BIOLOGICAL CONTROL OF WHITE ROOT DISEASE OF RUBBER TREES

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ABSTRACT

White root disease caused by *Rigidoporus microporus* (Sw.) Overeem is one of the important disease of rubber trees and it causes economically important losses in rubber plantation. In this study, sample collection was taken in the southern part of Thailand at Narathiwat, Trang, and Surat Thani provinces. The symptom of white root disease is appeared as changed in leaves color from green turn to yellow at one or a few branches or whole canopy or dead trees with fruiting bodies at the collar of the dead stem. Fifty isolates of *R. microporus* were isolated from infected roots of rubber trees and fruiting bodies. All isolates were proved for their pathogenicities on the rubber tree variety RRIM600. The symptom was observed within 3 months and all isolates were pathogenic on the rubber trees. The most virulent isolates were SNK02, SNK03 and SND08 which obtained from Narathiwat province.

The molecular phylogenetic study was confirmed to be a taxon of *R. microporus* by DNA sequencing at internal transcribed spencer (ITS) region. This was firstly recorded about ITS technique of *R. microporus*. The tree derived from parsimony analysis ITS confirmed that *R. microporus* is distincted from *R. ulmarious* but similar correlated to each other. The genetic variation among isolates of *R. microporus* was determined using ISSR. Cluster analysis based on ISSR characters grouped the isolates according to geographical origins and the results showed two main distinct groups, designated as A and B rooting from out group, *Ganoderma* sp. GM101. Group A contained isolates collected from Surat Thani and Trang provinces and group B of those collected from Narathiwat province.

Thirty isolates of promising antagonistic fungi were isolated from soil which collected from rubber plantation at Narathiwat and Surat Thani province. They were isolated by baiting and

soil plate technique which included 2 isolates of *Acremonium fusidioides,* 2 isolates of *Aspergillus niger*, 2 isolates of *Chaetomium aureum,* 7 isolates of *Ch. bostrychodes*, 1 isolate of *Ch. cochliodes*, 1 *isolate* of *Ch. cupreum*, 5 isolates of *Ch. fusiforme*, 2 isolates of *Ch. indicum,* 1 isolate of *Penicillium canescens*, 2 isolates of *Trichoderma hamatum*, 2 isolates of *T. harzianum*, and 3 isolates of *T. viride.* These fungi were tested for their ability to inhibit the growth of *R. microporus* by dual culture and found that all isolates of *A. niger*, *Ch. elatum*, *Ch. cochliodes, Ch. bostrychodes*, *Ch. cupreum*, *T. hamatum*, *T. harzianum*, and *T. viride* gave percentage of growth inhibition (PGI) over 50%. Among them the tested *Trichoderma* species gave the highest values of PGI followed by the tested *Aspergillus* species and the tested *Chaetomium* species. However, *T. viride* STN04, STN05, and *T. hamatum* STN07 gave the best results with PGI at 89.5%.

Ten effective isolates of antagonistic fungi from dual culture as follows:- *A. niger* SN71 and SN72, *Ch. bostrychodes* BN08, BN11, and BS01, *Ch. cupreum* RY202, *T. hamatum* STN07, *T. harzianum* STN01 and STN02 and *T. viride* STN04 were extracted for antagonistic substances as crude extracts and tested for their ability to inhibit the growth of *R. microporus* by plate assay. The results showed that crude hexane extract from *Ch. cupreum* RY202 gave the highest inhibition of mycelial growth of *R. microporus* with percentage of colony inhibition of 82.0% and the effective dose (ED_{50}) of 170 µg/ml followed by crude methanol extract from *T. hamatum* STN07 and crude ethyl acetate extract from *Ch. cupreum* RY202 with percentage of colony inhibition of 80.0 and 78.0% and the effective dose (ED_{50}) at 187 and 402 µg/ml, respectively. The bioactive compound produced from *Ch. cupreum* named rotiorinol was also tested for its ability to inhibit the growth of *R. microporus* and found that this compound could inhibit the mycelial growth at concentration of 250 and 500 µg/l with effective dose (ED_{50}) 26 µg/ml.

Chaetomium cupreum RY202 was formulated as powder and oil form. These formulations were applied to inhibit the growth of *R. microporus* in the pot experiments. The results showed that *Ch. cupreum* RY202 could reduce the white root disease of rubber trees with disease reduction in the treatment of powder and oil from of 60 and 80%, respectively.

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บทคัดย่อ

โรครากขาวเกิดจากเชื้อ *Rigidoporus microporus* (Sw.) Overeem เป็นโรคที่สำคัญของยางพารา และเป็นสาเหตุที่ทำให้เกิดความเสียหายทางเศรษฐกิจกับแหล่งปลูกยางในหลายประเทศ รวมทั้งประเทศไทย ในการศึกษาครั้งนี้ แหล่งเก็บตัวอย่างโรครากขาวอยู่ที่ภาคใต้ของประเทศไทย ที่จังหวัด นราธิวาส ตรัง และ สุราษฎร์ธานี อาการของโรครากขาวที่เห็นได้คือ ใบของยางพาราจะเปลี่ยนจากสีเขียวเป็นสีเหลือง ซึ่งใบจะเปลี่ยนสีเพียงข้างเดียวของทรงพุ่มหรือบางกิ่ง หรือทั้งต้นหรือตาย และอาจจะสร้างดอกเห็ดที่โคนต้นที่ตาย

เชื้อสาเหตุโรครากขาว *R. microporus* จำนวน 50 ไอโซเลท (isolate) แยกจากรากยางพาราที่เป็นโรค และจากฟรุทติ้ง บอดี้ (fruiting body) ของเชื้อ จำนวนไอโซเลทของเชื้อสาเหตุทั้งหมดนำไปทดสอบความสามารถในการทำให้เกิดโรคกับยางพาราพันธุ์ RRIM600 พบว่าอาการของโรคจะสังเกตุได้ภายในระยะเวลา 3 เดือนหลังการทดสอบ และทุกไอโซเลทสามารถทำให้เกิดโรคกับต้นยางพาราได้ สายพันธุ์ที่รุนแรงคือ SNK02 SNK03 และ SND08 ที่ได้จากจังหวัดนราธิวาส

การศึกษาไฟโลจินิทิคทรี (Phylogenetic tree) เพื่อยืนยันเชื้อ *R. microporus* โดยใช้เทคนิคดีเอ็นเอ ซีเควนซิ่ง (DNA sequencing) ที่ตำแหน่งไอทีเอส (ITS) การศึกษานี้เป็นรายงานครั้งแรกเกี่ยวกับดีเอ็นเอ ซีเควนซิ่ง ของ *R. microporus* ไฟโลจินิทิคทรีจาก ไอทีเอส ยืนยันว่า *R. microporus* แตกต่างจาก *R. ulmaroius* แต่มีความสัมพันธ์ซึ่งกันและกัน การศึกษาความแปรปรวนทางพันธุกรรมของสายพันธุ์ของ *R. microporus* โดยใช้เทคนิคไอเอสเอสอาร์ (ISSR) พบว่า ไอโซเลททั้งหมดแสดงออกสองกลุ่มหลัก ตามพื้นที่ที่ได้มา คือ กลุ่มเอ (A) และ บี (B) ซึ่งกลุ่มเอประกอบด้วยสายพันธุ์ทั้งหมดที่ได้จาก จังหวัด สุราษฎร์ธานี และตรัง ส่วนกลุ่มบีประกอบด้วยสายพันธุ์ที่ได้จากจังหวัดนราธิวาส

การศึกษาการคัดเชื้อราต่อต้าน (antagonistic fungi) ที่มีผลในการยับยั้งการเจริญของเชื้อ *R. microporus* โดยแยกจากดินที่เก็บบริเวณรากของยางพารา จากจังหวัดนราธิวาส และจังหวัด

นครศรีธรรมราช โดยวิธี baiting และ soil plate ปรากฏว่าได้เชื้อรา จำนวน 30 ไอโซเลท ดังนี้ *Acremonium fusidioides* 2 ไอโซเลท, *Aspergillus niger* 2 ไอโซเลท, , *Chaetomium aureum* 2 ไอโซเลท, *Ch. bostrychodes* 7 ไอโซเลท, *Ch. cochliodes* 1 ไอโซเลท, *Ch. cupreum* 1 ไอโซเลท, *Ch. fusiforme* 5 ไอโซเลท, *Ch. indicum* 2 ไอโซเลท, *Penicillium canescens* 1ไอโซเลท, *Trichoderma hamatum* 2 ไอโซเลท, *T. harzianum* 2 ไอโซเลท, และ *T. viride* 3 ไอโซเลท เชื้อราทั้งหมดนี้นำไปทดสอบความสามารถในการควบคุมการเจริญของของเชื้อ *R. microporus* โดยวิธีเลี้ยงเชื้อบนอาหารร่วม (dual culture) ผลปรากฏว่า จำนวนไอโซเลททั้งหมดของเชื้อ *A. niger*, *Ch. cochliodes*, *Ch. bostrychodes*, *Ch. cupreum*, *T. hamatum*, *T. harzianum*, และ *T. viride* ให้เปอร์เซ็นต์การยับยั้งการเจริญมากกว่า 50% ในจำนวนนี้ *Trichoderma* ให้ค่าเปอร์เซ็นต์การยับยั้งการเจริญสูงที่สุด รองลงมาคือ *Aspergillus* และ *Chaetomium* โดยเชื้อ *T. viride* STN04, STN05 และ *T. hamatum* STN07 ให้ค่าเปอร์เซ็นต์การยับยั้งการเจริญโตสูงที่สุด คือ 89.5%

เชื้อราต่อต้านจำนวน 10 ไอโซเลท ที่มีประสิทธิภาพในการยับยั้งการเจริญของเชื้อ *R. microporus* ดังนี้ *A. niger* SN71 และ SN72, *Ch. bostrychodes* BN08, BN11, และ BS01, *Ch. cupreum* RY202, *T. hamatum* STN07, *T. harzianum* STN01 และ STN02 and *T. viride* STN04 นำไปสกัดเพื่อให้ได้สารสกัดหยาบ และทดสอบความสามารถในการควบคุมการเจริญของ *R. microporus* พบว่า สารสกัดหยาบที่สกัดด้วยสาร hexane จากเชื้อ *Ch. cupreum* RY 202 ให้ผลดีที่สุดในการควบคุมการเจริญของเส้นใย *R. microporus* โดยสามารถควบคุมการเจริญของเส้นใยบนอาหาร PDA เท่ากับ 82.0% และ ED_{50} เท่ากับ 170 µg/ml รองลงมาคือ สารสกัดหยาบที่สกัดด้วย methanol จากเชื้อรา *T. hamatum* STN07, และสารสกัดหยาบที่สกัดด้วย ethyl acetate จากเชื้อรา *Ch. cupreum* RY 202 โดยสามารถควบคุมการเจริญของเส้นใยบนอาหาร PDA เท่ากับ 80.0 และ 78.0% และ ED_{50} เท่ากับ 187 และ 402 µg/ml ตามลำดับ สาร rotiorinol ซึ่งเป็น bioactive compound ที่สกัดจาก *Ch. cupreum* นำมาทดสอบความสามารถในการควบคุมการเจริญของ *R. microporus* และพบว่าสารนี้สามารถควบคุมการเจริญของเส้นใยของ *R. microporus* ได้ โดยที่ความเข้มข้นที่ 250 และ 500 µg/ml เส้นใยของ *R. microporus* ไม่สามารถเจริญได้ และให้ค่า ED_{50} เท่ากับ 26 µg/ml.

การทดสอบประสิทธิภาพของ *Ch. cupreum* RY202 ในรูปแบบผง และน้ำมัน ในการยับยั้งเชื้อ *R. microporus* ในกระถาง ปรากฏว่า *Ch. cupreum* RY202 ในรูปแบบผงและน้ำมันสามารถลดการเกิดโรครากขาวของยางพาราได้ โดยการใช้ *Ch. cupreum* RY202 ในรูปแบบผงและน้ำมันสามารถลดการเกิดโรคได้ 60 และ 80% ตามลำดับ

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CHAPTER I

INTRODUCTION

1.1 Statement and Significance of the Problems

The genus *Hevea* belongs to the family Euphorbiaceae. There are many species of *Hevea* such as *Hevea benthamiana* Muell-Arg., *H. brasiliensis* (Willd.) Muell.-Arg., *H. camargoana* Pires, *H. camporum* Ducke, *H. guianensis* Aubl., *H. microphyll* Ule, *H. nitida* Mart. ex Muell-Arg., *H. pauciflora* Muell-Arg., *H. rigidifolia* Muell Arg., and *H. spruceana* Muell Arg. etc. Among them, only three species yield usable rubber as follows:- *H. benthamiana*, *H. brasiliensis* and *H. guianensis*. However, *H. brasiliensis* is the only one species which planted commercially and yielded the source of natural rubber (Wycherley. 1992; Orwa *et al.* 2009). There are many common names of *H. brasiliensis* for example; hevea, jebe, rubber, para rubber, rubber tree, rubber wood, seringueira-branca, siringa, caoutchouc tree, etc (Orwa *et al.* 2009). Rubber tree was first found in the Amazon basin and it is a native plant of Brazil. However, Brazil was not the site of the successful commercialization of rubber (Law. 2009; FAO. 2001). It is introduced to many countries in Asia, such as China, India, Indonesia, Malaysia, Papua New Guinea, Sarawak, Sri Lanka, Thailand, and Vietnam as well as in Africa such as Cameroon, Côte d'Ivoire, Gabon, Liberia and Nigeria (Law. 2009). The production of natural rubber is now concentrated in Southeast Asia especially in Indonesia, Malaysia and Thailand which produce more than 92 percent of the world. Two other Asian producers are Sri Lanka and India and two African producers are Liberia and Nigeria (Law. 2009).

