOGENESIS

Oleh

NURUL ATIKA BINTI MOHAMAD REMLI

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Penyakit antraknos yang berpunca daripada spesis Colletotrichum merupakan penyebab utama kepada kerosakan cili di serata dunia. Ia telah menyebabkan kerugian besar kepada pengusaha cili di seluruh dunia dan mungkin memberi kesan lebih teruk tanpa kawalan yang baik yang kini masih bergantung kepada penggunaan racun kulat. Peningkatan kesedaran terhadap kesan penggunaan racun kulat terhadap kerosakan alam sekitar dan kesihatan telah membawa kepada pentingnya memahami mekanisme terjadinya jangkitan penyakit ini bagi membolehkan strategi kawalan yang lebih berkesan dilaksanakan. Kajian in dijalankan bagi menyelidik mekanisma yang membawa kepada patogenesiti spesis Colletotrichum terhadap buah cili di mana pengetahuan mengenai faktor yang menyumbang kepada patogenisiti ini belum diketahui. Tiga puluh lima pencilan kulat telah dipencilkan daripada lesi antraknos dari lokasi geografi yang berlainan di Malaysia. Keupayaan kulat tersebut untuk menghasilkan enzim yang terbaik telah disaring dan pengeluar enzim yang terbaik dipilih untuk kajian selanjutnya. Pencilan kulat CP2 diperiksa berdasarkan ciri-ciri morfologi, molekul dan patogenisiti. Berdasarkan keputusan yang diperoleh, pencilan kulat CP2 dikenalpasti sebagai Colletotrichum truncatum. Ujian patogenisiti menunjukkan bahawa C. truncatum CP2 adalah patogenik terhadap perumah asal dan dikenalpasti sebagai penyebab utama kepada antraknos cili. Bagi mengetahui strategi jangkitan yang diaplikasikan oleh C. truncatum CP2, proses jangkitan kulat ini pada cili dikaji menggunakan mikroskop cahaya, mikroskop elektron pengimbasan dan mikroskop elektron penghantaran. C. truncatum CP2 mempamerkan fasa biotropik selama 48 jam, sebelum beralih kepada fasa nekrotropik bermula 72 jam selepas inokulasi. Fasa biotrofi dalam C. truncatum CP2 dapat dikenalpasti melalui pembentukan tiub germa, appresorium dan hyphae. Fasa nekrotropi pula dapat dikenalpasti melalui pembentukan lesi dan penghasilan acervuli yang banyak pada buah cili. Peranan enzim pengurai dinding sel di dalam membantu C. truncatum CP2 untuk kolonisasi perumah asal dikenalpasti dengan mengambil kira perubahan dalam komposisi morfologi dan komposisi kimia cili.

Keputusan eksperimen aktiviti enzimatik menunjukkan bahawa polygalakturonase (PG) merupakan enzim pengurai dinding sel pertama yang dikesan dan aktiviti yang diperoleh lebih tinggi (0.24 ± 0.10 U / mL) daripada enzim lain, yang kemudiannya muncul dan dalam jumlah yang lebih rendah. Perubahan penting dalam pektin (jumlah kandungan uronida meningkat dari 50.33% - 71.85%) dan kandungan selulosa (menurun dari 11.45% - 12.32%) dalam cili yang dirawat dengan PG dan gabungan PG dan selulase menunjukkan peranan utama enzim ini dalam membantu C. truncatum CP2 semasa patogenesis pada buah cili. Menurut analisis inframerah transformasi Fourier, terdapat perubahan yang luar biasa pada getaran selulosa (3290 cm-1 dan 2924 cm-1) dan getaran pektin (1581, 1337 dan 1029 cm-1) di dinding sel cili dirawat dengan PG dan campuran kedua-dua enzim. Untuk memahami peranan sebenar enzim PG dalam patogenesis, enzim PG dari C. truncatum CP2 ditulenkan menggunakan sistem penulenan dwi-fasa. Keadaan penulenan optimum PG dicapai melalui penggunaan 22% (w/w) polietilen glikol dan 15% (w/w) sodium sitrat yang mengandungi enzim sebanyak 16% (w/w) pada pH 7.0 dengan tambahan 1.0% (w/w) natrium klorida. Kebolehan enzim PG yang telah ditulenkan untuk menguraikan buah cili kemudiannya diuji. Pembentukan lesi lebih cepat pada cili yang dirawat dengan PG yang ditulenkan telah mengesahkan penglibatan enzim ini dalam antraknos cili. Sebagai kesimpulan, C. truncatum CP2 mempunyai ciri-ciri yang boleh dianggap sebagai patogen antraknos yang serius dengan kehadiran faktor-faktor virulensi seperti enzim PG. Hasil daripada kajian ini memberi gambaran yang lebih baik mengenai interaksi C. truncatum CP2 dan buah cili dan dapatan kajian ini boleh digunakan dalam pembangunan strategi pengurusan penyakit yang lebih baik di Malaysia.

