UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF Trichoderma harzianum T3.13 AND CHARACTERIZATION OF ITS INTERACTION WITH Neoscytalidium dimidiatum U1, A PATHOGENIC FUNGUS ISOLATED FROM DRAGON FRUIT (Hylocereus polyrhizus (F.A.C. Weber) Britton & Rose)

WAN RUSMARINI BINTI WAN ZULKIFELI

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By

WAN RUSMARINI BINTI WAN ZULKIFELI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the degree of Master of Science

July 2016

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

IDENTIFICATION OF *Trichoderma harzianum* T3.13 AND CHARACTERIZATION OF ITS INTERACTION WITH *Neoscytalidium dimidiatum* U1, A PATHOGENIC FUNGUS ISOLATED FROM DRAGON FRUIT (*Hylocereus polyrhizus* (F.A.C. Weber) Britton & Rose)

By

WAN RUSMARINI BINTI WAN ZULKIFELI

July 2016

Chairman: Associate Professor Umi Kalsom Md Shah, PhD
Faculty: Biotechnology and Biomolecular Sciences

Endophytes are potential biological control agents. They produce enzymes which facilitate their initial colonisation of plant tissues and direct interactions with microbial pathogens. *Trichoderma* species have been broadly used in the effort to defeat soil-borne pathogens. The objectives of this study were to identify isolated endophytic fungi and pathogen of dragon fruit, and measure enzyme activity of chitinase from *Trichoderma harzianum* T3.13 grown in different types of medium. This work was also done to profile genes encoding chitinase, β-glucanase and N-acetylglucosamine from *Trichoderma harzianum* T3.13 against pathogen *Neoscytalidium dimidiatum* U1 and control pathogen *Colletotrichum gloerosporioides* by using reverse transcription-polymerase chain reaction (RT-PCR). In this study, endophytic fungi from the stem of healthy dragon fruit (*Hylocereus* spp.) was successfully identified as *Trichoderma harzianum* T3.13. *T. harzianum* T3.13 was shown to have the ability to produce antifungal activity against *N. dimidiatum* U1, a pathogen fungus from the stem of unhealthy dragon fruit. Mycoparasitic interactions between the two fungi were observed by scanning electron microscopy. *T. harzianum* T3.13 hyphae tangled with the hyphae of *N. dimidiatum* U1. The chitinolytic activities of *T. harzianum* T3.13 were 0.194 U/ml and 0.1 U/ml in a medium containing 3% (w/v) of colloidal chitin and 0.5 % (w/v) dried cell wall of *N. dimidiatum* U1 as sole carbon source, respectively. This study profiled the expression of genes encoding chitinase (*chit42*), β-glucanase (*bgn13*.1) and N-acetylglucosamine (*exc1*) from *T. harzianum* T3.13. Semi-quantitative RT-PCR was used to quantify the expression patterns of the genes during the interaction of *T. harzianum* T3.13 with pathogen *N. dimidiatum* U1 and control pathogen *C. gloeosporioides*, respectively. The expression patterns of these genes were profiled before and after the interactions occurred. The expression of the *exc1* and *chit42* genes were observed to be present before and after the interaction occurred in the presence of *N. dimidiatum* U1. However, the expression of the *bgn13.1* gene increased after 24 hours up to 96 hours of interaction

in the presence of *N. dimidiatum* U1. In the presence of *C. gloeosporioides*, the expression of *bgn13.1* and *chit42* gradually decreased during the interaction although the expression of the *exc1* gene did not change. The results suggested that the endophytic fungus *T. harzianum* T3.13 has the potential as a good biological control agent against *N. dimidiatum* U1 and *C. gloeosporioides.* Thus, the study provided an insight into cellular and molecular interactions between *T. harzianum* T3.13 and pathogenic fungi.

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

PENGENALAN *Trichoderma harzianum* T3.13 DAN PENCIRIAN INTERAKSINYA DENGAN *Neoscytalidium dimidiatum* U1, KULAT PATOGENIK DIASINGKAN DARIPADA DRAGON FRUIT (*Hylocereus polyrhizus* (F.A.C. Weber) Britton & Rose)

Oleh

WAN RUSMARINI BINTI WAN ZULKIFELI

Julai 2016

Pengerusi: Profesor Madya Umi Kalsom Md Shah, PhD
Fakulti: Bioteknologi dan Sains Biomolekul

