CONTROL OF RUBBER TREE PATHOGENS BY ENDOPHTIC FUNGI

SAKUNTALA SIRI-UDOM

DOCTOR OF PHILOSOPHY
IN APPLIED MICROBIOLOGY

GRADUATE SCHOOL
CHIANG MAI UNIVERSITY
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Sakuntala Siri-Udom

หัวข้อดุษฎีนิพนธ์	การควบคุมเชื้อก่อโรคในยางพาราโดยราเอนโดไฟท์	
ผู้เขียน	นางสาวศกุนตลา ศิริอุดม	
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บทคัดย่อ

คัดแยกเชื้อราเอนโดไฟท์จำนวน 271 ไอโซเลตจากพืชสมุนไพรจำนวน 13 ชนิดและยางพารา (*Hevea brasiliensis* Müll.Arg.) ในภาคเหนือและภาคอีสานของประเทศไทย ราเอนโดไฟท์ส่วนใหญ่คัดแยกได้มาจากพญายอ *(Clinacanthus nutans* (Burm.f.) Lindau, 24.4%), ทองพันชั่ง (*Rhinacanthus nasutus* (Linn.) Kurz, 10.3%) และลิ้นงูเห่า *(C. siamensis Bremek*, 8.9%) ตามลำดับ โดยพบราเอนโดไฟท์ในใบ (53.1%) มากกว่าในก้าน/ลำต้นและราก ราเอนโดไฟท์ไอโซเลต AL1T1 และ AL1T2 ซึ่งคัดแยกจากลำต้นของว่านหางจระเข้ (*Aloe vera* L.) มีฤทธิ์ยับยั้งเชื้อราได้หลายชนิด โดยยับยั้งการเจริญของ *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. *vasinfectum, Phellinus noxius, Rigidoporus microporus* และ *Rhizoctonia solani* ได้มากกว่า 50% เมื่อทดสอบด้วยเทคนิค dual culture สารสกัดหยาบของไอโซเลต AL1T1 และ AL1T2 มีฤทธิ์ยับยั้งเชื้อราก่อโรคในพืชเมื่อทดสอบเปรียบเทียบกับยาฆ่าเชื้อรา tridemorph และ carbendazim สารสกัดหยาบของไอโซเลต AL1T1 สามารถยับยั้งเชื้อรา *P. noxius* อย่างมีนัยสำคัญ ($P<0.05$) โดยมีค่า MIC เท่ากับ 0.31 มก./มล. ซึ่งน้อยกว่าค่า MIC ของยาฆ่าเชื้อรา tridemorph (0.62 มก./มล.) ขณะที่สารสกัดหยาบของไอโซเลต AL1T2 สามารถยับยั้งเชื้อรา *F. oxysporum* f. sp. *vasinfectum* อย่างมีนัยสำคัญ ($P<0.05$) โดยมีค่า MIC เท่ากับ 0.16 มก./มล. ซึ่งน้อยกว่าค่า MIC ของยาฆ่าเชื้อรา carbendazim (0.31 มก./มล.) การตรวจสอบฤทธิ์ยับยั้งเชื้อราของสารสกัดหยาบด้วยวิธี TLC-autobiography พบว่า สามารถแยกสารประกอบที่มีฤทธิ์ยับยั้งเชื้อราของไอโซเลต AL1T1 ได้ เมื่อใช้ตัวทำละลายเคลื่อนที่คือ dichloromethane: methanol, 95:5 (v/v) โดยมีค่า Rf อยู่ในช่วง 0.22-0.33 ส่วนไอโซเลต AL1T2 พบว่า สร้างสารประกอบที่มีฤทธิ์ยับยั้ง

เชื้อราที่มีค่า Rf อยู่ในช่วง 0.20-0.33 โดยสามารถยับยั้งการเจริญของเส้นใยและการงอกของสปอร์เชื้อรา *F. oxysporum* f. sp. *vasinfectum* ได้ นอกจากนี้ราเอนโดไฟท์ไอโซเลต BS1B201, C4IV301 และ PS1IV102 สามารถสร้าง indole acetic acid (IAA) ได้ในปริมาณ 40.7±0.5 ไมโครกรัม/มล., 23.4±0.1 ไมโครกรัม/มล. และ 16.8±0.1 ไมโครกรัม/มล. ตามลำดับ สามารถจำแนกราเอนโดไฟท์ไอโซเลต AL1T1 และ AL1T2 โดยอาศัยลักษณะสัณฐานวิทยาและลักษณะทางพันธุกรรมอยู่ในไฟลัม Ascomycota ในอันดับ Sordariales โดยมีความเหมือนของลำดับเบสของยีนบริเวณ ITS rRNA เท่ากับ 99-100%

จากการทดลองสามารถคัดแยกราเอนโดไฟท์ที่สร้างไอระเหยอินทรีย์จากใบของยางพาราได้จำนวน 3 ไอโซเลต ซึ่งราเอนโดไฟท์ทั้งหมดจัดจำแนกอยู่ในสกุล *Muscodor* วงศ์ *Xylariaceae* โดยอาศัยลักษณะทางสัณฐานวิทยาและลักษณะทางพันธุกรรม เชื้อราทุกไอโซเลตสามารถสร้างไอระเหยอินทรีย์ (VOCs) ที่มีฤทธิ์ยับยั้งจุลินทรีย์ได้หลายชนิด เช่น แบคทีเรีย, ยีสต์ และราเส้นสาย เชื้อราไอโซเลต RTM5IV3 มีความเหมือนกับลำดับเบสของยีน ITS rRNA กับเชื้อรา *Muscodor* สปีชีส์อื่นน้อยกว่า 86% ดังนั้นจึงถูกตั้งเป็นสปีชีส์ใหม่ โดยให้ชื่อว่า *Muscodor heveae* sp. nov. ซึ่งสร้างไอระเหยอินทรีย์ที่มีสาร 3-methylbutan-1-ol เป็นสารหลักและ 3-methylbutyl acetate และอนุพันธ์ของ azulene เป็นสารรอง VOCs ที่สร้างโดยเชื้อรา *Muscodor* มีศักยภาพในการควบคุมเชื้อจุลินทรีย์ก่อโรคทั้งแบคทีเรีย, ยีสต์ และราเส้นสาย ยิ่งกว่านั้นไอระเหยอินทรีย์ของ *M. heveae* สามารถยับยั้งเชื้อราก่อโรค *P. noxius* และ *R. microporus* ซึ่งเป็นสาเหตุของโรคในระบบรากของยางพารา

การรมด้วยไอระเหยอินทรีย์ (biofumigation) เป็นการตรวจสอบประสิทธิภาพของ *M. heveae* ในการควบคุมโรครากขาวในยางพารา โดยไอระเหยอินทรีย์ของ *M. heveae* สามารถยับยั้งการเจริญของเชื้อรา *R. microporus* ในระดับห้องปฏิบัติการด้วยเปอร์เซ็นต์ยับยั้งการเจริญเท่ากับ 100 ส่วนการทดลองในระดับโรงเรือนโดยใช้หัวเชื้อ *M. heveae* รมดินที่มีเชื้อรา *R. microporus* เจริญอยู่ ทำการบันทึกค่าการเกิดโรค (disease score) เมื่อพืชอายุครบ 150 วัน และวิเคราะห์ผลทางสถิติ การทดลองประกอบด้วย 7 กรรมวิธี การรมด้วยไอระเหยอินทรีย์ของ *M. heveae* สามารถยับยั้งการเกิดโรคได้เป็นอย่างดี โดยกรรมวิธี RMH40 และ RMH80 ซึ่งมีหัวเชื้อ *M. heveae* ปริมาณ 40 ก./1 กก. และ 80 ก./1 กก. ตามลำดับ ให้ผลไม่แตกต่างกันอย่างมีนัยสำคัญ ($P<0.05$) เมื่อเปรียบเทียบกับกรรมวิธี RT ที่ใส่ยาฆ่าเชื้อรา tridemorph และชุดควบคุมที่ไม่ได้ใส่เชื้อราก่อโรคพืช กรรมวิธี RMH40 และ RMH80 ให้ผลแตกต่างกันอย่างมีนัยสำคัญ ($P<0.05$) กับกรรมวิธี R ที่ใส่เฉพาะเชื้อราก่อโรค โดยกรรมวิธี RMH40 และ RMH80 มีค่าการเกิด

โรคเท่ากับ 1.3 ±0.5 และมีอัตราการอยู่รอดของยางพาราเท่ากับ 100% ขณะที่กรรมวิธี R มีค่าการเกิดโรคเท่ากับ 4.8±0.5 และมีอัตราการอยู่รอดของยางพาราเท่ากับ 25 % อาการสำคัญที่แสดงการเกิดโรคคือ พบ rhizomorphs สีขาวของเชื้อ *R. microporus* บนรากพืชที่มีการติดเชื้อ การทดสอบฤทธิ์ยับยั้งของสารสังเคราะห์ไอระเหยอินทรีย์ 3-methylbutan-1-ol, 3-methylbutyl acetate และ 2-methylpropanoic acid พบว่า สามารถยับยั้งการเจริญของเชื้อรา การเจริญของรากและต้นของพืชทดสอบได้ แต่ไม่ส่งผลต่อยางพาราเมื่อสังเกตจากการทดลองการควบคุมโรคด้วยไอระเหยอินทรีย์ของ *M. heveae* ในระดับโรงเรือน ดังนั้นไอระเหยอินทรีย์ที่มีฤทธิ์ทางชีวภาพจากเชื้อรา *M. heveae* สามารถใช้เป็นวิธีทางเลือกสำหรับการควบคุมโรครากขาวในยางพารา และสามารถนำไปประยุกต์ใช้ทางการเกษตรและอุตสาหกรรมต่อไป

คำสำคัญ ราเอนโดไฟท์, ฤทธิ์ยับยั้งเชื้อรา, indole acetic acid, *Muscodor*, ยางพารา, ไอระเหยอินทรีย์ที่มีฤทธิ์ยับยั้งจุลินทรีย์

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ABSTRACT

A total of 271 endophytic fungi were isolated from 13 medicinal plants and the rubber tree (*Hevea brasiliensis* Müll.Arg.) in northern and northeastern of Thailand. Most endophyte were isolated from *Clinacanthus nutans* (Burm.f.) Lindau (24.4%), followed by *Rhinacanthus nasutus* (Linn.) Kurz (10.3%) and *C. siamensis* Bremek (8.9%), respectively. Endophytic fungi were more prevalent in the leaves (53.1%) than the branches/stems and the roots. Isolate AL1T1 and AL1T2 from stem of *Aloe vera* L. exhibited board range of antifungal activity above 50% growth inhibition by dual culture technique toward to *Colletotrichum gloeosporioides, Fusarium oxysporum* f. sp. *vasinfectum, Phellinus noxius, Rigidoporus microporus* and *Rhizoctonia solani* AG-2. Compared with the positive control tridemorph and carbendazim, crude extract of AL1T1 and AL1T2 showed a strong antifungal activity on the above phytopathogens. Crude extract of AL1T1 significantly inhibited *P. noxius* with MIC 0.31 mg/ml ($P<0.05$) and less than MIC value of tridemorph (0.62 mg/ml). While, AL1T2 crude extract significantly inhibited *F. oxysporum* f. sp. *vasinfectum* with MIC 0.16 mg/ml ($P<0.05$) and less than MIC value of carbendazim (0.31 mg/ml). The antifungal activity of AL1T1 and AL1T2 crude extract were examined by TLC-bioautography. The results showed the separation of active compounds and Rf values ranged from 0.22-0.33 of AL1T1 (separated using 95:5 (v/v) dichloromethane: methanol) was found to inhibit mycelial growth of *P. noxius*. The active compounds of AL1T2 showed Rf values ranged from 0.20-0.33 and inhibited mycelial growth and spore germination of *F. oxysporum* f. sp. *vasinfectum*. Isolate BS1B201, C4IV301 and PS1IV102 produced plant growth hormone,

indole acetic acid (IAA) at the concentration of 40.7±0.5 µg/ml, 23.4±0.1 µg/ml and 16.8±0.1 µg/ml respectively. Endophytic fungi isolates AL1T1 and AL1T2 belong to phylum Ascomycota in the order Sordariales based on morphological characteristic and the sequence similarities of ITS rRNA gene (99-100% sequence similarity).

Volatile metabolite-producing endophytic fungi were isolated from leaves of rubber tree and their antimicrobial competence were studied. Three isolates was obtained, and their phenotypic and phylogenetic relationship with the genus *Muscodor* in the family *Xylariaceae* was studied. All isolates could produce volatile metabolites with apparent antimicrobial activity against diverse tested microbes (bacteria, yeast and filamentous fungi). An isolate, RTM5IV3, with <86% similarity with ITS rRNA gene compared to other species of the genus *Muscodor,* was proposed as a novel species with the name, *Muscodor heveae* sp. nov. Its bioactive volatile metabolites included 3-methylbutan-1-ol as a major component, followed by 3-methylbutyl acetate and azulene derivatives. The volatile organic compounds (VOCs) produced by the *Muscodor* isolates have potential for biological control of pathogenic microorganisms such as bacteria, yeast and filamentous fungi. Furthermore, the VOCs of *M. heveae* were active against the pathogenic fungi, *P. noxius* and *R. microporus* causing root disease of rubber tree.

The bioactive compounds of *M. heveae* were examined by the process of biofumigation for the purposes of controling white root rot disease in the rubber trees. VOCs of *M. heveae* possess antimicrobial activity against *R. microporus in vitro* with 100% growth inhibition. *In vivo* tests were carried out under greenhouse conditions using *M. heveae* inoculum fumigated soil that had been inoculated with *R. microporus* inoculum. After which, all 7 treatments were compared. Significant differences were observed with a disease score at 150 days after treatment. Biofumigation by *M. heveae* showed great suppression of the disease. Biocontrol treatments; RMH40 (40 g 1 kg^{-1} *M. heveae* inoculum) and RMH80 (80 g 1 kg^{-1} *M. heveae* inoculum) were not found to be significantly difference when compared with fungicide treatment (RT) and the non-infested control, but results were found to be significantly difference from those of the untreated inoculated control (R) ($P<0.05$). RMH40 and RMH80 revealed a low disease score of 1.3 ±0.5 with a high survival rate of rubber tree seedling (100%), while R showed

the highest disease score of 4.8±0.5 with 25% survival rate of rubber tree seedling. A sign of disease is the presence of rhizomorphs of *R. microporus* on the infected roots, appearing as a white color. The artificial volatile compounds test confirmed that the major component, 3-methylbutan-1-ol, and the minor compounds, 3-methylbutyl acetate and 2-methylpropanoic acid, inhibited mycelial growth of fungal pathogens and root and shoot elongation in the tested plant but had no effect on the rubber tree when fumigation was demonstrated and observed *in vivo*. The bioactive VOCs of *M. heveae* would be an alternative method for the control of white root rot disease in rubber trees, while there is potential to expand these findings toward further agricultural and industrial applications.

Keywords Endophytic fungi, antifungal activity, indole acetic acid, *Muscodor*, rubber tree, volatile antibiotics

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LIST OF ABBREVIATION

µl	microlitre
cm	centimeter
g	gram
kg	kilogram
h	hour
ml	millilitre
mm	millimeter
min	minute
M	molar
mM	millimolar
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	round per minute
sec	second
v/v	volume by volume

LIST OF SYMBOLS

°C	degree Celsius
%	percent
β	Beta

ข้อความแห่งการริเริ่ม

1. วิทยานิพนธ์นี้ได้นำเสนอการคัดแยกราเอนโดไฟท์ในพืชสมุนไพรไทยและยางพารา เพื่อศึกษาถึงฤทธิ์ยับยั้งจุลินทรีย์ก่อโรคพืชของสารปฏิชีวนะและไอระเหยอินทรีย์ของราเอนโดไฟท์ รวมทั้งสมบัติในการส่งเสริมการเจริญเติบโตของพืช เพื่อการนำไปใช้ประโยชน์ทางการเกษตรและอุตสาหกรรมต่อไป

2. เพื่อนำเสนอวิธีทางชีวภาพในการควบคุมโรคในยางพาราโดยใช้ประโยชน์จากราเอนโดไฟท์ซึ่งการรมด้วยไอระเหยอินทรีย์จากราเอนโดไฟท์ที่มีฤทธิ์ยับยั้งจุลินทรีย์ก่อโรคพืชถูกนำมาประยุกต์ใช้

STATEMENT OF ORIGINALITY

1. This study provides the isolation and screening endophytic fungi from Thai medicinal plants and the rubber tree that capable of producing antibiotics, volatile metabolites and plant growth promoting metabolites and expand these findings toward further agricultural and industrial applications.
2. Discovery of alternative technique to control plant pathogens by biofumigation using antagonistic volatile metabolites from endophytic fungi.

CHAPTER 1

General introduction and thesis outline

1.1 Introduction

Rubber tree (*Hevea brasiliensis* Müll.Arg.) is an economic crop that produces natural latex as a major raw material for rubber manufacturers. Most of plantation areas of rubber tree are located in South East Asia such as Malaysia, Indonesia and Thailand (van Beilen and Poirier, 2007). Especially, in Thailand is optimally located to supply the natural rubber demands of the Asia Pacific region. Although recent farming practices for rubber tree are well developed to earn high yield of latex, the plant diseases that cause reduction of latex yield and lethal effect of rubber tree are still often found (Jayasuriya and Deacon, 1995; Evueh and Ogbebor, 2008). One of the main pathogens in rubber plantation is *Rigidoporus* sp., which causes white root disease. This is the most destructive root disease in rubber plantations which kill the tree irrespective of age or health status and results in economic losses to the latex industry (Guyot and Flari, 2002; Ogbebor *et al*., 2015). Although, many chemicals such as metalazyl and tridermorph were used to control fungal disease but they have a negative effect on human health, cause environmental pollution, leave residues in agricultural soil (Harman *et al*., 2004; Jayasinghe, 2010) and induce chemical resistance of several plant pathogenic fungi (Kim and Hwang, 2007).

Fungal endophytes are determined by their in *planta* life without causing apparent disease on the host plants (Chaverri and Gazis, 2011; Prakash, 2015). Along with the symbiotic life, they have been recognized for their advantages in sustaining plant growth and plant defensive system (Strobel and Daisy, 2003; Prakash, 2015). With such benefits, they become one of the promising microbial resources as for biocontrol agents appliled in agricultura purposes. A mode of action of endophytes in prevention and/or suppress of plant diseases caused by phytopathogens is to form antimicrobial substances. Moreover, some endophytic fungi that belong to the families *Xylariaceae* of the phylum Ascomycota

have been reported their capacity to form volatile metabolites with antimicrobial activity. Among of endophytes, *Muscodor albus* is the first known fungal endophyte isolated from *Cinnamomum zeylanicum* which produces bioactive volatile metabolites (Strobel *et al*., 2001; Ezra and Strobel, 2004).

Thus, alternative methods with emphasis on biological control using microorganism have been suggested as the most sustainable long-term solution. Natural products from microorganism are the most important source for new and potential pharmaceutical agents. Specifically, fungal endophytes are expected to be a potential source of new natural bioactive agents that potential kill a wide variety of harmful disease-causing agents including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses and protozoans that affect humans and animals (Strobel and Daisy, 2003). The efficiency of biological control depend on interactions between biological control agent and pathogen (Viterbo *et al*., 2007) such as physical contact, synthesis of hydrolytic enzyme, toxic antibiotic compound, competition, and induction of resistance in plant host (Benítez *et al*., 2004). Furthermore, volatile metabolites-producing endophytes may be an alternative biological approach as biofumigation in control of plant diseases. It is known that sapwood and leaf fragments of rubber tree are a rich source of fungal endophytes (Evueh and Ogbebor, 2008; Rocha, 2010; Gazis, 2012), but only few studies revealed antimicrobial activity of them (Evueh and Ogbebor, 2008; Rocha, 2010). Moreover, no volatile metabolites-producing endophyte has yet been reported from rubber tree host.

1.2 Thesis objectives

1.2.1 To isolate and identify endophytic fungi from Thai medicinal plants and the rubber tree

1.2.2 To screen the effective fungi that capable of producing bioactive agents, plant growth promoting metabolites and volatile metabolites against rubber tree pathogens

1.2.3 To purify and characterize the effective bioactive agents and volatile metabolites

1.2.4 To investigate biological control of rubber tree pathogens *in vivo* by using endophytic fungi

1.3 Usefulness of the thesis

1.3.1 The possibility to discover the effective/novel endophytic fungi for controlling rubber tree pathogens or other pathogenic microorganisms.

1.3.2 Possible discovery of new species of endophytic fungi in the rubber tree.

1.3.3 Discovery of modify method or technique to control pathogens in rubber plantation.

1.4 Plan of the thesis

This thesis was initiated to collect 13 kinds of medicinal plants and the rubber tree from various locations in northern and northeastern of Thailand. Endophytic fungi was isolated, identified and screened for their antagonism. Setting the background to this work, in Chapter 2 reviews the knowledge about endophytic fungi, their ability to produce bioactive compounds and volatile organic compounds (VOCs), including the diversity of endophytic fungi in the rubber tree.

Chapter 3 describes the isolation, identification and screening endophytic fungi from Thai medicinal plants that capable of producing antimicrobial substances and plant growth promoting metabolite.

Chapter 4 describes the isolation and identification base on morphology, molecular, and volatile composition in genus *Muscodor* isolated from the rubber tree.

Chapter 5 describes the antimicrobial activity and phytotoxic activity of VOCs from *Muscodor*.

Chapter 6 describes the use of VOCs from *Muscodor* to control white root disease cause by *Rigidoporus microporus*.

Chapter 7 describes the summarization and discussion of data in this thesis.

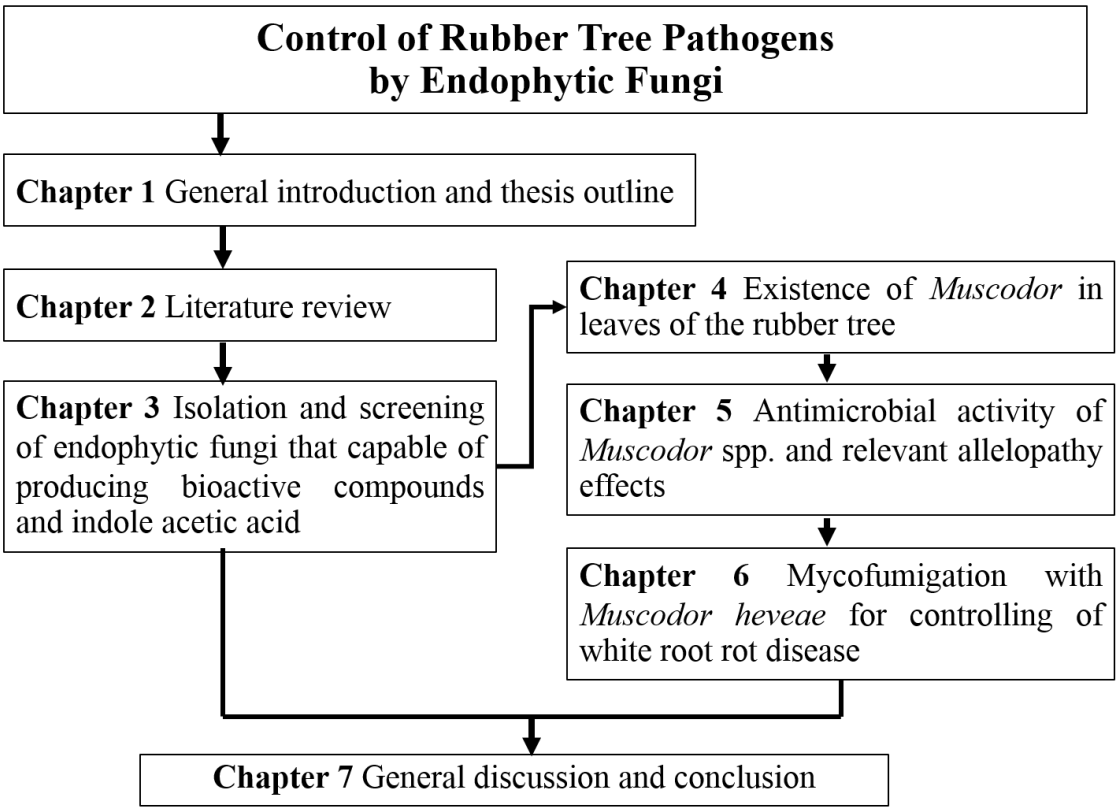


Figure 1.1 Schematic presentation of the relationships between chapters of the thesis.

CHAPTER 2

Literature review

2.1 Introduction of endophytic fungi

Endophytic fungi are the fungi that colonize living in plant tissue without causing any symptoms or negative effects (Huang *et al*., 2001). They have been found in every plant species and may live in roots, stems and/or leaves, emerging to sporulate at plant tissue (Stone *et al*., 2004) (Figure 2.1). Two endophytic groups, Clavicipitaceous (C-endophytes) and nonclavicipitaceous (NC-endophytes) have been recognized based on phylogenetic analysis, plant hosts and ecological functions (Table 2.1). Although the systemic, C-endophytes and NC-endophytes are considered endophytes but they differ in important ways (Table 2.2). C-endophytes were lived in some grasses, while NC-endophytes are highly diverse fungi. They can be found in asymptomatic tissues of nonvascular plants, fern and allies, conifers, and angiosperms (Rodriguez *et al*., 2009). C-endophytes, class 1 represent the phylogenetic characteristic related clavicipitaceous species which limited to some cold and warm season grass (Rodriguez *et al*., 2009). Endophytes of nongrass hosts in class 2-4 represent a broader range taxa from several orders and families of Ascomycetes and some Basidiomycetes families (Stone *et al*., 2004). The unique ability of Class 2 is their ability to confer habitat-adapted stress tolerance to host plant (Rodriguez *et al*., 2008; Rodriguez *et al*., 2009). Transmission of Class 1 endophytes is primary vertical, with maternal plants transfer fungi to offspring by seed infection. Class 1 endophyte frequently increase plant biomass, drought tolerance and produce active compounds that protect plants from herbivores (Clay, 1988).

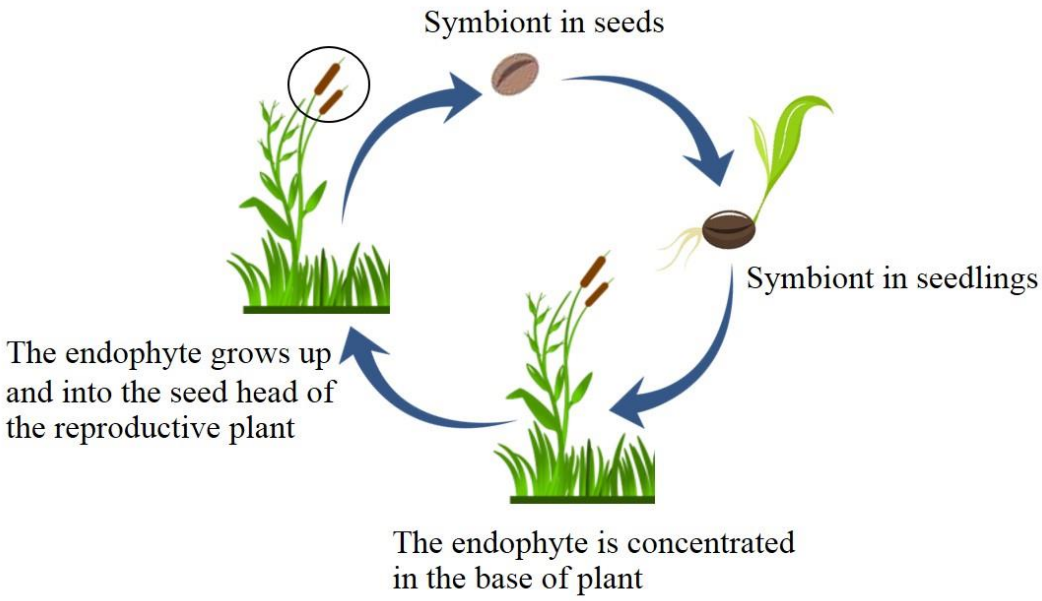


Figure 2.1 Endophyte asexual life cycle.

Table 2.1 Symbiotic criteria used to characterize endophytic fungi classes (Rodriguez *et al*., 2009).

Characteristic	Clavicipitaceous	Nonclavicipitaceous		
Criteria	Class 1	Class 2	Class 3	Class 4
Host range	Narrow	Broad	Broad	Broad
Tissue(s) colonized	Shoot and Rhizome	Shoot, root and rhizome	Shoot	Root
In planta colonization	Extensive	Extensive	Limited	Extensive
In planta biodiversity	Low	Low	High	Unknown
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Fitness benefits*	NHA	NHA and HA	NHA	NHA

*Nonhabitat-adapted (NHA) benefits such as drought tolerance and growth enhancement are common among endophytes regardless of the habitat of origin. Habitat-adapted (HA) benefits result from habitat-specific selective pressures such as pH, temperature and salinity.

All three classes in NC-endophytes have broad host ranges. Class 2 endophytes may live in both above- and below-ground tissues. Class 3 and 4 endophytes are limited to above-ground tissue and roots. The colonization of host tissues also differs, Class 2 and 4 endophytes are ability of extensive plant tissue colonization while Class 3 endophytes form highly localized infections (Rodriguez *et al*., 2009).

Table 2.2 Comparison of characteristics of C-endophytes and NC-endophytes (Stone *et al*., 2004).

C- endophytes	NC-endophytes
1. Few species, only Clavicipitaceae	1. Many species, taxonomically diverse
2. Extensive internal colonization	2. Restricted internal colonization
3. Occurring in several host species	3. Most species with limited host species
4. Systemic, seed transmitted	4. Nonsystemic, spore transmitted
5. Host colonized by only one species	5. Hosts infected by several species

Recently, it shows that endophytes are not host specific (Cohen, 2006) and single endophytic fungi can infect a wide host range. Some strains of the same endophytic fungi isolated from different part of the same host showed different ability to utilized different substances. So, endophytic fungi can be isolated from different plants belongs to the different families (Jalgaonwala *et al*., 2011). C-endophytes show hyphae colonization in all grass tissues and is found both in the seed coat and the embryo (Stone *et al*., 2004). While, NC-endophytes are limited infection in grass tissues but there are two genera *Phialophora* and *Gliocladium* can be seed-borne. However, there also are seed-borne endophyte in non-grass hosts. The dominant endophyte colonization in healthy tissue often is limited to no more than few cells. Endophytic fungi are viewed as outstanding source of secondary metabolite bioactive compounds and used as biocontrol agents applied in agriculture. A mode of action of endophytic fungi in prevention and/or suppression of plant diseases caused by phytopathogens is the production of antimicrobial substances. Moreover, endophytic fungi from medicinal plants could be rich source of active compounds (Sappapan *et al*., 2008). It was found that symbiotic plant stimulates

defense system more quickly than non-symbiotic plants after pathogen infection (Jalgaonwala *et al*., 2011).

2.2 Natural products from endophytic fungi

Endophytic fungi are source of bioactive compounds with antimicrobial activities and also protect against insect, pest and plant pathogens (Jalgaonwala *et al*., 2011). The world's first billion dollar anticancer drug, taxol is a highly functionalized diterpenoid (Stierle *et al*., 1993) (Figure 2.2). It is an antitumor agents but it is used to treat other human tissue-proferating disease as well (Jalgaonwala *et al*., 2011). *Taxomyces andreanae* is an endophytic fungus of the class Hyphomycete. It is the first report of an endophytic fungus from *Taxus brevifolia* that produce taxol (Stierle *et al*., 1993). Recently, several endophytic fungi show ability to produce taxol such as *Fusarium redolens* isolated from *T. wallichiana* (Garyali *et al.*, 2013) and the 3 taxol-producing fungi; *Guignardia mangiferae*, *Fusarium proliferatum* and *Colletotrichum gloeosporioides* isolated from *T. media* (Xiong *et al*., 2013).

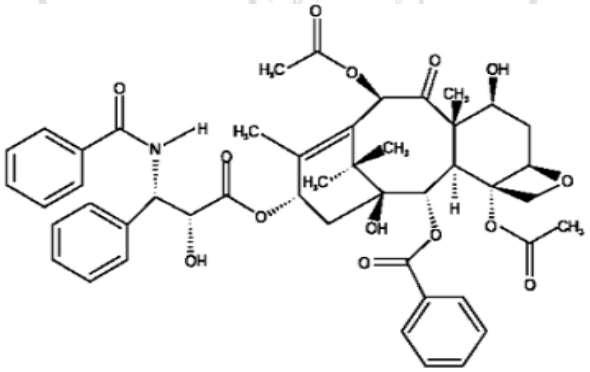


Figure 2.2 The world's first billion dollar anticancer drug, taxol (Jalgaonwala *et al*., 2011)

Antioxidant secondary metabolites are often produced by endophytic fungi. The previous report showed antioxidant capacity of endophytic fungi isolated from wheat (*Triticum durum*) with antimicrobial activity against bacteria, yeast and phytopathogenic fungi (Sadrati *et al*., 2013). Moreover, many bioactive compounds with antimicrobial properties have been successfully discovered from several endophytic fungi such as *Phomopsis* sp. isolated from the leaves of *Vitex negundo* L. showed significant antimicrobial activity against human pathogenic bacteria (Desale and Bodhankar, 2013). Another isolate from mangrove cloud produce cytosporone B and cytosporone C with

antifungal activities against *Candida albicans* and *F. oxysporum* (Huang *et al*., 2008). Furthermore, *P. cassia* isolated from *Cassia spectabilis* produced ethyl 2, 4-dihydroxy-5, 6-dimethylbenzoate and phomopsilactone displayed antifungal activity against fungal pathogens; *Cladosporium cladosporioides* and *C. sphaerospermum* (Silva *et al*., 2005). An endophytic fungi, *Pestalotiopsis adusta* produced 2 active compounds, pesralachlorides A ($C_{21}H_{21}Cl_2NO_5$) and pesralachlorides B ($C_{20}H_{18}Cl_2O_5$). These compounds showed significant antifungal activities against *F. culmorum, Gibberella zeae* and *Verticillium alboatrum* (Li *et al.,* 2008). *Aspergillus fumigatus* LN-4 isolated from stem bark of *Melia azedarach* produced 2 new alkaloids, 12β-hydroxy-13α-methoxyverruculogen TR-2 and 3-hydroxyfumiquinazoline A with potent antifungal activities against several plant pathogens such as *Alternaria alternata, Al. solani, Botrytis cinerea, C. gloeosporioides, G. saubinettii, F. oxysporum* f. sp. *niveum, F. oxysporum* f. sp. *vasinfectum* and *F. solani* (Li *et al*., 2012). While, *Aspergillus* sp. isolated from *Panax notoginseng* produced averythrin, versicolorin B and averantin with antifungal activities against *F. solani.* Moreover, averythrin and averantin also showed antibacterial activities against *Bacillus subtilis* (Liu *et al*., 2014). A new α-tetralone derivative, (3S)-3, 6, 7-trihydroxy-α-tetralone, together with cercosporamide, β-sitosterol and trichodermin were produced by *Phoma* sp. isolated from *Arisaema erubescens*. These compounds were obtained from *Phoma* species for the first time except trichodermin and displayed antimicrobial activities (Wang *et al*., 2012). *Chaetomium globosum* isolated from *Ginkgo biloba* produced 6 active compounds, chaetoglobosin A, C, D, E, G and R with inhibitory activity against 2 phytogenic fungi *Rhizopus stolonifer* and *Coniothyrium diplodiella* (Zhang *et al*., 2013). Three know compounds 6, 8, 1′-tri-*O*-methyl averantin, aversin and 6, 8-di-*O*-methyl versiconol were produced by *Penicillium purpurogenum.* These compounds displayed antifungal activities against *B. cinerea* which cause grey mold disease (Li *et al*., 2014a).

Some antiviral agents are reported from endophytic fungi such as cytonic acid A and B have been isolated from *Cytonaema* sp. with antiviral activities to inhibit human cytomegalovirus (hCMV) protease (Guo *et al.,* 2000). Isoindolones derivatives compounds, emerimidine A and B produced by *Emericella* sp. (HK-ZJ) isolated from *Aegiceras corniculatum* showed anti-influenza A viral (H_1N_1) activities (Zhang *et al*., 2011). Endophytic fungi from Thai medicinal plants have been reported their antiviral

activities against Herpes simplex virus type 1 including anticancer activities (Wiyakrutta *et al*., 2004). Other natural product were produce by endophytic fungi, nodulisporic acid was isolated from *Nodulipotium* sp., *Bontia* and *Daphnoides* with insecticidal activities against the larvae of the blowfly (Verma *et al*., 2007). Nematicidal or insecticidal compounds, 1, 3 oxazinane derivatives from *Geotrichum* sp. AL4, isolated from leaves of *Adenocarpus indica* showed effective nematicidal activity against nematodes (Jalgaonwala *et al*., 2011). Furthermore, anti-parasitic agent, cercosporin has been produced by endophytic fungus *Mycosphaerella* sp. associated with *Psychotria horizontalis* in Panama (Moreno *et al*., 2011).