ACKNOWLEDGEMENTS

First of all, I would like to thank Allah Almighty, for his blessing and grace, for me to complete this research project at the Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, Serdang.

I would like to express my sincere appreciation to my main supervisor, Assoc. Prof. Dr. Umi Kalsom Md Shah for giving her advice, motivation and support throughout this research. My sincere gratitude and appreciation also to my co-supervisors, Prof. Dr. Arbakariya Ariff, Dr. Mohd Termizi B. Yusof and Dr. Jeffrey Lim Seng Heng, for their valuable discussion and assistance throughout my research.

I also would like to express my sincere thanks to staff and officers of Faculty of Biotechnology and Biomolecular Sciences. And, as always my appreciation to all my friends especially Maya Emira, Saja Mahdey, Kazeem Muinat and Wan Rusmarini for their generous guidance and help since I started my work.

Mostly, my deepest appreciation to my loving and supportive husband, Mohd Shaifuddin bin Wahab, my kids (Ahmad Qayyum and Aisyah Humaira) and also to my family for their love, patience and encouragement throughout this work. Acknowledgement is also to those who are involved directly and indirectly in the completion of this study.

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

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LIST OF ABREVIATIONS

ADF	Acid detergent fiber
ADL	Acid detergent lignin
ANOVA	Analysis of Variance
Ap	appresorium
ATPS	Aqueous two phase system
ATR	Attenuate total reflectance
C	conidium
Cu	cuticle
CBM	Carbohydrate binding module
CMC	Carboxy methyl cellulose
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicyclic acid
FAO	Food and agriculture organization
FAOSTAT	The statistics division of food and agriculture organization
FTIR	Fourier Transform Infrared
Hai	hour after inoculation
g	gram
mg	milligram
µg	microgram
g/g	Gram per gram substrate
g/L	Gram per liter
GF	gel filtration
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
IU	International unit
Ha	acre
HAI	hour after inoculation
Hg/ha	Hectogram per acre
IEX	Ion exchange
ITS	Internal transcribed spacer
KCl	Potassium chloride

KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Potassium hydrogen phosphate
kV	kiloVolt
$MgC1_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
$MnSO_4.H_2O$	Magnesium sulphate hydrate
mm	milimetre
MOA	Ministry of agriculture
$NaNO_3$	Sodium nitrate
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information
NDF	Neutral detergent fiber
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
PG	polygalacturonase
PL	pectin lyase
PNL	pectate lyase
PME	pectin methylesterases
pp	penetration peg
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electron microscope
T ha^{-1}	Tons per acre
TEM	Transmission electron microscope
UF	Ultrafiltration
v/v	volume per volume
UV	Ultraviolet
w/v	weight per volume
%	percent
°C	Degree celcius