Endofitik berpotensi sebagai ejen kawalan biologi. Ia menghasilkan enzim yang memudahkan pengkolonian awal mereka terhadap tisu tumbuhan dan berinteraksi secara langsung dengan mikrob patogen. Spesies *Trichoderma* telah digunakan secara meluas dalam usaha untuk menghapuskan patogen tanah. Objektif kajian ini adalah untuk mengenalpasti kulat endofitik dan patogen pada buah naga yang telah diasingkan dan mengenalpasti aktiviti enzim kitinase dari *Trichoderma harzianum* T3.13 yang hidup dalam media yang berbeza. Kajian ini juga dijalankan untuk memprofil gen kitin, β-glukan dan N-acetylglukosamine dari *Trichoderma harzianum* T3.13 apabila bertindak-balas dengan patogen *Neoscytalidium dimidiatum* U1 dan patogen kawalan *Colletotrichum gloerosporioides* dengan menggunakan tindakbalas rantaian polimerase-transkripsi berbalik. Dalam kajian ini, kulat endofitik dari batang pokok buah naga (*Hylocereus* spp) yang sihat telah berjaya dikenal pasti sebagai *Trichoderma harzianum* T3.13. *T. harzianum* T3.13 telah terbukti mempunyai keupayaan untuk menghasilkan aktiviti antikulat terhadap *N. dimidiatum* U1, iaitu kulat patogen dari batang pokok buah naga yang tidak sihat. Interaksi mikoparasitik antara kedua-dua kulat diperhatikan dengan menggunakan pengimbas mikroskop elektron. Hifa *T. harzianum* T3.13 didapati berpaut dengan hifa *N. dimidiatum*. Aktiviti kitinolitik *T. harzianum* T3.13 adalah 0.194 U/ml dan 0.1 U/ml, masing-masing dalam medium yang mengandungi 3% (w/v) kitin koloid dan 0.5% (w/v) dinding sel kering daripada *N* . *dimidiatum* sebagai sumber karbon tunggal. Kajian ini memprofilkan ekspresi gen kitin (*chit42*), β-glukan (*bgn13*.1) dan N-acetylglukosamine (*exc1*) daripada *T. harzianum* T3.13. Semi-kuantitatif RT-PCR digunakan untuk mengukur ekspresi dari gen yang berbeza apabila *T. harzianum* T313 berinteraksi dengan patogen *N. dimidiatum* U1 dan patogen kawalan *C. gloeosporioides*. Ekspresi gen ini telah diprofil sebelum dan selepas interaksi berlaku. Ekspresi gen *exc1* dan *chit42* wujud sebelum dan selepas interaksi berlaku dalam kehadiran *N. dimidiatum* U1. Namun, ekspresi bagi gen *bgn13*.1 mula meningkat selepas

24 jam interaksi sehingga 96 jam interaksi dalam kehadiran *N. dimidiatum* U1. Dalam kehadiran *C. gloeosporioides*, ekspresi gen *bgn13*.1 dan *chit42* berkurangan secara beransur-ansur semasa interaksi tetapi ekspresi gen *exc1* tidak berubah. Hasil kajian ini mencadangkan bahawa endofitik kulat *T. harzianum* T3.13 mempunyai potensi untuk menjadi agen kawalan biologi terhadap patogen *N. dimidiatum* U1 dan *C. gloeosporioides*. Oleh itu, kajian ini memberi pengetahuan tentang interaksi selular dan molekular antara *T. harzianum* T3.13 dan kulat patogenik.

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Finally, the greatest accomplishment is not in never falling, but in rising again after you fall. Fall forward.

Barokallahufikum.

Thank you.

I certify that a Thesis Examination Committee has met on 26 July 2016 to conduct the final examination of Wan Rusmarini binti Wan Zulkifeli on her thesis entitled "Identification of *Trichoderma harzianum* T3.13 and Characterization of its Interaction with *Neoscytalidium dimidiatum* U1, a Pathogenic Fungus Isolated from Dragon Fruit (*Hylocereus polyrhizus* (F.A.C.Weber) Britton & Rose)" 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

Hidayah binti Ariffin, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Ho Chai Ling, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Sahilah Abd. Mutalib, PhD
Associate Professor
Universiti Kebangsaan Malaysia
Malaysia
(External Examiner)

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 22 November 2016

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

Umi Kalsom Md Shah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd Puad Abdullah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Tan Geok Hun, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

_____
BUJANG KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
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Signature: _____
Name of Chairman of
Supervisory Committee: Associate Professor Dr. Umi Kalsom Md Shah

Signature: _____
Name of Member of
Supervisory Committee: Associate Professor Dr. Mohd Puad Abdullah