2.3 Volatile producing-endophytic fungi

Volatile organic compounds (VOCs) are carbon-based solids and liquids which enter the gas phase by vaporizing at 0.01 kPa at 20 °C (Pagans *et al*., 2006). Fungi produced VOCs in both primary and secondary metabolism pathways (Korpi *et al.*, 2009). Previous reports showed the mixture of fungal volatile compounds that have been identified were aldehydes, alcohols, benzene derivatives, cyclohexanes, ketones, hydrocabons, heterocycles, phenol, thioalcohols, thioesters and their derivatives (Mercier *et al*., 2007; Morath *et al*., 2012). Fungal VOCs have special odors and macrofungi such as mushrooms could produce distinctive odors with mixture of different VOCs while the microscopic fungi produced musty odors (Morath *et al*., 2012) (Table 2.3). In agriculture, fungal VOCs may be used as part of biological control strategies to control plant disease in the termed "mycofumigation". Moreover, these VOCs were interested in the plant-growth promoting properties and used to prevent post-harvest fungal contamination. VOCs from *Phoma* sp. significantly enhanced the growth of tobacco seedlings (Naznin *et al*., 2014). In addition, volatile producing endophytic fungi able to detect the plant host and induce their colonization process by producing canonical plant growth regulating substance such as auxins and/or cytokinins (Ortiz-Castro *et al*., 2009). Thus VOCs play a role in plant morphogenetic process (Kanchiswamy *et al.*, 2015). The VOCs produced by endophytic fungi have not been commonly reported. But some endophytic fungi that belong to the families *Diaporthaceae, Hypocreaceae*, and *Xylariaceae* of the phylum Ascomycota are notable for their capacity to form volatile metabolites with antimicrobial activity (Stinson *et al.*, 2003b; Suwannarach *et al*., 2013b). Endophytic fungus in family

Stachybotriaceae, *Myrothecium inunduatum* cloud produce bioactive VOCs with antifungal activity against plant pathogenic fungi. Moreover, in microaerophilic conditions, this fungus produced fuel related hydrocarbons such as octane and 1, 4-cyclohexadiene, 1-methyl- and cyclohexane, (1-ethylpropyl) (Banerjee *et al*., 2010). *Phomopsis* sp. isolated from *Odontoglossum* sp. produced unique VOCs with antifungal properties against phytopathogenic fungi including sabinene, the compound that previously known in higher plant (Singh *et al*., 2011) (Table 2.4).

Table 2.3 Structure, odors and functions of VOCs produced by fungi (Morath *et al*., 2012).

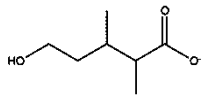
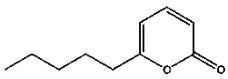
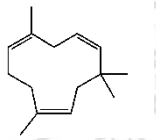
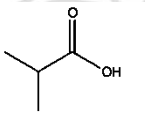
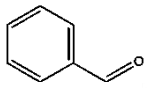
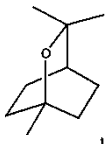
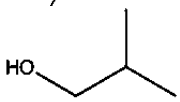
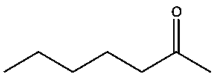
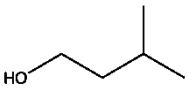
Volatile compounds	Structure	Odor and functions
1-butanol-3-,methyl-, acetate		banana odor, antifungal
6-pentyl-a-pyrone		coconut odor, antibiotic
β-caryophyllene		woody-spicy odor, plant-growth promoting
isobutyric acid		rancid cheese-like odor, antifungal
benzyl aldehyde		almond odor, antimicrobial
1,8-cineole		camphor-like odor, antifungal
2-methyl-1-propanol		mild alcohol odor, fungivore attractant
2-heptanone		cheese odor, unknown
3-methyl-butanol		component of truffle odor, unknown

Table 2.4 List of non-systemic endophytic fungal taxa that produce VOCs (Zhi-Lin *et al*., 2012).

Taxon	Classification	Host	Major compounds
Acremonium sp.	Hypocreales	*Brachiaria brizantha*	Unidentified antibiotic compounds
Aspergillus niger	Trichocomaceae	*Rosa damacaena*	2-phenylethanol
Botrytis sp. BTF21	Sclerotiniaceae	*Musa spp.*	Butane 2-methyl; β-butyrolactone, 2-butenedinitrile
Cladosporium sp. MIF01	Capnodiales	*Mimosa pudica*	Butane 2-methyl, 1-propanol 2-methyl
Fusarium sp. CID124	Nectriaceae	*Centaurea stoebe*	Unidentified sesquiterpenes
Gliocladium roseum, G. catenulatum	Hypocreales	Unknown	Hydrocarbons (benzene, heptane, 1-octene, octane, m-xylene, 3-methylnonane, dodecane, tridecane, hexadecane and nonadecane)
Meliniomyces variabilis	Helotiaceae	*Pinus sylvestris*	Ethanol; acet-aldehyde
Myrothecium inunduatum	Hypocreales	*Acalypha indica*	3-octanone; 3-octanol; 7-octen-4-ol
Nodulisporium sp.1	Xylariaceae	*Cinnamomum loureirii*	β-elemene; β-selinene; α-selinene; 1-methyl-1,4-cyclohexadiene
Nodulisporium sp.2	Xylariaceae	*Olearia argophylla*	1,3,8-p-Menthatriene; caryophyllene; eucalyptol
Phialocephala fortinii	Helotiaceae	*Pinus sylvestris*	Ethanol, acet-aldehyde, toluene
Phoma sp.	Pleosporales	*Larrea tridentata*	alpha.-humulene (sesquiterpene); alcohols; reduced naphthalene derivatives; trans-caryophyllene
Phomopsis sp.	Diaporthales	*Odontoglossum* sp.	Sabinene (monoterpene); 1-butanol, 3-methyl; benzeneethanol; 1-propanol, 2-methyl; 2-propanone

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The genus *Muscodor* is classified into the family *Xylariaceae*. The first species, *Muscodor albus* was isolated from *Cinnamomum zeylanicum* in Honduras, Central America which produces bioactive volatile compounds (Worapong *et al*., 2001; Strobel *et al*., 2001; Ezra *et al*., 2004). The low-molecular weight volatile compounds have broad antimicrobial property, inhibiting or killing fungi and bacteria (Strobel *et al*., 2001). This fungus lacked spores and spore-producing structures (Ezra *et al*., 2004). Recently, 18 species of the genus *Muscodor* were isolated from diverse plants in different countries (Table 2.5). *Muscodor albus* produced strong bioactive VOCs with antimicrobial activity. These volatile compounds could be used for non-agricultural and agricultural biofumigation such as they were investigated to control common building molds (Mercier and Jiménez, 2007) and control *Pythium ultimum* both *in vitro* and *in vivo* (Worapong and Strobel, 2009). Furthermore, the effectiveness of *M. albus* volatile to control pathogens was supported by the composition of the substrates (Ezra and Strobel, 2003). Desiccated rye grain culture was found similar to fresh culture for control of *Penicillium expansum* at 21°C for 24 h in postharvest fumigation and isobutyric acid was the fungicidal agent (Mercier *et al*., 2007). Biofumigation by volatiles metabolites from *Muscodor* was developed as a postharvest treatment. The first demonstration of biofumigation to control fungal decay was placed fresh rye culture of *M. albus* in closed plastic boxes with fruit samples (Mercier and Jiménez, 2004). It showed significant results to control blue mold and gray mold of apple, including brown rot of peaches (Mercier and Jiménez, 2004). Furthermore, *M. albus* volatile was used to control barley seed contamination with *Ustilago hordei* (Pers.) Lagerh. It showed 100% growth inhibition of *U. hordei* in the plant grown from the seed that fumigated with *M. albus* for 4 days (Strobel *et al*., 2001).

Soil-borne disease affects crop production and it often used fungicide to control pathogens. Volatile-producing endophytic fungi may be an alternative method as biofumigation in control of soil-borne disease. For example, solid-state culture of *M. albus* infested artificially to soil or potting mix for control *Verticillium* wilt of eggplants (Stinson *et al*., 2003a), *Phytophthora* root rot of pepper (Mercier and Manker, 2005). Including, another species of *Muscodor*, *M. cinnamomi* displayed potential biofumigation to control *Rh. solani* AG-2 damping-off disease (Suwannarach *et al*., 2012).

2.4 Plant growth promoting endophytic fungi

The fungi are considered to promote plant growth directly or indirectly. The common characteristic include production of plant growth promoting secondary metabolites such as auxin, gibberellin, cytokinin, ethylene, including siderophore (Ahmad *et al*., 2005; Hamayun *et al*., 2010). The ability to promote plant growth of fungal endophytes may be due to capacity to produce higher amounts of growth promoting regulators (Hamayun *et al*., 2010). The one of the most physiologically active auxins is indole acetic acid (IAA). It is a common product of L-tryptophan metabolism by plant growth promoting endophytic fungi. In addition, endophytes could enhanced plant growth by uptake of nutritional elements such as N and P (Ryan *et al*., 2008; Kumar *et al.*, 2011). Several endophytic fungi were produced plant growth promoting metaboites such as *Heteroconium chaetospira*, the root endophyte significantly increased biomass of Chinese cabbage due to nitrogen transfer (Usuki and Narisawa, 2007). *Phoma glomerata* and *Penicillium* sp., which isolated from roots of cucumber could produce gibberellins and IAA to significantly promote the growth of Gas-deficient rice (Waqas *et al*. 2012). Endophytic fungus, *Phoma* spp. from *Tinospora cordifolia* and *Calotropis procera* could promote the growth of *Zea mays* seedlings in pot experiments (Kedar *et al*., 2014). *Absidia* and *Cylindrocladium*, endophyes from rice showed significant increases in rice height, fresh and dry weight (Atugala and Deshappriya, 2015).

Table 2.5 Major volatile compounds and bioactivity compare with related *Muscodor* spp.

Taxon	Host	Collection site	Major compounds	Bioactivity
M. albus cz620[1]	*Cinnamomum zeylanicumin*	Honduras	2-methylpropanoic acid; naphthalene and azulene derivative	antifungal, antibacterial
M. vitigenus[2]	*Paullinia paullinoides*	Lake Sandoval, Peru	naphthalene	anti-insect
M. roseus[3]	*Grevillea pteridifolia*	North of Australian	ethyl 2-butenoate and 1,2,4-trimethylbenzene	antifungal
M. crispans[4]	*Ananas ananassoides*	Bolivian Amazon Basin	2-methylpropanoic acid	antifungal, antibacterial
M. yucatanensis[5]	*Bursera simaruba*	Yucatan Peninsula, Mexico	1*R*,4*S*,7*S*,11*R*-2,2,4,8-tetramethyltricyclo [5.3.1.0(4,11)]undec-8-ene	phytoinhibitory activity
M. fengyangensis[6]	*Actinidia chinensis*	Southeast of China	2-methylpropanoic acid	antifungal, antibacterial
M. cinnamomi[7]	*C. bejolghota*	Chiang Mai, Thailand	ethyl 2-methylpropanoate	antifungal, antibacterial
M. sutara[8]	*Prestonia trifidi*	Columbian tropical Pacific rainforest	2-Methylpropanoic acid	antifungal
M. kashayum[9]	*Aegle marmelos*	West Ghats of India	3-Cyclohexen-1-ol, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	antifungal, antibacterial
M. equiseti[10]	*Equisetum debile*		2-methylpropanoic acid	antifungal, antibacterial

Table 2.5 (continue)

Taxon	Host	Collection site	Major compounds	Bioactivity
M. musae[10]	*Musa acuminata*	Chiang Mai, Thailand	2-methylpropanoic acid	antifungal, antibacterial
M. oryzae[10]	*Oryza rufipogon*	Chiang Mai, Thailand	3-methyl-butan-1-ol	antifungal, antibacterial
M. suthepensis[10]	*C. bejolghota*	Chiang Mai, Thailand	2-methylpropanoic acid	antifungal, antibacterial
M. strobelii[11]	*C. zeylanicum*	South of India	4-octadecylmorpholine	antifungal, antibacterial
M. darjeelingensis[12]	*C. camphora*	Northeast of Himalayas, India	2, 6-Bis (1, 1-dimethylethyl)-4-(1-oxopropyl) phenol	antifungal, antibacterial
M. tigerii[13]	*C. camphora*	Tiger Hill area of Darjeeling, India	4-octadecylmorpholine	antifungal, antibacterial
M. ghoomensis[14]	*C. camphora*	Darjeeling, West Bengal, India	4-octadecylmorpholine	antifungal, antibacterial
M. indicus[14]	*C. camphora*	Darjeeling, West Bengal, India	1, 6-dioxacyclododecane-7, 12-dione and 4-octadecyl morpholine	antifungal, antibacterial

Worapong *et al*., 2001[1]; Daisy *et al.,* 2002[2]; Worapong *et al.,* 2002[3]; Mitchell *et al*.,2008[4]; Gonzalez *et al*., 2009[5]; Zhang *et al.,* 2010[6]; Suwannarach *et al*., 2010[7]; Kudalkar *et al*., 2011[8]; Meshram *et al*., 2013[9]; Suwannarach *et al*., 2013a[10]; Meshram *et al*., 2014[11]; Saxena *et al*., 2014[12]; Saxena *et al*., 2015[13]; Meshram *et al*., 2015[14]

2.5 Para rubber tree and natural rubber

The rubber tree or pará rubber tree (*Hevea brasiliensis* Müll.Arg.) is an economic crop that produces natural rubber as a major raw material for rubber manufacture. It is a medium to large-sized plant and native plant in rainforests of the Amazon region of South America including Brazil, Venezuela, Ecuador, Columbia, Peru and Bolivia. Recently, most of the world' s plantation areas of rubber trees are located in countries in South East Asia such as Malaysia, Indonesia and Thailand (van Beilen and Poirier 2007). The genus *Hevea* is belong to the family *Euphorbiaceae*. There are 3 species, *H. brasiliensis, H. guianensis* and *H. benthamiana* could produce natural rubber. But, *H. brasiliensis* is the only species planted commercially viable quantities of high-quality rubber (30–50% by volume of fresh latex) (Ko *et al*., 2003).

Description: *H. brasiliensis* is a deciduous tree, the plantation tree usually 15-25 m tall with a leafy crown and typically 30-40 tall in the wild (Figure 2.3A). The leaves are in spirals with 3 leaflets. The leaf stalk (petioles) usually 7.5-10 cm long and leaf shape is obovate (Figure 2.3B). The flowers are small with no petals. They are either male or female flower but both are found in the same tree (monoecious). The flower color is bright or cream-yellow. The bark is pale to dark brown with a smooth surface and inner bark is pale brown with white or cream colored latex. The fruit is an exploding 3-lobed capsule, usually 5-6 cm in diameter with gray-brown color (Figure 2.3B).

Figure 2.3 Rubber tree plantation (A) Leaves and seeds of *Hevea brasiliensis* (B).

The rubber seeds propagated and released explosively up to 15 m from the parent plant and can reach a height of over 44 m in the wild (Dickson *et al*., 2011). Rubble tree in plantation is small for 2 reasons. First, it grow more slowly when latex was tapped from the rubber tree. Second, latex production of rubber tree declines after 30 years then the trees are cut. The original crop of rubber tree is in tropical and humid climate areas. However, it can be found in deeply flooded land, acidic boggy sites and drained upland (Gazis, 2012). For optimum growth and high productivity, it required temperature ranging from 26-30 °C and a fairly-distributed rainfall ≥ 1,250 ml per year. Rubber tree can grown on a board range of soil type but the soil should contained the major nutrients such as organic matter (N) > 4.5%, phosphorous (P) > 20 ppm and potassium (K) > 150 ppm. Moreover, it requires a fairly deep surface soil of at least 1 m and pH 5-7. Flat or level lands are good for rubber planting. Rubber tree plantation spans from South American to Southeast Asia that is rubber tree has an exceptional adaptability to a variety of an environmental condition. However, clones adaptation do not always present the same performance in different regions (Martins and Zieri, 2003).

Seedling production of rubber tree concerns rootstock growth in nurseries, stalk formation and grafting operations. Seeds are utilized mainly for rootstocks production (Cardinal *et al.,* 2007). High quality seeds produce healthy rubber tree seedlings. In addition, rootstocks from hybrid cultivars seeds are very healthy than collected seeds in native areas. In rootstock formation, the seeds are germinated to small seedlings that call "spider paw" then they are transferred to the nurseries or plastic bags contained artificial substrate for seedling productions. In stalk formation, clonal gardens have the objective to obtain plant buds for grafting. They have time period estimate 5 years with an objective obtain "brown stalk" buds and 12 years to obtain "green stalk" buds. In grafting operation, the selection of "green stalk" bud is essential for success in grafting because bud formation is rapidity and longevity in the clonal garden. Then, vigorous grafted seedlings are ready to transplant in the field (Cícero and Marcos-Filho, 2010). The previous study showed there were no significant differences in the anatomy and morphology of the leaf from rubber tree clone, GT1 compare with RRIM 600 clone which grafted on the same rootstock and same condition. However, the clone GT1 presented cell thickness in palisade parenchyma and amount of sclerenchyma fibers greater than the RRIM 600 clone (Martins and Zieri, 2003).

The natural rubber (cis-1, 4-polyisoprene) is a secondary metabolite which the producing plant uses natural rubber to protect it from insect and disease (Kaewchai, 2013). It is produced in special cells called laticifers and located inside the phloem. When the bark is tapped, the cytoplasm of laticifer cells are explelled in the form of latex. It contains 30-50% (w/w) of natural rubber which is a raw material in commercial defense and transportation industries (Ko *et al*., 2003). The primary product of rubber are used for the automotive, shipping, agriculture, chemicals, pharmaceutical and consumer industries. Secondary product is lumber and latex products such as globes, vehicle tires, medical tubing and footwear. In addition, the seeds were used as food by Amazonian people and used as an alternative to petroleum fuel (Gazis, 2012). Latex extraction and harvesting is possible from 6-7 years after crop and it is the primary source of natural rubber. Rubber tree produce latex until 25-30 years.

In 2005, the large-scale plantation areas located in Southeast Asia and Africa (Gazis and Chaverri, 2010). About 90% of natural rubber in foreign markets is produced in Southeast Asia. Thailand is the leader of the world to produce and export the rubber products with product capacity of 3.1-3.2 million tons per year. In 2009, rubber plantations in Thailand covered 2.7 million ha. South of Thailand is the largest rubber plantation areas cover 2.1 million ha (77.8%) and the remaining 0.60 million ha (22.8%) are plated in the areas in Northeast, North and Central region, respectively (Saengruksawong *et al*., 2012). In 2012, rubber plantation areas in Thailand increased to 22.2 million ha and plantation area in South of Thailand increased to 13.9 million ha.

2.6 Endophytic fungi in para rubber tree

Natural rubber is synthesized by many plant species belonging to 300 genera but *H. brasiliensis* produce the best quality with 90% of the world's natural rubber production (Gazis and Chaverri, 2010). Previous report showed diversity of endophytic fungi isolated from leaves and sapwood of *H. brasiliensis* grown in Peru, one native planted area. The most frequently isolated were the genera belong to phylum Ascomycota, *Penicillium*, *Pestaloiopsis* and *Trichoderma* (Gazis and Chaverri, 2010). Different endophyte species were dominated in different parts of the plant. Leaf samples display the great number of endophyte species because they have less infection barriers when compared with sapwood (Arnold and Lutzoni, 2007). Commonly, the genera that often found in *H.*

brasiliensis were *Alternaria, Botryosphaeria, Colletotrichum, Fusarium, Pestalotiopsis/Pestalotia* and *Xylaria* (Cannon and Simmons 2002; Gamboa *et al*. 2002; Santamaria and Bayman, 2005). But, Gazis and Chaverri (2010) found the other genera that are not commonly isolated from *H. brasiliensis* such as *Corallomycetella, Fimetariella, Perisporiopsis*, *Rubrinectria* and *Trichoderma*. Moreover, the new endophyte spececies, *T. amzonicum* was found in sapwood and leaves of *H. brasiliensis* and *H. guianensis* (Chaverri and Gazis, 2011) while *Trichoderma* species such as *T. cacao* and *T. gileri* have been isolated from sapwoods and *T. harzianum* was isolated from leaves and sapwoods (Gazis and Chaverri, 2010). However, a diversity study of the genus *Muscodor* that lives in association with rubber trees has not been reported previously.

2.7 Rubber tree disease

Although recent farming practices for the rubber tree have achieved a high yield of latex, plant diseases that cause reduction of latex yield and mortality in rubber trees are still common (Jayasuriya and Deacon 1995; Evueh and Ogbebor 2008). Early detection of disease helps prevent their spread and damage in nurseries and plantations.

2.7.1 Leaf disease

2.7.1.1 Leaf and seedling blight caused by *Fusarium oxysporum* f. sp. *vasinfectum*.

The fungus usually cause vascular wilt of cotton (Hering *et al.,* 1999) but also infects to another plant leaf and seedling including the rubber tree. The pathogen can survive for many years in the form of chlamydospore in plant debris or the soil (Moricca *et al*., 1998). Culture characteristic is cottony whitish to purple (Figure 2.4A), conidiophores simple and branching. Macroconidia, several-celled slightly curved or bent at the pointed ends, multiseptate, typically canoeshaped, hyaline. Microconidia, single-cell in chains and clusters. It infects both seedling and trees in plantations. After infection, the symptom shows irregular brown lesions on leaf surface or seedling stem (Figure 2.4B) and branch with a pinkish, shiny, cottony growth on the infected area.

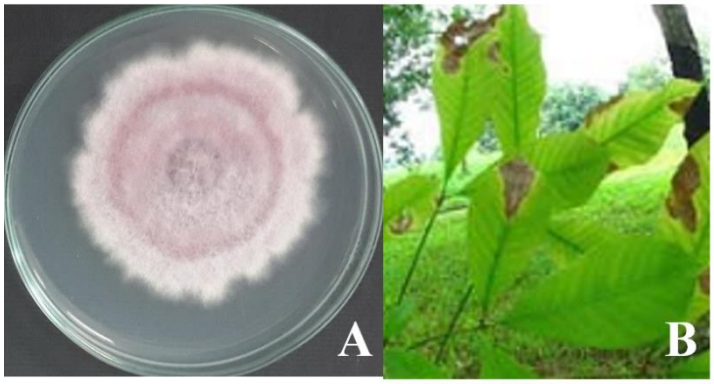

Figure 2.4 Colony of *Fusarium oxysporum* f. sp. *vasinfectum* on PDA (A) symptom of seedling blight (B) (Tangonan *et al*., 2008).

2.7.1.2 Leaf and seedling blight caused by *Rhizoctonia solani* Kuhn.

The fungus infects start from the tip of the leaf with a chlorotic lesion. It is easily seen mycelial growth at the lower part of the leaves. The symptom shows discoloration and rotting of vascular tissue (Figure 2.5). In addition, seedling blight of rubber caused by *Rh. solani* associated with the presence of nematodes that infected stem of budded rubber seedlings.

Figure 2.5 The symptom of Leaf and seedling blight caused by *Rhizoctonia solani* Kuhn (Tangonan *et al*., 2008).

2.7.1.3 Seedling blight caused by *Phytophthora* spp.

The fungus infects the rubber in nurseries. Symptom shows that lower portion of the plant have discolored tissue around the base. Brown strips that extend to the last bud are observed when cut. The pit shrink, then plants dies.

2.7.1.4 Anthracnose leaf spot and leaf blight

This disease is caused by *Colletotrichum gloeosporioides*. The fungus produces 10-15 μm x 5-7 μm conidia with hyaline, one-celled, ovoid to oblong, slightly curved or dumb-bell shape. The spore masses showed pink or orange colored. The infection start from the edge of the leaf and spread to the center. Inside infected tissue appear the waxy acervuili, typically with setae and simple, short, erect conidiophores. The disease affects mainly young budded plants, seedling and young trees. The symptom shows brown and irregular-shaped lesions on leaf surface. Moreover, the infected lesions merge to form concentric lines and then malformation occurs (Figure 2.6).

Figure 2.6 Anthracnose leaf spot disease on rubber tree leaves (Tangonan *et al*., 2008).

2.7.2 Stem and branches diseases

2.7.2.1 Die back disease

Various species of fungi such as *Phytophtora, Colletotrichum* and *Botrydiplodia* are caused this disease. The progressive death of branches start from the terminal bud to the main stem or trunk, then the whole tree is dead. It is observed by leafless branches at the upper part of the canopy (Tangonan *et al*., 2008).

2.7.3 Root disease

2.7.3.1 White root rot disease

It is the most serious problem of rubber tree plantation and causing slow death in all stages from seedling to mature trees (Tangonan *et al*., 2008). This disease is present in Congo, Cote d'Ivorire, India, Malasia, Nigeria, Sri Lanka and Thailand (Rajalakshmy and Jayarathnam, 2000; Omorusi, 2012; Kaewchai, 2013). White root rot is responsible for yield losses of up to 50% in rubber plantations in West Africa (Ogbebor *et al.,* 2013). In Thailand, previous report showed that 55% disease trees in rubber plantations located in Phangnga province are white root rot disease (Kaewchai, 2013). *Rigidoporus microporus* (Syn. *R. lignosus*, *Fomes lignosus*) is major causing agent of this disease by decay lignin and polysaccharide in root tissues (Geiger *et al.,* 1986). The process of disease infection are 3 stage; penetration, colonization and degradation. The fungus penetrates and colonizes root tissues. After degrade the host cell, the mycelium repeatedly carry out penetration and colonization of the host cell wall. Fungal mycelium can observed as intra and inter interactions of cell wall. The pathogen produces range of cell wall degrading enzymes such as CM-cellulase, pectinase and laccase, which correspond to the diverse polymers in host plant cell wall (Omorusi, 2012).

The disease detection is difficult at the early stage because symptoms of root disease in the above ground level are different from the below ground level. In early stage, the symptom showed slightly off-green leaves, then the green leaves in unaffected branches turn to yellow and the whole canopy is destroyed, it is possible indication of root disease (Tangonan *et al*., 2008) (Figure 2.7A). Typical symptom of the disease is the present of rhizomorphs on infected roots with white color but becomes yellow or reddish brown as they grow old. The rhizomorphs are flattened mycelium with 1-2 mm thick which grow firmly attached to the root or tree trunk surface (Omorusi, 2012) (Figure 2.7B). In addition, fungal rhizomorphs with whitish or yellowish colored grown on top or upper layers of the soil and decaying leaves and branches around the base of tree trunks that are sources of fungal inoculum.

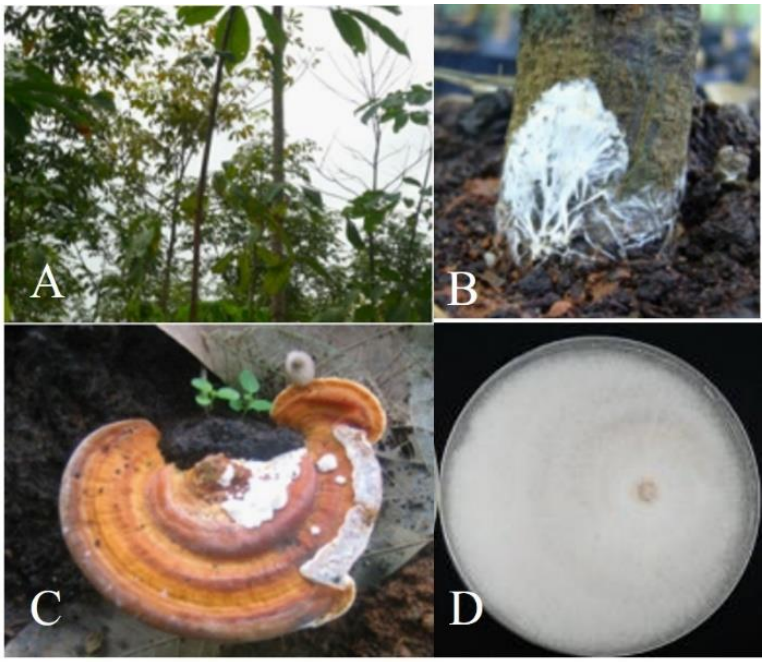

Figure 2.7 Early stage of white root rot disease (A), typical symptom of the disease is the present of rhizomorphs (B), fruiting body of *Rigidoporus microporus* (C) (Kaewchai, 2013) and mycelial growth on potato dextrose agar (PDA) (D).

Fruiting body commonly grow at the collar or near the disease tree base and on decaying roots or stumps (Figure 2.7C). It looks like bracket fungus attached to host. The growth and development of *R. microporus* often occur during rainy season and can survive both in living hosts and nonliving substrate. In the disease cycle, the infection and colonization of *R. microporus* occur in root system of young rubber trees which grow from stumps of infected trees referred to as primary inoculums. The other aspect is the progress of the *R. microporus* along roots from infected trees as secondary inoculums toward healthy rubber trees (Figure 2.8). Disease development and the death of infected trees are most rapid between the first few years after planting.

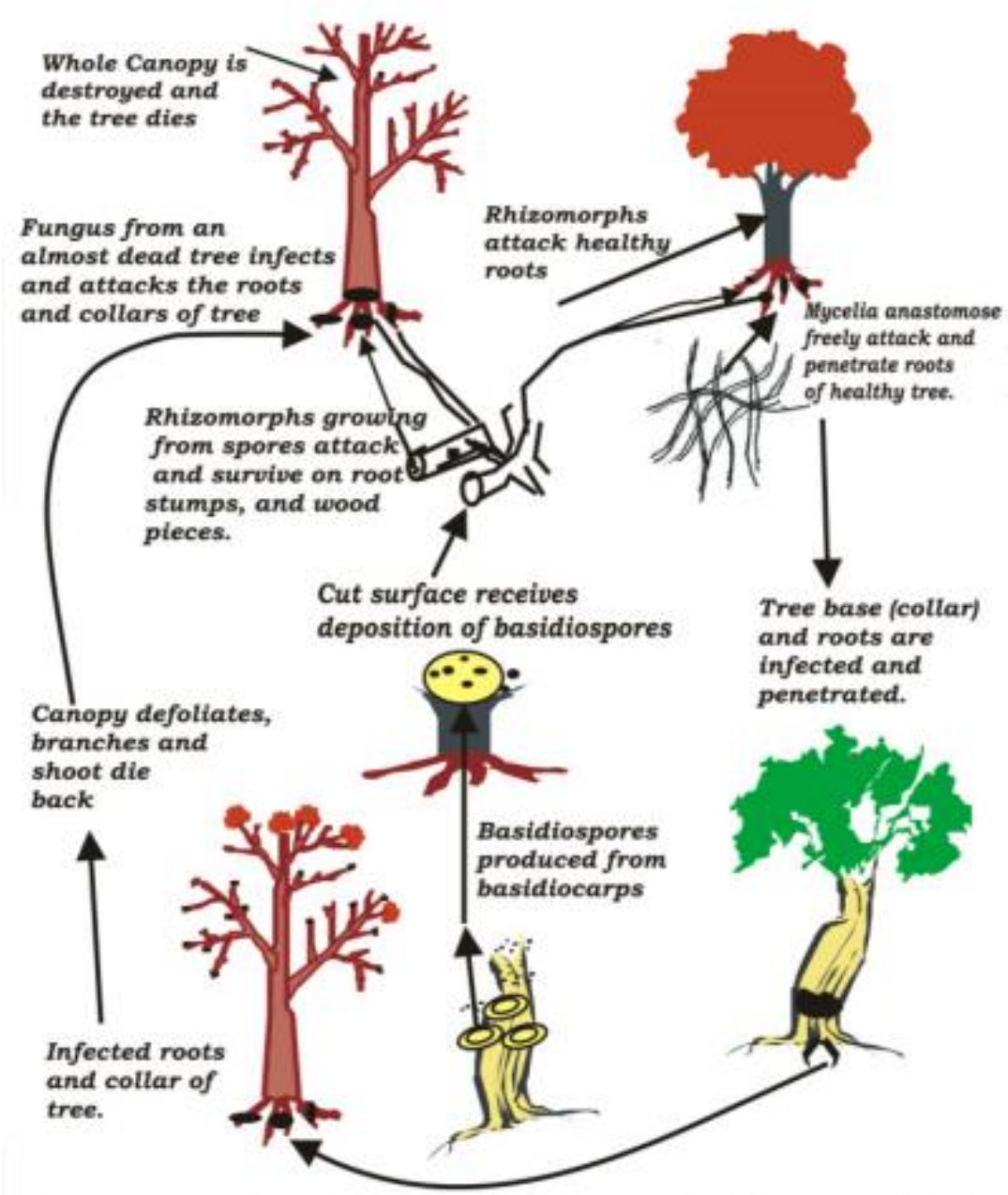

Figure 2.8 White root rot disease cycle (Omorusi, 2012).

2.7.3.2 Brown root rot disease

Phellinus noxius, a fungus which require acidic, high temperature and humid conditions. It can infect root in several trees of all ages and health conditions and has a variety of host plant worldwide (Ann *et al.,* 2002). The fungus produces a white colony which turns brown on PDA at the optimal temperature, 30 °C (Figure 2.9A). Arthrospores and staghorn-like hyphae are also observed in culture

(Figure 2.9B-C). The vegetative hyphae have no clamp connections. *P. noxius* is one of species that are strong virulence while another species act as saprobe in nature or weak pathogens on trees.

The rhizomorphs in the infected roots is tawny brown, becoming dark color with age. The infected roots develop a very rough and irregular surface. The symptom showed pale brown root rot in early stage, then brown zigzag line display in the wood and brown line network can also be seen on the wood surface. The disease tree exhibit reduced growth, discoloration and wilting of leaves and dieback of branches, then whole plant eventually dies (Sahashi, 2013). However, leaf and root symptoms are unique and different from the other root rot pathogen, *R. microporus* and it degrades the polysaccharide fraction but not lignin (Kirk, 1971). The fruiting body is rarely observed on disease trees. It is dark brown bracket fungus while the underside is dark gray.

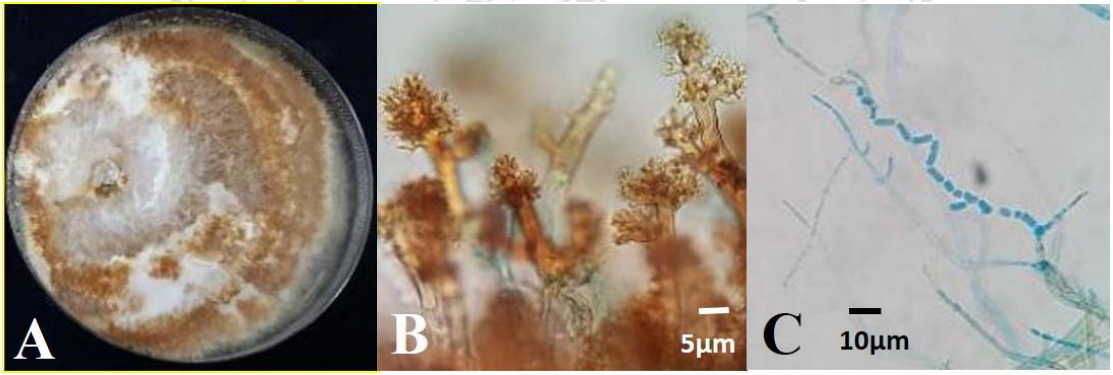

Figure 2.9 Colony of *Phellinus noxius* on PDA (A), staghorn-like hyphae (B) and arthrospores (C).

2.7.3.3 Red root rot disease

The disease is caused by *Ganoderma pseudoferreum*. It produce red shiny mycelium cover infected root. The growing edge of the fungus is commonly white while the red colors is formed only a few centimeter. The rot is pale brown in the early stage, then advance stage, the fungus produces fruiting body. It is hard woody bracket with dark reddish brown but the upper surface is wrinkle with ash white under the bark of the root.

2.8 Management of rubber tree disease

2.8.1 Chemical control

The application of chemical to control the disease was done by spraying for leaf disease and soil drenching for root rot disease. However, it is unworkable and costly due to the tree height and the wide area. Chemical control affect to environment, animal and human health. Moreover, several phytopathogenic fungi have developed resistance to chemical fungicides (Kim and Hwang, 2007). The chemicals such as mancozeb, hexaconazole, difenoconazole and tridemorph (calixin) were applied to control root rot disease (Tangonan *et al*., 2008). The application dosage follow by the manufactures but it should be applied to the root every 3 months. When detected early stage, dig the soil around the base of the tree, then scrape the fungal rhizomorphs. Drench with chemicals for the rubber diseases. Build canals around the effected trees to protect spreading the disease to healthy trees. The fungal rhizomorphs was inhibited and the treated trees showed complete recovery. However, the infected tree at advanced stage of *R. microporus* did not recover. It also added the powdered sulphur in the plating hole, when it is known to promote fungal growth antagonistic to inhibit fungal root disease. However, clearing methods are used to reduce root rot disease such as uprooting and poisoning of old trees. *Phytophthora* black strip was control by 4 effective chemicals, mancozeb, metalaxyl, oxadixyl and benomyl.