In Thailand, rubber trees were first introduced to Trang province from Malaysia by Phraya Ratsadanupradit Mahison Phakdi, Governor of Trang Province in 1899. It becomes one of the major crop in Thailand. The planting area of rubber tree has nowadays expanded all over the country. There are more than 80% of the rubber plantations are found in the southern regions. The south has fertile growing climate which result in highly conducive to rubber tree cultivation. This climate helps farmers achieve high yields roughly 1.76 tons of rubber per hectare. Since 1991, Thailand has become the world supplier of natural rubber production. In 2006, nearly 90% of natural rubber production or over 2,771,673 tons and estimated US$5.41 billion of natural rubber were exported. The rubber products are exported worldwide and produce significant revenue for

the country. The leading export markets for Thai rubber are Japan, Malaysia, USA, China and South Korea. Ten percent of all the rubber produced in Thailand is used for domestic consumption. Of this portion, 65% is processed into value-added goods, such as tires and tubes for motorcycles, airplanes, cars and bicycles (46-51%), gloves (13-15%), rubber bands (8-10%), and elastic (8-9%). Rubber wood, a renewable resource that presents an attractive alternative to hardwoods timber, is an increasingly important product in the domestic market and now one of the major resources for making furniture for export (Anonymous. 2009).

Ecology of rubber tree is ranging from subtropical to tropical. The rubber tree requires hot temperatures, high humidity and well-drained and fertile soils. Rubber trees can grow in over 20 meters above sea level, but growing best on flat low land with over 2,000 mm of rain a year. Rubber tree is a quick-growing tree, about 25-30 meters height in plantations (Orwa *et al.* 2009). It takes about seven years for their first cash crop depending on climate and location. The trees are planted in rows with approximately three meters in between the trees and six meters in between the rows. In Thailand, rubber tapping normally takes place early in the morning when the internal pressure of the tree is highest. Cutting the bark of this tree releases the latex. The latex is located in the inner bark of the tree and flows in the vessels of the tree. Latex is thought to be a defense against insect predators for the tree (The International Rubber Research and Development Board. 2007). The economic time of the trees about 10 - 20 years depends on how well the tapping is carried out. Older trees produce more latex, and stop producing latex after 26-30 years. Thereafter, the trees were cut and the rubber timbers were taken for production of furniture and indoor building components. With the increasing in plantation area, research in this area emphasize in improving the varieties which yield both high quality latex and timber (Rogers. 1981).

Many diseases are known to attack *Hevea* trees which classified as 1) leaf diseases for example, leaf spot caused by *Botryodiplodia elactica*, *B. theobromae*, anthracnose caused by *Colletotrichum gloeosporioides*, bird's eye spot leaf caused by *Bipolaris heveae*, powdery mildew caused by *Oidium heveae*, Corynespora leaf disease caused by *Corynespora cassiicola*, leaf fall caused by *Phytophthora* sp. 2) stem diseases for example, black stripe caused by *Phytophthora botryosa* Chee., *P. palmivora* (Butler) Butler, die-back caused by *P. palmivora*, mouldy rot caused by *Ceratocystis fimbriata*, pink disease caused by *Pellicularis salmonicolor*, and 3) root diseases for example, white spongy rot caused by *Polystichus occidentalis* and *P. personii*, white root disease caused by *Rigidoporus microporus* (Sw.) Overeem, brown root disease caused by *Phellinus noxius* (Corner) G.H. Gunn) and red root disease caused by *Ganoderma pseudoferreum*

(Wakef) (Duke. 1983; Nandris *et al.* 1987; Nicole and Benhamou. 1991; Law. 2009). Among these diseases, root diseases are the most serious diseases especially white root disease (Nicole and Benhamou. 1991).

White root disease is controlled using an integration of cultural and chemical methods by clearing the land of old rubber tree to reduce the source of inoculum before replanting and after planting, cutting away diseased tissue and applying chemical fungicide. However, chemical fungicides have been known to have a negative effect on human health, cause environmental pollution, leave residues in the agricultural soil, and several plant pathogenic fungi have developed resistance (Deahl and Demuth. 1993). To avoid the negative effect or harmful of chemical use, biological control would therefore be taken as an alternative save and sound measure for controlling this disease by reducing the inoculum sources, as well as to inhibiting the disease spread.

1.2 Objectives

The main objectives of this study were as follows:-

1. To collect, isolate and test for pathogenicity of white root rot pathogen.

2. To confirm the species and study the pathogenic variability among isolates of the white root rot pathogen.

3. To investigate the efficiency antagonistic fungi for controlling the white root disease.

4. To test the efficiency of antagonistic fungi against white root rot pathogen in pot and field trials.

1.3 Scope of the Study

1. Collection the causing agent of white root disease in the southern part of Thailand.

2. Isolation and study the characteristic and test for their pathogenicities of white root rot pathogen.

3. Study the molecular techniques for white root pathogen isolates.

4. Isolation and screening the antagonistic fungi from rhizosphere soil in rubber plantation to control white root pathogen and test the efficiency of selected antagonistic fungi in pot experiment.

CHAPTER II

LITERATURE REVIEW

2.1 History of White Root Disease

White root disease caused by *Rigidoporus microporus* (Sw.) Overeem (Synonyms, *R. lignosus*, *Fomes lignosus*) (Hood. 2006). There are many common names of this disease such as white thread, root rot disease, white cocoa root disease, white hevea root disease, white root rot. White root disease is reported to aggressively kill trees, fruit trees, and several agriculture crops (Louanchi *et al.* 1996), for examples oil palm, cocoa, durian, sugar apple, neem, long gong, bitter bean, champeak, jackfruit, tea, mangosteen, coconut, pepper, citrus, coffee, chili, egg plant, cassava, sweet potato, and bamboo (Srinara. 2010). White root disease is the most serious of all diseases found in rubber plantation all over the world for example Malaysia, Indonesia, Thailand, India, Sri Lanka, West and Central Africa. The young plantations in Malaysia, it causes greater losses than those caused by other diseases and pests. In Sri Lanka, the average loss is about 10%, in the Ivory Coast, the average rate of infection is about 2% (Guyot and Flori. 2002). If untreated with cultural methods or chemical fungicides, it can result in substantial death of trees and sometimes losses of a whole stand. Rubber tree losses due to white root disease reached more than 50% in old plantation, then resulted in damage in large patches of dead trees (Nandris *et al.* 1988).

Rigidoporus microporus forms many white, flattened mycelial strands 1-2 mm thick that can grow on the surface of the root bark. The rhizomorphs of this fungus grow rapidly and extend several meters through the soil in the absence of any woody substrate. The main source of inoculum is the remnants of infected wood, either pieces of roots or stumps left buried in the soil. The healthy rubber trees can be infected by free rhizomorphs growing from stumps or infected woody debris buried in the ground as well as by root contacting with the infected root of the neighboring tree and the disease spreads to the tree collar and to the other lateral roots through the fungal rhizomorphs thus white root diseases transmitted from tree to tree especially along the planting rows (The international rubber research and development board. 2007; Nandris *et al.* 1987). The rhizomorphs infect the roots and degrading extracellular enzyme able to decay the wood (Nandris *et al.* 1987). The first visible symptom is the change in color of the leaves from

green to yellow. In the first stage, yellow leaves are only observed on one or a few branches but eventually the whole canopy would be affected, then the branches die back and the tree dies (Guyot and Flori. 2002). The incidence of white root disease is more severe in replanted areas which have previously been planted with rubber trees than plantings in former jungle areas (The international rubber research and development board. 2007). Mycelial growth rates on the superficial roots of *Hevea* are estimated to be 2.5 m per year. Because trees are planted every 3 m in rows and 6 m apart, the pathogen spreads within row rather than between rows. The fungus attacks the root and collar region and kills trees at any growth stage (Nandris *et al.* 1987). Not only damages the tree, but involves heavy expenditure for its control. When symptoms begin to appear in the aerial parts, it notes that it is too late to save the trees (Hamidson and Naito. 2004) and disease has already spread to the next trees. Then a good early detection of diseased trees is needed especially in young crops (Guyot and Flori. 2002).

The cycle of this disease begin with the stumps of the rubber tree, were infected by the basidiospore (primary inoculum) then the root system of rubber trees is infected and colonized by mycelial filaments growing from stumps of infected trees remaining on or under the ground. The propagation along roots of the pathogen from infected rubber trees (secondary inoculum) infect toward the root of neighboring healthy rubber trees. The healthy trees are infected with the pathogen and the tree die. The disease spread from tree to tree. The fruiting bodies are produced at the collar of the dead stem and release basidiospores as seen in Figure 2.1 (Nicole *et al.* 1986).

The characterization of fruiting bodies shows broad (to 20 cm wide), relatively thin, annual to less frequently perennial, leathery, broadly attached shelf, clustered often imbricate; upper surface, concentrically furrowed, initially orange-red-brown, faintly velvety, later smooth, faded; lower surface bright orange-brown, eventually paling, pores fine (6-9 per mm). In section, context pale colored. Monomitic, generative hyphae thin walled, with cross walls (septa), without clamps connection, hyaline (colorless). Hymenium with cystidioles, basidiospores sub-globose to globose, thin walled, colorless, smooth, non-staining in Melzer's reagent, 3.5-4.5 x 3.5-4 um. Cause a root disease of planted trees e.g. rubber trees, produces white, branching mycelial cords and a white rot (Hood. 2006). The orange-yellow fruiting body formed mainly during rainy season at the base of the infected trees. This fruiting body produce a large number of basidiospores, even in the dry season but seem to have a limited role in disseminating disease. This has been controversial points in the biology of *R. microporus* (Nandris *et al.* 1987).

Ryvarden (1991) reported the taxonomy of *R. microporus* (Sw.) Overeem that belongs to Kingdom Fungi, Phylum Basidiomycota, Class Basidiomycetes, Order Polyporales, Family Meripilaceae, Genus Rigidoporus, Species *R. microporus* (Sw.) Overeem.

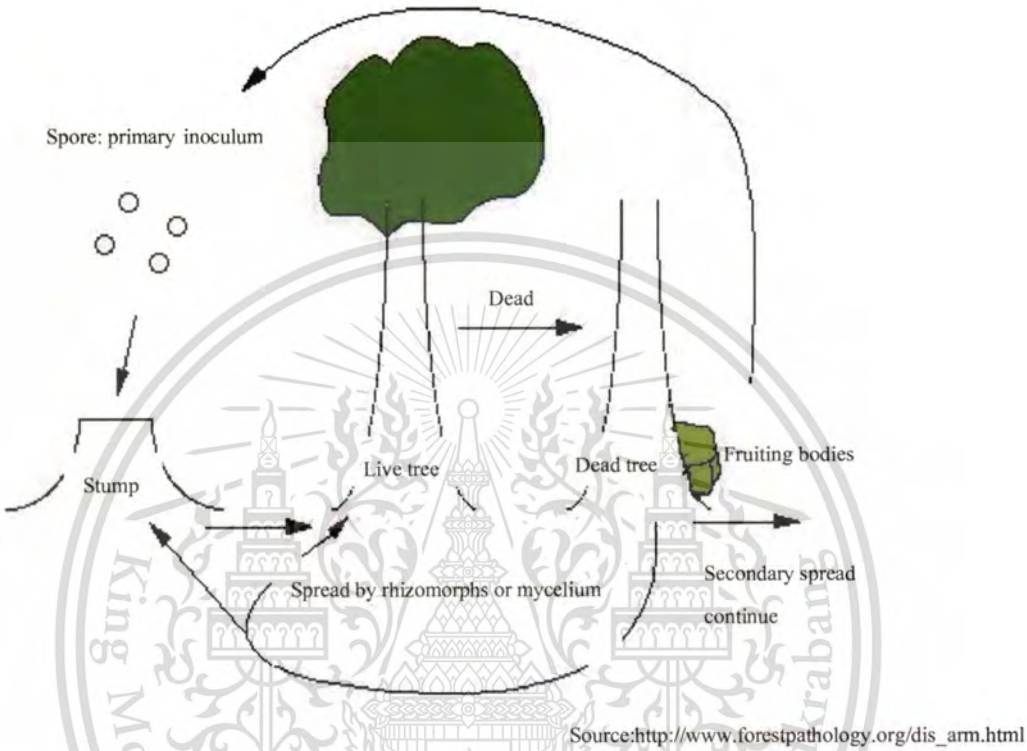


Figure 2.1 Disease cycle of white root disease of rubber trees.

The most important and potent wood destroying organisms is white rot fungi which caused by Basidiomycetes (Highley and Dashek. 1997). *Rigidoporus microporus* is one of the causing agents of white rot fungi. It can attack various components of the wood cell wall. The mechanisms involve in the penetration of the tree roots by the mycelium. This pathogen normally develops two types of mycelium which are different from each other morphologically and metabolically including their enzymatic secretions. One type occurs in the rhizomorph and is probably specialized for superficial dissemination, while the other for infection. The fungi colonized cork cells, and penetrated by a combination of mechanical and enzymatic action (Nicole *et al.* 1986). Enzymatic degradation has been well described for cellulose, hemicellulose, and lignin (Highley and Dashek. 1997). White-rot fungi secrete one or more of three extracellular enzymes that are essential for lignin degradation. The three enzymes comprise: peroxidase, lignin

peroxidase and laccase. White-rot fungi cause the wood become pale and whitish mass. The name white-rot derived from the appearance of wood attacked by fungi, where lignin removal results in a bleached appearance to the substrate (Pointing. 2001). White- rot fungi are the only known organisms that are capable of completely degrading lignin to carbon dioxide and water (Highley and Dashek. 1997).

2.2 The Importance of Molecular Phylogeny in Identification

One of parasitic basidiomycetes cause important tree diseases as white root disease caused by *Rigidoporus microporus* or *Phellinus noxius*. The mycelium is normally hidden within the substrate, and it is generally only the fruiting body or basidiocarp that is visible at the surface. This fruiting body tends to show the greatest morphological variation and is used to distinguish between species. The identification of these fungi has been based on morphology, substrate utilization, and reproductive structures. This method is complicated because the fruiting bodies are frequently absent or difficult to detect in culture and also often show wide ranges of variability in physiologic characteristics, appearances and abilities (Hibbett and Donoghue. 2001). The morphological descriptions are used to identify cultivars; however, this approach lacks objectivity and reliability (Moukhamedov *et al.* 1994). Some species have been defined mainly on host disease symptoms or host association. However, the use of fruiting body morphology in distinguishing between species is sometimes augmented by other methods such as the form of the fungus in pure culture; physiological factors, such as degree of virulence to different hosts, environmental growth preferences (nutrient, temperature); and by relationships at the molecular level and DNA techniques (Hood. 2006). Among the several techniques for fungal identification, DNA techniques are valuable tools. DNA techniques are a valuable tool in the identification of fungi when it is not producing fruiting bodies, the morphological taxonomic characters form the basis of the species description, the fungi that are difficult to grow in culture, or fail to key distinguishing features when grown in culture (Glen. 2006). The advances in molecular technology appear to offer a rapid method to identify fungi base on more objective evaluations (Hibbett *et al.* 1997). The molecular techniques are already being used to identification of unknown species, genetic variability, characterization and relatedness of fungal isolates and species and pathogen detection (Wang *et al.* 2005; Glen. 2006).