Signature: _____
Name of Member of
Supervisory Committee: Dr. Tan Geok Hun

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide-nucleotide BLAST
bp	base pair
BSA	Bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNS	3,5-dinitrosalicyclic acid
dNTPs	Deoxynucleotides
E value	Expect value
EtBr	ethidium bromide
g	gram
g	gravity
HCl	hydrochloric acid
Kg	kilogram
M	molarity
mL	Milliliter
mM	Millimolar
mRNA	messenger ribonucleic acid
MW	molecular weight
NAG	N-acetyl-D-glucosamine
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	Nanogram
nM	Nanomolar
OD	optical density
PCI	Phenol: chloroform: isoamylaalcohol
PCR	polymerase chain reaction
PDA	Potato dextrose agar
RNA	ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
rRNA	ribosomal RNA
RT-PCR	Reverse transcription polymerase chain reaction
SEM	scanning electron microscopy
T_a	annealing temperature
TAE	Tris acetate EDTA
T_m	melting temperature
U	Unit
U/mL	Unit per mL
UV	ultraviolet
V	voltage
v/v	Volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Dragon fruit is a cactus fruit crop that's have a high demand at national and international levels. This fruit has been planted in over 10 countries worldwide (Valencia-Botín *et al*., 2013). Since dragon fruit starts to be farmed in commercial farms, some symptoms of rotting and spots in stems and fruits were being identified (Valencia-Botín *et al*., 2013). Studies on stem soft rot diseases were begun at 1990s in Mexico because of the diseases caused by two types of Enterobacteria. While fungi such as *Botryosphaeria dothidea* Ces. & de Not also identified as causing agent of the spots on stems diseases (Valencia-Botín *et al*., 2013). One more fungal disease become violent as it affects fruits and stems is anthracnose disease. Researcher in United State of America (USA) and Japan revealed their report in etiological study found that the causing agents of anthracnose disease are fungus *Colletotrichum gloesporioides* Penz (Poonpolgul and Kumphai, 2007). In Peninsular Malaysia, red-fleshed dragon fruit (*Hylocereus polyrhizus*) is very popular and widely cultivated (Masyahit *et al*., 2009). However, anthracnose symptoms start to be detected on the stems of dragon fruit in the states of Kedah and Penang in August 2010 and January 2011, respectively (Iskandar Vijaya *et al*., 2015).

The anthracnose disease was found in commercial plantations in the Malaysia. Prevention could be initiated for controlling plant disease through earlier detection and accurate identification of plant pathogens. It is important to prevent the introduction and distribution of new pathogens in a growing area where has not present yet. Therefore, the availability of fast, sensitive and accurate methods for detection and identification of fungal pathogens is required to improve disease management measures (Capote *et al*., 2012).

Day now, the old-style agricultural practices have many problems such as disease, drought, and low soil fertility as a result of use of pesticides, harmful chemical, pest, pollution and global warming (Pratibha, 2011). Therefore, there is a requirement for eco-friendly biological control agents that can help in undertaking the above problems. There are many types of bacteria and fungi that's involves in biological control activities. Today, *Trichoderma* was identified has a high potential in controlling the plant diseases. *Trichoderma* species can produce different kinds of enzymes which stand as an important role in biological control activities, like cell wall degradation, hypha growth, biotic and abiotic stress tolerance and antagonistic activity against plant pathogens (Hasan, 2014). They can colonize aboveground and belowground plant organs or grow between living cells (endophytes). They can live in soil organic matter as saprophytes and could be appear in plant litter and mammalian tissues (Rai and Mehra, 2015).

Trichoderma species are known for their assembly of cell wall degrading enzymes such as chitinase. Chitinase enzyme has been established massive attention because of their potential use in biological control for phytopathogenic fungus and other phytopathogenic organisms that containing chitin such as insects (Souza *et al*., 2003). In these respect, the fungi that's produce chitinase enzyme has intensively studied as biological control agents.

Genes play a major role in the biological control process by regulating signals and leads the secretion of some enzymes that help in the degradation of the pathogens. Increased in the expression of the genes such as chitinase and glucanase gene, helps in enhanced the biological control activity, promoting the plant growth and prevents the plant from pathogen attack (Massart and Jijakli, 2007). Therefore, gene such as chitinase gene can be cloned and produced in a large amount for commercial applications (Pratibha, 2011).

In the preliminary study, the endophytic *Trichoderma* from healthy stem dragon fruit and pathogen *Neoscytalidium* from unhealthy stem dragon fruit are isolated. They are shows an antagonistic activities between each other. Therefore this study has been done to initiate the identification for the genus level for *Trichoderma* and *Neoscytalidium*. The production of chitinase enzyme by *Trichoderma harzianum* T3.13 has been evaluated too in order to obtain substantial chitinase enzyme activities. Meanwhile, the profile expressions of *T. harzianum* T3.13 genes such as chitinase gene, β-glucanase gene and N-acetylglucosamine gene are also profiled during antagonistic activity against pathogens *Neoscytalidium dimidiatum* U1 and *Colletotrichum gloerosporioides*.

The objectives of this study are:

1. To identify isolated endophytic fungi and pathogen of dragon fruit plants.
2. To measure the enzyme activity of chitinase from *Trichoderma harzianum* T3.13 grown in different types of medium
3. To profile genes encoding chitinase, β-glucanase and N-acetylglucosamine from *Trichoderma harzianum* T3.13 against pathogen *Neoscytalidium dimidiatum* U1 and control pathogen *Colletotrichum gloerosporioides* by using reverse transcription-polymerase chain reaction (RT-PCR).

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