2.8.2 Biological Control

Biological control is the using of resistant varieties and the manipulation of antagonists and biological competitors (Tangonan *et al*., 2008). It is the method avoids problems from chemical control such as the development of the chemical resistance of pathogens. Although, it can't completely control the pathogens as rapidly as chemical control but it can be an important component in the development of agriculture management system. Basidiomycete fungi was used to control the growth of *R. lignosus* under nursery and plantation. Likewise, *T. harzinum* was applied to control the develop of fungal rhizomorph on rubber plant. It gave an effect in suppressing *R. lignosus* and found to have different results with the chemical control. *Trichoderma harzianum* showed highly antagonistic by completely overgrowing the pathogen (Tangonan *et al*., 2008). In

2010, Kaewchai and Soytong reported that the crude extract and rotiorinol, a bioactive compound produced from *Cheatomium cupreum* could inhibit mycelial growth of *R. microporus* and the application of *Ch. cupreum* in poweder and oil form showed significantly inhibit the growth of *R. microporus* in the rubber tree. In addition, *T. hamatum* STN07 and *T. harzianum* STN01 were rapidly grown over the pathogen colony. However, the application of antagonistic fungi in the field needs to develop for effective results and sustainable (Kaewchai, 2013). In 2015, Ogbebor *et al*. showed antifungal activity of *Hypocrea jecorina* to control *R. lignosus* with 86.83% growth inhibition. Moreover, *H. virens, H. jecorina, H. lixii, T. spirale* and *Trichoderma* sp. were also effective on *R. lignosus*. *In vivo,* seedling in *H. jecorina* treatment at 60 days and *H. virens* treatment at 150 days after inoculations, respectively had highest length of stem and length of tap rot, lowest plant death, and least foliar symtomp compared to the control.

CHAPTER 3

Isolation and screening of endophytic fungi that capable of producing bioactive compounds and indole acetic acid

3.1 Introduction

At least one million species of endophytic fungi are found in the plant tissue (Dreyfus and Chapela, 1994). Natural select the evolution of beneficial endophyte strains and several endophyte are found to produce bioactive compounds that protect plant from insect pests and pathogens (Saikkonen *et al*., 2004). They play an important role in plant defense including the function as growth promoter and enable the host survival under extreme conditions (Rosa *et al*., 2012). Recently, the development and spread of drug-resistant pathogens are still a global problem and there is a need to search for new active agents with antimicrobial activity (Espinel *et al*., 2001). Thus, endophytic fungi are source of novel or bioactive metabolites for pharmacological and agricultural applications (Idris *et al*., 2013). Natural products from fungal endophyte showed antagonistic activity to inhibit several pathogenetic organism such as bacteria, fungi, viruses and protozoans. Furthermore, they could be plant growth regulators such as indole acetic acid (IAA), one of the most physiologically active auxins. IAA is a product of L-tryptophan metabolism by various microorganism including Plant growth promoting rhizobacteria (PGPR) (Ahmad *et al*., 2005).

Plants that produce effective natural products had associated with endophytic fungi that produced the same compounds (Tan and Zou, 2001). Valuable plant products might be produced by their endophytic fungi, which were easier and more economical for large-scale production than plant sources (Strobel and Daisy, 2003). Thus, searching for active fungal endophyte, medicinal plants were a great source of endophyte based on their pharmaceutical properties.

Endophytic fungi colonized plant tissues without causing any symptoms. They were found in all terrestrial plants and healthy plants were interesting source for endophyte isolation.The large number of endophytic fungi played an important role on ecosystem with greatest biodiversity (Strobel, 2003). The biodiversity of plant species were reported in Thailand and it had a long traditional of herbal medicine (Panthong *et al.,* 1991). Thai medicinal plant might be another source of effective endophytic fungi with biological activity or produced novel bioactive compounds. The previous report showed that *Colletotrichum* sp. E5T9, an endophytic fungus from wild medicinal plants in Thailand produced asparagenase with cancer inhibitory property (Theantana *et al*., 2009). Furthermore, *Fusarium* spp. and *Acremonium* spp. were the predominate species of endophytic fungi from *Sesbania grandiflora* (L.) Pers. with antimicrobial activity against bacteria, yeast and molds (Powthong *et al*., 2013).

3.2 Materials and methods

3.2.1 Preparation and surface sterilization of plant materials

Fourteen healthy medicinal plant species and rubber tree were used as source for isolation of endophytic fungi. Three plants such as *Clinacanthus nutans* (Burm.f.) Lindau, *C. siamensis* Bremek and *Rhinacanthus nasutus* (Linn.) Kurz were collected from Medicinal Plant Garden, Doi Suthep-Pui National Park (18°48′22″N, 98°54′51″E, altitude 1,076 m), located in Chiang Mai Province. *Aloe vera* L., *Hippeastrum johnsonii* Bury*.*, *Muchlenbeckia platyclada* Meissn., *Pereskia grandiflora* Haw., *Jatropha podagrica* Hook., *Schefflera leucantha* R. Vig. and *Citrus hystrix* DC. were collected in different areas from Chiang Mai Province. *Bauhinia strychnifolia* Craib and *Betula alnoides* Buch.-Ham. ex G.Don were collected from Udon Thani province (17°24′54″N, 102°47′12″E). *Pseuderanthemum palatiferum* (Nees) Radlk. was collected from Nakhon Ratchasima province (14°58 ′16″N, 102°5′59″E) and *Hevea brasiliensis* Müll.Arg. was collected in planting areas from Nong Bua Lamphu province (17°12′14″N, 102°26′26″E).

Plant materials were cut randomly into small segments (leaf, 5 mm x 5 mm; branch 5 mm long). All segments were sterilized by soaking in 75% ethanol for 30 s, 2% sodium hypochlorite for 3 min, and 95% ethanol for 30 s under a laminar flow hood (Suwannarach *et al*., 2010).

3.2.2 Isolation of endophytic fungi

Each plant materials were placed on 2% malt extract agar containing rose bengal (0.033 g/l) and chloramphenicol (50 mg/l) (Theantana *et al*., 2007). Petri dishes were sealed with Parafilm® M (Bemis company, Inc., USA) and incubated at 25±2 °C for 2 weeks. The hyphal tips were cut when the fungi growing out from the samples and transferred to potato dextrose agar (PDA). The medium was also be used for subculture and stock culture.

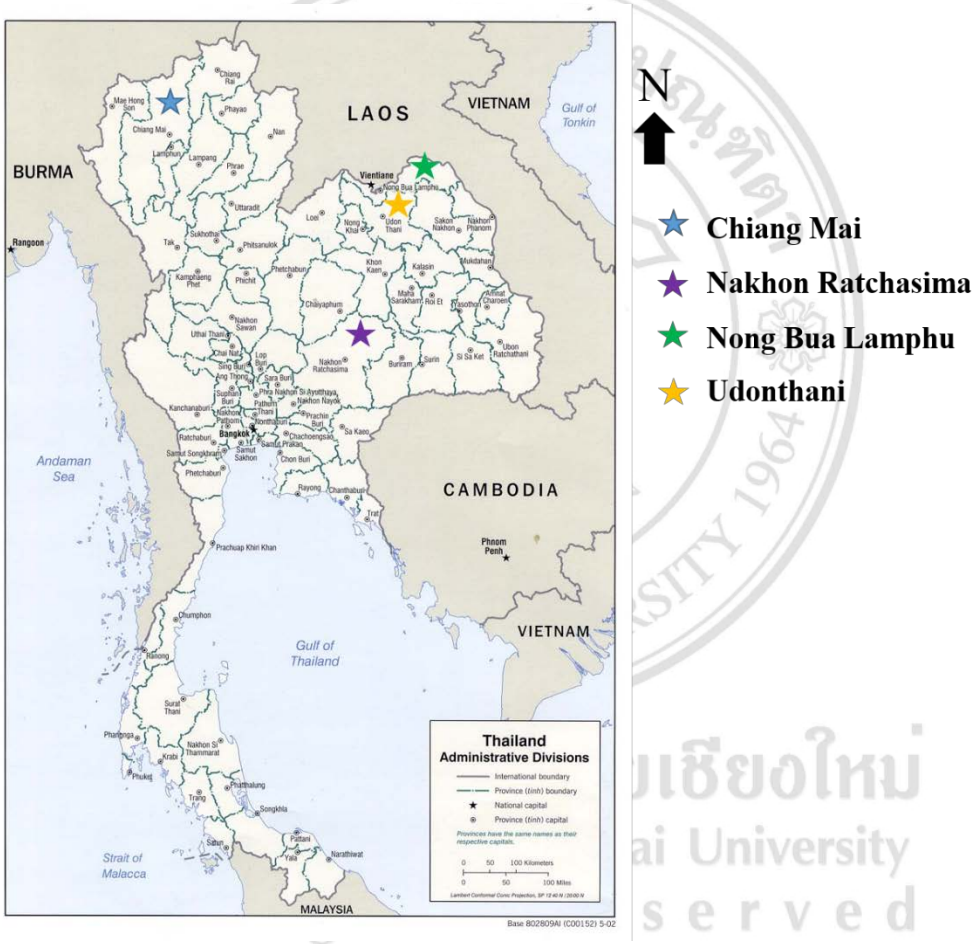

Figure 3.1 Colleting sites (star marks) of selected plants in Thailand where used in this study.

3.2.3 Isolation and pathogenicity test of phytopathogenic fungus

Leaf and seedling blight disease of the rubber tree were sampled from rubber tree planting area in Nong Bua Lamphu province. Stem containing lesions were cut into small pieces (5 mm long) and surface sterilized according to the method in section

3.2.1, then placed on PDA and incubated at room temperature (25±2 °C) for 2 weeks. The hyphal tips were cut when the fungi grew out from the samples and transferred to PDA. The PDA medium was used for subculture and stock culture**.**

Pathogenicity tests was carried out in plastic boxes. Healthy leaf and stem of the rubber tree seedlings were surface sterilized with 70 % ethanol. Each mycelial plug of fungal isolates (6 mm diameter) were placed on leaf and stem for pathogenicity test with moistened cotton wool and incubated at room temperature (25±2 °C) for 2 weeks. Then, disease symptom on leaf and stem were recorded.

3.2.4 Phenotypic and genotypic classification

Identification of endophytic fungi was carried out on the basis of morphological and molecular characteristics (Huang *et al.*, 2008). The fungal isolates were grown on potato dextrose agar (PDA) and incubated at room temperature to observe morphological characteristic such as color, shape and size of spores. For molecular identification, the effective fungal endophytes were grown on PDA at 25±2 °C for 10 day. The aerial mycelium of each isolate was scraped from the PDA surface. This fungal biomass was then freeze-dried and ground into a fine powder with a pestle and mortar. A modified SDS-CTAB method (Suwannarach *et al.*, 2013a) was used for the DNA extraction. The internal transcribed spacer regions 1 and 2 including 5.8S rDNA (ITS1-5.8-ITS2 rDNA) was a targeted nucleotide sequence for the amplification using a pair of universal primers (ITS4 and ITS5). The amplification was carried out using a GeneAmp 9700 thermal cycler (Applied Biosystems) with the following polymerase chain reaction (PCR) conditions: initial denaturization at 95 °C (2 min), 30 cycles of reaction [denaturization at 95 °C (30 s), annealing at 50 °C (30 s), and extension at 72 °C (1 min)], and final extension at 72 °C (10 min). The reaction mixture (25 µ L) contained 1 µ L of the DNA template, 0.2 µ M dNTP, 0.2 µ L of FastTaq (Applied Biosystems), 0.2 µ M each primer, 2.5 µ L of MgCl 2 buffer, and sterile water to bring the final volume to 25 µ L. The PCR product was confirmed by gel electrophoresis and purified with a PCR cleanup Gel Extraction NucleoSpin® Extract II Purification Kit (Macherey-Nagel, Germany) following the manufacturer' s manual. The purified PCR product was then sequenced by 1st Base, Malaysia. All DNA sequences obtained were aligned and compared to the available sequences in the GenBank database using the BLASTN facility

within the NCBI website (http://www.ncbi.nlm.nih.gov/ BLAST/). Phylogenetic analysis of all sequences was conducted by the neighbor-joining method using MEGA6 software (Tamura *et al*., 2013).

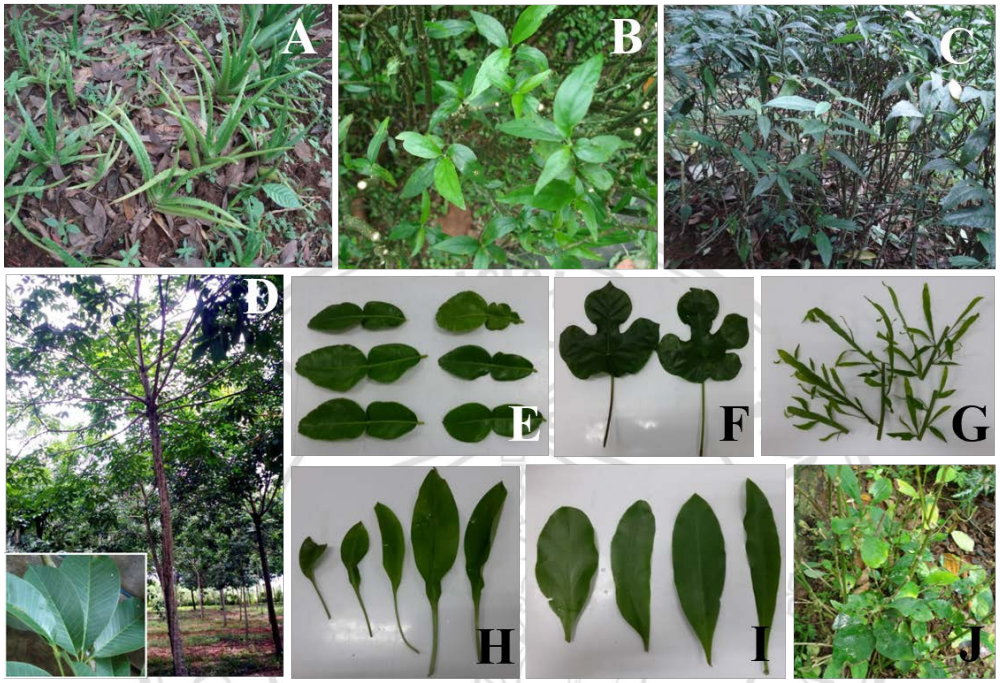

Figure 3.2 Plant samples in this study; A, *Aloe vera*; B, *Clinacanthus nutans*; C, *Clinacanthus siamensis*; D, *Hevea brasiliensis*; E, *Citrus hystrix*; F, *Jatropha podagrica*; G, *Muchlenbeckia platyclada*; H, *Hippeastrum johnsonii*; I, *Pereskia grandiflora*; J, *Rhinacanthus nasutus*.

Table 3.1 Collection details of selected plants in this study.

Plant host	Collection site	Collection time
Aloe vera	Chiang Mai Province	June, 2013
Bauhinia strychnifolia	Udon Thani Province	September, 2012
Betula alnoides	Udon Thani Province	September, 2012
Clinacanthus nutans	Medicinal Plant Garden, Chiang Mai Province	June, 2011
Clinacanthus siamensis	Medicinal Plant Garden, Chiang Mai Province	August, 2011
Hevea brasiliensis	Rubber tree planting area, Nong Bua Lamphu Province and Payao Province	May, 2011-December, 2012
Citrus hystrix	Chiang Mai Province	September, 2013
Jatropha podagrica	Chiang Mai Province	August, 2013
Muchlenbeckia platyclada	Chiang Mai Province	August, 2013
Hippeastrum johnsonii	Chiang Mai Province	August, 2013
Pereskia grandiflora	Chiang Mai Province	August, 2013
Pseuderanthemum palatiferum	Nakhon Ratchasima Province	June, 2012
Rhinacanthus nasutus	Medicinal Plant Garden, Chiang Mai Province	June, 2011
Schefflera leucantha	Chiang Mai Province	June, 2013

3.2.5 Screening for antagonism against rubber tree pathogens by dual culture method

Antagonistic activity of all fungal endophytes was tested against a set of pathogenic fungi listed in Table 3.2. The antagonistic analysis was observed from the interaction between fungal endophytes and the pathogens using dual culture technique. An agar plug (6 mm diameter) 4-day-old fungal endophytes growing on PDA was inoculated on PDA part of the petri dish, then an agar plug of fungal pathogens was inoculated opposite side of the petri dish. The control plates were inoculated with either pathogens. All Petri dishes were wrapped with Parafilm® M (Bemis company, Inc., USA) and incubated at room temperature (25±2 °C) for 5 days. The percentage of inhibition of fungal growth after the dual culture test was calculated with the following equation: [(R1-R2)×100] ÷ R1 , where R 1 was the average colony radius of each tested fungi measured in the control plates (without the tested fungi), and R 2 was the average colony radius that calculated from the tested plates.

Table 3.2 The list of pathogenic fungi used in this study.

Pathogenic fungi	Source
Colletotrichum gloeosporioides *Fusarium oxysporum* f. sp. *vasinfectum* *Rhizoctonia solani* AG-2	The Sustainable Development of Biological Resources (SDBR) Lab, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand
Phellinus noxius BCC26237 *Phytophthora parasitica* BCC15560	The BIOTEC (National Center for Genetic Engineering and Biotechnology) Culture Collection (BCC), Bangkok, Thailand.
Rigidoporus microporus	Department of Microbiology and Parasitology, Faculty of Science, Naresuan University, Phitsanulok.

3.2.6 Crude extract preparation

The crude extract of effective endophytic fungi was prepared according to the modified solid state fermentation method of Sharmin, *et al*. (2013). Fungal endophytes were grown on PDA at room temperature (25±2 °C) for 5 days. Three pieces (0.5x0.5 cm^2) of mycelial plugs were inoculated on PDA at room temperature (25±2 °C) for 7 days. The medium from all the petri dishes were cut into small pieces and collected in beakers containing ethyl acetate (1:1 v/v) for 3 days. The filtrates was extracted three times with ethyl acetate and evaporated to dryness using a rotary vacuum evaporator. Then, the extract was resuspended with 50% methanol (MeOH).

3.2.7 Bioassay of crude extract by Agar well diffusion method

The crude extracts were dissolved in 50% methanol at the concentration of 5 and 10 mg/ml. An agar plug of pathogenic fungi was placed on the center of PDA plates. Then, 35 µl of each resuspended fungal supernatant was separately loaded into 0.6 mm diameter wells cut around the mycelial plug of pathogenic fungus. The plates were incubated at room temperature (25±2 °C) for 7 days and the results were recorded and calculated the percentage of inhibition of fungal growth according to the method in section 3.2.4. Tridemorph (2, 6-Dimethyl-4-tridecylmorpholine) and carbendazim (Methyl *1H*-benzimidazol-2-ylcarbamate) were used as fungicide to control *R. microporus* and *P. noxius*, and *F. oxysporum* f. sp. *vasinfectum*, respectively. In addition, 50% methanol was used as negative control.

3.2.8 Determination of minimum inhibitory concentrations (MICs) of crude extract

The crude extracts were determined their MIC by using 2-fold serially diluted crude extract from 0.04 to 5.00 µg/ml with 50% methanol. Then, 35 µl of each concentration was loaded into 0.6 mm diameter wells that were cut around the mycelial plug of pathogenic fungus. The plates were incubated at room temperature (25±2 °C) for 7 days. The lowest concentration that inhibited mycelial growth was recorded as the MIC. The tests were conducted in three replicates and SPSS program version 17.0 for Windows was used to analyze the experimental data with one-way analysis of variance (ANOVA).

3.2.9 Antifungal activities assay by TLC-bioautography

Each 40 µl of crude extract in 50% methanol (5 mg/ml) was spotted on TLC sheet (TLC aluminium sheets, 10 cm x10 cm, silica gel 60GF254, Merck, Germany). Then, it was placed in a glass tank with solvent system using 95:5 (v/v) dichloromethane: methanol as mobile phase. After running the chromatogram, TLC plate were dried and viewed under UV light at different wave length of 254 nm and 365 nm (Prakash *et al.*, 2013). The Rf values of TLC plate were calculated.

Each active compound was examined for antifungal activity by performing bioautography coupled with thin-layer chromatography (TLC-bioautography). The modified bioautography method was performed according to the method that described by Bhavya *et al.* (2011). Six mycelial plugs (6 mm diameter) of 4-day-old *P. noxius*, and *F. oxysporum* f. sp. *vasinfectum* were placed on PDA plates and incubated at room temperature (25±2 °C) for 1 day. While, spore suspension of *F. oxysporum* f. sp. *vasinfectum* ($1x10^6$ spore/ml) was swab on PDA plate to detect the inhibition of spore germination. The chromatograms were developed, as described and were placed on PDA plate between the colonies of *P. noxius* and *F. oxysporum* f. sp. *vasinfectum*, respectively (Figure 3.3). After 1 hour diffusion process, TLC sheets were removed and incubated at room temperature (25±2 °C) for 3-7 days. The inhibition zone was observed and recorded.

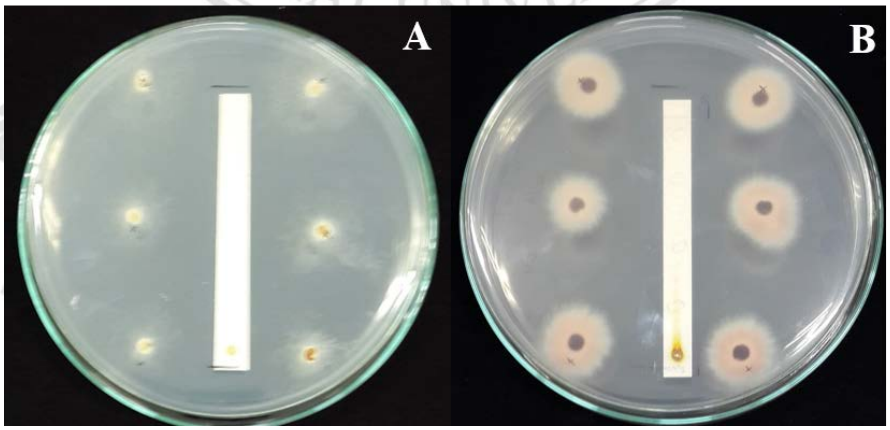

Figure 3.3 Antifungal activities assay by TLC-bioautography. The developed chromatogram were placed on PDA plate between the colonies of *P. noxius* (A) and *F. oxysporum* f. sp. *vasinfectum* (B), respectively.

3.2.10 Preliminary assay of hemolysis

An agar plug (6 mm diameter) of 4-day-old fungal endophytes growing on PDA was inoculated on 5% Sheep blood agar. All Petri dishes were wrapped with Parafilm® M (Bemis company, Inc., USA) and incubated at room temperature (25±2 °C) for 7-15 days. The crude extracts were dissolved in 50% methanol at the concentration of 5 and 10 mg/ml. Then, 35 µl of each resuspended fungal supernatant was separately loaded into 0.6 mm diameter wells on 5% Sheep blood agar. The plates were incubated at room temperature (25±2 °C) for 7 days. Type of hemolysis on blood agar was observed and recorded.

3.2.11 Screening for indole acetic acid (IAA) production

All isolates of endophytic fungi were inoculated in 5 ml Potato dextrose broth (PDB) with L-tryptophan (2 mg/ml) and incubated in the dark at room temperature (25±2 °C) with shaking at 150 rpm on a shaker for 5 days. The broth cultures were filtrated by two layers of gauze cloth to separate the broth cultures and mycelia. A modified method described by Admad *et al.*, 2005 was used for screening IAA production. All filtrates (1 ml) were mixed with 2 ml of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$; 50 ml of 35% perchloric acid ($HClO_4$)) and incubated in the dark for 30 min. The development of pink color indicated IAA production and the absorbance at 530 nm was measured. The level of IAA production was estimated by standard IAA graph (Appendix B).

3.2.12 Extraction of crude IAA and Thin layer chromatography (TLC)

The broth cultures of endophytic fungi were prepared according to the method in section 3.2.9 and filtrated by two layers of gauze cloth. Then, the filtrates were acidified to pH 2.5 to 3.0 with 1 N HCl and extracted twice with ethyl acetate at double volume of the filtrates. The ethyl acetate fraction was evaporated to dryness using a rotatory evaporator. The crude extracts were dissolved in 1 ml of methanol and spotted on TLC sheets (TLC aluminium sheets, silica gel 60GF254, Merck, Germany) with standard IAA and standard IBA (indole-3-butyric acid). Then, TLC sheets were developed in chloroform:methanol:water (84:14:1, v/v/v). Spots with Rf values identical to authentic IAA were identified under UV light at wave length of 254 nm by spraying

with Ehmann's reagent (mixed with 1 volume of 50 ml of 0.2 % (w/v) *p*-dimethylaminobenzaldehyde in conc. HCl added with 50 ml of absolute ethanol and 3 volumes of 2.03 g of $FeCl_3.6H_2O$ in 500 ml of water and 300 ml of conc. H_2SO_4) and Salkowski's reagent (Ehmann, 1977).

3.3 Results

3.3.1 Isolation of endophytic fungi

A total of 271 endophytic fungi were isolated from 14 plant species, the most of them were isolated from *C. nutans* (24.4%), followed by *R. nasutus* (10.3%) and *C. siamensis* (8.9%), respectively (Table 3.3). Most of them colonized in leaf (53.1%) and only 10 isolates colonized in root of plant sample (Table 3.3). Some of them did not produce spore and belonged to mycelia sterilia group. While, endophytic fungi isolate JP1B1-1 and JP1B1-2 were isolated from branch of *J. podagrica* with whitish colony and fast-growing mycelium (Figure 3.4).

Table 3.3 Number of endophytic fungi isolated from medicinal plants and the rubber tree.

Plant species	Leaf		Stem/Branch	Root	Total
	Intervein	Vein			
Aloe vera	3*	3*	7	1	11
Bauhinia strychnifolia	3	1	5	0	9
Betula alnoides	1	2	3	0	6
Clinacanthus nutans	17	14	29	6	66
Clinacanthus siamensis	4	9	11	0	24
Hevea brasiliensis	0	3	20	0	23
Citrus hystrix	15	3	1	0	19
Jatropha podagrica	12	2	8	0	22
Muchlenbeckia platyclada	8	3	0	0	11
Hippeastrum johnsonii	12	6	2	0	20
Pereskia grandiflora	3	6	0	0	9
Pseuderanthemum palatiferum	3	5	2	0	10
Rhinacanthus nasutus	0	0	25	3	28
Schefflera leucantha	0	9	4	0	13

(*) = vein absent

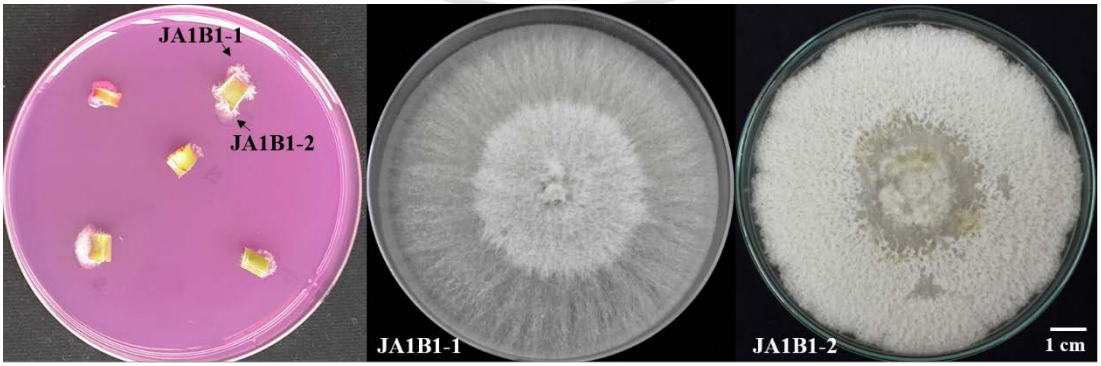

Figure 3.4 Fungal isolate JP1B1-1 and JP1B1-2 grew out from branch segment of *Jatropha podagrica*.

3.3.2 Isolation and pathogenicity test of pathogenic fungus causing leaf and seedling blight in the rubber tree

Fusarium oxysporum f. sp. *vasinfectum* (PRT3-1) was isolated from rubber tree seeding with observation of the disease symptom**.** Culture characteristic is cottony whitish to purple (Figure 3.5A), conidiophores simple and branching. Macroconidia, several-celled slightly curved or bent at the pointed ends, multiseptate, typically canoeshaped, hyaline (Figure 3.5C). Microconidia, single-cell in chains and clusters (Figure 3.5D). The fungal isolate was classified and identified base on morphological and molecular characteristic. It shared relatively sequence similarity of ITS rRNA gene (100%) with *Fusarium oxysporum* f. sp. *vasinfectum* (Figure 3.6).

Phytopathogenicity test showed that *Fusarium oxysporum* f. sp. *vasinfectum* (PRT3-1) infected leaf and branch of rubber tree seedling. The symptoms showed irregular brown lesions on leaf and branch surface with a cottony growth on the infected area (Figure 3.7).

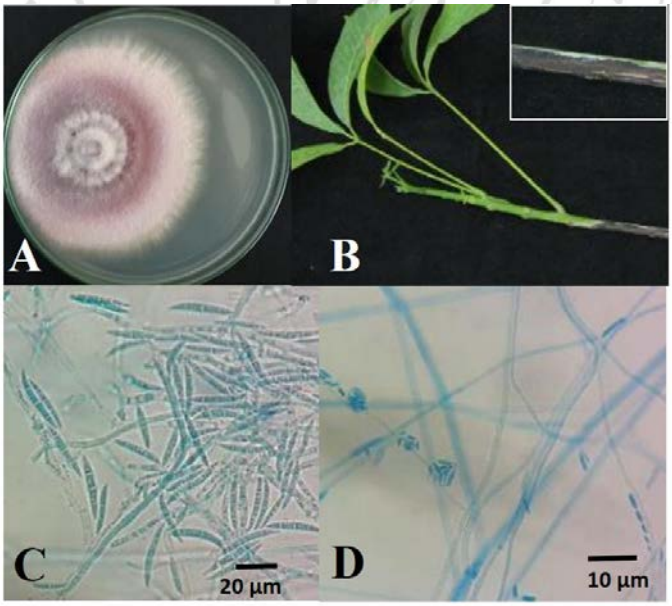

Figure 3.5 Colony of *Fusarium oxysporum* f. sp. *vasinfectum* (A), symptom of seedling blight (B), macroconidia (C) and microconidia (D).

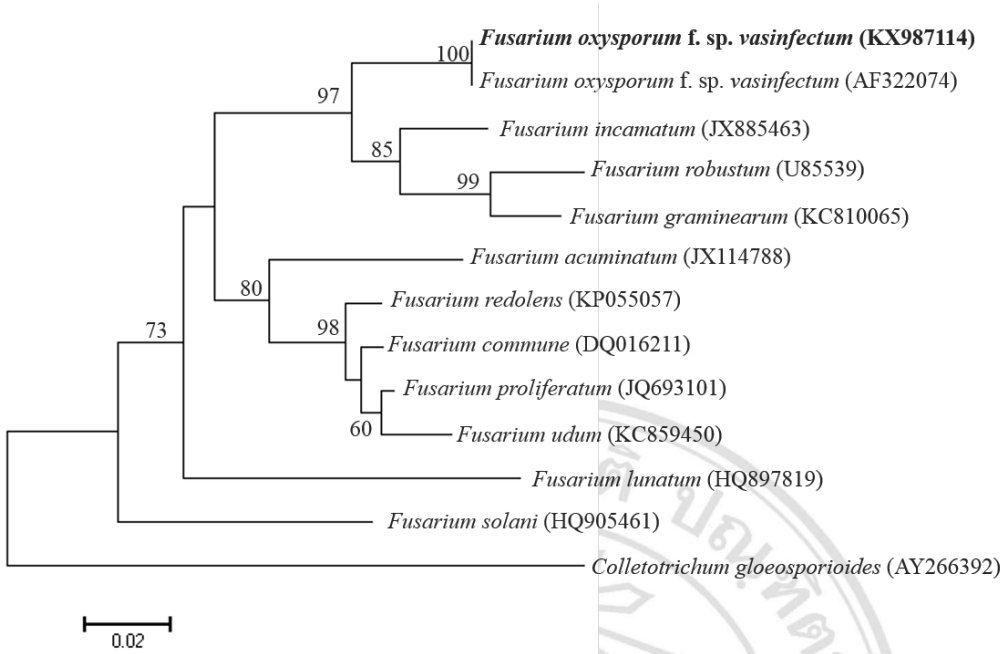

Figure 3.6 Neighbor-joining (NJ) phylogenetic tree of *Fusarium* spp. based on ITS rRNA sequence alignment of 13 sequences. *C. gloeosporioides* was used as an out group. The isolate PRT3-1 was proposed as *F. oxysporum* f. sp. *vasinfectum*. The codes indicated in parentheses and refered to the accession numbers of the sequences available in the GenBank database.

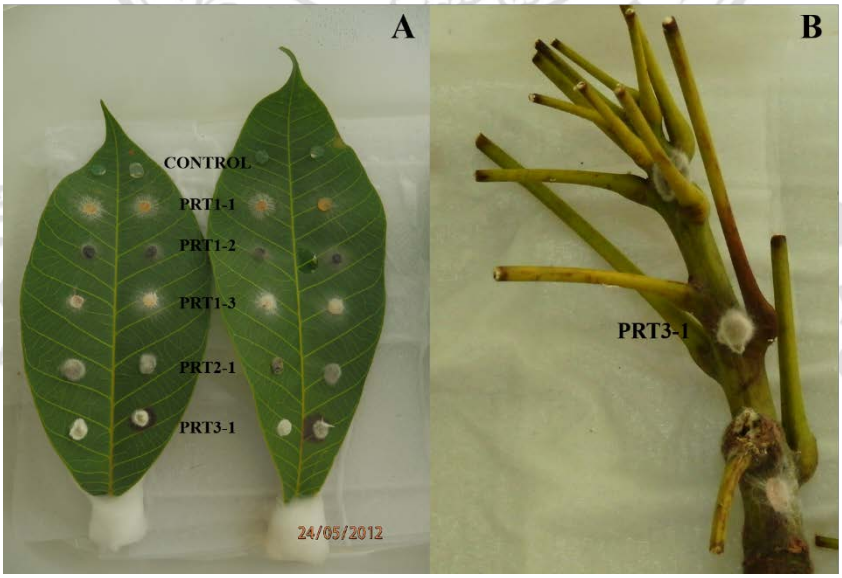

Figure 3.7 Pathogenicity test of *Fusarium oxysporum* f. sp. *vasinfectum* (PRT3-1) on leaf and branch of rubber tree seedling. The symptom showed irregular brown lesions on leaf (A) and branch surface (B).

3.3.3 Genotypic classification of *Rigidoporus microporus*

The white root rot pathogen, *Rigidoporus* spp. (Nakaew *et al*., 2015), was classified and identified using morphological and molecular characteristics (Figure 3.8-3.9). It belongs to the Polyporales and the mycelial morphology on PDA was found to be a whitish colony with radially growing flattened mycelial growths. It also produced a slightly yellowish undersurface. This isolate (KR076793) showed 100% sequence similarity of the ITS rRNA gene to *R. microporus*. However, it has been associated with three distinctive clades corresponding to Africa (clade I), South East Asia (Clade II) and South America (Clade III) (Figure 3.9). The African clade, South East Asian clade and South American clade consisted of isolates from Nigeria, Malaysia and Peru, respectively. The phylogenetic tree dendrogram supported the node separating the isolate (KR076793) in the South East Asian clade from the African clade with 99% bootstrap support (Figure 3.9).