The molecular techniques have been accepted to be used in many areas of applied mycology. The molecular or DNA data has led a major study in development of the systematic, biochemistry and ecology of fungi (Bridge. 2002). The development of molecular technology has provided approach which is already being used to identification of unknown species, genetic variability, characterization and relatedness of fungal isolates and species and pathogen detection (Bridge. 2002; Wang *et al.* 2005; Glen. 2006). Moreover, molecular methods have been now starting to give function of fungi in the environment, where they are exists, and how they interact with other organisms (Bridge. 2002). The selection of the most appropriate method depends on the application and the number of samples (Hassel and Gunnarsson. 2003).

Ribosomal DNA (rDNA) is the most conserved region in the genome (Iwen *et al.* 2002). The whole rDNA gene contains a small subunit (SSU) 18S rRNA, 5.8S rRNA, and a large subunit (LSU) 28S rRNA. Internal transcribed spacer (ITS) region I (ITSI) and ITSII are found between SSU rRNA and 5.8S rRNA and between 5.8S rDNA and LSU rRNA, respectively. The internal transcribed spacers (ITS) have been used widely in study on the relationships among species within a single genus or among infraspecific populations and study characterization in fungi (Hibbett and Donoghue. 2001). In particular, sequence analysis of ITS regions proved useful in studying phylogenetic relationships of species because of their comparative variability (Sreenivasaprasad *et al.* 1994, 1996; Moriwaki *et al.* 2002; Photita *et al.* 2005). Sequencing the ITS region of the ribosomal DNA turned out to the appropriate method for a data bank, because the whole sequence information is available for identification, and isolate variation (Schmidt and Moreth. 2002). The ITS region is useful study for characterization in fungi for four reasons as follows:-1) the ITS region is relatively short (500-800 bp) and can be easily amplified by polymerase chain reaction (PCR) using universal single primer (White *et al.* 1990), 2) the multicopy of the rDNA repeat makes the ITS region easy to amplify from small or dilute DNA samples, 3) the ITS region may be highly variable among morphologically distinct species, and 4) PCR-generated ITS species-specific probes can be produced quickly (Bridge and Arora. 1998).

The PCR primers for work with fungal Internal Transcribe Sequences (ITS) were ITS1 and ITS4. These two primers amplify the highly variable ITS1 and ITS2. They also amplify a wide range of fungal targets and work well to analyze DNA isolated from individual organisms. These primers were intended to be specific to fungi and basidiomycetes (Moriwaki *et al.* 2002; Photita *et al.* 2005).

DNA-ITS sequences is used for identification of rot fungi such as *Serpula lacrymans, S. himantioides, Meruliporia incrassata, Leucogyrophana mollusca, L. pinastri, Coniophora puteana, C. arida, C. marmorata, C. olivacea, Antrodia vaillantii, A. serialis, A. sinuosa, A. xantha, Oligoporus placenta, Donkioporia expansa, Gloeophyllum abietinum, G. sepiarium,* and *G. trabeum* (Schmidt and Moreth. 2002). Fulton *et al.* (1999) also studied the nucleotide sequence analysis of the ITS regions of rDNA of three brown rot pathogens *Monilinia laxa, M. fructicola* and *M. fructigena.* ITS region have been used for the characterization, identification and detection of *Verticillium albo-atrum* and *V. dabliae* and used to differentiate *V. tricorpus* from the other species of *Verticillium* (Moukhamedov *et al.* 1994).

During the last years different methods of DNA such as microsatellites (simple sequence repeats; SSR), inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), terminal restriction fragment length polymorphism (T-RFLP), micro-arrays and DNA sequencing have become available to detect genetic variation within populations and among individuals (McDonald. 1997; Hassel and Gunnarsson. 2003; Glen. 2006). These methods detect genetic variation directly at the DNA level (McDonald, 1997; Milgroom. 1996; Hassel and Gunnarsson. 2003; Brown. 1996) and require different genetic markers due to important features, such as genomic abundance, level of polymorphism, locus-specificity, codominance of alleles, reproducibility, labor-intensity, technical requirements, operation costs, development costs, quantities of DNA required, and amenability to automation. Each molecular method has its own advantages and disadvantages as shown in Table 2.1. The most appropriate genetic marker depends on the specific application, the presumed level of polymorphism, and the presence of sufficient technical facilities and know-how, time constraints and financial limitations (Spooner *et al.* 2005).

The ISSR method was first developed by Zietkiewicz *et al.* (1994) and developed from SSR technique (Yu *et al.* 2006). Although SSR analysis technique provides a codominant and high reproductivity but development needs time-consume and laborious (Chadha and Gopalakrishna. 2007). ISSR technique which involves polymerase chain reaction (PCR) amplification of DNA segments using a primer composed of a microsatellite region (Ratnaparkhe *et al.* 1998). ISSR do not require genome sequence information, quickly and easy operates as same as RAPD but give more high reproductivity than those with RAPD (Bornet and Branchard. 2001; Spooner *et al.* 2005). The ISSR is detects a higher level of polymorphism than those

detected with RFLP or RAPD (Godwin *et al.* 1997). The main advantage of ISSRs is that no sequence data for primer construction are needed and only low quantities of template DNA are required (5–50 ng per reaction). Furthermore, ISSRs are randomly distributed throughout the genome. Weakness of this technique is multilocus technique (Gupta *et al.* 1994; Zietkiewicz *et al.* 1994; Godwin *et al.* 1997).

ISSR method was first reported as a technique for analysis the genetic variation in plant and animals and later used to obtain DNA markers in fungi (Bornet and Branchard. 2001; Bayraktar *et al.* 2008). It is power tool for investigating genetic variation within closely related species (Yu *et al.* 2008) and also for studying of genetic population on fungi (Menzies, *et al.* 2003; Chadha and Gopalakrishna. 2007). This technique is reliable and suitable for population genetics analysis of intraspecies (Yu *et al.* 2006) and study on intraspecific genetic variation of fungi pathogenic provide useful information for controlling disease (Takatsuka. 2007). ISSR analysis can be applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Gupta *et al.* 1994; Zietkiewicz *et al.* 1994; Godwin *et al.* 1997). ISSR was successfully to study the diversity and molecular relationships of fungi such as *Colletotrichum* isolates from the Iwokrama forest in Guyana (Lu *et al.* 2004) and study detection of genetic variation and population structure among isolates of *Fusarium oxyaporum* f.sp. *ciceris* on Chickpae inTurkey (Bayraktar *et al.* 2008).

The genetic diversity and gene flow within or between pathogen populations will lead to an understanding of how the pathogen is likely to adapt or evolve in the environmental change such as exposure to abiotic stresses, fungicides and plant resistant (McDonald. 1997). Thus, understanding the genetic variation in populations of the causal agent of disease would be useful in the improvement of disease management system or help to develop cultivars with tolerance and/or resistance to disease (Stenglein and Balatti. 2006; Bayraktar *et al.* 2008).

Table 2.1 The characteristics of DNA techniques.

DNA techniques	RFLP	DNA sequencing	RAPD	Micro-satellites	ISSR	AFLP
Genomic abundance	High	Low	High	High	Medium-High	High
Level of polymorphism	Medium	Low	Medium	High	Medium	Medium
Locus-specificity	Yes	Yes	No	Yes	No	No
Codominance of alleles	Yes	Yes	No	Yes	No	No/yes
Reproducibility	High	High	Low	High	Medium-High	Medium-High
Labour-intensity	High	Low/High	Low	Low	Low	Medium
Technique demands	High	High	Low	Low-Medium	Low-Medium	Medium
Operational costs	High	High	Low	Low	Low-Medium	Medium
Development costs	Medium-High	High	Low-Medium	High	Low	Low
Quantity of DNA required	High	Low	Low	Low	Low	Medium
Amenability to automation	No	yes	yes	yes	yes	yes

Source: Spooner *et al.* (2005)

2.3 Disease Control Strategies

Biological control and its synonym biocontrol have been used in different fields of biology, most notably entomology and plant pathology. In plant pathology, the application of microbial antagonists to suppress the pathogen is referred to biological control agent (BCA). Moreover, the term of biological control also has referred to the use of natural products extracted from various sources (Pal and Gardenner. 2006). The use of microorganisms as biological control agents to control plant disease is a potentially powerful alternative method (Kulkarni *et al.* 2007) because of their rich diversity, complexity of interactions and numerous metabolic pathways. The microorganisms are an amazing resource for biological activity (Emmert and Handelsman. 1999; Alabouvette *et al.* 2006). Over the past 30 years, microorganisms have been described, characterized, and tested for their use as biological control agents against soil borne plant pathogens. Biological control agents especially antagonistic fungi have been used to control plant diseases with 90% of using different *Trichoderma* species e.g. *T. harzianum*, *T. virens*, *T. viride* (Benítez *et al.* 2004). Many species of *Chaetomium* e.g. *Chaetomium globosum*, *Ch. cochlioides*, *Ch. cupreum* can also be antagonistic against various soil microorganisms (Soytong *et al.* 2001; Kanokmedhakul *et al.* 2002, 2006). The others fungi have been reported as biological control agents such as *Ampelomyces quisqualis, Aspergillus niger, Candida oleophila, Coniohyrium minitans, Cryptococcus albidus, Gliocladium virens, G. catenulatum, Fusarium oxysporum, Phlebiosis gigantean, Pythium oligandrum, Rhodotorula glutinis,*(Boyetchoko *et al.* 1999; Butt *et al.* 1999; Hofstein and Chapple. 1999; Butt. 2000; Khetan. 2001; Ghisalberti. 2002; Fravel. 2005). An effective biological control agent should be genetically stable, effective at low concentrations, easy to mass produce in culture on inexpensive media, and be effective against a wide range of pathogens (Irtwange. 2006). The fungal biological control agent should also occur in an easily distributed form, be non-toxic to humans, have resistance to pesticides, be compatible with other treatments, and be non-pathogenic against the host plant (Fravel. 2005; Irtwange. 2006). Biological control of pathogens, the total or partial destruction of pathogen populations by other organisms, occurs routinely in the nature (Haggag and Moamed. 2007). The fungi which used as biological control agents against plant pathogen were describes below.

Chaetomium species can be antagonistic against various plant pathogens. The application of *Chaetomim* as a biological control agent to control plant pathogens first commenced when Martin Tviet and M.B. Moor found *Ch. globosum* and *Ch. cochliodes* occurring on oat seeds and

that these fungi provided some control of *Helminthosporium victoriae* (Tviet and Moor. 1954). *Chaetomium* species have been reported to be potential antagonists of various plant pathogens such as *Curvularia lunata*, *Pyricularia oryzae* and *Rhizoctonia oryzae* (Soytong and Quimio. 1989; Dhingra *et al.* 2003; Aggarwal *et al.* 2004; Park *et al.* 2005). Many species of *Chaetomium* suppress the growth of pathogen through competition (for substrate and nutrients), mycoparasitism, antibiosis, or combinations of these mechanisms (Marwah *et al.* 2007; Zhang and Yang. 2007). *Chaetomium cupreum* and *Ch. globosum* were reported to be antagonist and successfully used to reduce tomato wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici* in green house (Soytong *et al.* 2001). Moreove, they found to inhibit isolates of various plant pathogens such as root rot disease of citrus, black pepper, and strawberry, and damping off disease of sugar beet (Soytong *et al.* 2001; Tomilova and Shternshis. 2006). These taxa have been formulated in the form of powder and pellets as Ketomium®, a broad spectrum mycofungicide (Soytong *et al.* 2001). The mycofungicide Ketomium® which comprises a *Chaetomium* spore suspension has been evaluated for its effect on Siberian isolates of the phytopathogenic fungi *Botrytis cinerea*, *Didymella applanata*, *Fusarium oxysporum* and *Rhizoctonia solani.* It was found that Ketomium-mycofungicide was most efficient in suppressing raspberry spur blight caused by *Didymella applanata* and could also reduce potato disease caused by *R. solani,* increasing potato yield (Shternshis *et al.* 2005). Other species of *Chaetomium* which can act as biological control agents include *Ch. globosum* which can reduce soybean stem canker disease caused by *Diaporthe phaseolorum* f.sp. *meridionalis* (Dhingra *et al.* 2003) and *Ch. cochliodes* which has activity against *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt, and *Phytophthora parasitica* causing citrus root rot (Phonkerd *et al.* 2008).

Gliocladium spècies have been reported to be biological control agent of many plant pathogens (Viterbo *et al.* 2007), for example, *Gliocladium catenulatum and G. virens. G. catenulatum* has been reported to be parasitise of *Pythium ultimum*, *Rhizoctonia solani Sporidesmium sclerotiorum* and *Fusarium* spp. (Punja and Utkhede. 2004; Viterbo *et al.* 2007; Paulitz and Belanger. 2001). *G. virens* has been used as a biological control agent against a wide range of soil borne pathogens such as, *Pythium* and *Rhizoctonia* (Viterbo *et al.* 2007).

Trichoderma species have been used as biological control agents against a wide range of pathogenic fungi e.g. *Rhizoctonia* spp., *Pythium* spp., *Botrytis cinerea*, and *Fusarium* spp. *Phytophthora palmivora*, *P. parasitica* (Benítez *et al.* 2004) and has efficacy against a wide range of plant pathogenic fungi including, *Botrytis cineria*, *Fusarium*, *Pythium*, *Rhizoctonia* in many

crops such as, corn, soybean, potato, tomato, beans, cotton, peanut, and various trees (Khetan. 2001; Paulitz and Belanger. 2001). There are many species of *Trichoderma* which reported to be biological control agents against plant pathogens for example *T. harzianum*, *T. viride*, *T. virens* (Benítez *et al.* 2004; Sunantapongsuk *et al.* 2006; Zeilinger and Omann. 2007). Among them, *T. harzianum* is reported to be most widely used as an effective biological control agent (El-Katathy *et al.* 2001; Szekeres *et al.* 2004; Abdel-Fattah *et al.* 2007) *Trichoderma* species have been very successfully used as biocontrol agent because they are fast growing, have high reproductive capacity, inhibit a broad spectrum of fungal diseases, have a diversity of control mechanisms, are excellent competitors in the rhizosphere, have a capacity to modify the rhizosphere, are tolerant or resistance to soil fungicides, have the ability to survive under unfavorable conditions, are efficient in utilizating soil nutrients, have strong aggressiveness against phytopathogenic fungi, and also promote plant growth. Their ability to colonize and grow in association with plant roots is known as rhizosphere competence (Tang *et al.* 2001; Benítez *et al.* 2004; Vinale *et al.* 2004).