CHAPTER 1

INTRODUCTION

1.1 General background

Capsicum annuum L. is commonly known as chilli, or chilli pepper has been a part of Malaysian diet. Chilli fruits often used as a spice added to various dishes due to their hot flavour. However, the production of chilli in Malaysia is often hampered by fungal diseases (Ali *et al*., 2014; Heng *et al*., 2011; Yun *et al*., 2009). Anthracnose disease caused by *Colletotrichum* species is one of the main causes for post-harvest fruit rot of chilli and resulted in high yield losses. It can be developed on the field, during long distant transport and cold storage and these attributed to the lower fruit quality and marketability. According to FAO report (2016), area harvested with chilli in Malaysia in the year 2013 was estimated to be around 4,104 ha with estimated yield of 145,651 Hg/Ha (FAOSTAT, 2014). However, this number decreased to 3,582 ha in the year 2014 with the total estimated yield of 113,124 Hg/Ha. The statistical data has showed a reduction of approximately 22% of the yield and hence indicating the severity of anthracnose disease in chilli. Higher yield loss due to anthracnose disease will be faced by farmers if the disease is not well managed (Stanley Freeman, 2008). Such losses would increase the market price of chilli and ultimately affect both growers and consumers.

In conventional agriculture, control of chilli anthracnose at post-harvest stage is usually done by spraying the whole plant including the fruits with synthetic fungicides. The synthetic fungicides such as manganese ethylenebisdithiocarbamate (Maneb) and carbendazim are generally recommended for controlling anthracnose disease. However in recent years, the consumers' concern regarding the environmental and health damage associated with the use synthetic fungicides have increased. Furthermore, the demand for safer storage methods and vegetables free from pesticide residues by worldwide consumers is growing day by day. As a result, the pesticide legislation is becoming stricter and only limited number of fungicides are allowed for post-harvest usages. Moreover, the development of fungicide resistance pathogen due to frequent use of fungicides causes difficulty to control the anthracnose disease. Therefore, there is a need to search for another alternative control method that is considered safe for human health and environment.

In order to develop effective disease management strategies, an understanding of the mechanism leading to fungal pathogenicity on a particular host is essential. In general, plant pathogenic fungi form infection structures that accomplish crucial stages of pathogenesis including adhesion, penetration, proliferation and nutrient. *Colletotrichum* species use different strategies to attack host tissues, either through an intracellular hemibiotrophic strategy, a subcuticular intramural strategy or a combination of both strategies (Kubo *et al*., 2016). Most members of the genus

Colletotrichum infected the host cells through intracellular hemibiotroph (Wharton *et al*., 2001). Through this method, they initially colonized the host biotrophically, where the fungi obtained nutrients from living plant cells, and then they turned into necrotroph, where the fungi cause an extensive degradation of host cells.

The infection of host plants by fungal pathogens is usually mediated by numerous cell wall-degrading enzymes. The secretion of cell wall-degrading enzymes by fungal pathogens is very important as it contributes to the pathogenicity of fungal pathogens during interaction with the host (Fernando *et al*., 2001; Kikot *et al*., 2009). Among the various cell wall-degrading enzymes released by fungal pathogens, most research has been focused on pectinases enzyme. Earlier studies have reported that pectinases are the only enzymes that have the ability to cause tissue maceration and cell death. However, some of other cell wall-degrading enzymes such as cellulases, chitinases and xylanases have also been reported as the pathogenic determinants in disease development (Kubicek *et al.*, 2014).

Even though some *Colletotrichum* species have been identified as causal agents of chilli anthracnose disease worldwide, the information on the mechanism of infection and disease development of *Colletotrichum* species on chilli is still lacking. The extracellular enzymes profile which may have potential roles in pathogenesis of *Colletotrichum* during anthracnose infection is also unknown. A view on the strategy of infection used by pathogens along with the secretion of cell wall-degrading enzymes occur in the chilli fruits during infection would provide valuable information needed to develop a new control strategy for chilli anthracnose.

1.2 Objectives

The general objective of this study was to investigate the mechanism leading to pathogenesis of *Colletotrichum* species during its interaction with chilli fruit.

The specific objectives of the present investigation were as follow:

1. To isolate and identify *Colletotrichum* species isolated from lesions of chilli anthracnose using morphological, biochemical and molecular characterization.
2. To characterize the pre- and penetration process of *C. truncatum* CP2 on chilli fruit during pathogenesis.
3. To evaluate the cell wall-degrading enzymes produced by *C. truncatum* CP2 during pathogenesis on chilli.
4. To characterize the properties of cell wall-degrading enzymes produced by *C. truncatum* CP2.

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