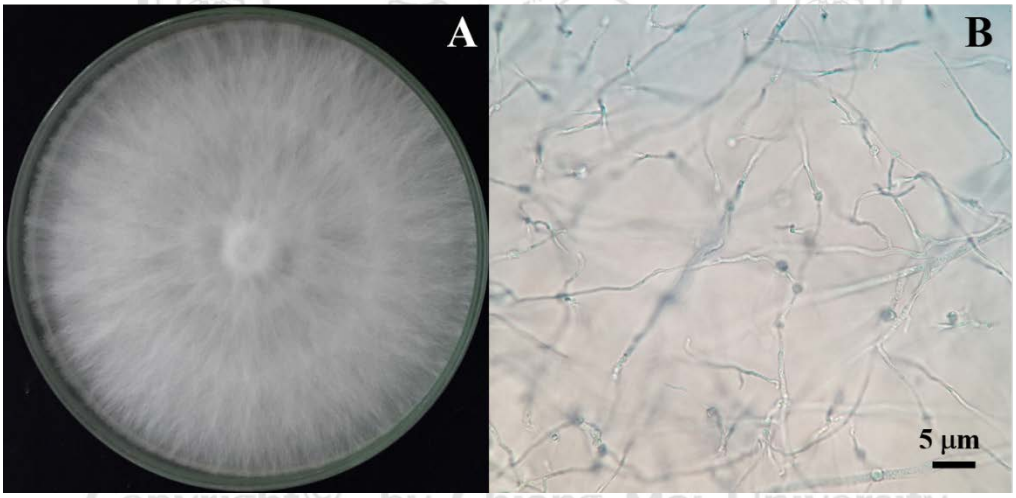

Figure 3.8 Characteristic of *Rigidoporus microporus*, colony on PDA (A) and hyphae (B).

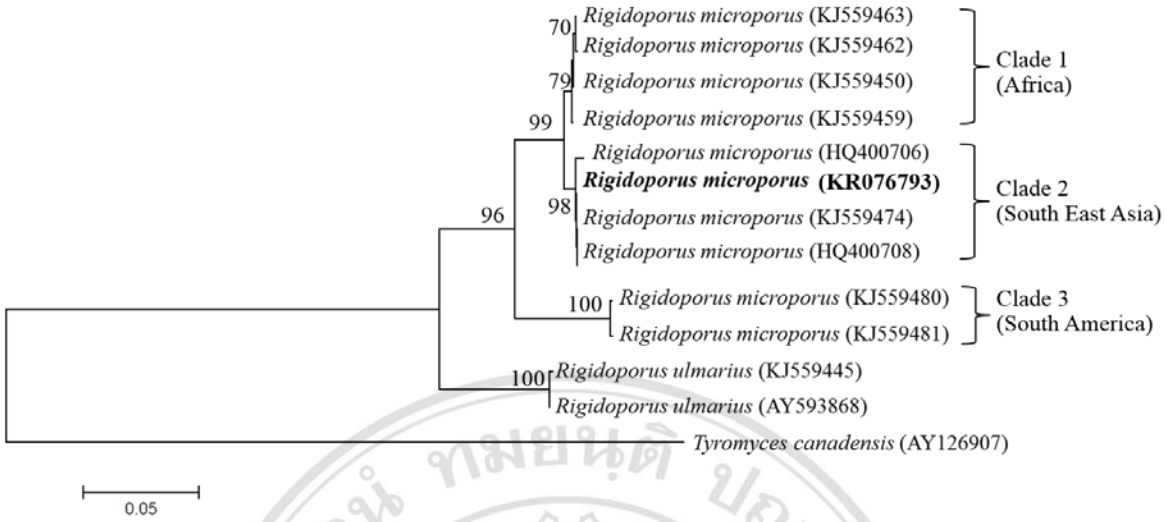

Figure 3.9 Neighbor-joining (NJ) phylogenetic tree of *Rigidoporus microporus* based on ITS rRNA sequence alignment of 13 sequences. *Tyromyces canadensis* was used as an out-group. The codes indicated in parentheses and refered to the accession numbers of the sequences available in the GenBank database.

3.3.4 Screening for antagonism against rubber tree pathogens by dual culture method

Based on the results, 7 isolates of endophytic fungi could inhibit the mycelial growth of fungal pathogens by showing of inhibition zone. Only 3 isolates showed the percentage of inhibition above 50%. Isolate AL1T1 showed the greatest percentage of inhibition of mycelial growth with *C. gloeosporioides* (60.0 %), followed by *P. noxius* (59.4%) and *R. microporus* (58.3%), respectively (Figure 3.10 and Table 3.4). Isolate AL1T2 showed the greatest percentage of inhibition of mycelial growth with *F. oxysporum* f. sp. *vasinfectum* (65.0 %), followed by *P. noxius* (60.7%) and *R. microporus* (54.4%), respectively (Figure 3.9 and Table 3.4). In addition, isolate JA1B1-2 showed highest antifungal activity by overgrowth of all tested fungal pathogens (Figure 3.11 and Table 3.4).

Table 3.4 Antifungal activity of endophytic fungi from medicinal plant.

Plant	Isolate number	Percentage of inhibition of microbial growth					
		R. microporus	*P. noxius*	*F. oxysporum* f. sp. *vasinfectum*	*C. gloeosporioides*	*Ph. parasitica*	*Rh. solani*
Aloe vera	AL1T1	58.3	59.4	-	60.0	20.4	51.0
	AL1T2	54.4	60.7	65.0	44.0	42.0	40.5
	AL1R2	53.3	52.4	25.0	48.0	32.5	44.5
Bauhinia strychnifolia	BS1B201	32.6	-	-	-	-	-
Clinacanthus nutans	C4V202-1	20.0	-	-	19.0	-	-
	C4R301	20.0	-	-	14.5	-	-
Schefflera leucantha	SC1V1	57.8	54.7	40.0	56.4	40.0	60.0
Hippeastrum johnsonii	HI1V3	70.2*	78.3*	67.2*	50.0*	63.8*	44.5*
	HI5IV1-1	65.5*	32.4*	56.4*	45.0*	57.5*	55.3*
	HI5IV1-2	63.1*	32.4*	55.3*	45.4*	56.7*	45.6*
Pseuderanthemum palatiferum	PS1IV102	37.1	-	-	-	-	-
Jatropha podagrica	JA1B1-1	51.8*	61.1*	28.6*	47.4*	41.5*	44.2*
	JA1B1-2	82.1*	81.5*	71.4*	60.4*	80.8*	65.9*
	JA1B1-6	53.6*	55.6*	28.6*	42.1*	53.7*	53.8*

(*) = Inhibition of pathogen by overgrowth of pathogen.

(-) = No visible sign of inhibition of pathogenic fungi.

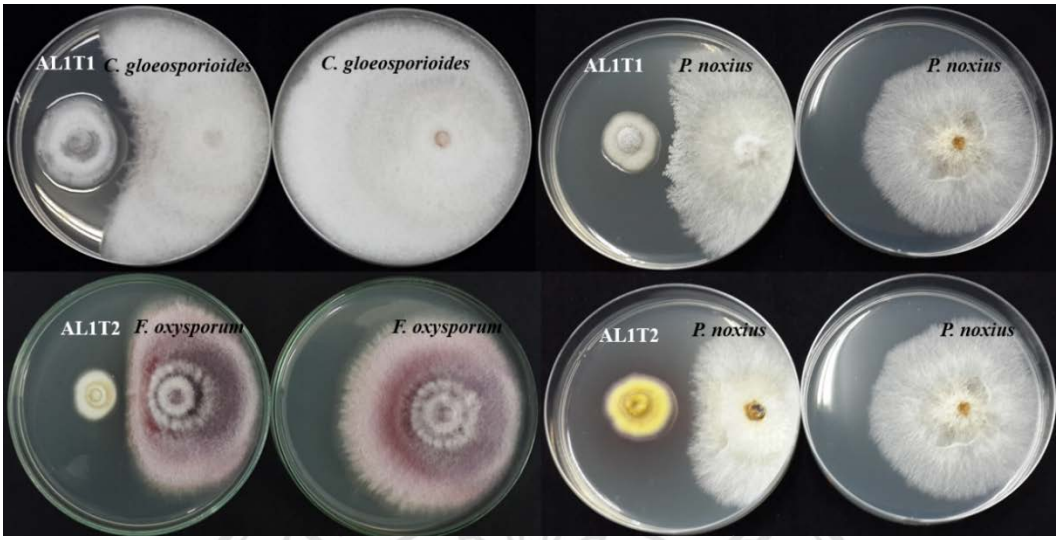

Figure 3.10 Preliminary testing for antifungal activity of endophytic fungi (isolate AL1T1 and AL1T2) against plant pathogenic fungi by dual culture technique.

Figure 3.11 Antifungal activity of endophytic fungus (isolate JA1B1-2) against *Rigidoporus microporus*, *Phellinus noxius*, *Phytophthora parasitica* and *Rhizoctonia solani* AG-2 by overgrowth of all tested fungal pathogens.

3.3.5 Bioassay of crude extract by agar well diffusion method

Two isolates of endophytic fungi, AL1T1 and AL1T2 showed antagonistic activity with high percentage of inhibition against almost tested fungi especially, fungal pathogens caused of root rot disease (*R. microporus* and *P. noxius*) and leaf and seedling blight disease (*F. oxysporum* f. sp. *vasinfectum*) in the rubber tree. They were cultured and extracted for their active metabolites. The crude extract of AL1T1 at the concentration of 10 mg/ml and 5 mg/ml inhibited mycelial growth of *P. noxius* as 65.8±5.7% and 63.3±3.8, respectively (Figure 3.12 and Table 3.5). It inhibited mycelial growth of *R. microporus* as 57.7±1.8% and 55.8±3.0%, respectively. While, the crude extract of AL1T2 at the concentration of 10 mg/ml inhibited mycelial growth of *F. oxysporum* f. sp. *vasinfectum*, *P. noxius* and *R. microporus* as 63.5±1.8%, 57.5±4.3% and 54.7±2.3%, respectively (Figure 3.12 and Table 3.5). In addition, mycelial growth of *P. noxius* under light microscope showed many swollen mycelia and staghorn-like hyphae (trichocysts). These structures were occurred abundantly at colony edge (Figure 3.13).

Table 3.5 Percentage of inhibition of mycelial growth after treated with crude extract of AL1T1 and AL1T2.

Fungal pathogens	Percentage of inhibition of mycelial growth			
	AL1T1		AL1T2	
	10 mg/ml	5 mg/ml	10 mg/ml	5 mg/ml
R. microporus	57.7±1.8	55.8±3.0	54.7±2.3	50.7±1.8
P. noxius	65.8±5.7	63.3±3.8	57.5±4.3	55.0±4.3
F. oxysporum f. sp. *vasinfectum*	-	-	63.5±1.8	58.4±1.8

(-) = No visible sign of inhibition of pathogenic fungi.

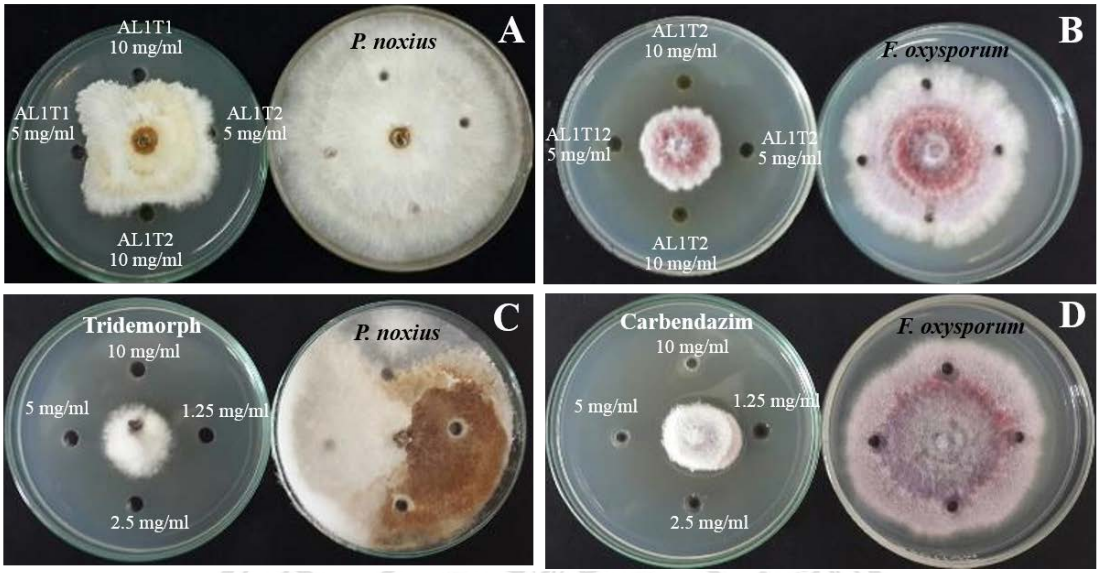

Figure 3.12 Bioassay of crude extract by agar well diffusion method. Mycelial growth of *Phellinus noxius* and *Fusarium oxysporum* f. sp. *vasinfectum* after treatment with crude extracts of AL1T1 and AL1T2 at different concentration, respectively (A-B). Mycelial growth of *P. noxius* and *F. oxysporum* f. sp. *vasinfectum* after treatment with tridemorph and carbendazim, the fungicides used as the control *P. noxius* and *F. oxysporum* f. sp. *vasinfectum*, respectively (C-D).

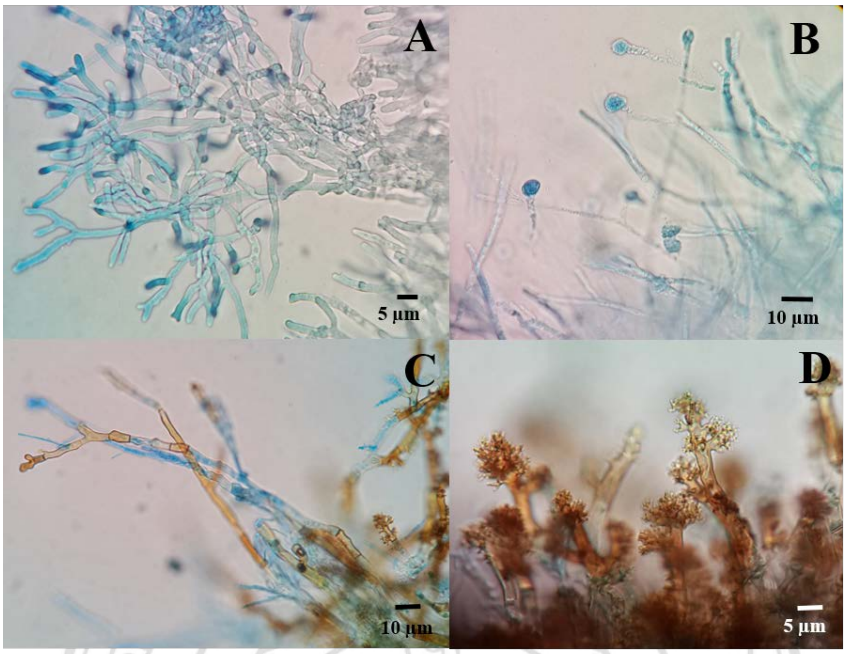

Figure 3.13 Mycelial growth of *Phellinus noxius* under light microscope. Mycelial growth on PDA in normal growth condition (A). Mycelial growth on PDA after treatment by crude extract of AL1T2; many swollen mycelia were occurred at colony edge (B) and staghorn-like hyphae (trichocysts) were occurred abundantly at colony edge (C-D).

3.3.6 Determination of minimum inhibitory concentrations (MICs) of crude extract

The active crude extracts were determined for their MIC. The MIC values of AL1T1 and AL1T2 ranged from 0.16-0.62 mg/ml. Crude extract from AL1T1 significantly inhibited *P. noxius* with MIC value of 0.31 mg/ml ($P<0.05$) (Figure 3.14 and Table 3.6). While, crude extract from AL1T2 significantly inhibited mycelial growth of *P. noxius* and *F. oxysporum* f. sp. *vasinfectum* with MIC value of 0.62 mg/ml and 0.16 mg/ml, respectively ($P<0.05$) (Figure 3.14 and Table 3.6). In addition, tridemorph and carbendazim showed MIC value at 0.62 mg/ml and 0.31 mg/ml, respectively (Table 3.6).

Table 3.6 Determination of minimum inhibitory concentrations (MICs) of crude extract and commercial fungicide.

Concentration of crude extract and fungicide (mg/ml)	Radial growth (cm)				
	P. noxius			*F. oxysporum* f. sp. *vasinfectum*	
	AL1T1	AL1T2	Tridemorph[1]	AL1T2	Carbendazim[2]
Control	4.0±0.1a	4.0±0.1a	4.5a	3.9±0.1a	3.9±0.1a
5	1.9±0.1b	1.9±0.3b	1.5b	1.4±0.2b	1.7±0.1b
2.50	2.0±0.1b	1.9±0.1b	2.0±0.1c	1.6±0.3b	2.0c
1.25	2.0±0.1b	2.0±0.5b	3.2±0.1d	1.8±0.1b	2.1±0.1c
0.62	2.1±0.1b	2.3±0.4b	3.1±0.1d	1.9±0.1b	2.8±0.1d
0.31	2.8±0.3b	3.2±0.4a	4.4±0.1a	2.1±0.1b	3.2e
0.16	3.7±0.2a	3.5±0.2a	4.4±0.1a	2.3±0.2b	3.8±0.1a
0.08	3.8±0.1a	3.8±0.1a	4.5a	3.5±0.6a	3.8±0.1a
0.04	3.8±0.1a	3.6±0.1a	4.4±0.1a	3.7±0.6a	3.9±0.1a

All tests were performed in triplicate, the radial growth is shown with mean ± SD. Means with different letters are significantly different according to Tukey's Test ($P < 0.05$). Two commercial fungicides, tridemorh[1] and carbendazim[2] for controling *P. noxius* and *F. oxysporum* f. sp. *vasinfectum*, respectively, were used as positive control and 50% methanol was used as negative control.

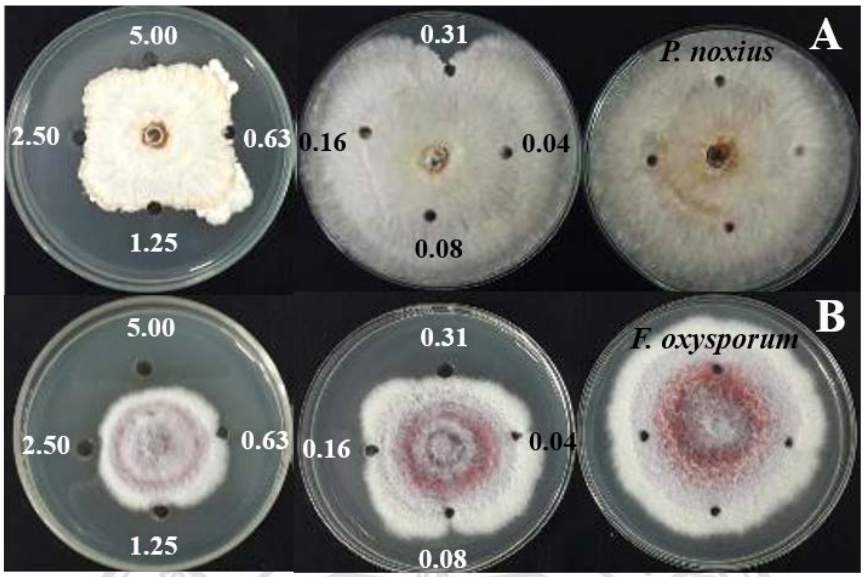

Figure 3.14 Minimum inhibitory concentrations (MICs) value of crude extracts ranged from 0.16-0.62 mg/ml. AL1T1 and AL1T2 showed antifungal activity against *Phellinus noxius* (A) and *Fusarium oxysporum* f. sp. *vasinfectum* (B), respectively.

3.3.7 Antifungal activities assay by TLC-bioautography

Among 2 wave length of UV light (254 nm and 365 nm), various spots were observed and Rf value of AL1T1 and AL1T2 crude extracts were measured at 0.22 to 0.83 and 0.20 to 0.78, respectively. In bioautography of AL1T1, 4 spots at Rf 0.22, 0.28, 0.30 and 0.33 showed antifungal activity against *P. noxius* (Figure 3.15). While, in AL1T2 bioautography, 3 spots at Rf 0.20, 0.26 and 0.33 inhibited mycelial growth and spore germination of *F. oxysporum* f. sp. *vasinfectum* (Figure 3.16).

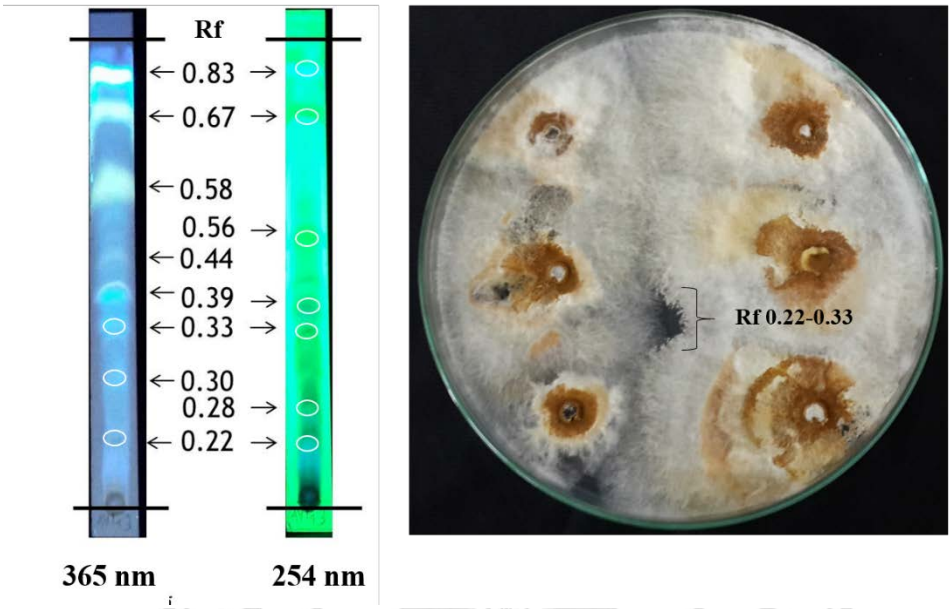

Figure 3.15 TLC-bioautograph of AL1T1 crude extract against mycelial growth of *Phellinus noxius*.

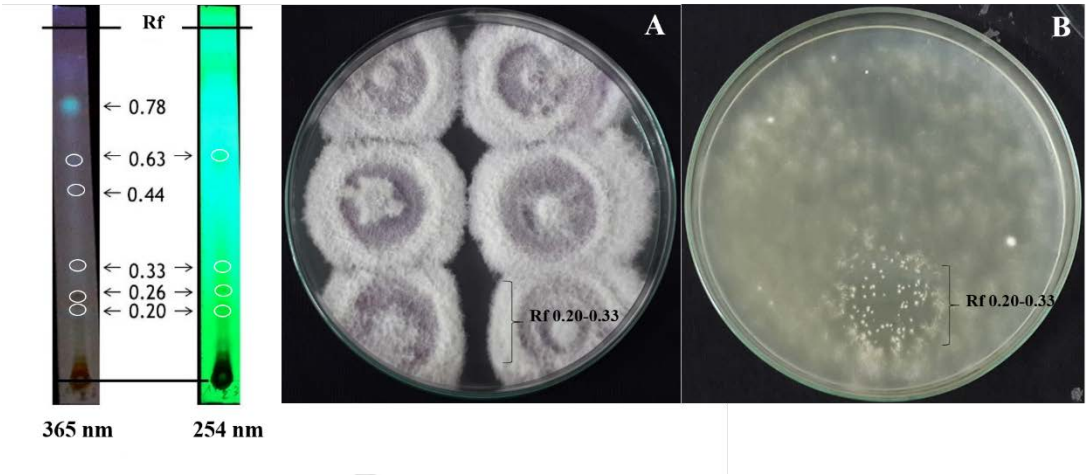

Figure 3.16 TLC-bioautograph of AL1T2 crude extract against mycelial growth (A) and spore germination (B) of *Fusarium oxysporum* f. sp. *vasinfectum*.

3.3.8 Preliminary assay of hemolysis

Isolate AL1T1 grown on 5% Sheep blood agar with beta-hemolysis. The clear zone was 1.9-2.1 cm (diameter). However, no change of the medium under and surrounding crude extract of AL1T1 after tested toxicity on 5% Sheep blood agar (Figure 3.17). While, isolate AL1T2 showed non-hemolysis after grown on 5% Sheep blood agar and tested with crude extract (Figure 3.17).

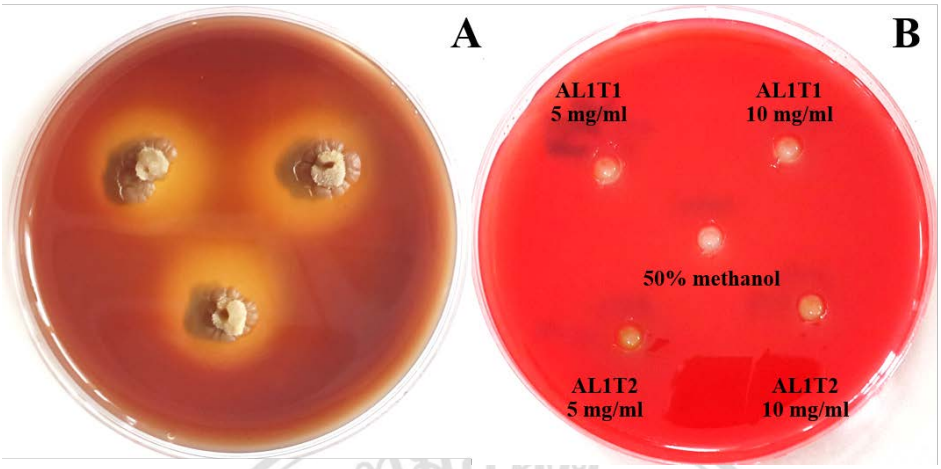

Figure 3.17 Preliminary assay of hemolysis. Isolate AL1T1 showed beta-hemolysis after grown on 5% Sheep blood agar for 15 days (A). Non-hemolysis on 5% Sheep blood agar after tested with crude extract of AL1T1 and AL1T2 (B).

3.3.9 Screening for indole acetic acid (IAA) production

Twelve isolates of endophytic fungi showed their ability to produce IAA in preliminary test. The range of IAA production was 9.4±1.2 µg/ml to 40.7±0.5 µg/ml. Endophytic fungus, isolate BS1B201 from branch of *Bauhinia strychnifolia* produced maximum concentration of IAA, following by isolate SC1V1 and C4V202-1, respectively (Table 3.7).

Table 3.7 Screening for IAA production of endophytic fungi.

Plant	Isolate number	IAA (µg/ml)
Aloe vera	AL1T1	22.6±6.0
Bauhinia strychnifolia	BS1B201	40.7±0.5
Clinacanthus nutans	C4V202-1	29.4±0.2
	C4IV301	23.4±0.1
	C4R301	19.6±1.1
Jatropha podagrica	JA1B1-1	19.9±2.0
	JA1B1-2	14.7±2.4
	JA1B1-6	9.4±1.2
Pseuderanthemum palatiferum	PS1IV102	16.8±0.1
Schefflera leucantha	SC1V1	33.6±5.0
Hevea brasiliensis	RTM5IV1	13.9±0.2
	RTM5IV3	18.8±0.2

3.3.10 Extraction of crude IAA and thin layer chromatography (TLC)

The chromatogram showed the compounds of isolate C4IV301, C4V202, RTM5IV1, PS1IV102 and BS1B201 closed to Rf value of standard IAA and standard IBA (Appendix C). Then, they were confirmed by TLC again with different mobile phase according to the method in section 3.2.10. The culture spots, standard IAA and standard IBA showed the same result after spray with Ehmann's reagent and Salkowski's reagent. Only 3 isolates of endophytic fungi, C4IV301, PS1IV102 and BS1B201 showed the same Rf value with standard IAA and standard IBA (Figure 3.18).

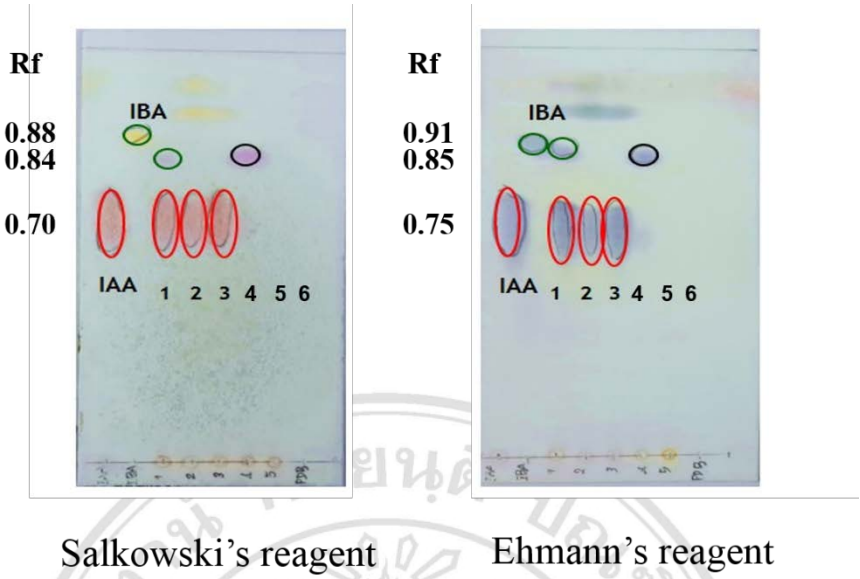

Figure 3.18 Thin layer chromatography (TLC) of IAA crude extract such as IAA standard, IBA standard, 1 = C4IV301, 2 = PS1IV102, 3 = BS1B201, 4 = C4V202-1, 5 = RTM5IV1 and PDB (negative control).

3.3.11 Phenotypic and genotypic classification of active endophytic fungi

Endophytic fungi isolate AL1T1 and AL1T2 colonized in stem of *Aloe vera* based on DNA analysis of ITS rRNA gene, it is suggested that both AL1T1 and AL1T2 belong to the phylum Ascomycota, order Sordariales (Figure 3.21 and Table 3.8). Colony color of isolate AL1T1 on PDA was whitish in the young age and becomes grayish in the old age (Figure 3.19A). The hyphae with septum (Figure 3.19B). Mycelium forms swollen cells (8.2-10.0 μm diameter, Figure 3.19C). Hyphae (1.0-1.3 μm thick) with coils (11.9-21.3 μm diameter, Figure 3.19D).

Colony color of isolate AL1T2 on PDA was yellowish. It produces water-soluble pigment and excretes around the colony with reddish color (Figure 3.20A). Mycelium forms swollen cell (Figure 3.20B) and produces chain of conidia (2-6 conidiospore on single conidiophore, Figure 3.20C). Spore shape is ovate (1.3x2.6 μm). Spore color is hyaline. Hyphae (1.0-1.8 μm thick) with coils (6.1-8.5 μm diameter, Figure 3.20D).

Table 3.8 Closest related taxa of AL1T1 and AL1T2 based on the sequence similarities of ITS rRNA gene.

Isolate number	Sequence length (nt)	Closest related taxa in the GenBank database	% Sequence similarity
AL1T1	481	Sordariales sp. JX243892	100
		Sordariales sp. JX243747	99
		Sordariales sp. JX244061	99
		Sordariales sp. JX244012	99
AL1T2	465	Sordariales sp. JX243892	100
		Sordariales sp. JX243747	99
		Sordariales sp. JX244061	99

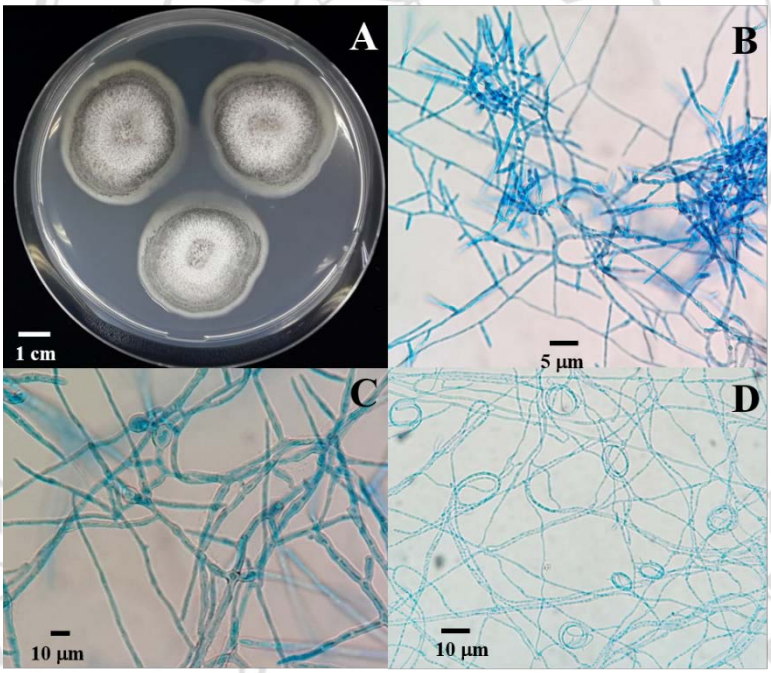

Figure 3.19 Morphological characteristics of isolate AL1T1. Colony morphology was observed after growing on PDA for 7 days (A). The hyphal morphology of isolate AL1T1 was observed further with light microscopes. The hyphae with septum (B), the swollen hypha (C) and formation of coiling hypha (D).

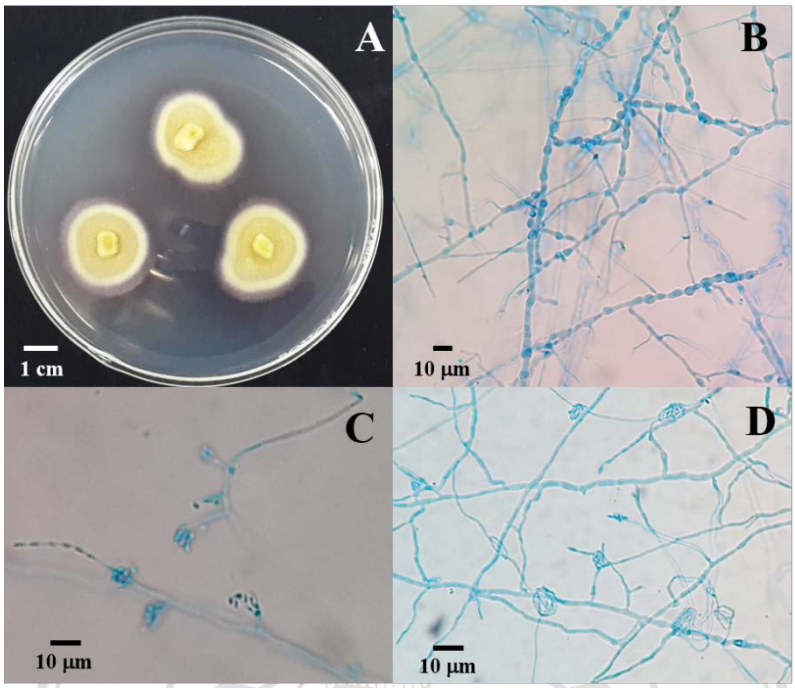

Figure 3.20 Morphological characteristics of isolate AL1T2. Colony morphology was observed after growing on PDA for 7 days (A). The hyphal morphology of isolate AL1T2 was observed further with light microscopes. The swollen hypha (B), Chain of conidia (C) and formation of coiling hypha (D).

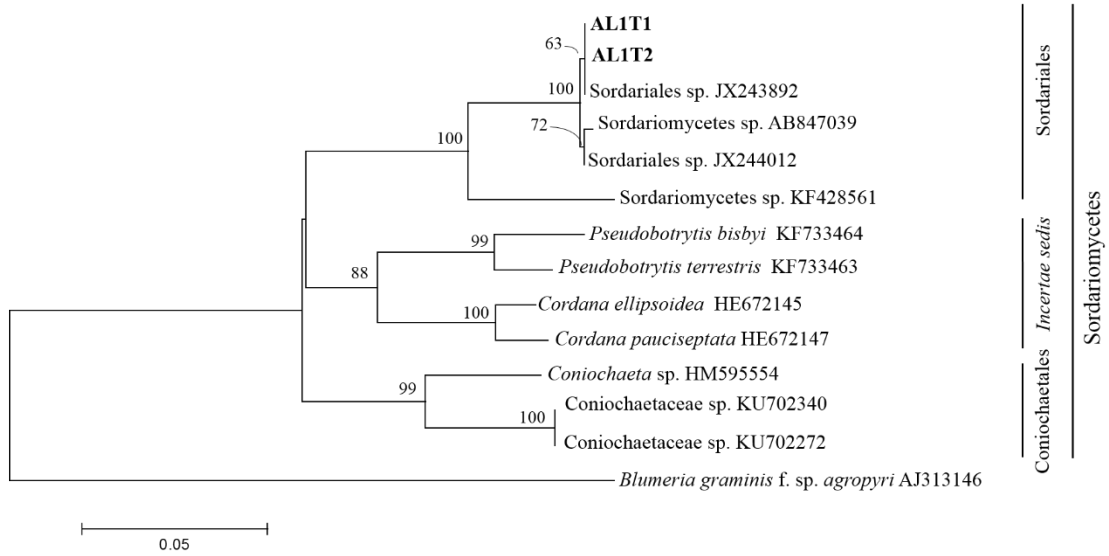

Figure 3.21 Neighbor-joining (NJ) phylogenetic tree of isolate AL1T1 and AL1T2 based on ITS rRNA sequence alignment of 14 sequences. *Blumeria graminis* f. sp. *agropyri* was used as an out group. The isolate AL1T1 and AL1T2 belonged to order Sordaiales. The codes indicated in parentheses refer to the accession numbers of the sequences available in the GenBank database.