Other fungi that can be used as biological control agents are *Penicillium* species, *Aureobasidium pullulans*, *Ulocladium atrum*, *Clonostachys rosea*. *Aureobasidium pullulans*. *Ulocladium atrum* have been tested for the control of *Botrytis aclada* which causes onion neck rot (Köhl *et al.* 1997). *Clonostachys rosea* was reported as a biological control agent against *Moniliophthora roreri* in cocoa crops (Hidalgo *et al.* 2003). *Ampelomyces quisqualis* showed the ability to reduce the growth and kills powdery mildews (Kiss. 2003). This fungus was the first organism reported to be a hyperparasite of powdery mildew and it can be easily found associated with powdery mildew colonies (Paulitz and Belanger. 2001). *Coniothyrium minitans* has been reported to be a mycoparasite of *Sclerotinia minor*, *S. sclerotiorum*, *S. trifoliorum* and *S. cepivorum* (Yang *et al.* 2007; Viterbo *et al.* 2007; Whipps *et al.* 2008). It has been applied successfully to control disease in many crops including lettuce (Jones and Whipps. 2004), oil oilseed rape (Li *et al.* 2006), peanut (Partridge *et al.* 2006) and alfalfa (Li *et al.* 2005). The genus *Fusarium* includes both plant-pathogenic and non-pathogenic races (Larkin and Fravel. 1999). The non-pathogenic species are known to have effective biocontrol activity (Whipps. 2001; Harman *et al.* 2004; Kvas *et al.* 2009). The use of non-pathogenic strains of *Fusarium oxysporum* to control Fusarium wilt has been reported for many crops (Fravel *et al.* 2003; Kvas *et al.* 2009). Similarly the genus *Rhizoctonia* contains both plant-pathogenic and non-pathogenic species and the latter can act as biocontrol agents (Harman *et al.* 2004). *Pythium oligandrum* has shown ability to reduce damping-off disease caused by *P. ultimum* in sugarbeet both in the laboratory

and in the field (Lewis *et al.* 1989; Khetan. 2001). This fungus has effects to control pathogens in the rhizosphere and has effects to induce plant resistance (Le Floch *et al.* 2003). *Pythium nunn* is also an antagonistic fungus of pathogens such as *Rhizoctonia solani*, *Phytophthora cinnamomi, P. parasitica*, *Pythium aphanidermatum, P. ultimum* and *P. vexans* (Khetan. 2001; Viterbo *et al.* 2007).

Biological control can result from many different types of interactions between biological control agents and plant pathogens (Pal and Gardener. 2006). These interactions were as follows:- physical contact and synthesis of hydrolytic enzymes, toxic compounds or anti-biotics, competition. Moreover they may induce resistance in the host plant (Benítez *et al.* 2004; Pal and Gardener. 2006). The mechanisms of biological control agents with the pathogen are many and complex. Mechanisms are influenced by soil type, temperature, pH, moisture content of soil and by the presence of other microorganisms (Howell. 2003). The understanding of mechanisms of biological control agent and plant diseases is critical to improve and use of biocontrol methods. Recent advances in the study of molecular genetics have provided a powerful tool that will aid in unraveling these basic mechanisms (Fravell. 1988). The mechanisms are classified as antibiosis, competition, mycoparasitism or lysis and induced resistance (Chet *et al.* 1990; Hoitink and Boehm. 1999; Irtwange. 2006).

Antibiosis is defined as the inhibition or destruction of the microorganism by substances which produced by microorganisms that inhibit the growth of another microorganism (Haggag and Mohamed. 2007). The substances were specific or nonspecific metabolites, antibiotics, or other toxic substances. Antibiotics can poison or kill other microorganisms (Pal and Gardener. 2006). Many biological control agents produce several types of antibiotics (Handelsman and Stabb. 1996). Some antibiotics have been shown to play a role in disease suppression, impede spore germination (fungistasis), or kill the cells (antibiosis) (Lewis *et al.* 1989; Benítez *et al.* 2004). *Gliocladium* and *Trichoderma* species produce a wide range of antibiotics and suppress disease by diverse mechanisms (Whipps. 2001; Harman *et al.* 2004) such as gliovirin, gliotoxin, harzianic acid, trichoviridin, viridin, viridiol, alamethicins, and Trichotoxin A50 (Suwan *et al.* 2000; Soytong. 2001; Howell. 2003; Vinale *et al.* 2008). These antibiotics are synergistic when combined with various cell wall degrading enzymes thus producing a strong inhibitory effect on many plant pathogens (Benítez *et al.* 2004). Many *Chaetomium* species produce many antibiotics substances such as *Chaetomium globosum* produces the chaetoglobusin C (Soytong *et al.* 2001) and chaetoviridins A (Park *et al.* 2005), *Chaetomium cupream* produces rotiorinol

(Kanokmedhakul *et al.* 2006). These abtibiotics have properties to control plant pathogens (Soytong *et al.* 2001; Park *et al.* 2005; Kanokmedhakul *et al.* 2006).

Several researchers have investigated the secondary metabolites or bioactive compounds that can control plant pathogens. There are many reports indicating that the extracts from fungi could inhibit spore production and mycelial growth of pathogens. For example the bioactive compounds extracted from *Trichoderma harzianum* PC01, *T. hamatum* PC02, *Chaetomium cupreum* CC, and *Ch. globosum* CG could inhibit spore production and mycelial growth of *Phytophthora parasitica*, *Colletotrichum gloeosporioides* and *C. paradoxa* (causing agent of fruit rot of Sala) (Meepeung and Soytong. 2004; Jitkasemkuk and Soytong. 2004). The bioactive compounds extracted form *Scleroderma critinum* and *Chaetomium globosum* could inhibit the mycelial growth and spore production of *Fusarium oxysporum* f.sp. *lycopersici*, *Phytophthora parasitica*, and *P. palmivora* (Srinon *et al.* 2004). The bioactive compounds extracted from *Chaetomium cochliodes*, *Chaetomella* sp and *Beauveria bassiana* could inhibit the mycelial growth and spore production of *Fusarium oxysporum* f.sp. *lycopersici* (Nuanjamrat and Soytong. 2004).

Competition between pathogens and non-pathogens for nutrients and space is important for limiting disease incidence and severity (Pal and Gardener. 2006). The competition for space and nutrient usually occurs in rhizosphere soil (Howell. 2003; Viterbo *et al.* 2007). Competition within and between species results in decrease growth and activities of weakness microorganism (Pal and Gardener. 2006). Therefore, competition between the biocontrol agent and the pathogen can result in displacement to the pathogen, competition for food and essential elements (Chet *et al.* 1990; Irtwange. 2006), for example, *Trichoderma harzianum* T-35could control *Fusarium* species on various crops occurs via competition for nutrients and rhizosphere colonization (Viterbo *et al.* 2007).

Mycoparasitism involves the complex process that includes the following steps: the chemotopic growth of the antagonist to the host; recognition of the host by mycoparasite; attachment; excretion of extracellular enzymes, lysis and exploitation of the host (Whipps. 2001; Viterbo *et al.* 2007). Many biological control agents produce and release lytic enzymes that able to lyse hyphae of pathogens (Pal and Gardener. 2006) such as chitinases, proteases, and β-1, 3 glucanases (Whipps. 2001). β-1, 3 glucanases have a good property for degrading cell walls, inhibiting mycelium growth and spore germination of plant pathogenic fungi (Sun *et al.* 2006; Lin. 2007). Chitinases play an important role in the degradation of chitin, the main cell wall

structure component of fungi (Cruz *et al.* 1992; Whipps. 2001). Proteases are involved in the degradation of pathogen hyphal membranes and cell walls (Elad and Kapat. 1999).

Plant respond to a variety of environmental stimuli, including gravity, light, temperature, physical stress, water and nutrient availability and also respond to a variety of chemical stimuli produced by soil-and plant associated microorganisms. These stimuli can either induce or condition to plant host defense through biochemical changes that enhance resistance against pathogens (Pal and Gardener. 2006). Induced resistance occurs in most plants in response to infestation by pathogens (Harman *et al.* 2004). Induced resistance by biocontrol agents involves the same suite of genes and gene products that involved in plant response known as systematic acquired resistance (SAR) (Handelsman and Stabb. 1996; Whipps. 2001). *Trichoderma* strains are capable to protect plants against many pathogens because they can induce resistance mechanisms that similar to the hypersensitive response (HR), systematic acquired resistance (SAR), and induced systematic resistance (ISR) in plants (Benitez *et al.* 2004; Haggag. 2008). Other fungal species can also induce resistant responses in plants, for example; *Chaetomium globosum* produces chaetoglobosin C that act to induce plant immunity for disease resistance in carrots, potatoes, sweet potatoes, tomatoes (Soytong *et al.* 2001; Kanokmedhakul *et al.* 2002). A non-pathogenic strain of *Fusarium*, *Pythium ultimun*, and *Rhizoctonia* could also induce plant resistance to pathogenic stains (Harman *et al.* 2004). Resistance may be resulted in an increase in the concentration of metabolites and enzymes related to defense mechanisms, such as phenyl-alanine ammonio-lyase (PAL) and chalcone synthase (CHS) (Viterbo *et al.* 2007). These enzymes are involved in the biosynthesis of phytoalexins, chitinases and glucanases. The metabolites produced by *Trichoderma* act as elicitors of plant resistance (Benitez *et al.* 2004).

Pathogens would be susceptible to one or more biocontrol agents, but practical implementation on a commercial scale has been constrained by many factors such as cost, convenience, efficacy and reliability of biological control. Cultural practices such as good sanitation, soil preparation, water management, host resistance can go a long way towards controlling many diseases. Biocontrol should be applied when cultural practices are insufficient for effective disease control. Research unreveals the various conditions needed for successful biocontrol of different diseases (Pal and Gardener. 2006).

The methods used to control root disease vary according to the region, type of culture, and the means available (Nandris *et al.* 1987). White root disease is being controlled by an

integration of cultural and chemical methods (Guyot and Flori. 2002). The example of cultural control are 1) clearing of the wood debris remaining in the soil before rubber trees are planted, 2) improving the soil conditions not favor to the growth of pathogen, 3) digging around the infected rubber tree to isolate disease and prevent spread of infection, and 4) exposing the roots, cutting out and burning of the infected parts of the trees (Nandris *et al.* 1987; Guyot and Flori. 2002). The chemical fungicide such as carbolineum which smeared the end cut of infected root, sulfur and PCNB which applied around the collar of the infected trees or applied at the time of planting by mixed with the soil, are recommended. Early application of sulfur can reduce white root disease incidence by up to 50%. However, these methods consume labor-intensive, difficult to apply on a large scale. The application of fungicides in large area was limited due to short duration of action, high cost of application, and low effectiveness. For economic reasons, chemical fungicides are not recommended for mature rubber trees (Nandris *et al.* 1987; Guyot and Flori. 2002). Moreover, chemical fungicides have been known to have a negative effect on human health, cause environmental pollution and leave residues in the agricultural soil (Soytong *et al.* 2005; Haggag and Moamed. 2007). Several plant pathogenic fungi have developed resistance to chemical fungicides (Benítez *et al.* 2004, Kim and Hwang. 2007). Sometimes it is too late for controlling white root disease by these methods because it has already spread to the next tree (Guyot and Flori. 2002). The important control strategies are to reduce the source of inoculum, as well as to inhibit disease spread, especially by the use of biological control agents. Biological control of pathogens, the total or partial destruction of pathogen populations by other organisms, occurs routinely in the nature (Agrios. 2005).

Antagonistic microorganisms for example *Aspergillus* species, *Penicillium* species, and *Trichoderma* species have been reported as biocontrol agents of white root disease (Nandris *et al.* 1987; Jayasuriya and Thennakoon. 2007). *Aspergillus* species was reported to be the effective agent against the white-rot Basidiomycetes (Nandris *et al.* 1987). Bruce and Highley (1991) stated that *Aspergillus* species could produce intense browning and lysis of the pathogen at the point of contact with an antagonist and this fungus did not overgrow and kill the Basidiomycetes when grown in competition on agar plates. *Trichoderma* strains were reported to be biological control agent. It quickly overgrew and killed Basidiomyctes causing wood decay fungi (Bruce and Heighley. 1991). *Trichoderma harzianum* was highly antagonistic against *R. microporus* on agar and rapidly overgrown on *R. microporus* colonies in vitro. The application of *T. harzianum* was able to recover the rate of the infected root 23.6%, while no recovery was recorded in control

in vivo (Jayasuriya and Thennakoon. 2007). The other species of *Trichoderma* which reported to be an effective antagonist for pretreatment to protect white rot fungi was *T. virens, T. viride, and T. hamatum* (Bruce and Heighley. 1991; Highley and Dashek. 1997; Jayasuriya and Thennakoon. 2007). *Trichoderma* species are known to be the effective antagonistic fungi because they posses a variety of mechanisms to combat other fungal species, these include fungistatic and fungicidal volatile and nonvolatile metabolite, high inoculum potential by rapid colonization and removal of available nutrients, and mycoparasitism by hyphal coiling associated with the production of a range of lytic enzyme including chitinase and β1-3 glucanase (Bruce and Highley. 1991; Highley and Dashek. 1997).