3.4 Discussion

Endophytic fungi colonized internal plant tissue without any symptom. The research about endophytic fungi expanded in recent year from isolation and identification of fungal endophyte, examining the nature of endophyte/plant interaction, including studying endophytic fungi from medicinal plant in order to discover active and novel compounds (Alvin *et al*., 2014). Endophytic fungi are presented in most of plant section, especially healthy leaf tissue (Karunai and Balagengatharathilagam, 2014) according to this study, most endophytic fungi were more prevalent in the leaves (53.1%) than the branches/stems and the roots. However, the endophytes population was affected by the environmental conditions under which the host is growing and the endophyte profile may be is more diversified in tropical areas (Karunai and Balagengatharathilagam, 2014). Isolate AL1T1 and AL1T2 were proposed as the effective fungi with antagonistic activity against a broad range of fungi. They were isolated from a very short stem of *Aloe vera*. This medicinal plant was widely known to use in traditional medicine as a skin treatment to relive minor burns and sunburn and help heal wounds. It served as a good model plants

for studying the effects of endophytic fungi colonization on secondary metabolism (Yadav *et al*., 2015). The previous studies showed that various fungi from leaves, stem and root of *A. vera* could produce extracellular enzyme and antagonistic activity towards microorganisms (Jalgaonwala *et al.*, 2010; Yadav *et al*., 2015). In addition, the genus *Phoma* was isolated from *A. vera* and could produce anti-cancer agent, taxol (Rebecca *et al*., 2011).

Root rot disease is the most serious problem of rubber tree plantation and present in many countries, including Thailand. In this study, the antagonistic test showed that the causing pathogen, *R. microporus* was susceptible to the extract of AL1T1 with the highest percentage of inhibition. While, the causing agent of brown root rot disease, *P. noxius* was susceptible to the extract of AL1T2 with the highest percentage of inhibition. However, AL1T2 showed greatest antagonistic activity to control *F. oxysporum* f. sp. *vasinfectum* according to the study of Yadav *et al*. (2015). Under the light microscope, there is swollen mycelia and staghorn-like hyphae (trichocysts) of *P. noxius* occurred abundantly at colony edge after treated by crude extract of AL1T2. It is still unknown about the function of trichocyst but it is the specialized hyphae of *P. noxius*, including arthospores and forming in pure culture (Ann *et al*., 2002).

The root rot disease spread to the healthy tree through root-to-root contact. After the tree removal, the pathogens can survive in colonized roots remaining in the soils for many years and also remain viable as mycelium in the rhizosphere for several months (Ann *et al*., 2002; Omorusi, 2012). Thus, the antagonistic activity of fungi to inhibit mycelial growth of the root rot pathogens was considered to control root rot disease in the field according to the previous studies such as the use of *Trichoderma hazianum* (Jayasuriya and Thennakoon, 2007) and *Chaetomium cupreum* to control *R. microporus in vivo* (Kaewchai and Soytong, 2010). Comparing to the positive control, tridemorph and carbendazim, crude extract of AL1T1 and AL1T2 showed a strong antifungal activity on the above phytopathogens, *R. micoporus*, *P. noxius* and *F. oxysporum* f. sp. *vasinfectum*. Crude extract of AL1T1 significantly inhibited *P. noxius* with MIC 0.31 mg/ml ($P<0.05$) which was less than MIC value of tridemorph (0.62 mg/ml). While, AL1T2 crude extract significantly inhibited *F. oxysporum* f. sp. *vasinfectum* with MIC 0.16 mg/ml ($P<0.05$) which was less than MIC value of carbendazim (0.31 mg/ml). The TLC-autobiography is

one of the more convenient method for testing crude extract of microorganisms, plant and purified bioactive compounds against pathogenic bacteria and fungi (Prakash *et al*., 2013). Isolate AL1T1 and AL1T2 were examined for their antifungal activity by TLC-bioautography. The results showed the separation of active compounds and Rf values that ranged from 0.22-0.33 of AL1T1 (separated using 95:5 (v/v) dichloromethane: methanol) and was found to inhibit mycelial growth of *P. noxius*. The active compounds of AL1T2 showed Rf values ranged from 0.20-0.33 and inhibited mycelial growth and spore germination of *F. oxysporum* f. sp. *vasinfectum*.

It was supported by the study of Li *et al*. (2012) who found that endophytic fungi from *Melia azedarch* had ability to produce active compounds to inhibit broad range of pathogenic fungi, including *F. solani*, *F. oxysporum* f. sp. *niveum* and *F. oxysporum* f. sp. *vasinfectum*. From TLC-autobiography assay, the extracellular fractions of both isolate were effective, which indicates that antifungal compounds from fungal endophytes are secreted extracellularly and may contribute in part to the plant protection systems against the pathogens (Abdel-Motaal *et al*., 2010).

In preliminary test of hemolysis, AL1T1 grown on 5% Sheep blood agar and produced enzyme or a substance that causes beta-hemolysis to breakdown of red blood cells. However, the crude extract of AL1T1showed non-hemolysis and should determine the general toxicity by another method to confirm the result such as the brine shrimp lethality assay, which is an effective and rapid assay to screen potential cytotoxic activity (Lu *et al*., 2012).

Twelve isolates of endophytic fungi (4.4%) showed ability to produce IAA in preliminary test but only 3 isolates exhibited the same Rf value with standard IAA and IBA after TLC tested. Isolate BS1B201 produced maximum IAA concentration. This result was supported by the previous study of Khan *et al*. (2016) who found that endophytic fungi, *Biporalis* and *Phoma* from medicinal plant produced IAA and increased rice seed germination and growth. Furthermore, in this study, all of them showed antagonistic activity to inhibit *R. microporus* but percentage of growth inhibition was less than 50%. Endophytic fungi isolates AL1T1 and AL1T2 belonged to phylum Ascomycota in the order Sordariales based on morphological characteristic and the sequence similarities of ITS rRNA gene (99-100% sequence similarity). The

phylogenetic tree showed that isolate AL1T1 and AL1T2 closed to Sordariales sp. JX243892 with 63 % bootstrap. However, both AL1T1 and AL1T2 could not be identified at genus and species level only ITS rRNA gene was used in DNA analysis for this work. It is not enough to indicate the species of both isolates, thus other regions such as large-subunit rRNA (LSU) and small-subunit rRNA (SSU) should be included in molecular analysis. In addition, investigation of morphology characters of both isolates showed differences, further analyses would be useful for the identification.

Sordariales is a large group of microfungi that occur as the degraders of dung, wood, plant debris, and soil (Cannon and Kirk, 2007). However, these fungi have been isolated as endophytuc fungi such as *Chaetomium* (Beena *et al*., 2000; Li *et al*., 2014b) and *Trichoderma* (Bailey *et al*., 2009; Mulaw *et al*., 2013; Hosseyni-Moghaddam and Soltani, 2014). They produce bioactive compounds with antimicrobial activity. The synthetic chemicals are costly and have harmful effect on human health and environment, including may induce pathogen resistance. Thus, biological control by using a cooperation of microorganisms such as endophytic fungi isolate AL1T1 or AL1T2 and isolate BS1B201 to prevent disease and promote plant growth offer an attractive alternative method for disease management without the negative impact of the chemical control.

CHAPTER 4

Existence of *Muscodor* in leaves of the rubber tree

4.1 Introduction

Microbial endophytes are bacteria or fungi which live within plants without causing apparent disease (Huang *et al*., 2001; Chaverri and Gazis, 2011). As symbionts most endophytes enhanced plant growth and a plant's defensive system (Strobel and Daisy, 2003; Tejesvi *et al*., 2007). Because of such benefits, endophytes are one of the promising microbial resources as biocontrol agents applied in agriculture. A mode of action of endophytes in prevention and/or suppression of plant diseases caused by phytopathogens is the production of antimicrobial substances. The bioactive compounds in the form of volatile metabolites produced by endophytes have not been commonly reported. Some fungi that belong to the families *Diaporthaceae, Hypocreaceae,* and *Xylariaceae* of the phylum Ascomycota are notable for their capacity to form volatile metabolites with antimicrobial activity (Stinson *et al*., 2003b; Suwannarach *et al*., 2013b). In the family *Xylariaceae, Muscodor albus* is the first known fungal endophyte isolated from *Cinnamomum zeylanicum*, which produces bioactive volatile metabolites (Strobel *et al*., 2001; Ezra *et al*., 2004).

The genus *Muscodor* does not produce spores and is classified into the family *Xylariaceae* based on the polyphasic approach (Kudalkar *et al*., 2011). Although morphological characteristics can be used to classify the members of *Muscodor* at the generic level, a more appropriate genotypic classification method is needed for identification at the species level and to understand their phylogenetic lineage. In addition, profiling and comparison of unique volatile metabolites produced by members of the genus *Muscodor* are important criteria for its classification (Strobel, 2006; González *et al*., 2009). The major volatile metabolite produced by the genus *Muscodor* is 2-methylpropanoic acid (Kudalkar *et al*., 2011; Suwannarach *et al*., 2013a), which differs from other endophytic genera in the same family, e.g. *Nodulisporium* spp. that produce

eucalyptol (Tomsheck *et al*., 2010). Recently, many novel species of the genus *Muscodor* (*M. cinnamomi*, *M. equiseti*, *M. musae*, *M. oryzae*, and *M. suthepensis*) were isolated from diverse plant hosts growing in northern Thailand (Suwannarach *et al*., 2010; 2013a). Other species of *Muscodor* have been isolated from tropical trees and vines in Australia, Central and South America, and Central, South and Southeast Asia (Meshram *et al*., 2013; Suwannarach *et al*., 2013a; Saxena *et al*., 2015).

While a previous report showed that *Penicillium*, *Pestalotiopsis* and *Trichoderma* were the most frequently isolated fungi from rubber trees (Gazis and Chaverri, 2010). However, a diversity study of the genus *Muscodor* that lives in association with rubber trees has not been reported previously. Volatile metabolite-producing endophytes may be an alternative biological approach as biofumigation in control of plant diseases and fungal contamination of rubber tree seedlings and their latex products. It is known that sapwood and leaf fragments of rubber trees are a rich source of fungal endophytes (Evueh and Ogbebor, 2008; Rocha *et al*., 2010; Gazis, 2012), but few studies have reported their antimicrobial activities (Evueh and Ogbebor, 2008; Gazis, 2012). Moreover, no volatile metabolite-producing endophytes have yet been reported from rubber trees.

4.2 Materials and methods

4.2.1 Preparation and surface sterilization of plant materials

Healthy leaves of rubber tree were used as a source for isolation of endophytic fungi. A total of 45 leaves were collected from rubber trees planted in Nongbualamphu province, northeastern Thailand (geographical location: 17°12′14″ N, 102°26′26″ E) (Figure 4.1**)**. They were placed in plastic bags and stored in an icebox before transported to the laboratory within 24-48 h after sampling. The age of rubber trees was approximately 6 years, which was considered as the mature phase for latex harvest. The leaves were prepared and surface sterilized following the method described by Suwannarach *et al.* (2010). Briefly, plant leaves were rinsed with tap water for 15 min and randomly cut into small segments (25 mm^2). All segments were sterilized by soaking in 75% ethanol for 30 s, 2% sodium hypochlorite for 3 min, and 95% ethanol for 30 s, respectively.

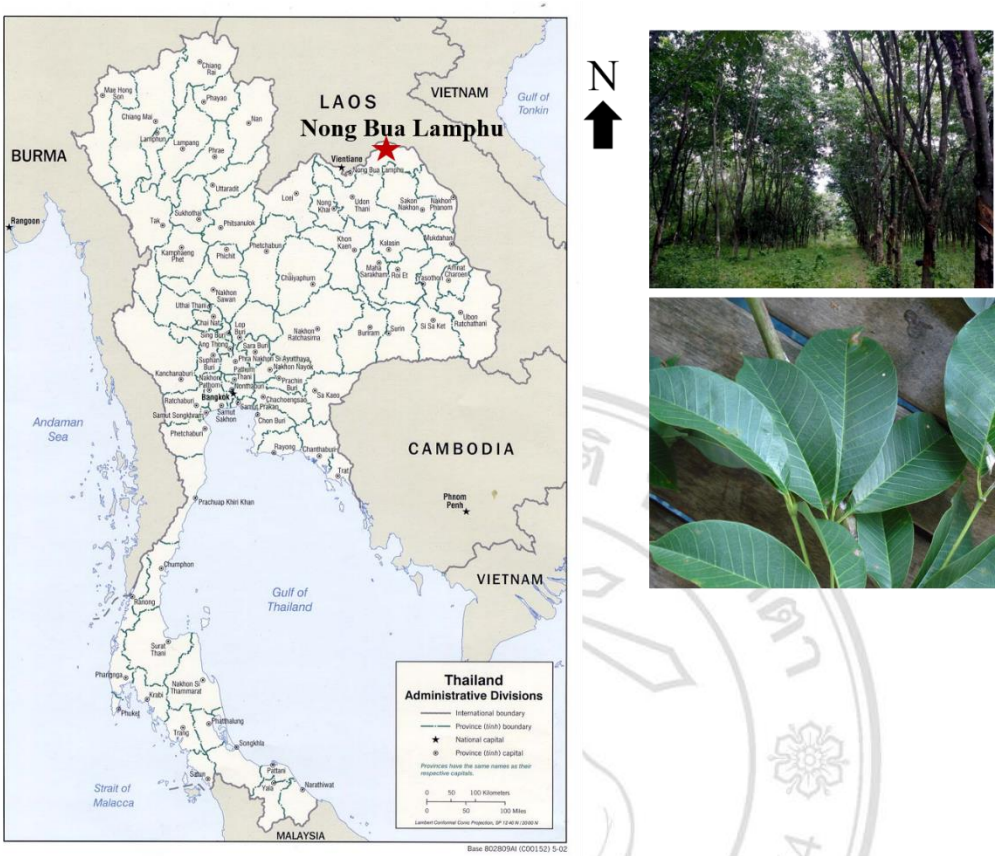

Figure 4.1 Colleting sites (star marks) of rubber tree in Nong Bua Lamphu province, Thailand.

4.2.2 Isolation of volatile metabolite-producing endophytic fungi

Endophytic fungi that form volatile metabolites were isolated using a parallel-growth isolation technique (Worapong *et al*., 2001). Briefly, a part of a two-compartment petri dish was filled with potato dextrose agar (PDA, Himedia Laboratories, India), while the other side contained a half strength PDA supplemented with rose bengal and chloramphenicol at final concentrations of 0.033 g L^{-1} and 50 mg L^{-1}, respectively. A strain producing volatile antibiotic metabolites, *M. cinnamomi* (MB518008) (Suwannarach *et al*., 2010) was used as a reference strain for this isolation. An agar plug (6 mm diam) of *M. cinnamomi* grown previously on PDA at ambient temperature (25 ± 2 °C) for 7 days was placed on the PDA side of the prepared two-compartment Petri dish. This inoculation was incubated at 25 ± 2 °C for 4 days. Then, five surface sterilized plant segments were placed on the half strength PDA, where they were exposed to the volatile metabolites produced by *M. cinnamomi*. The Petri dish was sealed with Parafilm® M

(Bemis company, Inc., USA), and incubated at 25 ± 2 °C for 2 weeks. The production of its VOCs facilitated selection pressure, allowing the growth of only fungal species that tolerated these volatiles (Figure 4.2). Then, the hyphal tips of endophytic fungi that grew out from the plant segments were aseptically transferred to a separated PDA plate. This technique could be used to select for other isolates of *Muscodor* (Worapong *et al*., 2001; Mitchell *et al*., 2008). The endophytic fungi were tested for antifungal activity following a protocol described by Strobel *et al*., 2001, if they produced volatile antibiotics. Spore production of endophytic fungi was studied on four different media including corn meal agar (CMA), malt agar (MA), PDA and water agar (WA). The microscopic structures like hyphal characteristics, cellular bodies and spore production were observed under a light microscope (Olympus SZ40, Japan). The colony texture, color and fruiting body were observed under a stereomicroscope (Olympus CH30, Japan). The pure cultures of endophytic fungi were subsequently stored in 20% (v/v) glycerol at -20 °C and deposited at the Sustainable Development of Biological Resources (SDBR) Lab, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand, and the BIOTEC Culture Collection (BCC), Bangkok, Thailand.

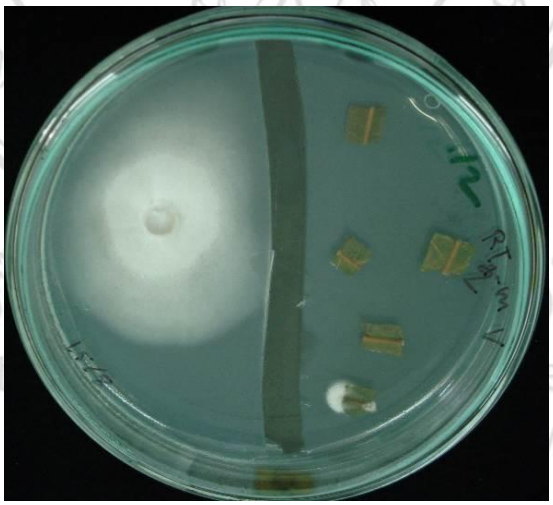

Figure 4.2 *Muscodor* grew out from the plant segment on half strength PDA.

4.2.3 Phenotypic classification

Colony and hyphal characteristics of all endophytic fungal isolates were initially observed with a light microscope (Olympus CH30, Japan). The morphological characteristics were observed also with a scanning electron microscope (JEOL JSM-5910LV, Japan), following a protocol described by Ezra *et al.* (2004) (Appendix D). All endophytic fungal isolates whose morphology was typical of the genus *Muscodor*, grouped and selected for further classification.

4.2.4 Genotypic classification

All *Muscodor* isolates were grown on PDA at 25 ± 2 °C for 10 d. The aerial mycelium of each isolate was scraped from the surface of PDA. This fungal biomass was then freeze-dried and ground into a fine powder with a pestle and mortar. A modified SDS-CTAB method (Suwannarach *et al.*, 2013a) was used for the DNA extraction. The internal transcribed spacer regions 1 and 2, including 5.8S rDNA (ITS1-5.8-ITS2 rDNA) was a targeted nucleotide sequence for the amplification, using a pair of universal primers (ITS4 and ITS5). The amplification was carried out using GeneAmp 9700 thermal cycler (Applied Biosystems) with the following PCR conditions: initial denaturation at 95 °C (2 min), 30 cycles of reaction [denaturation at 95 °C (30 sec), annealing at 50 °C (30 sec), and extension at 72 °C (1 min)], and final extension at 72 °C (10 min). The reaction mixture (25 µL) contained 1 µL of the DNA template, 0.2 µM dNTP, 0.2 µL of FastTaq (Applied Biosystems), 0.2 µM each primer, 2.5 µL of $MgCl_2$ buffer, and sterile water to bring the volume to 25 µL. The PCR product was confirmed by gel electrophoresis and purified with PCR cleanup Gel Extraction NucleoSpin® Extract II Purification Kit (Macherey-Nagel, Germany) following the manufacturer's manual. The purified PCR product was then sequenced by 1st Base, Malaysia. All DNA sequences obtained were aligned and compared to the available sequences in GenBank database using the BLASTN facility within the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis of all sequences was conducted by the neighbor-joining method using MEGA6 software (Tamura *et al*., 2013).

4.2.5 Profiling and comparison of volatile metabolites

Only the *Muscodor* isolate that was a possible novel species (having a <86% similarity to the ITS rRNA sequence of other members of the genus *Muscodor*), was analyzed for its profile of volatile metabolites. The mixture of volatile metabolites at headspace of the *Muscodor* isolate growing on PDA at 25 ± 2 °C for 10 days was identified by a modified gas chromatography-mass spectrometry (GC-MS) procedure with solid phase microextraction (SPME) (Strobel *et al*., 2001). The SPME equipped with a syringe consisting of gray hub fiber material made by divinylbenzene/carboxen (50/30) on polydimethylsiloxane affixed on a stable flex fiber, was used for trapping the volatile metabolites produced in the headspace of the *Muscodor* culture for 45 min. The syringe was inserted into the splitless injection port of an Agilent 7890A gas chromatograph equipped with mass spectrometer MSD 5975C (EI) mass selective detector. A DB-Wax capillary column (30 m × 0.25 mm I.D.) with a film thickness of 0.25 μm was used for separation of volatile metabolites. The column was initiated with a thermal program of 40 °C for 2 min, and increasing to 200 °C at a rate of 5 °C min^{-1}. Ultra-high purity helium was used as a carrier gas with an initial column head pressure of 60 kPa. Before trapping the volatile metabolites, the fiber material was conditioned at 250 °C for 34 min under a flow of helium gas. A 30-s injection time was used to introduce the adsorbed volatile metabolites into the GC. The volatile metabolites were identified through a library comparison with the NIST database by considering their quality match (80% or higher).

4.3 Results

4.3.1 Volatile metabolites-producing endophytic fungi of rubber tree leave

A total three isolates of volatile metabolites-producing endophytic fungi was isolated from leaves of rubber trees, using the parallel-growth isolation technique with *M. cinnamomi* as a reference strain. All isolates (100%) were classified morphologically into the genus *Muscodor*. The phylogenetic analysis of ITS rRNA sequences derived from all *Muscodor* isolates revealed the different closest related species (Table 4.1). Isolates RTM5IV1 and RTM5IV2 were closely related to *M. vitigenus* and *M. equiseti*, respectively. This phylogenetic relation was supported by 100% similarity of the rRNA gene sequence and morphological characteristics. Isolate RTM5IV3 showed the lowest sequence similarity (86%) to *M. albus* cz620,

M. cinnamomi, *M. musae*, and *M. oryzae*. The phylogenetic analysis of ITS rRNA sequences derived from all *Muscodor* isolates revealed the different closest related species and displayed three majorclades, A, B, and C, containing all known species of *Muscodor* and all isolates from this study with a high bootstrap support (91%) (Figure 4.3). Isolate RTM5IV3 showed the lowest sequence similarity (86%) to *M. albus* cz620, *M. cinnamomi*, *M. musae*, and *M. oryzae*. In clade A, it separated from *M. albus* group, *M. strobelii* and *M. suthepensis* with 93% bootstrap support (Figure 4.3). Isolates RTM5IV1 and RTM5IV2 showed a maximum homology of 100% with *M. vitigenus* and *M. equiseti*, respectively and were classified into clade B with 99% bootstrap support (Figure 4.3). Morphological data and bioinformatics were insufficient to place the *Muscodor* isolates into exact linages. Nevertheless, we could suppose that the isolates giving a % sequence similarity lower than 100% could be novel members of the genus *Muscodor*. With this hypothesis, one of the *Muscodor* isolates (33.3%) found in this work was possibly a novel species isolated from rubber tree leaves.

4.3.2 Volatile analysis of *Muscodor* spp.

Isolate RTM5IV3 produced a mixture of 12 volatile metabolites which identified by GC/MS through a library comparison with the NIST database (Table 4.2 and Figure 4.4). Its volatile profile was compared with its closest phylogenetic species; *M. albus* cz620, *M. cinnanomi*, *M. musae*, and *M. oryzae*. The volatile compound produced by isolate RTM5IV3 with the highest percentage peak area (11.26) was 3-methylbutan-1-ol, the same as produced by *M. albus* cz620 and *M. oryzae*. The minor volatile compounds were 3-methylbutyl acetate and 2-methylpropanoic acid. These volatile compounds are also produced by *M. albus* cz620. However, the volatile profile of isolate RTM5IV3 was different from other *Muscodor* spp. (Table 4.2). In addition, isolate RTM5IV3 produced two volatile compounds, 1,1,9-trimethyl-5-methylidenespiro[5.5]undec-9-ene and methyl (*Z*)-*N*-hydroxybenzenecarboximidate, that were not found in other *Muscodor* spp. (Table 4.2).

Table 4.1 Closest related species of *Muscodor* isolates based on the sequence similarities of ITS rRNA gene.

Isolate number	**Sequence length (nt)**	**Accession number**	**Closest related taxa in the GenBank database**	**% Sequence similarity**
RTM5IV1	584	KF850710	*M. vitigenus* AY100022	100
RTM5IV2	611	KF850711	*M. equiseti* JX089322	100
RTM5IV3	593	KF850712	*M. albus* cz620 AF324336	86
			M. cinnamomi GQ848369	
			M. musae JX089323	
			M. oryzae JX089321	

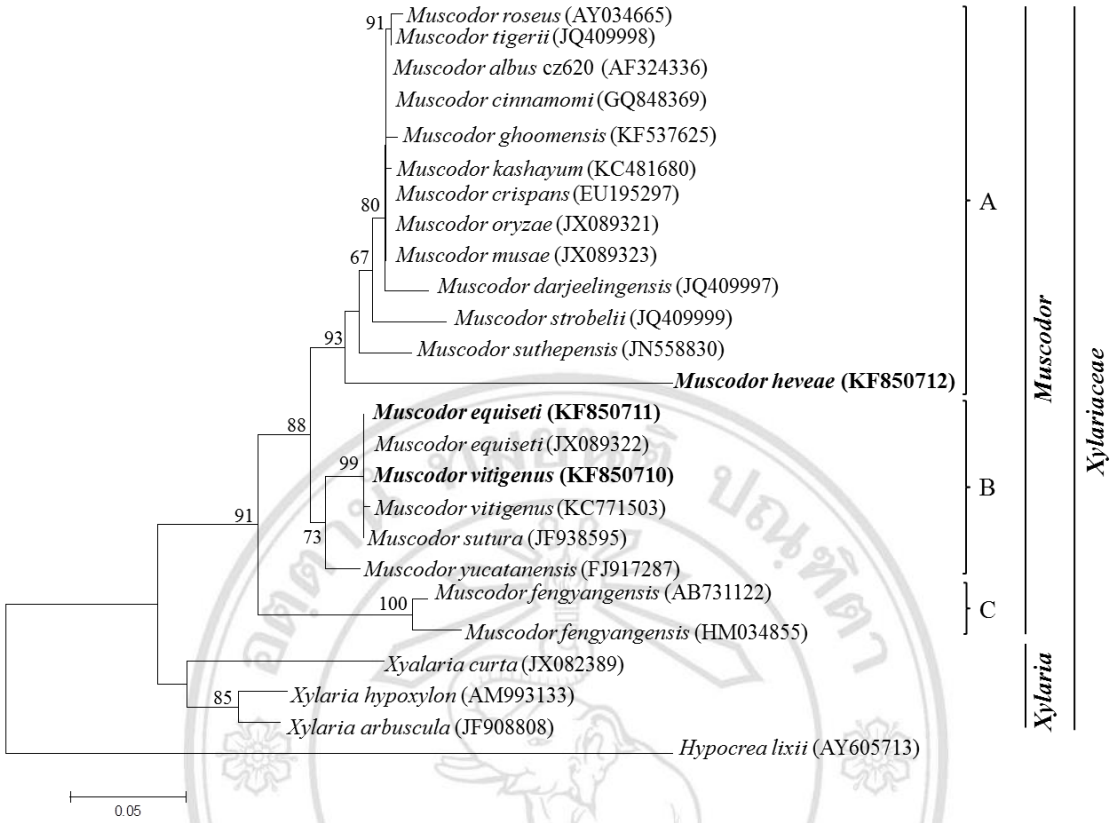

Figure 4.3 Neighbor-joining (NJ) phylogenetic tree of *Muscodor* spp. based on ITS rRNA sequence alignment of 25 sequences. *Hypocrea lixii* was used as an out group. The isolate RTM5IV3 (KF850712) was proposed as a novel species, *M. heveae* (see text for the description of novel species). The isolate RTM5IV1 (KF850710) and RTM5IV2 (KF850711) were proposed as *M. vitigenus* and *M. equiseti*, respectively. The codes indicated in parentheses refer to the accession numbers of the sequences available in GenBank database.

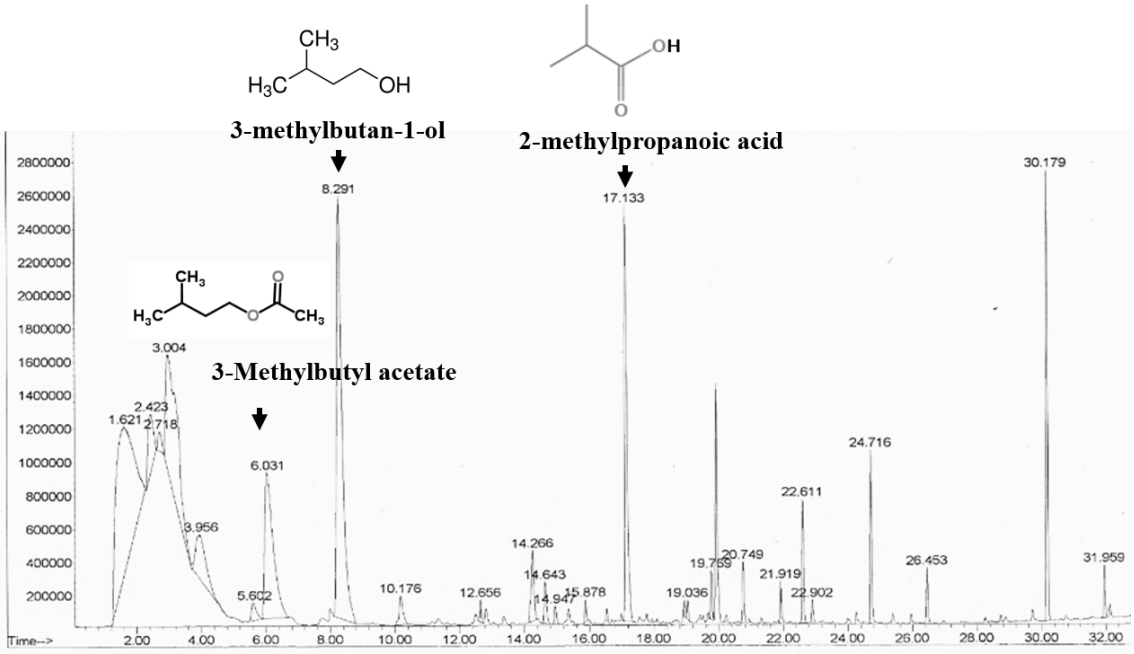

Figure 4.4 SPME-GC/MS profile of 3-methylbutan-1-ol, 3-methylbutyl acetate and 2-methylpropanoic acid produced by *Muscodor heveae* cultured on PDA.

Table 4.2 Composition of the volatile metabolites produced by *Muscodor* spp. by GC/MS.

RT (min)	Possible compound	Molecular formula	M/z	% Total area					
				Blank	MH[a]	MA[b]	MC[c]	MM[d]	MO[d]
5.41	cyclopentane	C_5H_{10}	7.69	0	0	0	7.69	0	0
5.56	ethyl acetate	$C_4H_8O_2$	88	0	0	7.63	0	0	0
5.60	2-methylpropan-1-ol	$C_4H_{10}O$	74	0	0.76/86*	0	0	0	0
6.03	3-methylbutyl acetate	$C_7H_{14}O_2$	130	0	6.92/90*	0	0	0	0
6.38	methyl 2-methylbutanoate	$C_6H_{12}O_2$	116	0	0	0	14.90	0	0
6.51	methyl propanoate	$C_4H_8O_2$	102	0	0	0.31	0	0	0
7.07	2-methylpropan-1-ol	$C_4H_{10}O$	74	0	0	0	0	0	3.48
7.16	ethanol	C_4H_6O	ND	0	0	6.24	0	0	0
7.77	3-methylbutanoyl acetate	ND	130	0	0	0	0	20.41	4.70
8.03	ethyl 2-methylpropanoate	$C_6H_{12}O_2$	116	0	0	2.07	0	0	0
8.29	3-methylbutan-1-ol	$C_5H_{12}O$	88	0	11.26/83*	0	3.12	0	0
10.32	2-methylbutyl 2-methylpropanoate	$C_9H_{18}O_2$	158	0	0	0	0	0.21	0

Table 4.2 (continue).

RT (min)	Possible compound	Molecular formula	M/z	% Total area					
				Blank	MH[a]	MA[b]	MC[c]	MM[d]	MO[d]
10.88	3-methylbutan-1-ol	$C_5H_{12}O$	88	0	0	0	0	13.34	32.69
11.45	2-methylpropyl 2-methylpropanoate	$C_8H_{16}O_2$	ND	0	0	0.58	0	0	0
12.05	2-methylpropan-1-ol	$C_4H_{10}O$	74	0	0	2.06	0	0	0
12.10	3-methyl-3-buten-1-ol	$C_5H_{10}O$	86	0	0	0	0	0	0.27
12.20	ethyl 2-hydroxy-2-methylpropanoate	$C_6H_{12}O_3$	132	0	0	0	0	0	0.88
12.50	3-methylbutyl acetate	$C_7H_{14}O_2$	ND	0	0	22.24	0	0	0
12.66	1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-2,4,6,8,10,12-hexaoxa-1,3,5,7,9,11-hexasilacyclododecane	$C_{12}H_{36}O_6Si_6$	445	0.67/91*	0	0	0	0	0
12.97	3-hydroxybutan-2-one	$C_4H_8O_2$	88	0	0	0	0	0.59	2.00
14.57	3-methylbutyl 2-methylpropanoate	$C_9H_{18}O_2$	ND	0	0	1.53	0	0	0
15.28	3-methylbutan-1-ol	$C_5H_{12}O$	88	0	0	22.99	0	0	0
15.88	benzaldehyde	C_7H_6O	106	4.61/96*	0	0	0	0	0
16.08	2-pentylfuran	$C_9H_{14}O$	138	0	0	0.29	0	0	0

Table 4.2 (continue).

RT (min)	Possible compound	Molecular formula	M/z	% Total area					
				Blank	MH[a]	MA[b]	MC[c]	MM[d]	MO[d]
16.19	2,4-dimethyl-1-heptene	C_9H_{18}	126	0	0	0	0	0	0.13
16.31	1-isobutoxy-2-ethylhexane	$C_{12}H_{26}O$	186	0	0	0	0	0	0.31
17.13	2-methylpropanoic acid	$C_4H_8O_2$	88	0	4.73/91*	0	0	0	0
19.04	(1*R*,4*E*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0] undec-4-ene	$C_{15}H_{24}$	204	0	0.41/93*	0	0	0	0
19.43	isopropyl-4-piperidone	$C_8H_{15}NO$	141	0	0	0	0	0.23	0
19.50	4,5-dimethyl-1,3-cyclopentanedione	$C_7H_{10}O_2$	126	0	0	0	0	0	0.37
19.77	1,1,9-trimethyl-5-methylidenespiro[5.5]undec-9-ene	$C_{15}H_{24}$	204	0	0.51/95*	0	0	0	0
20.38	nonan-2-one	$C_9H_{18}O$	142	0	0	0.41	0	0	0
20.75	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	2.03/99*	0	0	0	0
21.03	2-methylpropanoic acid	$C_4H_8O_2$	88	0	0	0	0	33.17	15.41

Table 4.2 (continue).

RT (min)	Possible compound	Molecular formula	M/z	% Total area					
				Blank	MH[a]	MA[b]	MC[c]	MM[d]	MO[d]
21.07	8α-methyl-4-methylidene-6-propan-2-ylidene-2,3,4α,5,7,8-hexahydro-1*H*-naphthalene	$C_{15}H_{24}$	204	0	0	0.3	0	0	0
21.69	[1*S*-(1. α.,4. α.,7. α)]-1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1methylethenyl)-azulene	$C_{15}H_{24}$	204	0	0	0	0	0.29	0
21.81	(4*Z*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	$C_{15}H_{24}$	204	0	0	0	0	0	2.19
21.92	methyl (*Z*)-*N*-hydroxybenzenecarboximidate	$C_8H_9NO_2$	151	0	0.31/83*	0	0	0	0
22.54	azulene	$C_{10}H_8$	204	0	0	1.51	0	0	0
22.61	2-phenylethyl acetate	$C_{10}H_{12}O_2$	164	0	0.9/83*	0	0	0	0
23.16	1-methyl-4-[(2*E*)-6-methylhepta-2,5-dien-2-yl]cyclohexene	$C_{15}H_{24}$	204	0	0	0.94	0	0	0
23.76	2-methylbutanoic acid	$C_{15}H_{10}O_2$	102	0	0	0	0	1.10	0
24.72	2-phenylethanol	$C_8H_{10}O$	122	0	1.28/97*	0	0	0	0
24.75	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	0	0	0	1.00	0.44

Table 4.2 (continue).

RT (min)	Possible compound	Molecular formula	M/z	% Total area					
				Blank	MH[a]	MA[b]	MC[c]	MM[d]	MO[d]
25.20	1*H*-3α,7-methanoazulene, 2,3,4,7,8,8α-hexahydro-3,6,8,8 tetramethyl-,[1*R*-(1.α., 4α.α., 8α.α.)]	$C_{15}H_{24}$	204	0	0	3.63	0	0	0
25.30	2-methylpropanoic acid	$C_4H_8O_2$	88	0	0	6.08	0	0	0
25.72	3,7-dimethyl-1,6-octadiene	$C_{10}H_{18}$	138	0	0	0	0	0.05	0
26.04	(1*R*,4*E*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	$C_{15}H_{24}$	204	0	0	0.48	0	0	0
26.45	phenol	C_6H_6O	94	1.80/95*	0	0	0	0	0
26.91	2-methyl-propanamide	C_4H_9NO	87	0	0	0	0	0.07	0
27.05	2-phenylethyl acetate	$C_{10}H_{12}O_2$	164	0	0	0	0	3.28	1.46
27.27	6-nitro-2-picoline	$C_6H_6N_2O_2$	138	0	0	0	0	0.12	0
27.42	(1*E*,5*E*)-1,4,4-trimethyl-8-methylidenecycloundeca-1,5-diene	$C_{15}H_{24}$	204	0	0	0	3.23	0	0
27.55	naphthalene,1,2,4α,5,6,8α-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,[1*R*-(1.α., 4α.α., 8α.α.)]	$C_{15}H_{24}$	204	0	0	0.34	0	0	0

Table 4.2 (continue).

RT (min)	Possible compound	Molecular formula	M/z	% Total area					
				Blank	MH[a]	MA[b]	MC[c]	MM[d]	MO[d]
28.34	(*R*)-11-methylene-3,7,7-trimethylspiro[5.5]undec-2-ene	$C_{15}H_{24}$	204	0	0	0.36	0	0	0
28.50	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	0	1.07	0	0	0
28.57	(2*R*)-8,8,8α-trimethyl-2-prop-1-en-2-yl-1,2,3,4,6,7-hexahydronaphthalene	$C_{15}H_{24}$	204	0	0	3.24	0	0	0
30.18	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	3.3/95*	0	0	0	0
30.89	azulene,1,2,3,5,6,7,8,8α-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1*S* (1.α.,7.α.,8α.β.)]-	$C_{15}H_{24}$	204	0	0	0	8.58	0	0
30.9	(3*R*,α *S*,α *R*)-α -methyl-5-methylidene-3-prop-1-en-2-yl-1,2,3,4,α ,6,7,8-octahydronaphthalene	$C_{15}H_{24}$	107	0	0	0	7.32	0	0
31.12	2-phenylethyl acetate	$C_{10}H_{12}O_2$	ND	0	0	1.74	0	0	0
31.72	2-(2-methyl-2-propenyl)-2-cyclohexane-1-one	ND	150	0	0	0	0	0.25	0

Table 4.2 (continue).

RT (min)	Possible compound	Molecular formula	M/z	% Total area					
				Blank	MH[a]	MA[b]	MC[c]	MM[d]	MO[d]
33.17	2-phenylethanol	$C_8H_{10}O$	122	0	0	1.06	0	0	0

The volatile metabolites produced by *M. heveae* (MH) and its closest phylogenetic species; *M. albus* cz620 (MA), *M. cinnamomi* (MC), *M. musae* (MM), and *M. oryzae* (MO) were identified by GC-MS. The results were reported with retention time (RT), possible compound (with IUPAC name), molecular formula, mass to charge ratio (M/z), % total area and % quality*. The data obtained from [a]this study were compared with the GC/MS database of volatile compounds produced by *Muscodor* spp. which reported by [b]Strobel (2011), [c]Suwannarach *et al*. (2010), and [d]Suwannarach *et al*. (2013a). The red highlight refers to the major composition in each profile of the volatile metabolites. Compounds presented in a control PDA plate are not included in this Table. The analysis for *M. heveae* was done in triplicate.

Table 4.3 Growing condition of *Muscodor* spp. and GC-MS system for analysis of their volatile metabolites.

	M. heveae[a]	***M. albus***[b]	***M. cinnamomi***[c]	***M. musae***[d]	***M. oryzae***[d]
Growing condition					
Media	PDA	PDA	PDA	PDA	PDA
Incubation time	10 days	14 days	10 days	7 days	7 days
Incubation temperature	25 ± 2 °C	23 °C	25 ± 2 °C	25 ± 2 °C	25±2 °C
GC-MS system					
Equipment : GC	SPME fiber insert into an Agilent 7890A gas chromatograph	SPME fiber insert into a gas chromatograph (Hewlett Packard 5890 series II Plus)	An Agilent Technologis GC 7890	SPME fiber insert into Gas chromatography GC 2010 (Shimadzu, Japan)	SPME fiber insert into Gas chromatography GC 2010 (Shimadzu, Japan)
Equipment : MS	MSD 5975C (EI) mass spectrometer	VG 70E-HF double focusing magnetic mass spectrometer	MSD 5973 (EI) mass spectrometer	MS-QP2010 (Shimadzu, Japan)	MS-QP2010 (Shimadzu, Japan)
Temperature program of GC column	40 °C for 2 min, increased to 200 °C at 5 °C min^{-1}	25 °C for 2 min, increased to 220 °C at 5 °C min^{-1}	32 °C for 2 min, increased to 220 °C at 5 °C min^{-1}	40 °C for 2 min, increased to 200 °C at 5 °C min^{-1}	40 °C for 2 min, increased to 200 °C at 5 °C min^{-1}
Condition of fiber	250 °C for 34 min under a flow of helium gas	240 °C for 20 min under a flow of helium gas	250 °C for 39.6 min under a flow of helium gas	250 °C for 57 min under a flow of helium gas	250 °C for 57 min under a flow of helium gas

The data obtained from [a]this study or be taken from [b]Strobel (2011), [c]Suwannarach *et al.* (2010), and [d]Suwannarach *et al.* (2013a).

4.3.3 Taxonomic description of a novel species

Muscodor isolate RTM5IV3 did not produce spores on any medium, like the original *Muscodor* and had rope-like mycelium with coiled structures (Strobel, 2011). Although the isolate formed pale orange colonies when exposed to the natural light similar to *M. cinnamomi* and *M. oryzae*, its mycelial morphology was wavy and hair-like at the mycelial edge and produced a unique unidentified structure (Figure 4.5 and Table 4.4). The isolate RTM5IV3 was classified and identified with the polyphasic approach. Both phenotype and genotype revealed that the isolate belongs to the genus *Muscodor*. It shared relatively low sequence similarity of ITS rRNA gene (86%) with many species of the genus *Muscodor*. The phylogenetic tree showed isolate RTM5IV3 was separated from the other 12 *Muscodor* spp. in clade A with high bootstrap support (Figure 4.3). The phylogenetic dendrogram also supported the node separating isolate RTM5IV3 in clade A from five species of *Muscodor* in clade B and C with 88 and 91% bootstrap support, respectively. The isolate RTM5IV3 was able to form volatile metabolites with antimicrobial activity against diverse tested microbes, which is a typical phenotype of the genus *Muscodor*. However, the profile of volatile metabolites produced by the isolate was different than its closely related phylogenetic species, but the major component of the volatile metabolites was 3-methylbutan-1-ol, which was the same as the type species of the genus *Muscodor*, *M. albus* cz620 (Tables 4.2). Based on the phenotypic, genotypic and phylogenetic evidence, isolate RTM5IV3 represents a novel species of the genus *Muscodor*, for which the name *Muscodor heveae* sp. nov. was proposed. The taxonomic description includes the following:

Taxonomy

Muscodor heveae S. Siri-Udom & S. Lumyong, sp. nov. (Figure 4.5)

MycoBank: MB809310.

Diagnosis: Pale orange colonies on PDA in natural light, hyphae 1.2 - 3.7 µm thick, coils 14.8 - 27.1 × 19.7 - 39.4 µm diam, and producing a fruity odor.

Etymology: *heveae*, refers to the name of the host plant, *Hevea brasiliensis*.

Holotype: THAILAND, Nongbualamphu province, Chiang Mai, Chiang Mai University, from a healthy leaf of *Hevea brasiliensis* (*Euphorbiaceae*) during May,

2011-December, 2012. (ex-type living culture BCC 70461 and SDBR-CMU RTM5IV3), GenBank sequence KF850712.

Teleomorph: Unknown.

Description: In nature, the fungus is associated with *Hevea brasiliensis*. It is an ascomycete and does not produce spores. Colony color of the fungus on PDA in natural light is pale orange but becomes whitish in the dark (Figure 4.5A). The edge of colony was wavy mycelium (Figure 4.5B). Hyphae (1.2 - 3.7 µm thick) with coils (14.8 - 27.1 × 19.7 - 39.4 µm diam; Figure 4.5C), commonly intertwining of mycelium into twisted cable-like strand and rope like strand (4.9 - 7.4 µm thick; Figure 4.5D, 4.5G). Mycelium forms unique unidentified structures (3.8 - 7.5 µm diam; Figure 4.5E) and swollen cells at hyphal tips (2.5 - 4.9 µm diam; Figure 4.5F). A culture on PDA produced a fruity odor, which contains 3-methylbutan-1-ol as a major component. Spores and other fruiting bodies did not appear under any of the tested conditions.

The other two isolates of *Muscodor* tested; isolate RTM5IV1 and RTM5IV2 were identified as *M. vitigenus* (Mycobank: MB373747) (Figure 4.6) and *M. equiseti* (Mycobank: MB800814) (Figure 4.7), respectively based on morphology and phylogenetic analysis. The description of *M. vitigenus* and *M. equiseti* were showed in previous publications of Daisy *et al.* (2002) and Suwannarach *et al.* (2013a), respectively.

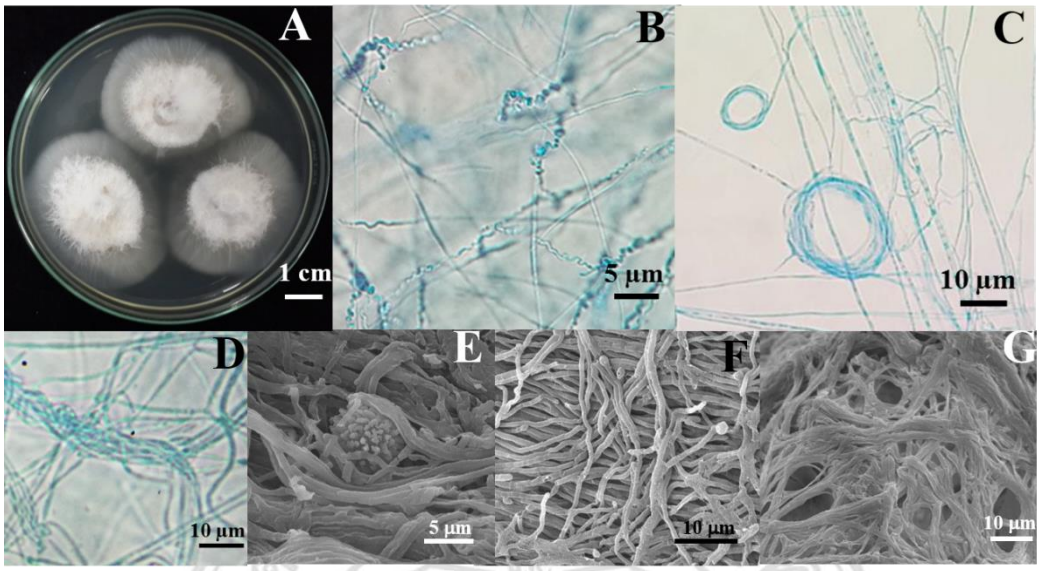

Figure 4.5 Morphological characteristics of *Muscodor heveae* (RTM5IV3) isolated from leaves of rubber tree. Difference in colony morphology was observed after growing on PDA in the dark (A) for 10 days. The hyphal morphology of *M. heveae* was observed further with light (B-D) and scanning electron (E-G) microscopes. The wavy mycelium from the colony edge (B), formation of coiling hypha (C), rope-like mycelium (D, G), unique unknown structure (E), and swollen like hyphal tips (F) were found as a typical morphology of *M. heveae*.

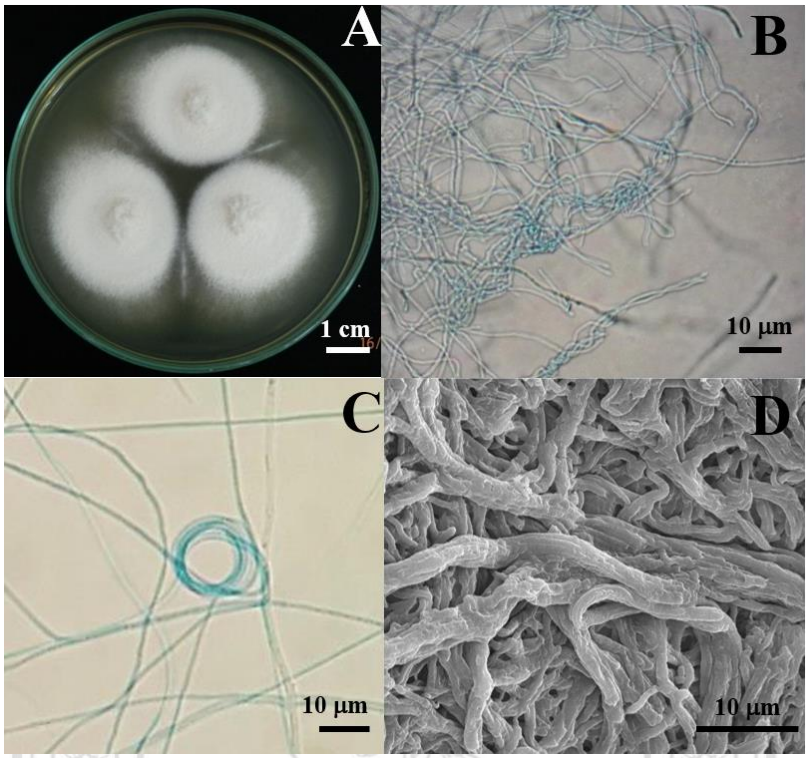

Figure 4.6 Morphological characteristics of *Muscodor vitigenus* (RTM5IV1) isolated from leaves of rubber tree. Difference in colony morphology was observed after growing on PDA in the dark (A) for 10 days. The hyphal morphology of *M. vitigenus* was observed further with light (B-C) and scanning electron (D) microscopes. The formation of rope-like mycelium (B, D), and coiling hypha were found as a typical morphology of *M. vitigenus*.

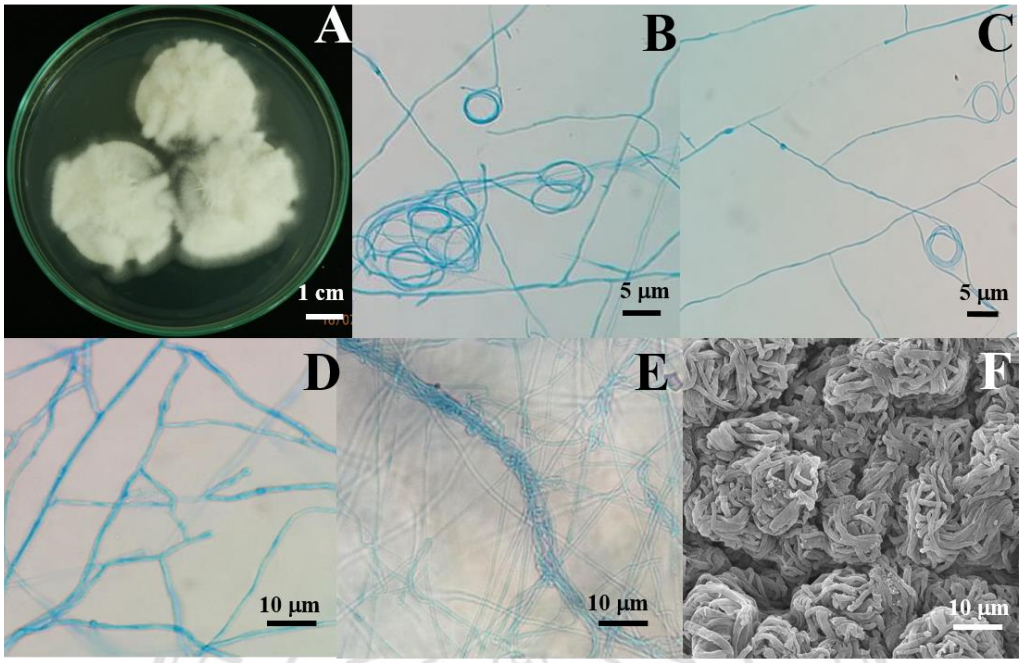

Figure 4.7 Morphological characteristic of *Muscodor equiseti* (RTM5IV2) isolated from leaves of rubber tree. Difference in colony morphology was observed after growing on PDA in the dark (A) for 10 days. The hyphal morphology of *M. equiseti* was observed further with light (B-E) and scanning electron (F) microscopes. The formation of coiling hyphae (B-C), triangular branching pattern (D), swollen cells (C-D), rope-like mycelium (E), and cottony-like mycelium (F) were found as a typical morphology of *M. equiseti*.

Table 4.4 Morphology and bioactivity of *Muscodor heveae* and its closely related phylogenetic species.

Morphology and bioactivity	***M. heveae***	***M. albus* cz620[1]**	***M. cinnamomi***	***M. musae***	***M. oryzae***
Mycelial growth	Rope-like strand and swollen cells with coils structure	Rope-like with coils structure	Rope-like with cauliflower-like bodies	Rope-like with coils structure	Rope-like with coils structure
Hyphal growth at colony front	Wavy and hairy-like mycelium	Straight	Straight	Straight and hairy-like mycelium	Straight
Pigment production					
in the light	Pale orange	Whitish	Pale orange	Whitish	Pale orange
in the dark	Whitish	ND	Whitish	Whitish	Pale orange
Host	*Hevea brasiliensis*	*Cinnamomum zeylanicumin*	*Cinnamomum bejolghota*	*Musa acuminata*	*Oryza rufipogon*
Bioactivity	Antifungal/antibacterial	Antifungal/antibacterial	Antifungal/antibacterial	Antifungal/antibacterial	Antifungal/antibacterial
Reference	In this work	Worapong *et al.* (2001)	Suwannarach *et al.* (2010)	Suwannarach *et al.* (2013a)	Suwannarach *et al.* (2013a)

ND refers to not determined.

4.4 Discussion

It is known that ascomycetes including the following notable genera, *Colletotrichum*, *Penicillium*, *Pestalotiopsis*, and *Trichoderma* are dominant fungal endophytes of rubber tree (Gazis and Chaverri, 2010; Gazis, 2012). In addition, Arbuscular mycorrhizal fungi (AMF) were first described on the root of rubber tree but rubber tree plantation expressed lowest numbers of AMF (Pereira *et al*., 2014). Fungal communities in rubber tree were affected by the physical and chemical properties of trees, tree ages, biological interaction, substrate preference, host preferences and geographical characters (Kodsueb *et al*., 2008; Monkai *et al*., 2016). However, there is no report of volatile metabolites-producing endophytes that live associated with rubber tree. We were the first to find that the rubber tree can be a source for discovering volatile metabolite-producing endophytes. The isolation technique used in this study was highly selective for the genus *Muscodor*. This was similar to a number of previous studies that used the same technique for isolation of volatile metabolite-producing endophytic fungi (Suwannarach *et al*., 2010; 2013a). Moreover, there was no contamination found during such isolations, suggesting that the volatile metabolites produced by either the reference strain and the emerging isolates exhibited strong and broad-spectrum antimicrobial activity. In addition, determining the distribution of volatile metabolites-producing endophytes in different plant tissues of rubber trees would be a further study in order to understand their spatial and temporal dynamics within the plant host.

In this study, we proposed a novel species, *M. heveae* that has a phenotypic and phylogenetic relationship with the genus *Muscodor*, but showing a distinct set of morphology, genotype, and profile of volatile metabolites produced. The phylogenetic analysis based on partial sequences of 18S rDNA, ITS1-5.8-ITS2 rDNA was required for accurate genetic classification. These sequences have been demonstrated to be highly conserved regions of DNA and very useful in the classification of fungi (von der Schulenburg *et al*., 2001). The sequence data obtained on *Muscodor* indicated that it was a xylariaceous fungus with incomplete molecular identity to other fungi in family *Xylariacea* (Ezra *et al*., 2004). In addition, a high percent similarity (99-100%) of ITS rRNA sequences among several emerging species of the genus *Muscodor* was often found (Suwannarach *et al*., 2010; Kudalkar *et al*., 2011). However, the authors are still confident

that their isolates could be proposed as the novel species. The most notable property of *Muscodor* was its ability to produce a mixture of volatile metabolites with antibiotic activity against variety of pathogenic fungi and bacteria (Ezra *et al*., 2004).

Thus, detailed phenotypes of the isolates, volatile gas composition analysis and comparison with their closely related phylogenetic species were priority criteria for assigning their novelty. At early stage, colony and mycelial characteristics were the only available phenotypes to identified *Muscodor* species (Zhang *et al*., 2010). Subsequently, analysis and comparison of volatile metabolites produced by *Muscodor* isolates have been used as an important tool for their classification and novelty determination. Variation of volatile metabolites-profile is commonly found across member species of the genus *Muscodor*, but they often share the same major volatile component (Kudalkar *et al*., 2011; Suwannarach *et al*., 2013a). The volatile profiles (Table 4.2) of four *Muscodor* spp. have been compared with *M. heveae*. All species are prevalent in Thailand except *M. albus* cz620. However, the previous reports showed that there were many isolates of *M. albus* existed in local host plant in many countries (Ezra *et al*., 2004; Atmosukarto *et al*., 2005; Strobel *et al*., 2007; Banerjee *et al*., 2010; Banguela-Castillo *et al*., 2015). Including, *M. albus* MFC2 have been isolated from *Myristica fragrans* in Thailand and produced bulnesene, ledol, (-)-globulol and azulene derivatives as major volatiles (Sopalun *et al*., 2003). The variation profile of volatile metabolites may be caused by diverse factors, including growing media and environment, and enzyme activity for biosynthesis of such metabolites by the test microbes (Morath *et al*., 2012).

The bioactive volatile components for the genus *Muscodor* were 2-methylpropanoic acid, 3-methylbutyl acetate and 3-methyl-1-butanol (3-methylbutan-1-ol) (Strobel, 2006; Mitchell *et al*., 2010), which were in agreement to the one detected in *M. heveae*. Volatile metabolites are formed as a mixture, and not all volatile metabolites exhibit antimicrobial activity. However, applying the mixture of volatile metabolites for antimicrobial activity has been suggested with more potent than using them as the individual compounds (Strobel *et al*., 2001). The major volatile metabolites in the mixture are not always the bioactive ones, and this is different between either species or taxa. In the genus *Muscodor*, the major volatile antimicrobial metabolite was 2-methylpropanoic acid (Mitchell *et al*., 2010). This bioactive volatile metabolite has been found to be

formed by a yeast *Saccharomyces cerevisiae*, but the most active compounds of its volatile mixture were 2-methyl-1-butanol and 3-methyl-1-butanol (Fialho *et al*., 2011).

CHAPTER 5

Antimicrobial activity of *Muscodor* spp. and relevant alleopathy effects

5.1 Introduction

Biological control is the control of destructive organisms by the use of other organisms. It suggests that endophytes can be used as biocontrol agents because they can help hosts tolerate harmful abiotic and biotic factors including pathogens. *Trichoderma* sp., one species of fungal endophyte was successfully used to control phytopathogens, *Colletotrichum gloeosporiodes* and *Rigidoporous lignosus* in rubber tree (Gazis and Chaverri, 2010). Endophytes could be used directly to treat seeds or transplants (Backman and Sikora, 2008), but the inoculation methods for endophyte associated with trees are limited. Syringes inoculation and soaking seeds in spore suspensions were used in the case of grasses and sprays of spore suspension have been used to introduce endophytes into beans and barley (Mejia *et al*., 2003).

Strobel *et al*. (2001) recently reported the volatile antibiotic from an endophytes, *Muscodor albus* which effectively inhibited and killed certain pathogenic fungi and bacteria. Volatile organic compounds (VOCs) are low molecular mass and usually hydrophobic compounds with high vapor pressure, i.e. they easily evaporate at room temperature. They can diffuse a long way from their point of origin and migrate in soil and aerial environments as well as through porous wood materials (Hung *et al*., 2013). Like plants and bacteria, fungi produce a large number of VOCs as mixtures of alcohols, ketones, esters, small alkenes, monoterpenes, sesquiterpenes, and derivatives (Korpi *et al*., 2009). The mixtures of VOCs produced by fungal endophytes have both antibacterial and antifungal activities (Strobel *et al*., 2001, 2006). The most effective group of inhibitory compounds was isoamyl acetate (Strobel and Daisy, 2003) and it benefit to use as mycofumigation to treat soil, seeds and plants. There are many *Muscodor* sp. that showed high activity in control pathogens such as *M. cinnamomi* exactly isolated from cinnamon in Thailand (Suwannarach *et al*., 2010).

Allelochemistry is the production and release of toxic substances produced by one species, which affect a receiving susceptible species (Saraf *et al.*, 2014). Allelopathy of microorganism is often demonstrated by testing allelochemical effects on seed germination. The previous research showed that *Arabidopsis thaliana* was used as a model to study the plant growth effects of natural mixtures of volatiles emitted by biocontrol, plant pathogenic (Splivallo *et al.*, 2009). Moreover, *A. thaliana* grown in the presence of VOCs from *T. viride* were taller, bigger and had more lateral roots (Hung *et al.*, 2013). However, there are few studies that explore the herbicide activity of the volatile and semi-volatile compounds from endophyte (Guzmán-Trampe *et al*., 2015).

5.2 Materials and methods

5.2.1 *In vitro* antimicrobial assay of VOCs

Antagonistic activity of all *Muscodor* isolates was tested against a set of pathogenic microbes listed in Table 5.1. The test microbes were comprised of bacteria, yeast and filamentous fungi. The parallel-growth isolation technique was adapted for the antagonism test. Briefly, a part of a two-compartment Petri dish was filled with PDA, while the other side contained nutrient agar for the test bacteria, yeast extract-malt extract agar for the test of yeast, or PDA for the test of filamentous fungi. An agar plug from the mycelial margin of each *Muscodor* isolate growing on PDA was inoculated on the PDA part of the Petri dish, and allowed to grow at room temperature (25 ± 2 °C) for 4 days. Then, each tested bacterium or yeast was individually streaked on its respective agar medium in the Petri dish. For the filamentous fungi test, an agar plug (6 mm diam) of their 4-day-old PDA culture was inoculated on PDA on opposite side of the Petri dish. All Petri dishes were wrapped with Parafilm® M (Bemis company, Inc., USA) and incubated at room temperature (25 ± 2 °C) for 6 days. The percentage of inhibition of fungal growth after the dual culture test was calculated with an equation: $[(R_1\text{-}R_2) \times 100] \div R_1$, where R_1 is the average of colony radiuses of each test microbe measured in the control plates (without *Muscodor* isolate), and R_2 is the average of colony radiuses calculated from the test plates. In the case of bacteria and yeast, the microbes were checked for visible growth and viability. The viability of the test microbes was observed by transferring them from the test plates and re-growing in fresh media (Strobel *et al.*, 2001).

Artificial volatile compounds including 3-methylbutan-1-ol (isoamy alcohol, Sigma-Aldrich, Germany), 3-methylbutyl acetate (isoamyl acetate, Merck, Germany) and 2-methylpropanoic acid (isobutyric, Sigma-Aldrich, Germany) were used to demonstrate the relative activity toward hyphal growth. They were found to be the most active volatile components produced by *M. heveae* according to the results in section 4.3.2, chapter 4. The artificial compounds were added individually at different concentrations ranging from 5-35 μl L^{-1} airspace into sterile filter paper in the compartments of the Petri dishes with an agar plug of *R. microporus* or *P. noxius* (Suwannarach *et al*., 2016). The Petri dishes were then sealed with Parafilm® M (Bemis company, Inc., USA), incubated at room temperature (25 ± 2 °C) for 6 days and the radial growth was measured.

5.2.2 Effect of VOCs from *Muscodor heveae* on root and shoot growth

Four dicots, *Arabidopsis thaliana* Ceol-0, garden pea (*Pisum sativum* L.), mung bean (*Vigna radiate* L.) and tomatoe (*Lycopersicon esculentum* Mill.), and three monocots, jasmine rice (*Oryza sativa* L.), ruzi grass (*Brachiaria ruziziensis*) and sweet corn (*Zea May* L. var. *saccharata*) were selected for this study. All plants were selected for their rapid growth and frequency of germination because tropical plants including rubber tree seeds germinate poorly. All of the seeds were commercial products acquired from the Chia Tai Co., Ltd., except for ruzi grass which was purchased from a local market in Nongbualamphu Province, Thailand and *A. thaiana* col-0 seeds that had been obtained from Professor Kenji Matsui at the Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan. An agar plug of *M. heveae* (6 mm Ø) was placed on the PDA medium in one of the compartments of divided Petri dishes (9 cm) and was grown at room temperature (25 ± 2 °C) for 4 days. The seeds were surface sterilized by soaking in 1-2% sodium hypochlorite for 3 min, and washed in sterile distilled water 3 times, while the seeds of *A. thaliana* col-0 were surface sterilized by soaking in 70 % ethanol for 3 min, 10% sodium hypochlorite for 12-13 min and washed in sterile distilled water for 5-7 times. Then, seeds were placed into the other compartment that contained 1% water agar except for *A. thaliana* col-0, which were placed on Murashige and Skoog medium (MS medium). All Petri dishes were sealed with Parafilm® M (Bemis company, Inc., USA), and incubated at room temperature (25 ± 2 °C) for a photoperiod of 12:12h fluorescent light in a plant growth chamber (Sanyo MLR-

351H, Japan) for 7 and 14 days (Macías-Rubalcava *et al*., 2010). The volatile assay with *A. thaliana* col-0 was incubated at room temperature (25 ± 2 °C) °C for a photoperiod of 16:8h fluorescent light for 14 days. Root and shoot length were measured and compared to the non-muscodor inoculated control with four replications per treatment.

Artificial volatile compounds including 3-methylbutan-1-ol (isoamy alcohol, Sigma-Aldrich, Germany), 3-methylbutyl acetate (isoamyl acetate, Merck, Germany) and 2-methylpropanoic acid (isobutyric, Sigma-Aldrich, Germany) were used to demonstrate the relative activity toward root and shoot growth. The artificial compounds were added individually at different concentrations ranging from 5-35 μl L^{-1} airspace into sterile filter paper in the compartments of the Petri dishes with seeds. The Petri dishes were then sealed with Parafilm® M (Bemis company, Inc., USA) incubated under suitable conditions and the root and shoot length were measured.

5.2.3 Statistical analysis

SPSS program version 17.0 for Windows was used to analyze the experimental data with T-test and one-way analysis of variance (ANOVA), along with Turkey's post hoc tests at different significant levels (P).

Table 5.1 The list of tested microbes used in this study.

Tested microbe	Source
Bacteria	
Bacillus cereus	The Central and Diagnostic Laboratory, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand
Methicillin-resistant *Staphylococcus aureus* (MRSA)	
Micrococcus luteus	
Enterobacter aerogenes	
Enterococcus faecalis	
Escherichia coli ATCC 35218	
Klebsiella pneumoniae (ESBL+)	
Proteus mirabilis	
Pseudomonas aeruginosa ATCC 27859	
Salmonella sp. Group D	
Yeast	
Cryptococcus neoformans	
Filamemtous fungi	
Aspergillus flavus	The Sustainable Development of Biological Resources (SDBR) Lab, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand
Aspergillus niger	
Colletotrichum gloeosporioides	
Fusarium oxysporum f. sp. *vasinfectum*	
Rhizoctonia solani AG-2	
Ganoderma australe BCC22321	The BIOTEC (National Center for Genetic Engineering and Biotechnology) Culture Collection (BCC), Bangkok, Thailand.
Phellinus noxius BCC26237	
Phytophthora parasitica BCC15560	
Rigidoporus microporus	Department of Microbiology and Parasitology, Faculty of Science, Naresuan University, Phitsanulok.

5.3 Results

5.3.1 Antimicrobial activity of volatile metabolites produced by *Muscodor* isolates

The volatile metabolites produces by different *Muscodor* isolates were tested for their antimicrobial activity against tested microbes (Table 5.2). The volatile metabolites produced by *Muscodor* isolates RTM5IV1 and RTM5IV3 exhibited antimicrobial activity against Gram positive and negative bacteria, yeast and filamentous fungi, while isolate RTM5IV2 inhibited only the growth of some Gram negative bacteria and filamentous fungi. Isolate RTM5IV3 showed the greatest antimicrobial activity with all tested organisms (Figure 5.1-5.2). *F. oxysporum* f. sp. *vasinfectum* was more susceptible to the volatile metabolites of RTM5IV3 than those of other isolates. *Ps. aeruginosa* ATCC 27859 was the most resistant to the tested microbe, was insensitive to the volatile metabolites produced by all isolate of *Muscodor*. All the *Muscodor* volatiles exhibited significant control of all tested pathogenic fungi of rubber tree (*C. gloeosporioides*, *P. noxius* and *R. microporus*) compared with other tested fungi.

Figure 5.1 Antifungal ability of VOCs from *Muscodor heveae* (MH) against *Aspergillus flavus*, *A. niger*, *Rhizoctonia solani* AG-2 and *Phytophthora parasitica*.

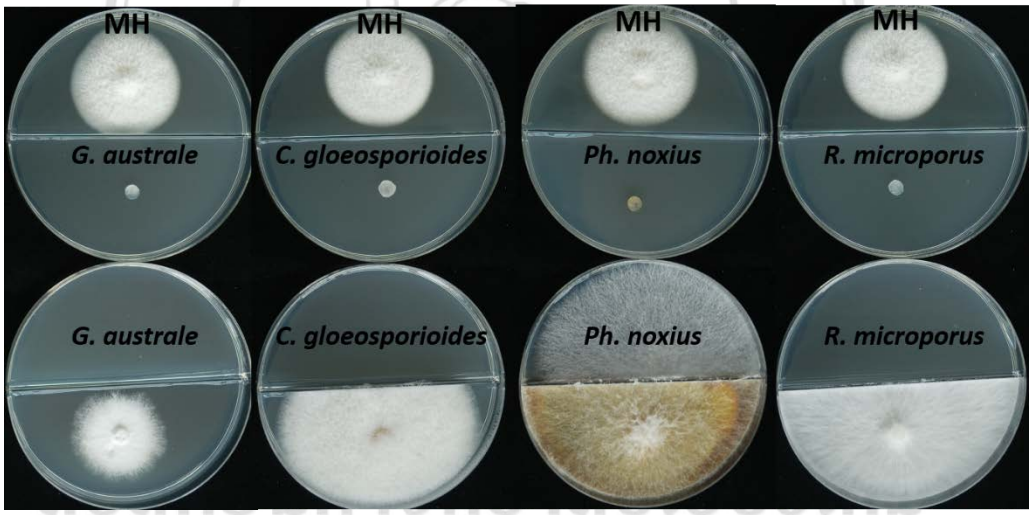

Figure 5.2 Antifungal ability of VOCs from *Muscodor heveae* (MH) against *Ganoderma australe*, *Colletotrichum gloeosporioides*, *Phellinus noxius* and *Rigidoporus microsporus*.

Table 5.2 Antimicrobial activity of volatile metabolites-producing *Muscodor* against human and plant pathogens.