The biological control provides a useful addition to the use of chemical preservatives for limiting fungal decay. The mechanisms of antagonistic fungi are complex, unlike the mechanisms of chemicals. Then antagonistic fungi may be subject to change under different environmental conditions (Bruce and Highley. 1991; Highley and Dashek. 1997).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Sample Collection, Isolation and Pathogenicity Test

3.1.1 Sample collection

White root disease is found to be the most seriously disease in the rubber tree plantation in the South of Thailand, then disease collection was collected in the south at Narathiwat, Surat Thani, and Trang provinces. The infected roots were collected from the infected trees which can be observed as yellow leaves and fruiting bodies on basal dead stem. The samples were kept in plastic bags and brought to laboratory for isolation

3.1.2 Isolation of pathogen

Rigidoporus microporus, causing agent of white root disease of rubber tree was isolated by tissue transplanting technique and direct isolation method from infected roots or fruiting bodies. For tissue transplanting technique, the infected root was cleaned with tap water and air dried. The small pieces of infected root were surface disinfested for 1 minute in 10% clorox, and washed in sterile water then transferred to water agar (WA; consisted of 20 g agar and 1,000 ml distilled water) and incubated at room temperature (27-30°C). The mycelia growing out of the plant tissues were then transferred aseptically to potato dextrose agar (PDA; consisted of 200 g potato, 20 g dextrose, 20 g agar, and 1,000 ml distilled water), isolated to pure culture. For fruiting bodies, they were cleaned with tap water then air dried. The fruiting body was cut, then inner tissue was transferred to WA by sterile needle. The mycelia growing out of tissues were then transferred to PDA, isolated to pure culture. The pure cultures were maintained in PDA slant at room temperature.

For direct isolation method, the infected roots were cleaned with tap water and air dried, then placed in the moist chamber and incubated at room temperature. The mycelia growing out of the samples were transferred to WA and PDA successively.

Pure cultures which obtained from tissue transplanting technique and moist chamber were transferred to PDA slants and kept at room temperature for study their pathogenicities, morphology, and molecular phylogeny.

3.1.3 Pathogenicity test

3.1.3.1 Inoculum preparation

All isolates of *Rigidoporus microporus* which obtained from 3.1.2 were cultured on PDA for 6 days, thereafter the agar plug of each isolate was transferred to sterile sorghum seed containing in glass bottle and incubated at room temperature until the mycelium of pathogen grew completely colonized sterile sorghum seeds. The 400 g mixed substrate (sawdust : rice bran : sugar; 100 : 3 : 2 w/w moistened with water) containing in plastic bag was sterilized in autoclave at 121°C, 15 lbs/inch^2 for 1 hr. Then, each isolate which colonized in sorghum seeds was separately transferred onto sterilized mixed substrate and incubated at room temperature for 30 days or until the myceliaum grew completely in the mixed substrate and called inoculum. The inoculum of each isolate was taken to test for pathogenicity test.

3.1.3.2 Inoculation technique (modified from Rodesuchit. 1998)

The experiment was done by Completely Randomized Design (CRD) with four replications. The 5 months old of rubber trees var. RRIM600 were planted in pots containing sterilized mixed soil (soil : sand : compost; 8 : 8 : 2, sterile at 121°C, 15 lbs/inch^2 for 1 hour) and inoculum of each isolate was inoculated in the pot near the root zone of rubber trees as seen in Figure 3.1. The inoculated rubber trees were maintained in the nursery and observed for disease incidence for 90 days. The Disease Index (DI) was determined 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

Level 5 = 76-100% yellow leaves

The virulent isolates was determined according to DI. After rating the disease incidence, the pathogen was reisolated from infected root. The most virulence or aggressive isolate was selected for screening the antagonistic fungi and studying the property of antagonistic fungi to

inhibit the growth of *Rigidoporus microporus* in the pot experiment. Treatment means were compared with Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

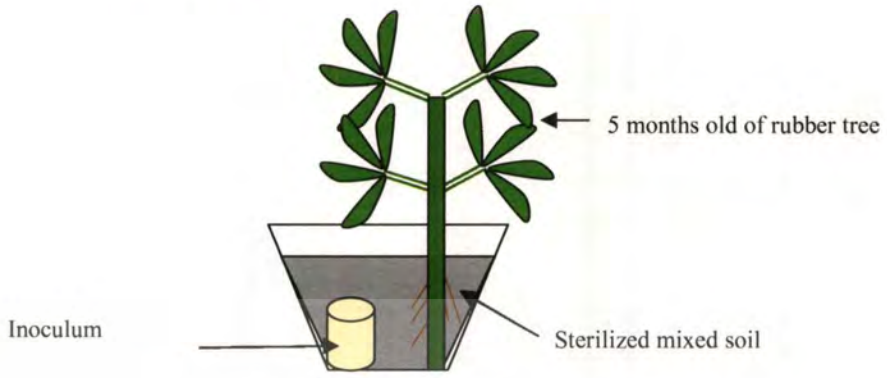


Figure 3.1 Inoculation technique.

3.2 Morphological and Molecular Phylogenetic Study

3.2.1 Morphological study

Mycelial discs of *R. microporus* was taken from the growing edge of colony, then placed onto PDA and incubated at room temperature. Colony diameter was recorded daily until the mycelium grew full the petri dish and the growth rate was calculated (mm per day). Colony colour, the characteristic of hypha and other specific structures were recorded and their photos were taken under compound microscope.

The fruiting body was also studied by culture each isolate in 800 g inoculum which prepared as same as 3.1.3.1. The plastic bag was removed, covered with soil, moistened every day until fruiting bodies were produced. The morphology of fruiting bodies such as shape, color, basidiospore were studied and their photos were taken.

3.2.2 Molecular phylogenetic study

The molecular phylogeny was studied to confirm the specie and to compare pathogenic variability among isolates of *R. microporus*. All isolates were done by the technique of DNA sequencing and inter-simple sequence repeats-polymerase chain reaction (ISSR-PCR).

3.2.2.1 DNA sequencing

DNA extraction: Mycelium of each isolate was separately grown on Petri dishes (90-mm diameter), containing 20 ml PDA and incubated at 25 °C for approximately 10 days. The mycelium was scraped from the surface of PDA, ground to fine powder using liquid nitrogen and transferred to 1.5 ml Eppendorf tube. The 600 µl Cetyltrimethylammonium bromide (CTAB) was added in the tube and incubated at 65 °C for 30 min. During incubated period, the tube was vortexed every 10 min. After that the tube was cooled for a few minutes then 600 µl chloroform : isoamyl alcohol (CIA; 24:1, v/v) was added, gently mixed and centrifuged at 7,000 rpm for 5 min at 4 °C. The aqueous layer was removed to new tubes and extracted again with CIA. Finally, DNA was precipitated by adding 300 µl isopropanol, mixed well then incubated at room temperature for 30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was decanted. The DNA pellet was suspended in 40 µl ddH2O.

PCR amplification and sequencing of ITS regions: The ITS regions were amplified using the universal primers ITS 1 (5′TCC GTA GGT GAA CCT GCG G 3′) and ITS 4 (5′TCC TCC GCT TAT TGA TAT GC 3′) (White *et al.* 1990). The amplifications were performed in reaction volumes of 50 µl containing 5 µl of 10x PCR buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 0.3 µl of 1.5 U *Taq* DNA polymerase, 1 µl of each primer, 1 µl genomic DNA and 37.7 µl of ddH_2O. PCR was carried out in a MyGene™ Series Peltier Thermal Cycler (Model MG96G) using the following program: 2 min initial denaturation at 94 °C followed by 35 cycles of 50 sec denaturation at 94 °C, 1 min annealing at 55 °C, 1 min extension at 72 °C, and final extension for 5 min at 72 °C. Amplification products were separated by 1% agarose gel in 1xTAE buffer strained with ethidium bromide which included in the agarose gel and visualized under UV fluorescence. PCR products were purified using the AxyPrep™ DNA Gel Extraction Kit (Axygen Scientific, Inc. USA) according to the manufacturer's instructions. DNA was sequenced by Shanghai Invitrogen Biotech Co., Ltd. (Shanghai, P.R China).

Data analysis: The ITS sequence from isolates and related species retrieved from GenBank (Table 3.1) were included in the analysed. *Auricularia delicata* was set up as out group. All sequences were edited and initial aligned by BioEdit, version 7.0.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit. html). Multiple sequence alignments were determined with the ClustalX version 1.83 program. Gaps were set as missing data. Phylogenetic analyses were performed from aligned sequences of data sets using PAUP 4.0 version 4.0 Beta 10

(Swofford, 2001). Optimality criterion was set to parsimony. Bootstrap analyses were based on 1000 replications.

3.2.2.2 Inter-simple sequence repeats-polymerase chain reaction (ISSR-PCR)

Inter-simple sequence repeats (ISSR) analysis was used to examine DNA polymorphism among the isolates. DNA extraction from the *Rigidoporus microporus* isolates was carried out as the same procedure as described as in 3.2.2.1.

Amplification conditions: Initially ten primers (Table 3.2) were used to screen for ISSR analysis. Only seven primers yielded polymorphic banding patterns. They were selected to analyze diversity among isolates of *Rigidoporus microporus*. Seven primers were follows: (AG)8C; (GA)8T, (TG)8A, (GA)8YG, (GT)8YC, GGGC(GA)8, and (CGA)5. To optimize reaction conditions, the concentration of $MgCl_2$ and the amount of 10 mM dNTP were determined. The annealing temperature was optimized to obtain a clear and reproducible patterns. The optimum reaction condition and G+C content of seven primers were shown in Table 3.3. The amplifications were performed in reaction volumes of 50 µl containing 5 µl of 10x PCR buffer, 2 µl of 10 µM primer, 3 µl of 25 mM $MgCl_2$ (for (TG)8A, (GA)8YG, GGGC(GA)8, and (CGA)5) and 4 µl of 25 mM $MgCl_2$ (for (AG)8C, (GA)8T and (GT)8YC), 1 µl of 10 mM dNTP, and 0.3 µl of 1.5 U Taq polymerase, 1 µl of genomic DNA, and 37.7 µl of ddH_2O. PCR was carried out in a MyGene™ Series Peltier Thermal Cycler (Model MG96G) under the following conditions: 2 min initial denaturation at 94 ^{0}C followed by 35 cycles of 1 min denaturation at 94 ^{0}C, annealing for 1 min at 52 ^{0}C, extension for 1.5 min at 72 ^{0}C, and final extension for 6 min at 72 ^{0}C. PCR products were separated by 1.5% agarose gel strained with ethidium bromide which included in the agarose and visualized under UV fluorescence.

Data analysis: The ISSR DNA bands obtained form each isolate were scored based on their presence (1) or absence (0). Only reliable and reproducible bands were considered for score. A similarity matrix was generated from the binary data using DICE similarities coefficient in SIMQUAL program of NTSYSpc Package (Rohlf. 2001). Dendrograms were constructed by cluster analysis based upon the unweighed pair group method with arithemetical mean (UPGMA) in the SAHN program of NTSYSpc package.

Table 3.1. Sequences from Genbank.

Taxon	Strain N0.	origin	Genbank acc. No.
Auricularia delicata		Costa Rica	AF291290
Fomes fasciatus	FP-1061048-T	USA	AM269766
Heterobasidion annosum	wb276	Austria	AF455496
Heterobasidion parviporum	E1	Latvia	FJ903330
Laetiporus gilbertsonii		USA	AM269785
Laetiporus sp.	6676	Uruguay	EU840673
Laetiporus sp.	6677	Uruguay	EU840674
Laetiporus sp.	6688	Uruguay	EU840675
Laetiporus sp.	6689	Uruguay	EU840676
Laetiporus sp.	6692	Uruguay	EU840677
Laetiporus sp.	6693	Uruguay	EU840678
Laetiporus sp.	6730	Uruguay	EU840681
Laetiporus sp.	5179	Uruguay	EU840682
Laetiporus sulphureus	TENN61397	USA	FJ596806
Oxyporus corticola	R-3714	USA	EF011122
Oxyporus corticola	R-3713	USA	EF011123
Oxyporus corticola	5385b	Estonia	DQ873641
Oxyporus corticola	C70	Latvia	FJ903327
Oxyporus cuneatus		Canada	DQ384575
Oxyporus latemarginatus	I239	Latvia	GU062267
Oxyporus latemarginatus	CTM10133	Tunisia	DQ000295
Oxyporus populinus	R-3716	USA	EF011121
Oxyporus subpopulinus	2313	China	FJ644281
Oxyporus subpopulinus	2251	China	FJ644282
Rigidoporus ulmarius		England	AY593868

Table 3.2 Ten primer sequences for ISSR technique.

Primer sequence	Primer length (bp)	%CG content
AGA GAG AGA GAG AGA GT	17	47.06
AGA GAG AGA GAG AGA GC	17	52.94
GAG AGA GAG AGA GAG AT	17	47.06
TGT GTG TGT GTG TGT GA	17	47.06
GAG AGA GAG AGA GAG AYG	18	52.78
GTG TGT GTG TGT GTG TYC	18	52.78
AGG GGA AGG GGA AGG GG	17	70.59
GGG CGA GAG AGA GAG AGA GA	20	60.0
CGA CGA CGA CGA CGA	15	66.67
GGC GGC GGC GGC GGC	15	100.00

Table 3.3 Primers sequences, the amount of $MgCl_2$ and dNTP, annealing time for ISSR analysis.

Primer sequence	$MgCl_2$ (µl)	dNTP (µl)	Annealing time (°C)
AGA GAG AGA GAG AGA GC	4	1	52
GAG AGA GAG AGA GAG AT	4	1	52
TGT GTG TGT GTG TGT GA	3	1	52
GAG AGA GAG AGA GAG AYG	3	1	52
GTG TGT GTG TGT GTG TYC	4	1	52
GGG CGA GAG AGA GAG AGA GA	3	1	52
CGA CGA CGA CGA CGA	3	1	52

Y= Pyrimidine

3.3 Screening Biological Control Agent Against *Rigidoporus microporus*

3..3.1 Isolation and identification of antagonistic fungi

3.3.1.1 Soil plate technique

Soil samples were collected from rubber trees plantation areas at Surat Thani, and Narathiwat provinces. The samples were kept in plastic bags and taken to the laboratory for isolation. Soil samples were ground and randomly taken at the weight of 0.005 g then placed in a sterile Petri dish (9.00 cm. diameter). Sterile glucose ammonium nitrate agar (GANA, containing 10 g glucose, 1 g yeast extract, 1 g NH_4NO_3, 0.5 g K_2HPO_4, 0.06 g Rose Bengal, 0.5 g $MgSO_4.7H_2O$, 20 g Agar, and 1,000 ml distilled water) was then poured into the Petri-dish of soil samples, mixed well and incubated in dark condition at room temperature and observed every day (Soytong. 1989). The single colony was transferred onto WA and PDA to be pure culture. Pure culture of each isolate was transferred onto PDA slant and kept at room temperature.

3.3.1.2 Baiting technique

Soil samples were placed in a sterile Petri dish and moistened with sterile water. The sterile pieces of rice straw and filter papers were placed on the top of soil. The Petri dish was incubated at the room temperature and periodically observed. The mycelium or the fruiting bodies of the fungus grown on the pieces of rice straw and filter papers were transferred to the isolating medium (water agar, WA). The hyphal tip was cut and transferred to PDA to get pure culture (Soytong. 1989).

All isolates obtained from soil plate and baiting techniques were morphologically identified by observing the characteristic of the hypha, spores, and other specific structures, measured and taken photo under compound microscope. Those isolates were taken to screen for biological control activities against the most aggressive isolate of *R. microporus* from 3.1.3.

3.3.2 Dual culture antagonistic test

The promising antagonistic fungi which obtained from 3.3.2.1 and 3.3.2.2 were tested for their antagonistic abilities to control the growth of *R. microporus*. The most aggressive isolate of *R. microporus* from 3.1.3 was used in this experiment. The experiment was carried out in the

laboratory using dual culture antagonistic test and using CRD with four replications. Means were compared by DMRT at $P = 0.05$ and $P = 0.01$. The promising antagonistic fungi were separately grown on PDA for 6 days at room temperature. The agar plug was removed with a sterile cork borer (3 mm. diameter) from the leading edge of colony and placed on the middle of a half of Petri dishes containing PDA (9-cm diameter). The agar plug of *R. microporus* at the age of 6 days peripheral was placed on the opposite side of Petri dish. The Petri dish which placed only the agar plug of *R. microporus* or promising antagonistic fungus was served as control. All tested Petri dish were incubated at room temperature for 10 days. Data collection was recorded as colony diameter (cm). Percentage of growth inhibition (PGI) was computed by following equation:-

$$PGI = \frac{Dc\text{-}Dd}{Dc} \times 100$$

where Dc is colony diameter of *R. microporus* in the control Petri dish.

Dd is colony diameter of *R. microporus* in the dual culture Petri dish.

The effective antagonistic fungi were selected according to PGI over 50%. Those were taken to study on antagonistic crude extract test.

3.3.3 Antagonistic crude extract test

Extraction method: The selected antagonistic fungi from 3.3.3 which gave PGI over 50% were separately cultured on PDA for 6 days. The agar plug of antagonistic fungus was transferred to Petri dish containing potato dextrose broth (PDB). The culture was incubated at room temperature for 30 days, thereafter filtered to yield mycelial mats. The mycelial mats were air dried, ground and weighted. The ground mycelium was extracted successively with hexane, ethyl acetate and methanol. Hexane was added into the flask containing ground mycelium and incubated for 5 days, then filtrated with filter paper. The filtrate was further extract through rotary vacuum evaporator to yield crude extract. The ethyl acetate was added into the marc and incubated for 5 days then filtered and evaporated the solvent to get crude extract. The methanol crude extract was as described above (Figure3.2) (Suwannapong. 2004). Crude extracts were kept in refrigerator until used.

Antagonistic crude extract test: The experiment was conducted using 3x6 factorials in CRD with 4 replications. Factor A was crude extracts of antagonistic fungus which extracted with

hexane, ethyl acetate, and methanol. Factor B was the concentration of crude extract at 0 (control), 10, 50, 100, 500, and 1,000 µg/ml. Each crude extract in each concentration was dissolved in Dimethylsulfoxide (DMSO), mixed with PDA before autoclaving at 121°C, 15 lbs/inch^2 for 15 min. The most virulent isolate of *Rigidoporus microporus* was cultured on PDA and incubated at room temperature for 6 days. The agar plug from actively growing colony was transferred and placed in the middle of PDA plate (5-mm diameter) incorporating with each concentration of crude extract, then incubated at room temperature for 5 days. The culture in PDA which mixed with DMSO was served as control. Data were collected as colony diameter (cm). Treatment means were compared using DMRT at $P = 0.05$ and $P = 0.01$. Effective dose (ED_{50}) of each crude extract was computed by probit analysis. The effect of antagonistic crude extract on mycelium of *R. microporus* was observed under compound microscope.

3.3.4 Bioactive compound test

Rotiorinol is a novel compound which discovered by Kanokmedhakul et al. (2006) which isolated from *Ch. cupreum*. Pure compound of rotiorinol was offered from Dr. Somdej Kanokmedhakul, Faculty of Science, Khon Kaen University. It was dissolved in DMSO before mixed to potato dextrose broth (PDB) in different concentrations and autoclaved at 121 °C, 15 pb/in^2 for 15 minutes. The agar plug of the most virulent isolate of *R.* microporus at 6 days were removed with a sterile cork borer (3 mm. diameter) from the leading edges of colony and transferred to the flask containing PDB which mixed with different concentrations of rotiorinol. The flasks were shaken at 100 rpm for 10 days. The fresh and dry weights of mycelium mass were recorded. The effect of rotiorinol on mycelium of *R. microporus* was also observed under compound microscope.

The experiment was conducted by Completely Randomized Design (CRD) with 4 replications. The treatments were different concentrations of rotiorinol as follows:- 0 (control), 10, 50, 100 and 250 µg/ml. Data were collected as fresh and dried weights which statistically computed analysis of variance. Treatment means were compared using DMRT at P = 0.05 and P = 0.01.

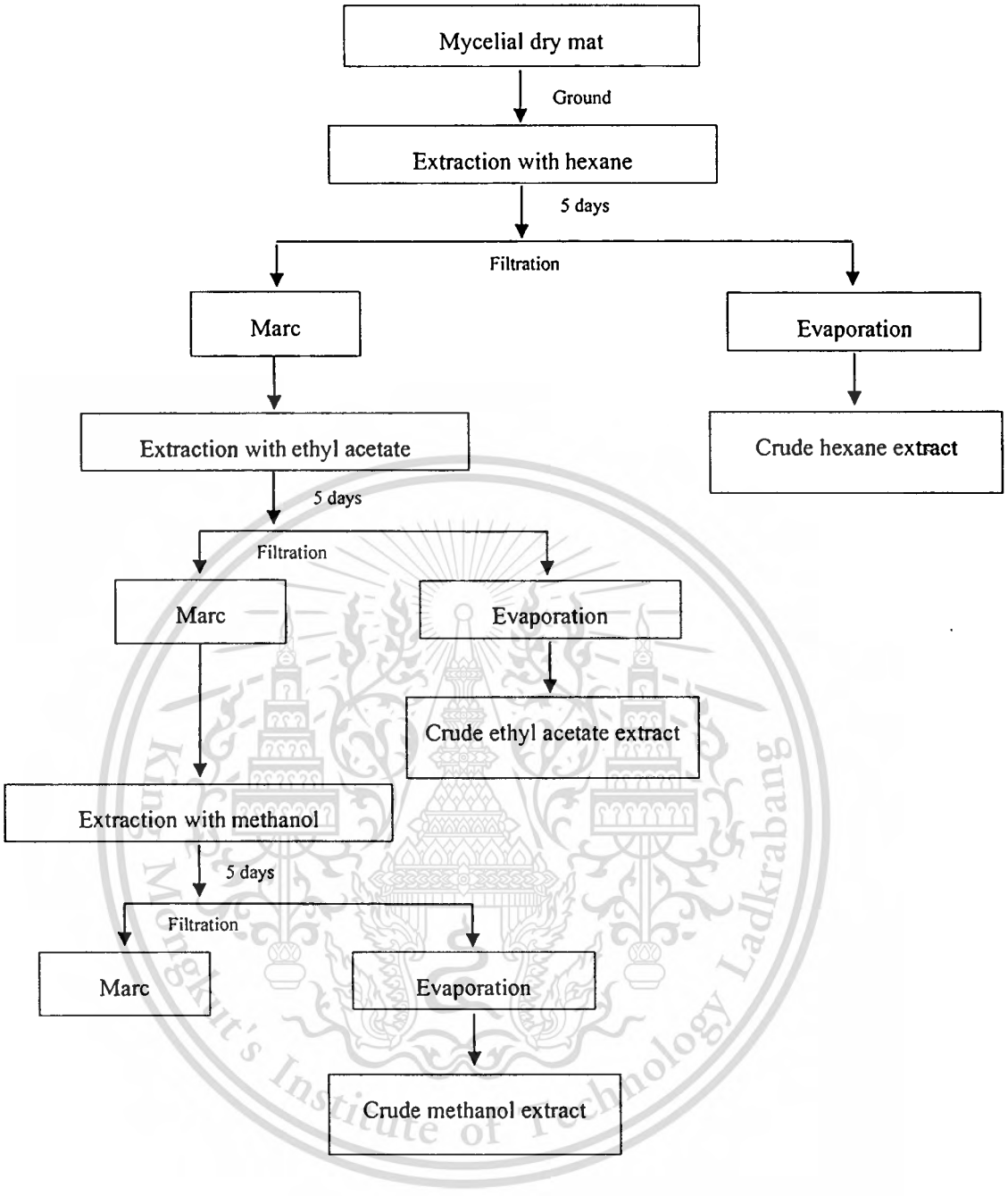


Figure 3.2 Crude extraction method.

3.4 Evaluation of Biofungicide to Control White Root Disease in the Pot Experiment

The experiment was designed by Completely Randomized Design (CRD) 4 replications. The treatments were as follows:-

T1 = non-treated one (control)

T2 = inoculated with pathogen (*R. microporus*)

T3 = treated with antagonistic fungus in powder form

T4 = treated with antagonistic fungus in the oil form

T5 = treated with antagonistic fungus in the powder form and pathogen

T6 = treated with antagonistic fungus in the oil form and pathogen

T7 = treated with fungicide (sulfur) and pathogen

The 100 g inoculum was prepared as same as previous experiment of pathoginicity test. The most virulent isolate of *R. microporus* from 3.1.3 was cultured on PDA for 6 days. The rubber tree variety RRIM 600 at the age of 5 months was planted in the pot containing sterilized mixed soil (soil : sand : compost; 8 : 2 : 2 v/v sterile at 121°C, 15 lbs/inch^2 for 1 hour). Thereafter, the inoculum was inoculated in the pot which planted rubber tree. The effective antagonistic fungus which gave the best results from 3.3.3, 3.3.4 and 3.3.5 was formulated in powder and oil formulation according to the methods of Dr. Kasem Soytong (unpublished data) at standard concentration 10^6 cfu/g for powder formulation and 10^6 cfu/ml for oil formulation. The oil and powder formulation were applied in the treatments as stated at the rate of 100 g or 100 ml/tree by mixing with planting soil. Thereafter, application the biofungicide in the powder and oil at the rate of 1 g/tree for powder form or 1 ml/tree for oil from every 2 weeks in T5 and T6. Sulfur was applied at the recommendation rate at 100 g/ tree by mixing with the planting soil.

Data collection as disease index (DI) was periodically recorded every 4 weeks. Infected root was also be observed and recorded. DI 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.

Data were statistically computed analysis of variance and treatment means were compared using DMRT at P = 0.05 and P = 0.01.

CHAPTER IV

RESULTS

4.1 Sample Collection, Isolation and Pathogenicity Test

4.1.1 Sample collection

White root disease was found in the rubber tree plantation in the South of Thailand where the climate is suitable for this disease. Sample collection was occurred at Narathiwat, Trang, and Surat Thani province (Figure 4.1). This disease can infect to young and the old trees. The visible symptom of white root disease was seen by changed in color the leaves from green to yellow. The yellowing leaves were observed on one or a few branches or whole canopy depend on the severity of disease (Figure 4.2A). The dead trees were also observed (Figure 4.2B). The causing agent produced the fruiting bodies at the collar of the dead stem. The fruiting bodies were broadly attached shelf and orange red brown in color (Figure 4.2C). The fruiting bodies were normally produced in the rainy season. In humid condition, there were rhizomorph of the pathogen at the infected root (Figure 4.2D). The rubber trees died in the large area if the disease severely occurred (Figure 4.2E). Infected roots and fruiting bodies (Figure 4.2C and D) of the pathogen were collected and taken to laboratory for isolation.

Figure 4.1 The area of sample collection in the South of Thailand.

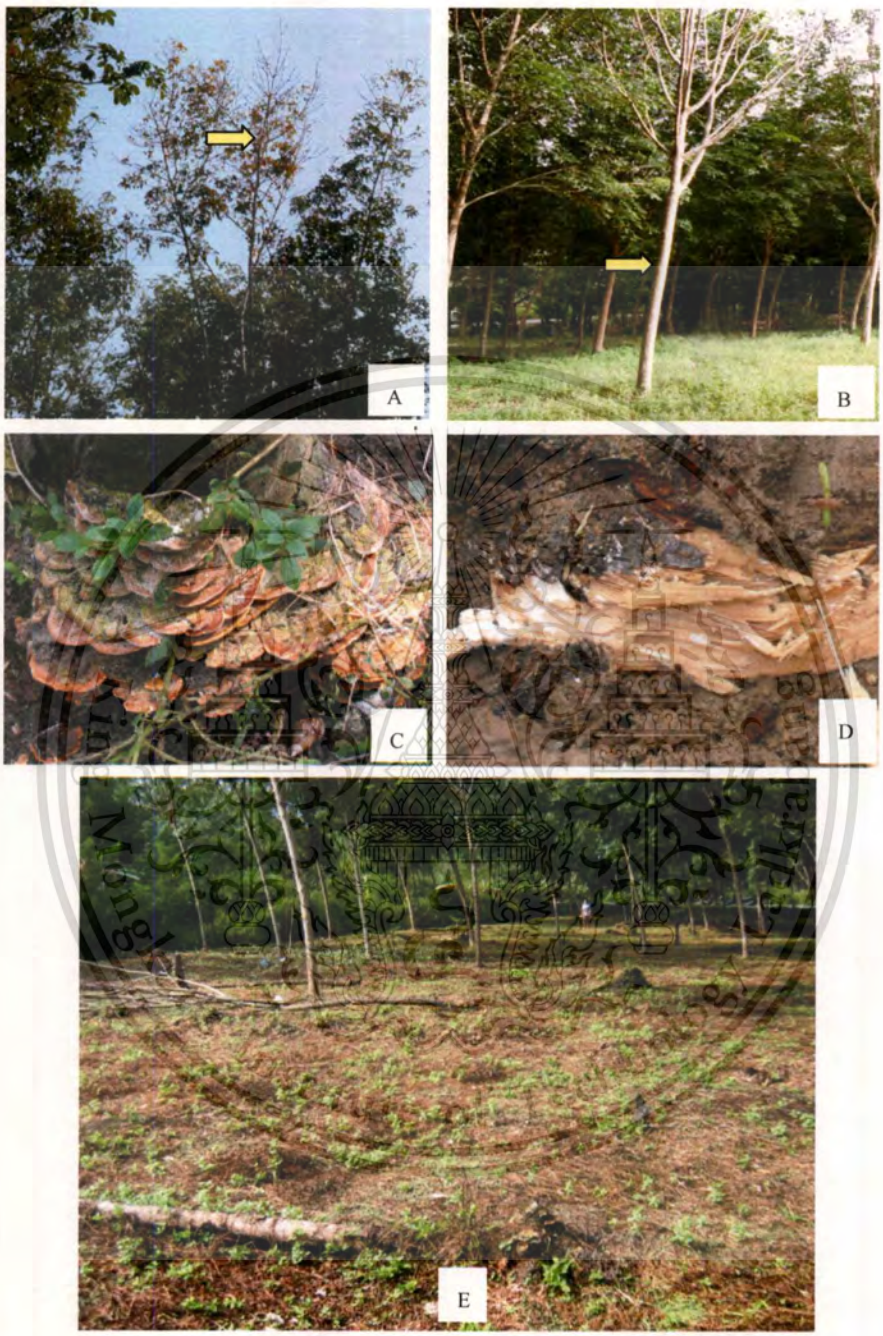


Figure 4.2 Symptom of white root disease in the field.