Test microbes	Inhibition activity of microbial growth (viability) after exposure by different isolates of *Muscodor* (%)		
	M. vitigenus	***M. equiseti***	***M. heveae***
Gram positive bacteria			
Bacillus cereus	100 (Dead)	0 (Alive)	100 (Alive)
Methicillin-resistant *Staphylococcus aureus* (MRSA)	0 (Alive)	0 (Alive)	100 (Dead)
Micrococcus luteus	0 (Alive)	0 (Alive)	100 (Dead)
Staphylococcus aureus ATCC 29213	100 (Dead)	0 (Alive)	100 (Dead)
Gram negative bacteria			
Enterobacter aerogenes	100 (Dead)	0 (Alive)	100 (Dead)
Enterococcus faecalis	0 (Alive)	100 (Dead)	100 (Dead)
Escherichia coli ATCC 35218	0 (Alive)	0 (Alive)	100 (Dead)
Klebsiella pneumoniae (ESBL+)	0 (Alive)	0 (Alive)	100 (Dead)
Proteus mirabilis	0 (Alive)	100 (Dead)	100 (Dead)
Pseudomonas aeruginosa ATCC 27859	0 (Alive)	0 (Alive)	0 (Alive)
Salmonella sp. Group D	0 (Alive)	0 (Alive)	100 (Dead)
Yeast			
Cryptococcus neoformans	100 (Dead)	0 (Alive)	100 (Dead)
Filamentous fungi			
Aspergillus flavus	100 (Alive)	98.4±1.3 (Alive)	100 (Dead)
Aspergillus niger	100 (Dead)	100 (Dead)	100 (Dead)
Colletotrichum gloeosporioides	100 (Dead)	100 (Dead)	100 (Dead)
Fusarium oxysporum f. sp. *vasinfectum*	39.8±2.2 (Alive)	47.4±1.5 (Alive)	62.8±2.2 (Alive)
Ganoderma australe BCC22321	100 (Dead)	100 (Dead)	100 (Dead)
Phellinus noxius BCC26237	100 (Dead)	100 (Dead)	100 (Dead)
Phytophthora parasitica BCC15560	100 (Dead)	97.1±1.3 (Alive)	100 (Dead)
Rhizoctonia solani AG-2	100 (Alive)	71.1±2.1 (Alive)	100 (Dead)
Rigidoporus microporus	100 (Dead)	100 (Dead)	100 (Dead)

All tests were done in triplicate and repeated twice, the % inhibition is shown with mean ± SD while without SD refered to the absence of mean difference. Alive = tested microbe re-growth on fresh media, Dead = tested microbe didn't growth on fresh media. Survival of each tested microbe was evaluated 6 days after exposure.

All active volatile compounds inhibited significantly hyphal growth of *R. microporus* and *P. noxius* ($P<0.05$). In addition, the major compound; 3-methylbutan-1-ol inhibited hyphal growth of *R. microporus* and *P. noxius* with 100% inhibition, respectively (Figure 5.3-5.4, Table 5.3).

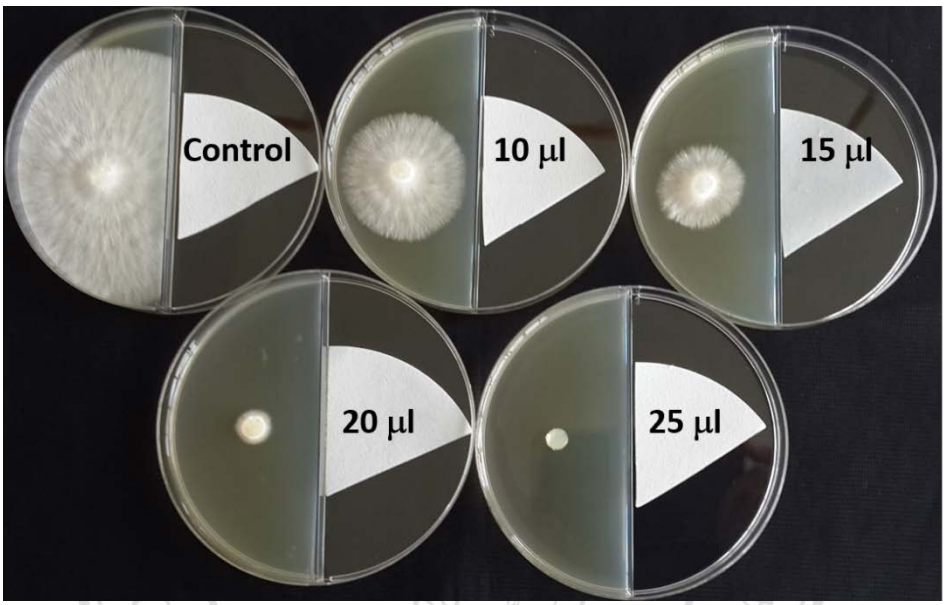

Figure 5.3 Effect of artificial volatile compounds, 2-methylpropanoic acid on the hyphal growth of *Rigidoporus microporus*.

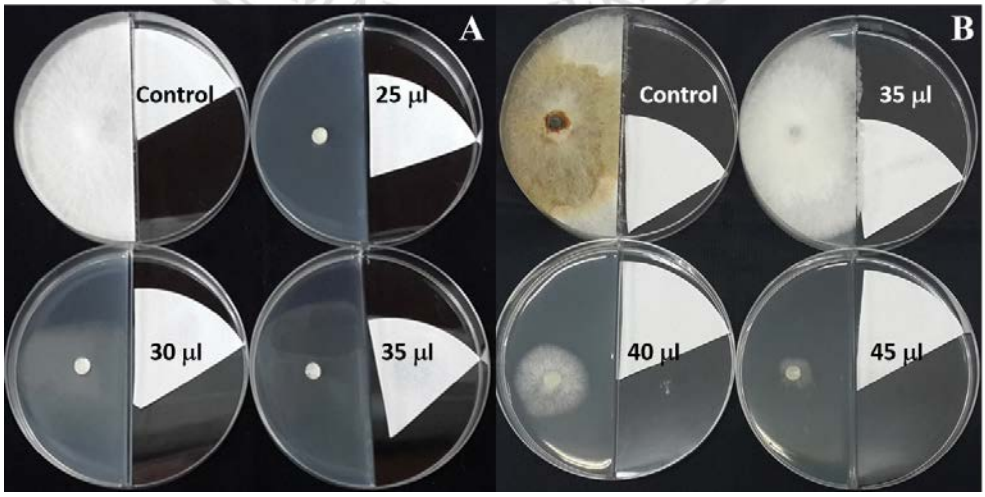

Figure 5.4 Effect of artificial volatile compounds, 3-methylbutyl acetate on the hyphal growth of *Rigidoporus microporus* (A) and *Phellinus noxius* (B).

Table 5.3 Effect of artificial volatile compounds on the hyphal growth of fungal pathogens.

Chemical agent	**Radial growth (cm)**	
(μl L^{-1} airspace)	***Rigidoorus microporus***	***Phellinus noxius***
Control	3.2a	3.5a
3-methylbutan-1-ol		
5	3.0±0.1a	3.2±0.1a
10	2.5±0.3b	1.8±0.1b
15	1.9±0.3c	1.5±0.9b
20	0d	0.3±0.1c
25	0d	0c
30	0d	0c
35	0d	0c
2-methylpropanoic acid		
5	1.7±0.1b	3.5±0.1a
10	1.3±0.1c	3.3±0.1ab
15	1.0±0.2d	2.8±0.1b
20	0.2e	2.1±0.1c
25	0f	0.8±0.1d
30	0f	0e
35	0f	0e

Table 5.3 (continue).

Chemical agent	**Radial growth (cm)**	
(μl L^{-1} airspace)	***Rigidoorus microporus***	***Phellinus noxius***
Control	3.2a	3.5±0.2
3-methylbutyl acetate		
5	2.2±0.2b	3.5 ±0.1a
10	2.1±0.1b	3.4 ±0.1a
15	1.1±0.2c	3.3±0.2ab
20	0d	2.9 ±0.1bc
25	0d	2.8 ±0.1cd
30	0d	2.4 ±0.3de
35	0d	2.3 ±0.1e
40	-	1.1 ±0.3f
45	-	0.9 ±0.3fg
50	-	0.6 ±0.1g
55	-	0.6 ±0.1g
60	-	0.2 ±0.1h
65	-	0h

All tests were done in triplicate, the radial growth is shown with mean ± SD while without SD refers to the absence of mean difference. Means with different letters are significantly different according to Tukey's Test ($P < 0.05$).

5.3.2 Effect of VOCs from *M. heveae* on root and shoot growth

The volatile metabolites affected root and shoot growth of the tested plant. They inhibited significantly root growth when compared with the control ($P<0.05$) (Table 5.4). However, shoot length of garden pea and jasmine rice did not different from the control after 7 day and 14 day exposure of VOCs (Table 5.4). Ruzi grass and *A. thaiana* col-0 were used for the artificial volatile compounds bioassay test as monocot and dicot sample, respectively. All volume of three active volatile compounds inhibited

significantly root and shoot growth (P<0.05). In addition, 3-methylbutan-1-ol and 2-methylpropanoic acid inhibited seed germination of ruzi grass and *A. thaliana* Ceol-0 with 100% inhibition at 15 μl L^{-1} airspace (Figure 5.5-5.6, Table 5.5).

Table 5.4 Effect of VOCs from *Muscodor heveae* on root and shoot growth.

Plant	**% Germination**	**Root length (cm)**		**Shoot length (cm)**	
		Control	*M. heveae*	Control	*M. heveae*
Monocotyledon					
Jasmine rice[2]	88.3	3.2±0.4*	0.7±0.4*	2.2±0.5	2.1±0.3
Sweet corn[1]	64.0	2.0*	0*	1.3±0.7*	0*
Ruzi grass[2]	70.0	1.3±0.4*	0.2±0.1*	2.8±0.5*	0.1±0.1*
Dicotyledon					
Arabidopsis thaliana Ceol-0[1]	95.0	1.0±0.4*	0*	1.2±0.1*	0*
Garden pea[2]	83.3	3.1±0.7*	0.8±0.1*	0.6	0.6±0.3
Mung bean[2]	88.0	6.3±1.1*	0.4±0.8*	8.4±0.9*	0*
Tomato[1]	85.0	5.7±0.5*	0.1±0.1*	4.9±1.0*	0*

[1]Measured after 7 days of exposure to fungal VOCs and [2]measured after 14 days of exposure to fungal VOCs. All tests were done in four replicates, the experimental data was analyzed with T-test (*P<0.05). Each value is the mean ± SD while without SD refers to the absence of mean difference.

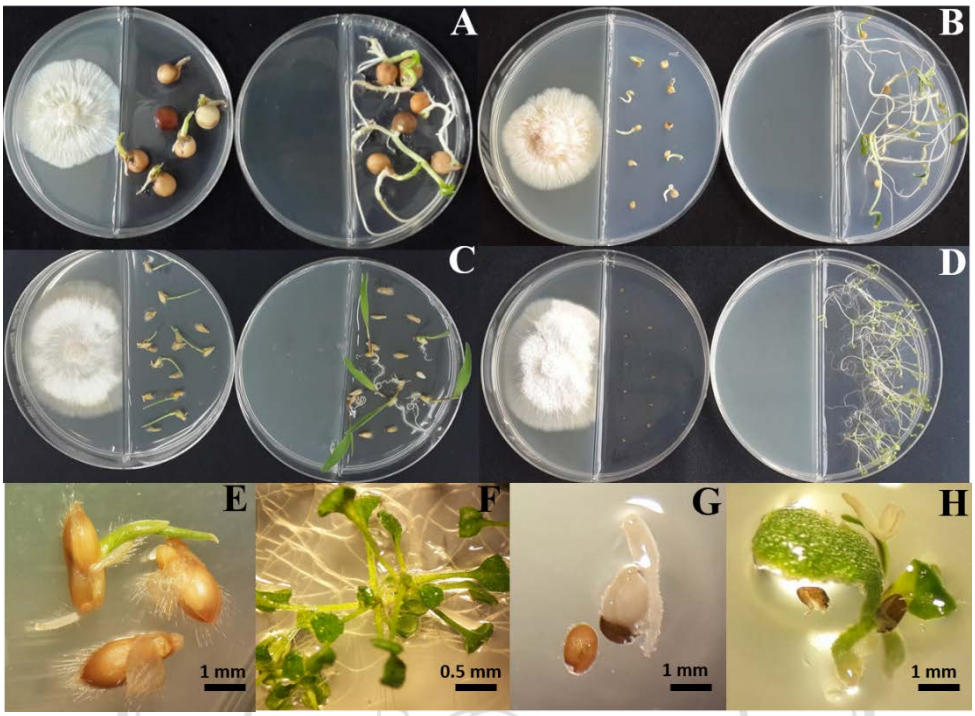

Figure 5.5 Effect of VOCs from *Muscodor heveae* on the root length and shoot length of garden peas over 7 days (A), tomato over 7 days (B), ruzi grass over 14 days (C), *Arabidopsis thaliana* Ceol-0 over 14 days (D). Effect of artificial volatile compounds on the root and shoot growth of ruzi grass, *A. thaliana* Ceol-0; 3-methylbutan 1-ol (E) and 3-methylbutyl acetate (F-H), respectively.

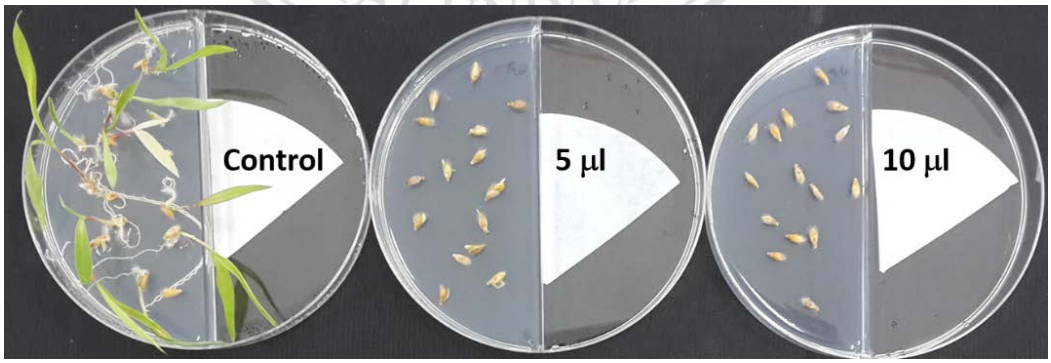

Figure 5.6 Effect of artificial volatile compounds, 3-methylbutan-1-ol on the root and shoot growth of ruzi grass.

Table 5.5 Effect of artificial volatile compounds on root and shoot growth of tested plant.

Chemical agent	**Ruzi grass**		***Arabidopsis thaliana* Ceol-0**	
(μl L^{-1} airspace)	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)
Control	2.6±0.3a	3.8±0.4a	1.8±0.3a	1.0a
3-methylbutan-1-ol				
5	0.8±0.8b	1.2±1.2b	0b	0b
10	0c	0.2±0.3b	0b	0b
15	0c	0b	0b	0b
20	0c	0b	0b	0b
25	0c	0b	0b	0b
30	0c	0b	0b	0b
35	0c	0b	0b	0b
2-methylpropanoic acid				
5	0.2b	0.4±0.2b	0b	0b
10	0.1b	0.1±0.1b	0b	0b
15	0b	0b	0b	0b
20	0b	0b	0b	0b
25	0b	0b	0b	0b
30	0b	0b	0b	0b
35	0b	0b	0b	0b

Table 5.5 (continue).

Chemical agent	**Ruzi grass**		***Arabidopsis thaliana* Ceol-0**	
(μl L^{-1} airspace)	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)
3-methylbutyl acetate				
Control	2.6±0.3a	3.8±0.4a	1.8±0.3a	1.0a
5	1.2±0.3b	2.4±0.2b	0.6b	0.4±0.1b
10	0.9±0.1bc	2.0±0.4b	0.3bc	0.3bc
15	0.9bc	1.7b	0.3bc	0.2cd
20	0.6bc	1.0±0.3c	0.2c	0.2d
25	0.7±0.3bc	1.0±0.1c	0.2c	0.2d
30	0.6±0.4bc	1.0±0.1c	0.2c	0.3d
35	0.4±0.1 c	0.4c	0c	0d

All tests were done in triplicate, the radial growth is shown with mean ± SD while without SD refers to the absence of mean difference. Means with different letters are significantly different according to Tukey's Test ($P < 0.05$).

5.4 Discussion

In nature, *Muscodor* spp. live within host plants under symbiotic condition, where they produce and expose their volatile metabolites into the surrounding environments of the host plants for preventing the invasion of pathogens (Mitchell *et al*., 2010; Zhang *et al*., 2010). A number of endophytes produced extremely biologically active compounds against pathogenic microbes (Yang *et al*., 1994). Thus, *Muscodor* spp. seemed to be beneficial to the host by providing protection from plant pathogens. Most pathogenic fungi in diverse economic plants were inhibited after exposure to the volatile metabolites produced by *Muscodor* isolates. The inhibitory mechanism on fungal growth was likely that volatile metabolites influence protein expression and the function of metabolic enzymes (Humphris *et al*., 2002; Fialho *et al*., 2011). *Muscodor heveae* produced active volatile compounds that are lethal to various microbes including rubber tree pathogenic fungi. The previous reports have shown that VOCs from *M. yucatanensis* inhibited seed germination and root elongation of monocots, barnyard grass and dicot,

amaranth and tomatoes (Macías-Rubalcava *et al*., 2010). However, the possible toxic effects of VOCs on seed germination and vegetative plant growth have received limited research attention (Lee *et al*., 2014). Volatile metabolites from *M. heveae* inhibited root and shoot growth in almost all tested plant except for jasmine rice and garden peas. The statistical comparison (T-test) demonstrated that the recorded shoot length of jasmine rice and garden peas was not significantly different from that of the controls ($P<0.05$).

The artificial volatile compounds test confirmed that the major compound, 3-methylbutan-1-ol and the minor compounds 3-methylbutyl acetate and 2-methylpropanoic acid of *M. heveae* showed antifungal and phytotoxic activity by inhibited significantly mycelial growth of pathogenic fungi and root and shoot growth of ruzi grass and *A. thaliana*, respectively. The volume of artificial volatile compounds to inhibited mycelial growth and showed phytotoxic activity associated with percentage of peak area from the analysis of volatile metabolites by GC/MS. However, the previous study showed that *A. thaliana* grown in the presence of *Trichoderma viride* volatile had more lateral roots (Hung *et al*., 2013).

Its volatile revealed 51 compounds of which isobutyl alcohol, isopentyl alcohol and 3-methylbutanal were most abundant, including 2-methyl-1-butanol. This bioactive compound showed antifungal (Fialho *et al*., 2011) and also phytotoxic activity (Macías-Rubalcava *et al*., 2010; Sánchez-Ortiz *et al*., 2016). Thus, the phytotoxic activity depended on the mixture of volatile and type of plant**.** Strobel *et al.* (2001) found that the effective inhibitory activity of VOCs depended on the mixture of volatile and type of the living organism. Such as, single volatile compound of *M. albus* had low activity in terms of inhibiting fungal and bacterial growth but as a volatile mixture, they inhibited and killed a broad range of microbes. Maintaining a balance between positive and negative effects of endophytic fungi on plant fitness may be important for multi-trophic interactions. Such as, *Alternaria* sp. CID62 and *Epicocum* sp. CID66 produce bioactive volatile to prevent *Centaurea stoeba* from insect herbivore, while *Alternaria* sp. can reduce flower development and *Epicoccum* sp. inhibit seed germination (Newcombe *et al*., 2009). Thus, more studies are necessary for understanding the significant allelochemical effects and mechanisms underlying these mode of action (Zhi-Lin *et al*., 2012).

Since bioactive volatile metabolites are found from the genus *Muscodor*, they have been considered for uses in agricultural, medical and industrial applications (Strobel, 2006). Biological control with the volatile metabolite-producing fungi could be a sustainable alternative method to prevent plant diseases at the nursery and postharvest stages, with reduced usage of chemical fungicides (Mercier and Manker, 2005). Biological fumigation was a strategy for applying the volatile metabolites-producing fungi, which has been approved for controlling diverse postharvest infections caused by *Botrytis cinerea, Penicillium expansum,* and *Sclerotinia sclerotiorum* (Ramin *et al*., 2005). The applications of biological fumigation using the genus *Muscodor* for controlling root diseases has also been demonstrated (Worapong and Strobel, 2009; Suwannarach *et al*., 2012). Based on the potent antimicrobial activity of the volatile metabolite-producing fungi that we observed, the *Muscodor* isolates could be used effectively for controlling infectious diseases in rubber trees. Further evaluation of the *Muscodor* isolates for their antimicrobial activity in applied biofumigation are required not only for agricultural aspects but also medical and industrial relevance. In addition, the phytotoxic activity of volatile compounds produced by *M. heveae* on root and shoot elongation of tested plant could be useful in biocontrol of weeds as potential herbicides.

CHAPTER 6

Mycofumigation with *Muscodor heveae* for controlling of white root rot disease

6.1 Introduction

White root rot disease caused by *Rigidoporus microporus* is the most serious of all problems faced by rubber tree (*Hevea brasiliensis*) plantations and causes slow death at all stages of growth from seedlings to mature trees (Tangonan *et al*., 2008). However, this disease is present in the Congo, Cote d'Ivorire, India, Malaysia, Nigeria, Sri Lanka and Thailand (Rajalakshmy and Jayarathnam, 2000; Omorusi, 2012; Kaewchai, 2013). It is responsible for yield losses of up to 50% in rubber plantations in West Africa (Ogbebor *et al*., 2013), 43% in a small-holdings survey in Malaysia and 96% of the results of rubber tree deaths at a rate of up to five trees per hectare per year in planting areas in Nigeria (Oghenekaro *et al*., 2015).

In Thailand, a previous report has shown that 55% of diseased trees in rubber plantations located in Phangnga Province suffered from white root rot disease (Kaewchai, 2013). *Rigidoporus microporus* syn. *R. lignosus* is a major economically important pathogen of rubber trees acts by decaying lignin and polysaccharides in the root tissues (Geiger *et al.*, 1986). Previous studies have reported on the high *R. microporus* inoculum density in the soil of rubber tree natural forests and plantations (Nandris *et al*., 1988). The process of disease infection involves 3 stages: penetration, colonization and degradation. Fungal pathogen live and can remain in the soil for a long period of time. It can infect the surface of the root bark and develop in differentiate rhizomorphs. After which time, it releases extracellular enzymes that are able to decay the wood and collar of host plants (Nakaew *et al.*, 2015). It can cause a significant amount of damage over a wide area. Normally, chemical fungicides have been recommended for controlling root rot disease but they are expensive for local farmers and are highly toxic to human health and the environment (Kaewchai and Soytong, 2010). The use of endophytic fungi as a biocontrol

agent can help hosts tolerate harmful abiotic and biotic factors including pathogens (Singh *et al*., 2011).

An endophytic fungus, *Muscodor* belonged to the family *Xylariaceae*. The hyphal characteristic is a sterile because no reproductive structure have ever been observed (Strobel, 2006). It has properties associated with producing active low-molecular weight volatile compounds with a broad antimicrobial activity to inhibit or kill most species of fungi and bacteria (Strobel *et al*., 2001; Mercier *et al*., 2007). In experiments, *Muscodor heveae*, did not produce spores on any medium and had rope-like mycelium with coiled structures. Its bioactive volatile metabolites included 3-methylbutan-1-ol as a major component, followed by 3-methylbutyl acetate and azulene derivatives. VOCs from *M. heveae* show strong activity for the biological control of various bacteria, yeast and filamentous fungi, especially rubber tree pathogens such as *R. microporus* and *Phellinus noxius*. Recently, volatile-producing endophytes have attracted great attention with their strong antibiotic activity or other fragrant volatiles can be used in the flavoring industries (Zhi-Lin *et al*., 2012). Mycofumigation involves the use of antimicrobial volatile compounds from fungi such as *Muscodor* that are applied to control other microorganisms (Stinson *et al*., 2003a). It is an alternative method for controlling soil-borne pathogens. In 2003, *M. albus* and *M. roseus* were used as mycofumigants to control *Rhizoctonia solani* AG-2, *Pythium ultimum* and *Aphanomyces cochlioides* (Stinson *et al*., 2003a). In addition, mycofumigation by *M. albus* was used to control *Verticillium* wilt and the black dot disease of the potato (*Solanum tuberosum* L.) caused by *Verticillium dahlia* and *Colletotrichum coccodes*, respectively (Grimme, 2008). Worapong and Strobel (2009) reported that *P. ultimum* which had caused root rot disease in kale (*Brassica oleracea*) was controlled by *M. albus*. Furthermore, *M. cinnamomi* was used as biofumigants to control *Rhizoctonia solani* AG-2 damping-off disease of plant seedlings (Suwannarach *et al*., 2012).

6.2 Materials and methods

6.2.1 Fungal cultures

Muscodor heveae was isolated from the leaves of rubber tree plants in Nongbualamphu Province, northeastern Thailand and stored in 20% (v/v) glycerol at

-20 °C at the Sustainable Development of Biological Resources (SDBR) Lab, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand (SDBR-CMU RTM5IV3), and the BIOTEC (National Center for Genetic Engineering and Biotechnology) Culture Collection (BCC), Bangkok, Thailand (BCC 70461). The morphological characteristics and antimicrobial activity of VOCs had been previously described in chapter 4.

A pathogenic *Rigidoporus microporus* was gently provided by Dr. Nareeluk Nakaew at the Department of Microbiology and Parasitology, Faculty of Science, Naresuan University, Phitsanulok, Thailand. The pathoginicity of the fungus was previously confirmed and described (Nakaew *et al*., 2015). The culture was stored in 20% (v/v) glycerol at -20 °C. The new cultures were transferred to a potato dextrose agar (PDA, Himedia Laboratories, India) plate and grown for 5–7 days at room temperature (25 ± 2 °C).

6.2.2 Preparation of *Muscodor heveae* and *Rigidoporus microporus* inocula

A modified method for solid media preparation was applied following that of Suwannarach *et al*. (2012). Rye grain was boiled for 25 min and 40 g of boiled grain were contained in 250 mL glass bottles. The specimens were then autoclaved at 121 °C for 30 min. The mycelium plugs (5 mm diam) of *M. heveae* were inoculated in each glass bottle and were then incubated in the dark at room temperature for 30 days (Figure 6.1). While, *R. microporus* was cultured on sterilized inoculum medium containing: 100 g sawdust, 3 g rice bran and 2 g sucrose moistened with water. The inoculum was contained in plastic bags and incubated at room temperature for 30 days (Kaewchai and Soytong, 2010) (Figure 6.2).

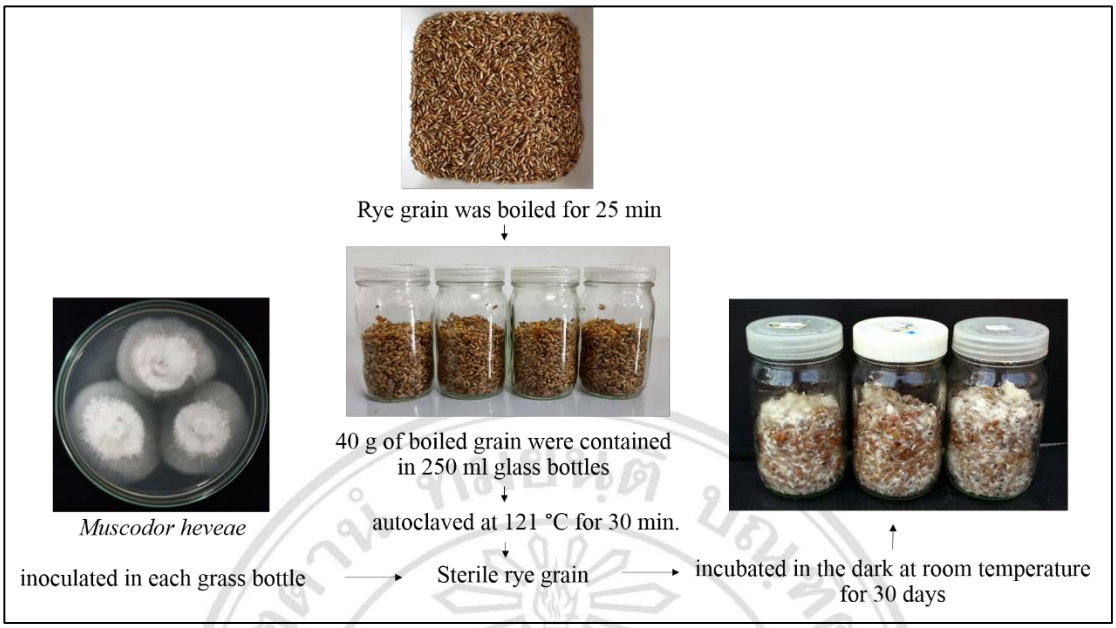

Figure 6.1 Preparation of *Muscodor heveae* inocula.

Figure 6.2 Preparation of *Rigidoporus microporus* inocula.

6.2.3 Effect of VOCs from *Muscodor heveae* on *Rigidoporus microporus in vivo*

Five-month healthy rubber tree seedlings (variety RRIM 600) were placed in 6 inch x 12 inch pots containing 3 kg sterilized soil (soil: commercial soil, 3:1). Four-week-old rye grain inoculum of *M. heveae* was scraped, cut and minced into the sterilized soil. The experiment was conducted in 7 treatments with 4 replicates under greenhouse conditions. The treatments conducted were as follows: non-infested control pots contained sterilized mixed soil. Pathogenic treatment involved the inoculation with 1 spawn of *R. microporus* (R). The *Muscodor* inoculated control was treated with *M. heveae* inoculum and sterilized mixed soil at a rate of 40 g / 1 kg^{-1} (MH40) and 80 g / 1 kg^{-1} (MH80), respectively (Figure 6.3). Biological treatments involved treating the pathogenic fungus with *M. heveae* inoculum and sterilized mixed soil at 40 g / 1 kg^{-1} (RMH40) and 80 g / 1 kg^{-1} (RMH80), respectively. Chemical treatment involved treating the pathogenic fungus with fungicide (75% EC tridemorph) every 2 weeks at 20 ml / L^{-1} pot^{-1}. Data collection as disease score was recorded at 150 days after treatment. The disease score was categorized as follows: level 1 = healthy green leaves, level 2 = 1-25% yellow leaves, level 3 = 26-50% yellow leaves, level 4 = 51-75% yellow leaves and level 5 = 76-100% yellow leaves. Furthermore, the survival rate (%) of rubber tree seedlings was calculated. The infected roots were observed and the presence of rhizomorph was recorded. The experiment was conducted in a completely randomized design (CRD) with four replicates during the period of July to November 2014 (Kaewchai and Soytong, 2010). The temperature was maintained from 28.4 to 33.5 °C and the relative humidity ranged from 50 to 60 %. The daily light intensity ranged from 2,840 to 8,170 lux.

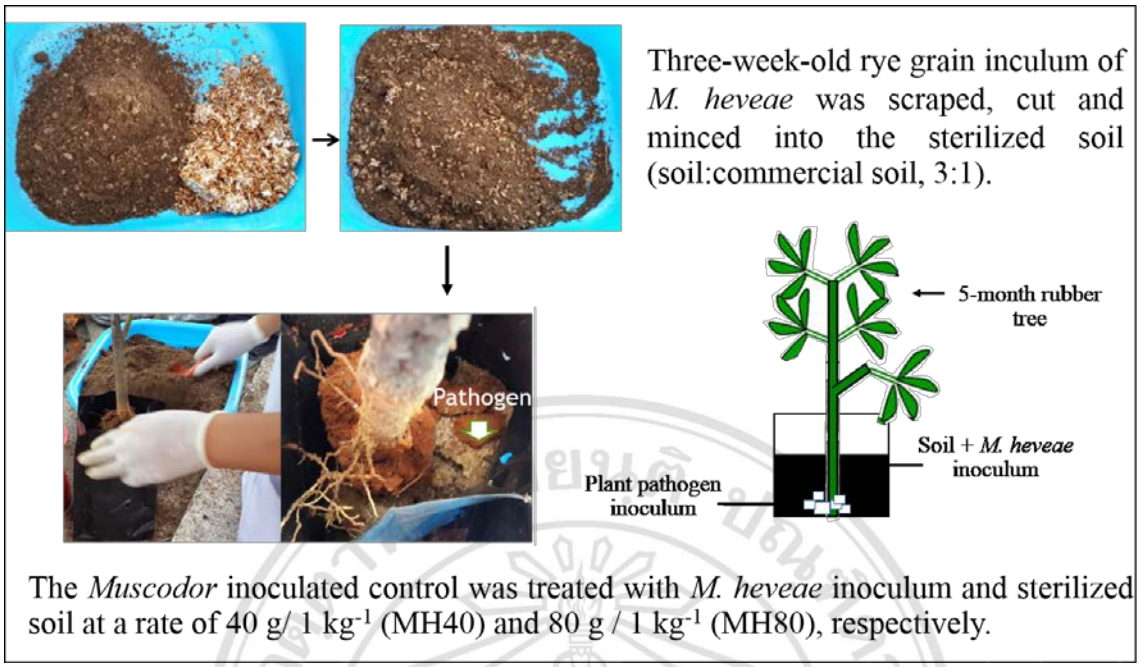

Figure 6.3 Inoculation method for investigation the effect of VOCs from *Muscodor heveae* on *Rigidoporus microporus in vivo*.

6.2.4 Soil analysis

Soil pH was directly measured by pH-meter (Sartorius PB-10, Germany). Organic matter, total inorganic nitrogen and potassium were analyzed following the method described by Sparks *et al.* (1996). The Bray II method (Houba *et al*., 1988) was used to measured available phosphorus.

6.2.5 Statistical analysis

SPSS program version 17.0 for Windows was used to analyze the experimental data with one-way analysis of variance (ANOVA), along with Turkey's post hoc tests at different significant levels (P).

6.3 Results

6.3.1 Effect of VOCs from *Muscodor heveae* on *Rigidoporus microporus in vivo*

The volatile metabolites produced by *M. heveae* exhibited antimicrobial activity against mycelial growth of *R. microporus* with 100% inhibition on tested plates

according to the results in section 5.3.1, chapter 5. Furthermore, no growth of *R. microporus* was observed when it was transferred to fresh PDA plates after 6 days of exposure to *M. heveae* volatile metabolites. *In vivo*, *R. microporus* was significantly controlled by soil fumigation with VOCs from *M. heveae* ($P<0.05$). The highest disease score (4.8) was observed in the pathogenic treatment (R) and this was associated with a low survival rate (25%) (Figure 6.4, Table 6.1). In the biocontrol treatments, RMH40 and RMH80 showed the lowest disease score (1.3) and a high survival rate (100%) that was greater than the chemical treatment (RT) and the non-infested control (Figure 6.4, Table 6.1). However, a statistical comparison demonstrated that RMH40 and RMH80 were not found to be significantly difference from RT and the non-infested control but were found to be significantly difference from the untreated inoculated control ($P<0.05$). In addition, MH40 and MH80 treatments showed no dead rubber trees after fumigated. The untreated inoculated control showed white mycelia of *R. microporus* colonized on infected root (Figure 6.5).

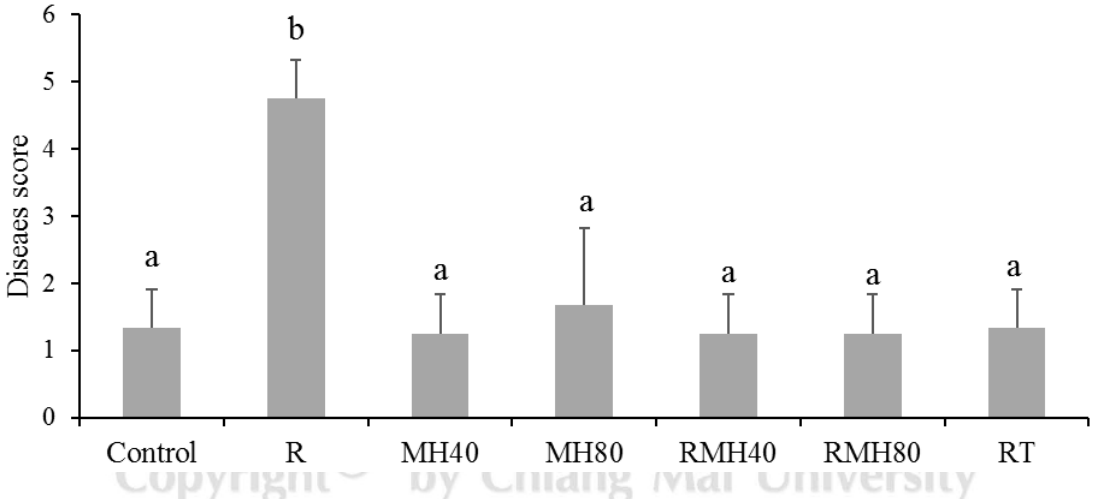

Figure 6.4 Comparison of disease score for different treatments. Data are presented as means of four replicates. The error bar at each point indicated that ± SD. The different letters above each graph indicated the means are significantly different by Turkey's test ($P<0.05$). The disease score was categorized as follows: level 1 = healthy green leaves, level 2 = 1-25% yellow leaves, level 3 = 26-50% yellow leaves, level 4 = 51-75% yellow leaves and level 5 = 76-100% yellow leaves.

Figure 6.5 Biofumigation test for suppression of white root rot disease caused by *Rigidoporus microporus* after 150 days. The treatment protocol were as follows; non-infested control, untreated inoculated control (R), fungicide treatment (RT), *Muscodor* treatment with 40 g 1 kg^{-1} *M. heveae* inoculum (MH40) and biocontrol treatment with 40 g 1 kg^{-1} *M. heveae* inoculum (RMH40), respectively (A) and the present of rhizomorphs on the infected roots displaying white color (B-C) compared with non-infested control (D).