A = yellowing leaves, B = dead tree, C = fruiting bodies at the collar of the dead stem, D = infected root and E = infested area.

4.1.2 Isolation of pathogen

A total of collection as the infected root and fruiting bodies were isolated by tissue transplanting technique and direct isolation method. Fifty isolates were obtained from three provinces including 27 isolates from Narathiwat province as follows:- SND04, SND05, SND07, SND08, SND10, SNK02, SNK03, SNK04, SNK05, SNK06, SNK09, SNK10, SNP02, SNP05, SNP06, SNP08, SNS01, SNS02, SNS03, SNS04, SNS05, SNS06, SNS07, SNS08, SNS09, SNS10, SNS11, 8 isolates from Trang province as follows:- STR01, STR02, STR03, STR04, STR05, STR06, STR07, STR08, and 15 isolates from Surat Thani province as follows:- SSS01, SST01, SST02, SST04, SST05, SST06, SST07, SST08, SST09, SST11, SST12, SST13, SST14, SST15, SST16 (Table 4.1). All isolates were maintained on PDA slant and kept at room temperature (27-30 ^{0}C). These isolates were morphologically studied and test for their pathogenicities followed Koch's Postulation.

Table 4.1 The isolates of *Rigidoporus microporus* in this study.

Province	Sites	Isolates[1]	Total
Narathiwat	Kokparimeng	SNK02, SNK03, SNK04, SNK05, SNK06, SNK09, SNK10	7
	Parulu	SNP02, SNP05, SNP06, SNP08	4
	Sungai-Padi	SNS01, SNS02, SNS03, SNS04, SNS05, SNS06, SNS07, SNS08, SNS09, SNS10, SNS11	11
	Todeng	SND04, SND05, SND07, SND08, SND10	5
Trang	Muang	STR01, STR02, STR03, STR04, STR05, STR06, STR07, STR08,	8
Surat Thani	Muang	SSS01	1
	Tachana	SST01, SST02, SST04, SST05, SST06, SST07, SST08, SST09, SST10, SST11, SST12, SST13, SST14, SST15, SST16	14
		Total	50

Code of isolate:- SNK = Saithong, Narathiwat, and Kokparimeng, SNP = Saithong, Narathiwat, and parulu, SNS = Saithong, Narathiwat, and Sungai-Padi, SND = Saithong, Narathiwat, and Todeng, STR = Saithong and Trang, SSS = Saithong, Muang and Surat Thani, SST = Saithong, Tachana and Surat Thani

4.1.3 Pathogenicity test

All isolates of *R. microporus* were tested for their pathogenicities with 5-months rubber tree variety RRIM600. The disease incidence was determined at 90 days. Disease index (DI) was recorded 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, level 5 = 76-100% yellow leaves (Figure 4.3).

The disease symptom of white root disease showed the yellowing leaves. There was rhizomoph of the pathogen at the root of the dead tree. It appeared white. During the pathogenicity test, there was the mycelium of the causing agent at the basal stem if it was in the moist condition. There was rhizomorph of the pathogen at the root of the dead rubber tree. It produced the fruiting bodies at the basal dead stem. The fruiting bodies showed orange-red-brown in color.

Pathogenicity test of all isolates on rubber trees was showed in Table 4.2. There was significantly different in disease incidence at $P = 0.05$. Base on the results, all isolates were grouped into three categories depended on DI as follows: low virulent isolates (DI = 1.0 – 2.0) moderately virulent isolates (DI = 2.1 – 4.0) and high virulent isolates (DI = 4.1 – 5.0). There were 25 isolates which grouped into low virulence as follows:- SND05, SND07, SNP05, SNK04, SNK09, SNK10, SNS01, SNS02, SNS04, SNS05, SNS06, SNS08, SNS09, SNS10, STR01, STR03, STR05, STR06, STR07, STR08, SSS01, SST08, SST09, SST11, and SST14. A total of 22 isolates were found moderately virulence as follows:- SND04, SND10, SNP02, SNP06, SNP08, SNK05, SNK06, SNS03, SNS07, SNS11, STR02, STR04, SST01, SST02, SST04, SST05, SST06, SST07, SST12, SST13, SST15, and SST16, and only 3 isolates showed high virulence as follows:- SND08, SNK02 and SNK03. These three high virulent isolates were obtained from Narathiwat Province. As a result, isolate SNK02 was selected for further study.

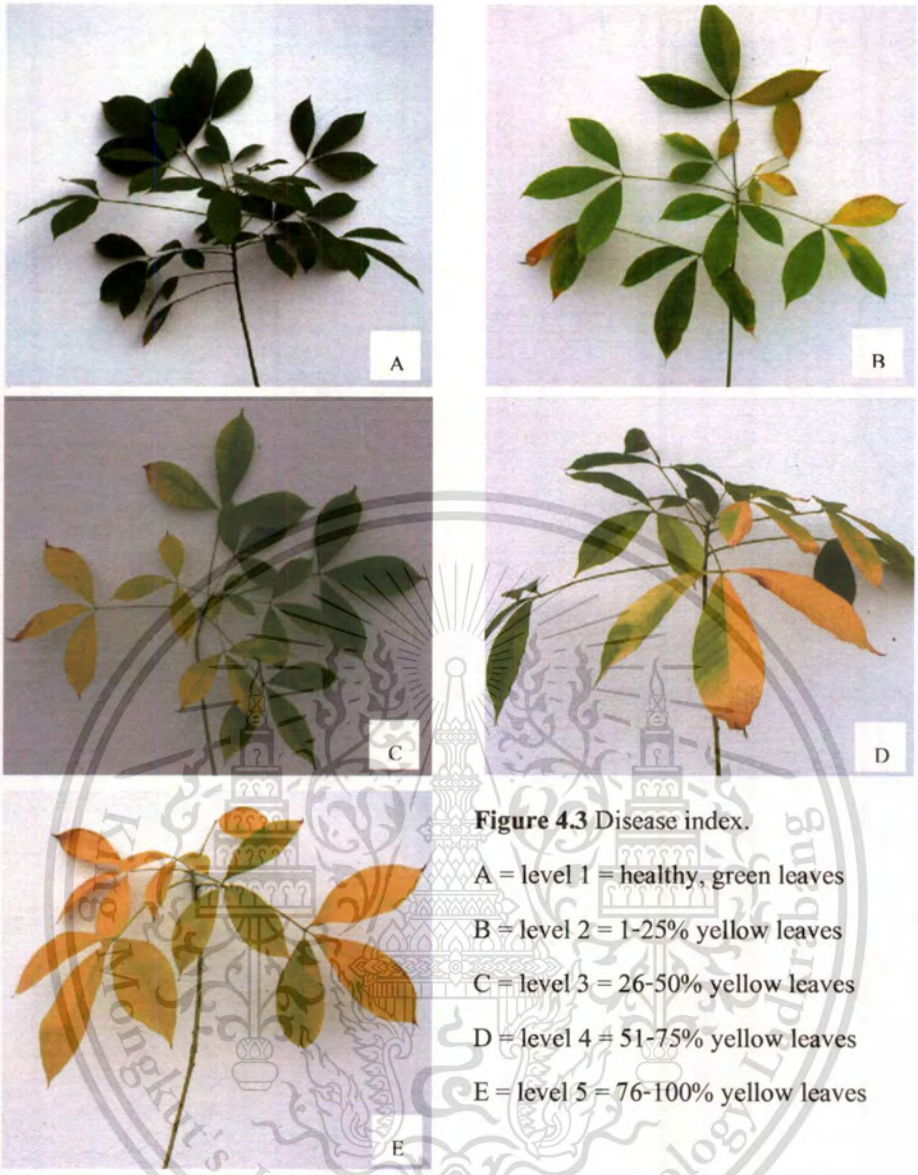


Figure 4.3 Disease index.

A = level 1 = healthy, green leaves

B = level 2 = 1-25% yellow leaves

C = level 3 = 26-50% yellow leaves

D = level 4 = 51-75% yellow leaves

E = level 5 = 76-100% yellow leaves

Table 4.2 Pathogenicity test of *Rigidoporus microporus*.

Virulent Group	Isolates	Disease Index (DI)[1]	Isolates	Disease Index (DI)
Low virulence	SND05	2.0bcd[2]	SND07	2.0bcd
	SNP05	2.0bcd	SNK04	2.0bcd
	SNK09	2.0bcd	SNK10	2.0bcd
	SNS01	1.3cd	SNS02	2.0bcd
	SNS04	1.3cd	SNS05	2.0bcd
	SNS06	1.0d	SNS08	2.0bcd
	SNS09	2.0bcd	SNS10	1.5cd
	STR01	1.0d	STR03	1.0d
	STR05	1.8bcd	STR06	1.0d
	STR07	1.8bcd	STR08	1.3cd
	SSS01	1.3cd	SST08	1.3cd
	SST09	2.0bcd	SST11	1.3cd
	SST14	2.0bcd		
Moderately virulence	SND04	3.8abcd	SNP02	3.0abcd
	SND10	2.8abcd	SNP06	3.0abcd
	SNP08	3.5abcd	SNK05	4.0abc
	SNK06	4.0abc	SNS03	2.5abcd
	SNS07	2.5abcd	SNS11	3.3abcd
	STR02	3.0abcd	STR04	2.8abcd
	SST01	3.0abcd	SST02	3.0abcd
	SST04	2.3abcd	SST05	4.0abc
	SST06	2.5abcd	SST07	2.3abcd
	SST12	2.5abcd	SST13	2.8abcd
	SST15	3.3abcd	SST16	4.0abc
High virulence	SND08	4.5ab	SNK02	5.0a
	SNK03	5.0a		

[1]DI:- 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

[2]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

4.2 Morphological and Molecular Phylogenetic Study

4.2.1 Morphological study

The isolates of *Rigidoporus microporus* were studied the morphology on the PDA medium and their fruiting bodies. The colony on PDA at room temperature (27-30 ^{0}C) was circular in shape, white and flattening, grew full the PDA plate within 6 days with growth rate of 1.3 cm per day. The hypha of this fungus showed hyaline, septum, no clamp connection, and possess many branches. The width of the hypha was vary from 2.8- 7.2 um. It was observed that all isolates were similar in morphology on PDA. The fruiting body of this fungus was also studied by culture each isolate in the mixed substrate in plastic bag which consisted of saw dust, rice band, and sugar and leaved them at room temperature for 30 days. After the mycelium grew cover the mixed substrate, the plastic bag was removed, covered with soil and watered every day. The fruiting bodies were produced after 2 months. The fruiting bodies were broad in shape with the width up to 20 cm depending on the age, thin, leathery, broadly attached shelf and no stalk. The upper surface was orange–red–brown, smooth and the lower surface orange–brown, and fine pores. In section, the hyphal system was monomitic, hyaline, thick walled with septate without clamp connection, hymenium with cystidioles. Basidiospores were globose, thin-walled, colourless, and smooth. The spore size was 3.6 - 4.1 µm. The morphological descriptions of 50 isolates were presented in Table 4.3. The character of colony on PDA and fruiting bodies of isolates SNK02, SNS05, SST02, STR07, SNP06 and SND04 were shown in Figures 4.4-4.9.

Table 4.3. Morphological character of *Rigidoporus microporus* isolates.

Isolates	Colony pattern	Hyphal character	Hyphal width (μm)	Fruiting body character	Fruiting body width (μm)	Growth rate (cm/day)
SND04	Circular, white and flattening	Hyaline, septum, no clamp connection	3.2-7.0	Broad shape, orange-red-brown, smooth	12.3	1.25
SND05	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-7.2	-		
SND07	Circular, white and flattening	Hyaline, septum, no clamp connection	2.8-6.9	Broad shape, orange-red-brown, smooth	10.2	1.26
SND08	Circular, white and flattening	Hyaline, septum, no clamp connection	2.9-7.0	Broad shape, red-brown, smooth	9.0	1.25
SND10	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-7.1	-		
SNK02	Circular, white and flattening	Hyaline, septum, no clamp connection	2.8-6.8	Broad shape, orange-red-brown, smooth	14.8	1.31
SNK03	Circular, white and flattening	Hyaline, septum, no clamp connection	3.2-6.9	Broad shape, orange-red-brown, smooth	9.0	1.27
SNK04	Circular, white and flattening	Hyaline, septum, no clamp connection	2.9-7.0	-		
SNK05	Circular, white and flattening	Hyaline, septum, no clamp connection	3.2-6.7	Broad shape, orange-brown, smooth	12.7	1.32
SNK06	Circular, white and flattening	Hyaline, septum, no clamp connection	3.1-7.2	-		
SNK09	Circular, white and flattening	Hyaline, septum, no clamp connection	2.8-7.0	-		
SNK10	Circular, white and flattening	Hyaline, septum, no clamp connection	3.2-69	-		
SNP02	Circular, white and flattening	Hyaline, septum, no clamp connection	3.2-7.0	-		
SNP05	Circular, white and flattening	Hyaline, septum, no clamp connection	3.1-6.9	-		
SNP06	Circular, white and flattening	Hyaline, septum, no clamp connection	2.8-7.1	Broad shape, orange-red-brown, smooth	11.7	1.33
SNP08	Circular, white and flattening	Hyaline, septum, no clamp connection	2.8-7.0	Broad shape, orange-red-brown, smooth	9.0	1.31
SNS01	Circular, white and flattening	Hyaline, septum, no clamp connection	2.9-7.1	Broad shape, orange-brown, smooth	20.0	1.27

Table 4.3. Morphological character of *Rigidoporus microporus* isolates (Continue).