6.3.2 Soil analysis

Soil pH after used in pot culture values ranged from 6.4 to 7.3. Organic matter was reduced from 5.0 g / 100g^{-1} into the values between 1.0 to 1.8 g 100 g^{-1}. The MH80 treatment showed increase values of total NPK at 0.3 g 100 g^{-1}, 69.8 mg kg^{-1} and 371.5 mg kg^{-1}, respectively. However, the highest exchangeable potassium value was 382.3 mg kg^{-1} in RMH80 treatment (Table 6.1).

Table 6.1 Survival rate (%) of rubber tree after biofumigation and chemical analysis of soil used in the pot culture.

Treatment	Survival rate (%)	Soil chemical analysis				
		pH	Organic matter (g/100 g)	N (g/100 g)	P (mg/kg)	K (mg/kg)
*Soil before used in the pot culture	-	7.1	5.0	0.2	68.1	356.0
R	25	6.6	1.0	0.3	67.7	360.1
MH40	100	7.3	1.6	0.1	58.1	304.2
MH80	75	6.4	1.4	0.3	69.8	371.5
RMH40	100	7.3	1.0	0.2	58.5	379.2
RMH80	100	6.8	1.0	0.2	60.8	382.3
RT	75	6.7	1.1	0.2	58.5	379.2

The treatment protocol were as follows; non-infested control, untreated inoculated control (R), *Muscodor* treatment with 40 g 1 kg^{-1} *M. heveae* inoculum (MH40), *Muscodor* treatment with 80 g 1 kg^{-1} *M. heveae* inoculum (MH80), biocontrol treatment with 40 g 1 kg^{-1} *M. heveae* inoculum (RMH40), biocontrol treatment with 80 g 1 kg^{-1} *M. heveae* inoculum (RMH80) and fungicide treatment (RT)**.**

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6.4 Discussion

Endophytic fungi can provide benefits to the host plant by providing protection from plant pathogens. It has been suggested that applying a mixture of VOCs is more effective than using them individually (Strobel *et al*., 2001). White root rot disease has been controlled *in vivo* by various biological control methods such as the application of *Chaetomium cupreum* in both powder and oil form (Kaewchai and Soytong, 2010), the utilization of active rhizospheric *Streptomyces sioyaensis* (Nakaew *et al*., 2015) and the use of antagonistic *Hypocrea virens* and *H. jecorina* (Ogbebor *et al*., 2015). This is the first report to demonstrate biofumigation for the control of white root rot disease in rubber trees. *Muscodor heveae* showed strong activity of VOCs in inhibiting *R. microporus in vitro* with 100% growth inhibition and 0% recovery of mycelium growth. There was no growth of *R. microporus* during the 6 day exposure to 10-day-old *M. heveae* culture. After transferred to fresh PDA plates, no growth of *R. microporus* was observed indicating that they were killed after the 6 day exposure of *M. heveae*.

Mycelium penetration and colonization are important stages of root disease infection. It was observed as intra and inner interactions of the plant's cell wall and it has produced degrading enzymes, which correspond to the diverse polymers in the cell wall of host plant (Omorusi, 2012). In the white root rot disease cycle, the infection and colonization of *R. microporus* occurred in the root system of young healthy rubber trees via rhizomorphs that grew from spore attacks or rhizomorphs that survive on the root stumps and wood pieces of infected trees (Omorusi, 2012). Soil fumigation could be an alternative method to inhibited rhizomorph penetration and colonization.

This is of particular interest with regard to the non-accumulation of toxic chemicals in soil over a long period of time. Although VOCs of *M. heveae* affected the root and shoot growth in the some test plants, but they had no effect on tropical plants when fumigation was demonstrated *in vivo*. Because, MH40 and MH80 treatments revealed no dead rubber trees after treatment. Furthermore, plant defense was enhanced by volatile compounds such as 1-octen-3-ol, a major fungal VOC with phytotoxic activity (Splivallo *et al*., 2007). When, *A. thaliana* was exposured to 1-octen-3-ol, the defense genes associated with wounding or ethylene and jasmonic acid signaling in *A. thaliana* were induced expression and inhibited *Botrytis cinerea* growth on infected leaves

(Kishimoto *et al*., 2007). The slightly off-green leaves were displayed as a symptom. Then, the green leaves in the unaffected branches turned yellow and the whole canopy was destroyed. This was a possible indication of root disease (Tangonan *et al.*, 2008). Thus, the disease score of the yellow leaf was related to disease control values in each treatment. However, a sign of disease is the presence of rhizomorphs on the infected roots with a white color but that becomes yellow or reddish brown, as the roots grow old (Omorusi, 2012). Only untreated inoculated control (R) showed a sign of root disease. Furthermore, soil chemical characteristics supported the claim that disease symptoms that occurred by the pathogen and did not occur by nutrient deficiency in the soil.

Biofumigation using the genus *Muscodor* for controlling root diseases was demonstrated such as biocontrol of damping-off by *M. albus* (Mercier and Jiménez, 2005), biocontrol of root rot of kale by *M. albus* (Worapong and Strobel, 2009) and *M. cinnamomi* (Suwannarach *et al*., 2012). Thus, *M. heveae* would be used effectively for controlling white root rot disease in rubber trees, while there was a potential to expand these findings toward further agricultural and industrial applications such as using to control post-harvest decay of fruits and vegetables.

CHAPTER 7

General discussion and conclusion

This study provide the effective endophytic fungi associated with medicinal plants and the rubber tree in Thailand. Non-volatile producing endophytic fungi were isolated and determined their antagonistic activity. Crude extract of endophytes were examined their antifungal activity by TLC-autobiography. Volatile producing endophytic fungi were analyzed their antagonistic volatile metabolites by GC/MS and used as biological control agents. In addition, plant growth promoting properties were also detected. Morphological characteristic and the rDNA sequence analysis were used to identify isolated endophytes. This chapter concluded the results, discusses the implications of the findings and proposes future work.

7.1 Endophytic fungi in medicinal plants and the rubber tree

Endophytic fungi from medicinal plants were attractive for bioactive metabolite or novel compound discover. Their diversity made a new area of research for novel drug or medicine (Mishra *et al*., 2015). The present study indicated that the fungal endophyte communites in leaves is diverse more than other part of host plant. However, fungal endophyte presented in all part of host plant (Hyde and Soythong, 2008) and could transfer from one plant to other by seeds (Aly *et al*., 2011). In this study, most fungal endophytes were isolated from *Clinacanthus nutans* in family *Acanthaceae* (Thai name: Phaya Yo). It was known to have various medicinal properties such as antimicrobial, anticancer, antioxidant and antiviral activities (Kunsorn *et al*., 2013; Yahaya *et al*., 2015) and colonized with endophytes such as fungi and bacteria (Jinffeng *et al*., 2014). While, the effective endophytic fungi with antifungal activity were isolated from short stem of *Aloe vera* L. in family *Lilaceae* (Thai name: Wan Hang Chora Khe). This medicinal plant showed anti-inflammatory activity, immune stimulatory activity and cell growth stimulatory activity, including antiviral and antifungal activity (Sitara *et al*., 2011).

According to previous studies, communities of endophytic fungi could be found in *A. vera* with antimicrobial activity (Jalgaonwala *et al.*, 2010; Bara *et al*., 2013; Yadav *et al*., 2015), including other endophyte such as bacteria (Akinsanya *et al*., 2015; Nongkhlaw and Joshi, 2015). The effective endophytic fungi, AL1T1 and AL1T2 belonged to phylum Ascomycota in the order *Sordariales* based on morphological characteristic and the sequence similarities of ITS rRNA gene (99-100% sequence similarity).

Non-volatile and volatile producing endophytic fungi were isolated from rubber tree (*Hevea brasiliensis* Müll.Arg.). According to the previous studies of endophytic fungi such as *Botryosphaeria*, *Colletotrichum*, *Phomopsis*, *Xylaria* (Gamboa *et al*., 2002; Arnold and Lutzoni, 2007), *Penicillium paxilli*, *Pestalotiopsis* aff. *palmarum*, *Trichoderma harzianum* (Gazis and Chaverri, 2010), *Trichoderma amazonicum* (Chaverri *et al*., 2011), including various species of arbuscular mycorrhizal fungi (AMF, Pereira *et al*., 2014) were found in this economic plant. However, it is the first report to isolated volatile-producing endophytic fungi, *Muscodor* spp. from leaves of the rubber tree.

7.2 Antifungal activity and plant growth promoting properties of endophytic fungi

The extracts from many types of local medicinal plants were used in traditional manner for treatments of various disease. The bioactive compounds were produced by the plant but in a mutualistic association with the host plant, endophytes may enhance bioactive metabolites that process bioactivity such as antibacterial and antifungal activity in host plant (Radu and Kqueen, 2002). It was supported by many studies, such as it found that hydroalcoholic, the extract from leaves of *A. vera* could inhibit mycelial growth of various fungi such as *Botrytris gladiolorum*, *Fusarium oxysporum*, *Heterosporium pruneti* and *Penicillium gladioli* (Jasso *et al*., 2005). While, *A. vera* gel extract was evaluated for their antifungal activity against *Aspergillus niger*, *A. flavus*, *Alternaria alternate*, *Drechslera hawaiensis* and *P. digitatum* (Sitara *et al*., 2011**)**. According to this study, isolate AL1T1 and AL1T2 showed strong antagonistic activity against several phytopathogenic fungi in the rubber tree, including *F. oxysporum* which have been inhibited by the extract from *A. vera* leaves (Jasso *et al*., 2005). Furthermore, Bara *et al*. (2013) showed that *Talaromyces worthmanii*, *A. vera* endophytic fungus could produce

bioactive compounds such as atropsiomer and worthmannin derivative with antimicrobial activity. Endophytic fungi, which was isolated from *Bauhinia strychnifolia* (BS1B201), *Clinacanthus nutans* (C4IV301) and *Pseuderanthemum palatiferum* (PS1IV102) exhibited plant growth promote property via produced indole acetic acid (IAA). This result was supported by the previous studies that showed several endophytic fungi, which produced IAA and increased seed germination and plant growth (Khan *et al*., 2012, 2015, 2016; Zhou *et al.,* 2013; Kedar *et al*., 2014). Future study on purification of effective metabolites and anti-cancer, anti-oxidant, anti-viral of their compounds will be evaluated.

7.3 Molecular and morphological identification of genus *Muscodor*

Volatile-producing endophytic fungus, *Muscodor* is classified into the family *Xylariaceae* and does not produced spores (Kudalkar *et al*., 2011). It could be isolated from several host plants and their VOCs are active against many pathogenic microorganisms such as bacteria, fungi, nematodes and insects. (Worapong *et al*., 2001; Suwannarach *et al*., 2013a; Saxena *et al*., 2015). Moreover, some species of *Muscodor* produced volatile metabolites which exhibit phytotoxic activity (Gonzalez *et al*., 2009; Macías-Rubalcava *et al*., 2010). It was difficult to classify *Muscodor* by using only morphological characteristics because it did not produce any reproductive structures. Thus, a more appropriate genotypic classification method was needed using more genes than ITS for identification at the species level and to understand their phylogenetic lineage. Moreover, profiling and comparison of unique volatile metabolites produced by members of the genus *Muscodor* were important criteria for its classification (Strobel, 2006; González *et al*., 2009). In this study, *M. vitigenus*, *M. equiseti* and a novel species, *M. heveae* were isolated from leaves of the rubber tree in northeastern Thailand. Based on morphological and physiological characteristics, profile of volatile metabolites produced and a molecular phylogenetic analysis. In addition, *M. vitigenus* and *M. qeuiseti* have been isolated from *Paullinia paullinoides* (Daisy *et al*., 2002) and *Equisetum debile* (Suwannarach *et al*., 2013a), respectively. Isolation of 3 species of *Muscodor* from the rubber tree indicates a possible susceptibility of this plant to colonize by members of this genus of mycelia sterilia endophytes.

7.4 Antagonistic volatile compounds of *Muscodor heveae* and revelant alleopathy

The mixture of volatiles with bioactive activity have been produced by several volatile-producing endophytic fungi such as *Botrytis* sp. (Ting *et al*., 2010), *Hypoxylon* sp. (Tomsheck *et al*., 2010), *Nodulisporium* sp. (Mends *et al*., 2012), *Phoma* sp. (Strobel *et al*., 2011), *Phomopsis* sp. (Singh *et al*., 2011), including *Muscordor* spp. (Worapong *et al*., 2001; Suwannarach *et al*., 2013a; Saxena *et al*., 2015). They had potential as harmful pesticides, fungicides and bactericides as well as genetic modification (Kanchiswamy *et al*., 2015). The present study indicated that VOCs from *Muscodor* spp. which isolated from leaves of the rubber tree inhibited growth of various fungi and bacteria. *F. oxysporum* f. sp. *vasinfectum* was more susceptible to the volatile metabolites of *M. heveae* than *M. vitigenus* and *M. qeuiseti*. The artificial volatile compounds test confirmed that the major component, 3-methylbutan-1-ol, and the minor compounds, 3-methylbutyl acetate and 2-methylpropanoic acid exhibited antifungal activity and phytotoxic activity in tested fungal pathogens and plants, according to previous studies of Gonzalez *et al*. (2009) and Macías-Rubalcava *et al*. (2010). Future study on the application of volatile metabolites from *M. heveae* in biocontrol of weeds *in vivo* as potential herbicides and controlling postharvest disease.

7.5 Controlling of white root rot disease in rubber tree using volatile compounds from *Muscodor heveae*

Mycofumigation involved the use of antimicrobial volatile compounds from fungi such as *Muscodor* that were applied to control other microorganisms, including soil-borne pathogens (Stinson *et al*., 2003a). VOCs of *M. albus* and *M. roseus* were used as mycofumigants to control *Rhizoctonia solani* AG-2, *Pythium ultimum* and *Aphanomyces cochlioides* (Stinson *et al*., 2003a). While, *M. albus* inoculum was used to control *Verticillium* wilt and the black dot disease of the potato (Grimme, 2008), including root rot disease in kale (Worapong and Strobel, 2009). Furthermore, *M. cinnamomi* were used as biofumigants to control *Rhizoctonia solani* AG-2 damping-off disease of plant seedlings (Suwannarach *et al*., 2012). In this study, a mixture of VOCs from *M. heveae* exhibited antagonistic activity against the mycelial growth of phytogenic fungi, which could be used as a biological control agent. The SPME-GC/MS analysis indicated that the major volatile compound produced by *M. heveae* with the highest percentage peak

area was 3-methylbutan-1-ol. The minor volatile compounds were 3-methylbutyl acetate and 2-methylpropanoic acid, respectively. It was supported by the previous studies, the bioactive volatile components for the genus *Muscodor* are 2-methylpropanoic acid, 3-methylbutyl acetate and 3-methyl-1-butanol (3-methylbutan-1-ol) (Strobel, 2006; Mitchell *et al*., 2010). Variation of volatile metabolites-profile was commonly found across member species of the genus *Muscodor*, but they often share the same major volatile component (Kudalkar *et al*., 2011; Suwannarach *et al*., 2013a).

Biofumigation by *M. heveae* showed great suppression of the disease. Biocontrol treatments; RMH40 (40 g 1 kg^{-1} *M. heveae* inoculum) and RMH80 (80 g 1 kg^{-1} *M. heveae* inoculum) were not found to be significantly different when compared with fungicide treatment (RT) and the non-infested control, but results were found to be significantly different from those of the untreated inoculated control (R) ($P<0.05$). According to the biofumigation of mixed volatile, including 2-methylpropanoic acid and 3-methylbutan-1-ol of *M. suthepensis* for controlling postharvest disease (Suwannarach, 2013). Further study on the optimal techniques of inocula production will be studied with the goal of finding low cost and easily maintained production methods and application of mixed culture of volatile-producing microorganism for biofumigants on a commercial agriculture scale in Thailand.

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LIST OF PUBLICATIONS

1. Siri-Udom S, Suwannarach N, Lumyong S (2015) Existence of *Muscodor vitigenus*, *M. equiseti* and *M. heveae* sp. nov. in leaves of the rubber tree (*Hevea brasiliensis* Müll.Arg.), and their biocontrol potential. *Annals of Microbiology* 66: 437-448.

APPENDIX A

Media

Various media were used in this study as isolation medium, storage medium or test medium and prepared as followed formula (per liter), dispensed into containers and sterilized by autoclaving at 121ºC for 15 min.

1. Corn Meal Agar (CMA) (Himedia Laboratories, India)

Corn meal	50 g
Agar	15 g, pH 6.0

2. Malt extract agar (MA)

Malt extract	30 g
Agar	15 g, pH 5.6

3. 2% Malt extract agar

Malt extract	20 g
Agar	15 g, pH 5.5

4. Nutrient agar (NA)

Beef extract	3 g
Peptone	5 g
Agar	15 g, pH 7

5. Potato dextrose agar (PDA)

Potatoes	200 g
Glucose	20 g
Agar	15 g. pH 5.6

6. Potato dextrose broth (PDB)

Potatoes	200 g
Glucose	20 g, pH 6.0

7. Water agar (WA)

Agar	15 g, pH 7.0

8. Yeast extract-malt extract agar (YMA)

Peptone	5 g
Yeast extract	3 g
Malt extract	3 g
Dextrose	10 g
Agar	15 g, pH 6.2

APPENDIX B

Standard curve of Indole acetic acid (IAA) preparation

1. Reagent

Salkowski's reagent (1 ml of 0.5 M $FeCl_3$; 50 ml of 35% $HClO_4$).

2. Method

Mixed IAA standard (1 ml) with Salkowski's reagent and incubated at room temperature (25±2 °C) in the dark for 30 min. The appearance of a pink color indicated IAA production and was measured at OD 530 nm, uninoculated media mixed with reagent used as a blank.

Table B OD530 nm of various concentration of standard IAA

IAA concentration (µg/ml)	OD 530 nm
0	0
10	0.036
20	0.048
30	0.086
40	0.116
50	0.150
100	0.316
150	0.498
200	0.068
250	0.900

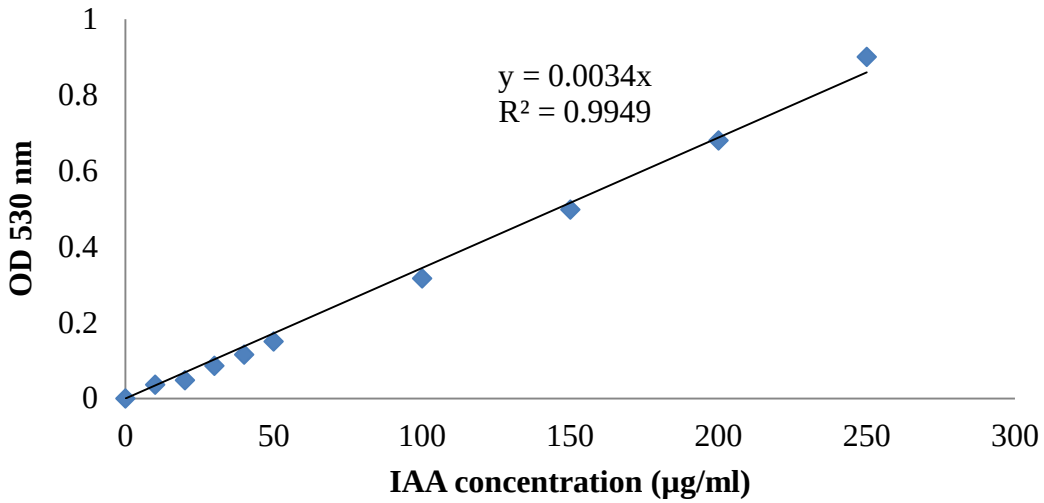


Figure B Standard curve of IAA concentration at OD 530 nm.

APPENDIX C

Extraction of crude IAA and Thin layer chromatography (TLC)

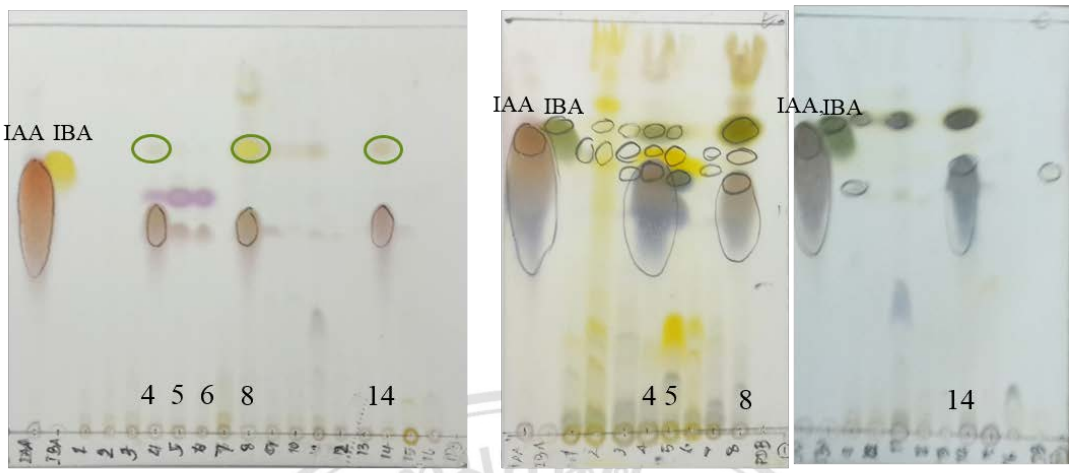
Method

The crude extracts of endophytic fungi were dissolved in 1 ml of methanol and spotted on TLC sheets (TLC aluminium sheets, silica gel 60GF254, Merck, Germany). Then, developed in ethyl acetate:chloroform:formic acid (55:35:10, v/v/v). Spots with Rf values identical to authentic IAA were identified under UV light at wave length of 254 nm by spraying with Ehmann's reagent and Salkowski's reagent (Ehmann, 1977).

Table C Rf values of crude IAA after developed by TLC

No.	Sample	Rf	
		Solawaski's reagent	Ehmann's reagent
	IAA	0.68	0.73
	IBA	0.70	0.77
4	C4IV301	0.58, 0.61, 0.74	0.64, 0.69, 0.73
5	C4V202-1	0.52, 0.61	0.63, 0.67, 0.73
6	RTM5IV1	0.52, 0.61	-
8	PS1IV102	0.57, 0.75	0.61, 0.67, 0.75
14	BS1B201	0.57, 0.73	0.67, 0.78

(-) = no data



Salkowski's reagent **Ehmann's reagent**

Figure C Thin layer chromatography (TLC) of IAA crude extract such as IAA standard, IBA standard, no. 4 = C4IV301, 5 = C4V202-1, 6 = RTM5I-V1, 8 = PS1IV102, 14 = BS1B201 and PDB (negative control).

APPENDIX D

Fungal preparation for scanning electron microscopy

1. Agar plug of 7-day-old fungal endophytes were placed into 2% glutaraldehyde in 0.1 M sodium carcodylate buffer (pH 7.2-7.4) with TritonX and left overnight.

2. All agar plugs were moved into dehydrating solutions. They were washed in seven 15 min changes of water-buffer, followed by a 15 min change in 5%, 10%, 15%, 20%, 40%, 50% and 70% ethanol. They were left overnight or longer in 70% ethanol.

3. They were rinsed for 15 min in 80%, 95% and 100% ethanol, followed by nine 15 min changes in acetone, followed by a 15 min change in 10%, 15%, 20%, 40%, 50%, 70%, 80%, 95% and 100% acetone.

The dehydration process was done slowly. For scanning electron microscopy the fungal material was critical-point dried, gold spotter-coated and images were recorded with a scanning electron microscope (JEOL JSM-5910LV, Japan).

APPENDIX E

Nucleotide sequence and GenBank accession number of ITS rRNA gene of *Muscodor vitigenus* (RTM5IV1)

```
LOCUS       KR076793  579 bp    DNA     linear   PLN 23-SEP-2015
DEFINITION  Rigidoporus microporus strain SDBR-CMU R1 internal
            Transcribed spacer 1, partial sequence; 5.8S ribosomal RNA
            gene, complete sequence; and internal transcribed
            spacer 2, partial sequence.
ACCESSION   KR076793
VERSION     KR076793.1
KEYWORDS    .
SOURCE      Rigidoporus microporus (Fomes lignosus)
  ORGANISM  Rigidoporus microporus
            Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina;
            Agaricomycetes; Polyporales; Rigidoporus.
REFERENCE   1  (bases 1 to 579)
  AUTHORS   Nakaew,N., Rangjaroen,C. and Sungthong,R.
  TITLE     Utilization of rhizosperic Streptomyces for biological
            control of Rigidoporus sp. causing white root disease in
            rubber tree
  JOURNAL   Eur. J. Plant Pathol. 142, 93-105 (2015)
REFERENCE   2  (bases 1 to 579)
  AUTHORS   Siri-Udom,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-APR-2015) Biology, Faculty of Science,
Chiangmai
            University, 239 Huay Kaew Road, Muang, Chiangmai 50200,
            Thailand
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /strain="SDBR-CMU R1"
                     /isolation_source="infected roots of diseased
                     tree"
                     /host="rubber tree; cultivar: RRIM600; age: 8-10
                     months"
                     /db_xref="taxon:219653"
                     /country="Thailand: Wangthong District,
                     Phitsanulok"
                     /lat_lon="16.8242 N 100.4286 E"
     misc_RNA        <1..>579
                     /note="contains internal transcribed spacer 1,
                     5.8S
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ribosomal RNA, and internal transcribed spacer 2"
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61 cacgcctcgc aatccatttc aaaccacact tgtgcacttc agaggggggag cctctcttgg
121 cctctccttc tttcatcact acaaaccact ttaaagtctt ttgtatttgt tggttaacta
181 taatgttaaa tacaactttc aacaacggat ctcttggctc tcgcatcgat gaagaacgca
241 gcgaaatgcg ataagtaatg tgaattgcag aattcagtga atcatcgaat ctttgaacgc
301 accttgcgct ccttggtatt ccgaggagca tgcctgtttg agtgtcgtgt aattctcaat
361 ctcaactttt tttgttgtgg attggatttg ggagcttgtc gtgtctcttt ctataatgaa
421 agaggttaga ctctccttga atgcattagc tcggtcacgt agtttgcccg acggttcacg
481 gtgtgatagt ctcacttcat cgccgttcta actgttggtg cctgtgtttt tgccggcttc
541 taatctctgg cctcttttc aaagtggcct ttacacttt

APPENDIX F

Nucleotide sequence and GenBank accession number of ITS1 rRNA gene of *Muscodor equiseti* (RTM5IV2)

LOCUS KF850711 611 bp DNA linear PLN 17-MAY-2016
DEFINITION *Muscodor* sp. RTM5-IV2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
ACCESSION KF850711
VERSION KF850711.1
KEYWORDS .
SOURCE *Muscodor* sp. RTM5-IV2
ORGANISM *Muscodor* sp. RTM5-IV2
Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Sordariomycetes; Xylariomycetidae; Xylariales; mitosporic Xylariales; *Muscodor*.
REFERENCE 1 (bases 1 to 611)
AUTHORS Siri-udom,S., Suwannarach,N. and Lumyong,S.
TITLE Existence of *Muscodor vitigenus*, *M. equiseti* and *M. heveae* sp. nov. in leaves of the rubber tree (*Hevea brasiliensis* Müll.Arg.), and their biocontrol potential
JOURNAL Ann. Microbiol. 66 (1), 437-448 (2016)
REFERENCE 2 (bases 1 to 611)
AUTHORS Siri-Udom,S. and Lumyong,S.
TITLE *Muscodor*, an endophytic fungus of *Hevea brasiliensis* (Para Rubber tree) from northeast of Thailand with volatile Antibiotic activities
JOURNAL Unpublished
REFERENCE 3 (bases 1 to 611)
AUTHORS Siri-Udom,S. and Lumyong,S.
TITLE Direct Submission
JOURNAL Submitted (08-NOV-2013) Biology, Faculty of Science, ChiangMai University, 239 Huay Kaew Road, Muang, ChiangMai 50200, Thailand
FEATURES Location/Qualifiers
source 1..611
/organism="Muscodor sp. RTM5-IV2"
/mol_type="genomic DNA"
/isolate="RTM5-IV2"
/isolation_source="healthy leaves"
/host="Hevea brasiliensis"
/db_xref="taxon:1457325"
/country="Thailand"
/lat_lon="17.2039 N 102.4406 E"
/collection_date="May-2011/Dec-2012"
/collected_by="Siri-Udom, S"

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                     /note="PCR_primers=fwd_name: its5, rev_name:
                     its4"
     misc_RNA        <1..>611
                     /note="contains 18S ribosomal RNA, internal
                     Transcribed spacer 1, 5.8S ribosomal RNA,
                     internal transcribed spacer 2, and 28S ribosomal
                     RNA"
ORIGIN
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tttctaaact
       61 cccaacccta tgtgaactta cctttgttgc ttcggcggcg gaggctaccc
tgcgggagaa
      121 taccacttag tggttaccct gtagtttcag gtacatcagc tacccggtag
tcatccccgc
      181 cggcggccaa ctaaactctg ttttctttgg aattctgaat aacaaactta
ataagttaaa
      241 actttcaaca acggatctct tggttctggc atcgatgaag aacgcagcga
aatgcgataa
      301 gtaatgtgaa ttgcagaatt cagtgaatca tcgaatcttt gaacgcacat
tgcgcccatt
      361 agcattctag tgggcatgcc tgttcgagcg tcatttcacc acttaagcct
tgttgcttag
      421 cgttgggagc ctacggcacg gcccgtagct ccttaaagtg attggcggag
ttggttctca
      481 ctctaagcgt agtaattata tctcgcttct gtagtggtcc cggcccctgc
cgtaaaaccc
      541 cttatacaaa ggttgacctc ggatcaggta ggaatacccg ctgaacttaa
gcatatcaat
      601 aagccggagg a
```

APPENDIX G

Nucleotide sequence and GenBank accession number of ITS1 rRNA gene of *Muscodor heveae* (RTM5IV3)

LOCUS KF850712 593 bp DNA linear PLN 17-MAY-2016
DEFINITION *Muscodor* sp. RTM5-IV3 internal transcribed spacer 1, Partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internaltranscribed spacer 2, partial sequence.
ACCESSION KF850712
VERSION KF850712.1
KEYWORDS .
SOURCE *Muscodor* sp. RTM5-IV3
ORGANISM *Muscodor* sp. RTM5-IV3
Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Sordariomycetes; Xylariomycetidae; Xylariales; mitosporic Xylariales; *Muscodor*.
REFERENCE 1(bases 1 to 593)
AUTHORS Siri-udom,S., Suwannarach,N. and Lumyong,S.
TITLE Existence of *Muscodor vitigenus*, *M. equiseti* and *M. heveae* sp. nov.in leaves of the rubber tree (*Hevea brasiliensis* Müll.Arg.), and their biocontrol potential
JOURNAL Ann. Microbiol. 66 (1), 437-448 (2016)
REFERENCE 2(bases 1 to 593)
AUTHORS Siri-Udom,S. and Lumyong,S.
TITLE *Muscodor*, an endophytic fungus of *Hevea brasiliensis* (Para Rubber tree) from northeast of Thailand with volatile Antibiotic activities
JOURNAL Unpublished
REFERENCE 3 bases 1 to 593)
AUTHORS Siri-Udom,S. and Lumyong,S.
TITLE Direct Submission
JOURNAL Submitted (08-NOV-2013) Biology, Faculty of Science, Chiang Mai University, 239 Huay Kaew Road, Muang, Chiang Mai 50200, Thailand
FEATURES Location/Qualifiers

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                     /mol_type="genomic DNA"
                     /isolate="RTM5-IV3"
                     /isolation_source="healthy leaves"
                     /host="Hevea brasiliensis"
                     /db_xref="taxon:1457326"
                     /country="Thailand"
                     /lat_lon="17.2039 N 102.4406 E"
                     /collection_date="May-2011/Dec-2012"
                     /collected_by="Siri-Udom, S"
                     /note="PCR_primers=fwd_name: its5, rev_name:
                     its4"
     misc_RNA        <1..>593
                     /note="contains internal transcribed spacer 1,
                     5.8S ribosomal RNA, and internal transcribed
                     spacer 2"
ORIGIN
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acctttggtt
       61 cccaaccggc ggcggaggct acccctgtgg gggataccac cggaggggtt
accccggaag
      121 ccccgagggc cataaatggc ttgccccgac atcttatccc cttacgacta
gctacccggt
      181 gggcctcccc tgcagggggc caaataaaat ctgtttttat tggaattctg
aattataaaa
      241 ttaataagtt aaaactttca aaaagggatc tcttgattct ggcatcgatg
aagaaggcaa
      301 cgaaatggct aaaactaatg agaattgcaa caattcagtg gaaccatcaa
atctttgaac
      361 gcacattgcg cccattagca ttttcactgg gcatgcctgt tgctaccgtc
atttcaccac
      421 ttaagccctg ttgcttagcg ccggaaacct acggcactgg cccggttctc
ccttaaagtg
      481 attggaacaa ttggttctca ctctaggggt accaaatcca tactctgcct
ccccctggta
      541 ggtttccagg cccctgccct aaaacccccc atatataaag gttgacctcg gat
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APPENDIX H

Nucleotide sequence and GenBank accession number of ITS1 rRNA gene of *Rigidoporus microporus* strain SDBR-CMU R1

```
LOCUS       KR076793  579 bp    DNA     linear   PLN 23-SEP-2015
DEFINITION  Rigidoporus microporus strain SDBR-CMU R1 internal
            transcribed
            spacer 1, partial sequence; 5.8S ribosomal RNA gene,
            complete
            sequence; and internal transcribed spacer 2, partial
            sequence.
ACCESSION   KR076793
VERSION     KR076793.1
KEYWORDS    .
SOURCE      Rigidoporus microporus (Fomes lignosus)
  ORGANISM  Rigidoporus microporus
            Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina;
            Agaricomycetes; Polyporales; Rigidoporus.
REFERENCE   1  (bases 1 to 579)
  AUTHORS   Nakaew,N., Rangjaroen,C. and Sungthong,R.
  TITLE     Utilization of rhizosperic Streptomyces for biological
            control of Rigidoporus sp. causing white root disease in
            rubber tree
  JOURNAL   Eur. J. Plant Pathol. 142, 93-105 (2015)
REFERENCE   2  (bases 1 to 579)
  AUTHORS   Siri-Udom,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-APR-2015) Biology, Faculty of Science,
            Chiangmai University, 239 Huay Kaew Road, Muang, Chiangmai
            50200, Thailand
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /organism="Rigidoporus microporus"
                     /mol_type="genomic DNA"
                     /strain="SDBR-CMU R1"
                     /isolation_source="infected roots of diseased
                      tree"
                     /host="rubber tree; cultivar: RRIM600; age: 8-10
                      months"
                     /db_xref="taxon:219653"
                     /country="Thailand: Wangthong District,
                      Phitsanulok"
                     /lat_lon="16.8242 N 100.4286 E"
     misc_RNA        <1..>579
                     /note="contains internal transcribed spacer 1,
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5.8S ribosomal RNA, and internal transcribed
spacer 2"
ORIGIN
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agaacatgtg
61 cacgcctcgc aatccatttc aaaccacact tgtgcacttc agaggggggag
cctctcttgg
121 cctctccttc tttcatcact acaaaccact ttaaagtctt ttgtatttgt
tggttaacta
181 taatgttaaa tacaactttc aacaacggat ctcttggctc tcgcatcgat
gaagaacgca
241 gcgaaatgcg ataagtaatg tgaattgcag aattcagtga atcatcgaat
ctttgaacgc
301 accttgcgct ccttggtatt ccgaggagca tgcctgtttg agtgtcgtgt
aattctcaat
361 ctcaactttt tttgttgtgg attggatttg ggagcttgtc gtgtctcttt
ctataatgaa
421 agaggttaga ctctccttga atgcattagc tcggtcacgt agtttgcccg
acggttcacg
481 gtgtgatagt ctcacttcat cgccgttcta actgttggtg cctgtgtttt
tgccggcttc
541 taatctctgg cctcttttc aaagtggcct ttacacttt

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Scholarship	2012 The Graduate School of Chiang Mai University, The Higher Education Commission, Thailand, under the National Research University (A1) program, Chiang Mai University 2013 Thailand Research Fund (RTA5580007)
Publication	Siri-Udom S, Suwannarach N, Lumyong S (2015) Existence of *Muscodor vitigenus, M. equiseti* and *M. heveae* sp. nov. in leaves of the rubber tree (*Hevea brasiliensis* Müll.Arg.), and their biocontrol potential. *Annals of Microbiology* 66: 437-448.