Isolates	Colony pattern	Hyphal character	Hyphal width (µm)	Fruiting body character	Fruiting body width (µm)	Growth rate (cm/day)
SNS02	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-7.2	-		
SNS03	Circular, white and flattening	Hyaline, septum, no clamp connection	2.9-7.0	-		
SNS04	Circular, white and flattening	Hyaline, septum, no clamp connection	2.8-7.0	-		
SNS05	Circular, white and flattening	Hyaline, septum, no clamp connection	3.2-7.2	Broad shape, orange-red-brown, smooth	12.3	1.34
SNS06	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-7.1	-		
SNS07	Circular, white and flattening	Hyaline, septum, no clamp connection	2.9-6.9	Broad shape, orange-red-brown, smooth	11.8	1.31
SNS08	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-6.8	--		
SNS09	Circular, white and flattening	Hyaline, septum, no clamp connection	3.1-7.1	Broad shape, orange-red-brown, smooth	8.6	1.26
SNS10	Circular, white and flattening	Hyaline, septum, no clamp connection	2.9-7.1	-		
SNS11	Circular, white and flattening	Hyaline, septum, no clamp connection	2.5-7.0	Broad shape, orange-red-brown, smooth	7.9	1.30
SSS01	Circular, white and flattening	Hyaline, septum, no clamp connection	2.7-7.2	Broad shape, orange-red-brown, smooth	9.0	1.30
SST01	Circular, white and flattening	Hyaline, septum, no clamp connection	2.6-7.2	-		
SST02	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-5.9	Broad shape, orange-red-brown, smooth	9.0	1.27
SST04	Circular, white and flattening	Hyaline, septum, no clamp connection	2.5-6.3	-		
SST05	Circular, white and flattening	Hyaline, septum, no clamp connection	2.8-7.1	-		
SST06	Circular, white and flattening	Hyaline, septum, no clamp connection	2.9-6.5	-		
SST07	Circular, white and flattening	Hyaline, septum, no clamp connection	3.4-6.8	Broad shape, orange-red-brown, smooth	11.0	1.30

Table 4.3. Morphological character of *Rigidoporus microporus* isolates (Continue).

Isolates	Colony pattern	Hyphal character	Hyphal width (μm)	Fruiting body character	Fruiting body width (μm)	Growth rate (cm/day)
SST08	Circular, white and flattening	Hyaline, septum, no clamp connection	3.2-5.9	Broad shape, orange-red-brown, smooth	10.5	1.31
SST09	Circular, white and flattening	Hyaline, septum, no clamp connection	2.5-7.2	-		
SST11	Circular, white and flattening	Hyaline, septum, no clamp connection	3.1-6.6	Broad shape, orange-red-brown, smooth	13.2	1.33
SST12	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-7.1	Broad shape, orange-red-brown, smooth	9.5	1.26
SST13	Circular, white and flattening	Hyaline, septum, no clamp connection	2.7-6.7	-		
SST14	Circular, white and flattening	Hyaline, septum, no clamp connection	2.4-6.5	-		
SST15	Circular, white and flattening	Hyaline, septum, no clamp connection	2.5-6.9	-		
SST16	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-6.6	-		
STR01	Circular, white and flattening	Hyaline, septum, no clamp connection	2.8-6.9	-		
STR02	Circular, white and flattening	Hyaline, septum, no clamp connection	2.5-6.7	-		
STR03	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-6.5	Broad shape, orange-red-brown, smooth	14.1	1.34
STR04	Circular, white and flattening	Hyaline, septum, no clamp connection	2.6-7.1	-		
STR05	Circular, white and flattening	Hyaline, septum, no clamp connection	3.2-6.5	Broad shape, orange-red-brown, smooth	9.0	1.25
STR06	Circular, white and flattening	Hyaline, septum, no clamp connection	3.0-7.1	-		
STR07	Circular, white and flattening	Hyaline, septum, no clamp connection	3.1-6.9	Broad shape, orange-red-brown, smooth	13.6	1.31
STR08	Circular, white and flattening	Hyaline, septum, no clamp connection	2.5-6.8	-		

- = No fruiting bodies

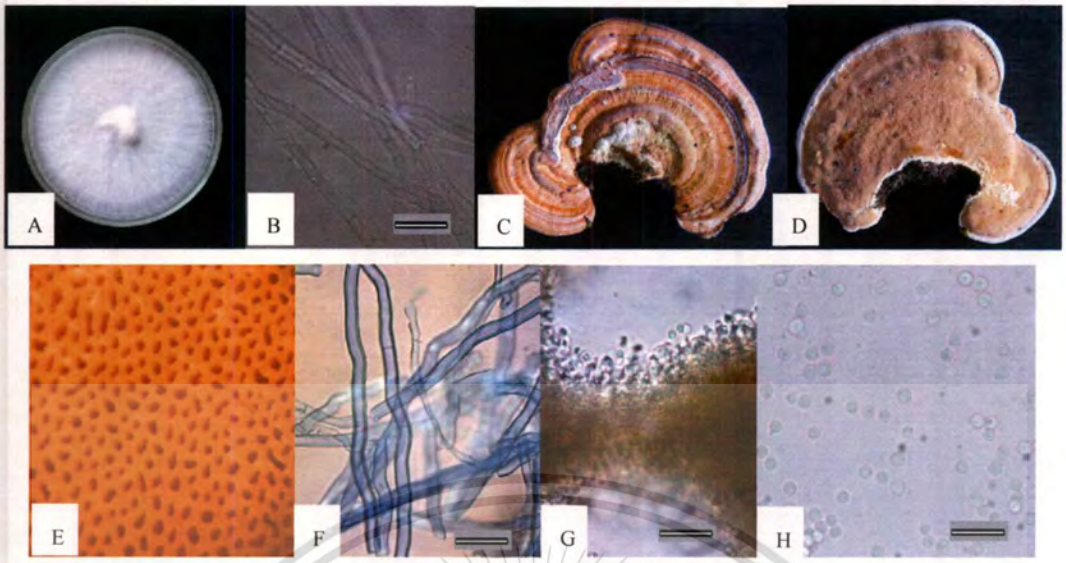


Figure 4.4 The characteristic of *Rigidoporus microporus* SNK02.

A = Colony on PDA at 6 days, B = hypha, C = fruiting body: upper surface, D = lower surface, E = pores at the lower surface, F = monomitic, generative hypha, G = hymenium and H = basidiospores. Bar. B, F, G, H = 10 µm

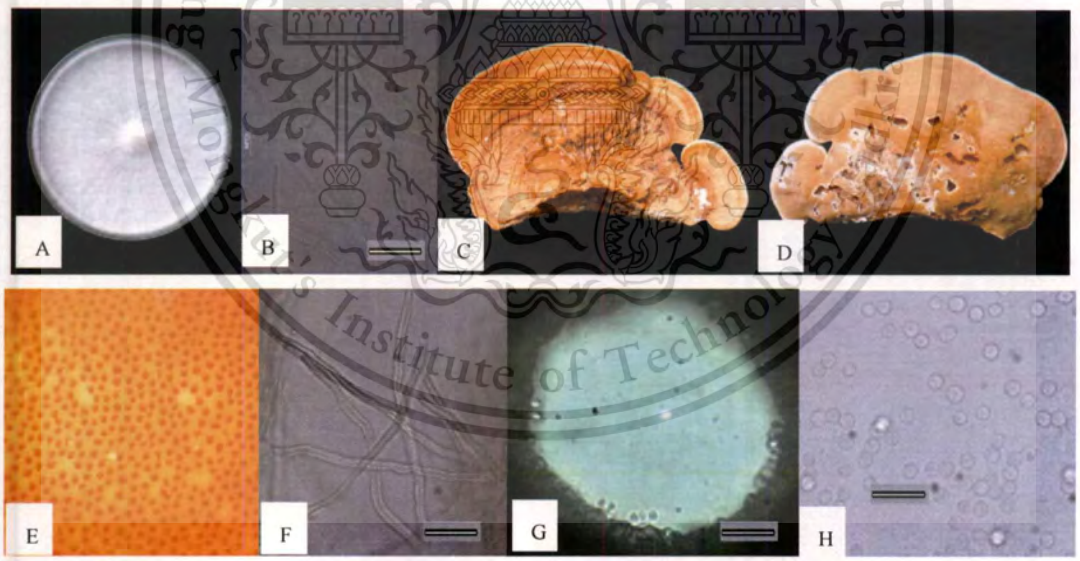


Figure 4.5 The characteristic of *Rigidoporus microporus* SNS05.

A = Colony on PDA at 6 days, B = hypha, C = fruiting body: upper surface, D = lower surface, E = pores at the lower surface, F = monomitic, generative hypha, G = hymenium and H = basidiospores. Bar. B, F, G, H = 10 µm

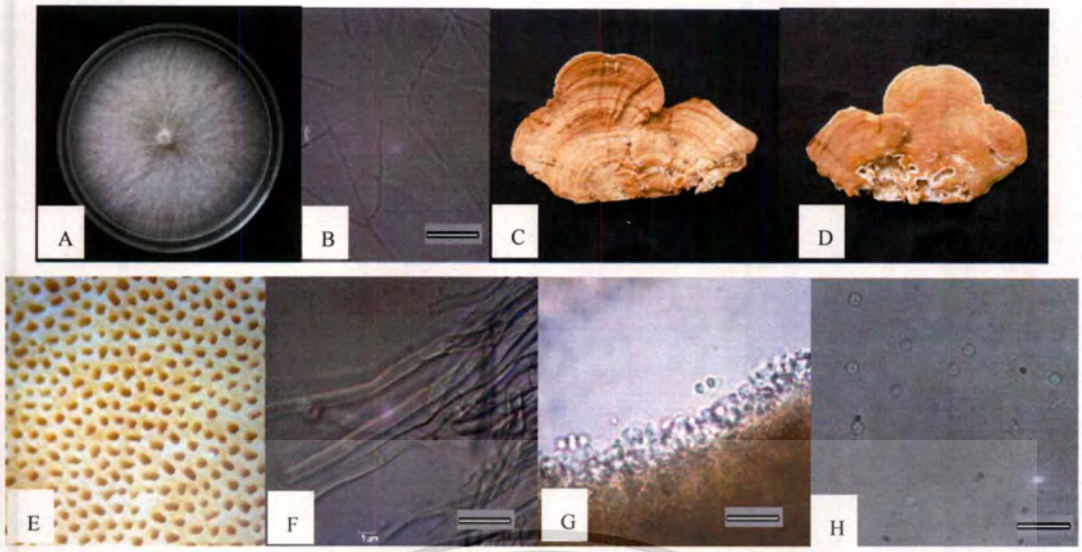


Figure 4.6 The characteristic of *Rigidoporus microporus* SST02.

A = Colony on PDA at 6 days, B = hypha, C = fruiting body: upper surface, D = lower surface, E = pores at the lower surface, F = monomitic, generative hypha, G = hymenium and H = basidiospores. Bar. B, F, G, H = 10 µm

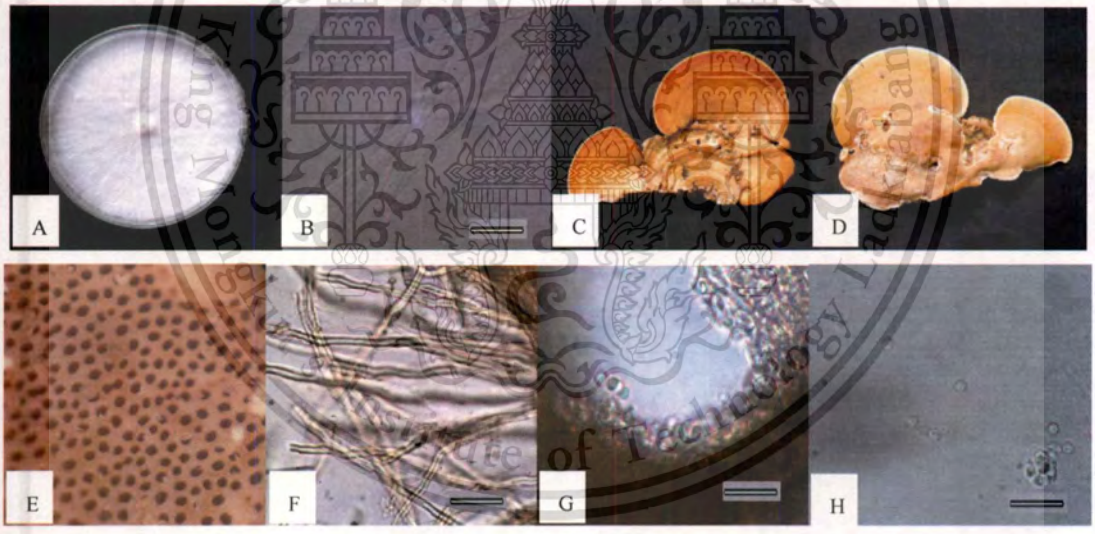


Figure 4.7 The characteristic of *Rigidoporus microporus* STR07.

A = Colony on PDA at 6 days, B = hypha, C = fruiting body: upper surface, D = lower surface, E = pores at the lower surface, F = monomitic, generative hypha, G = hymenium and H = basidiospores. Bar. B, F, G, H = 10 µm

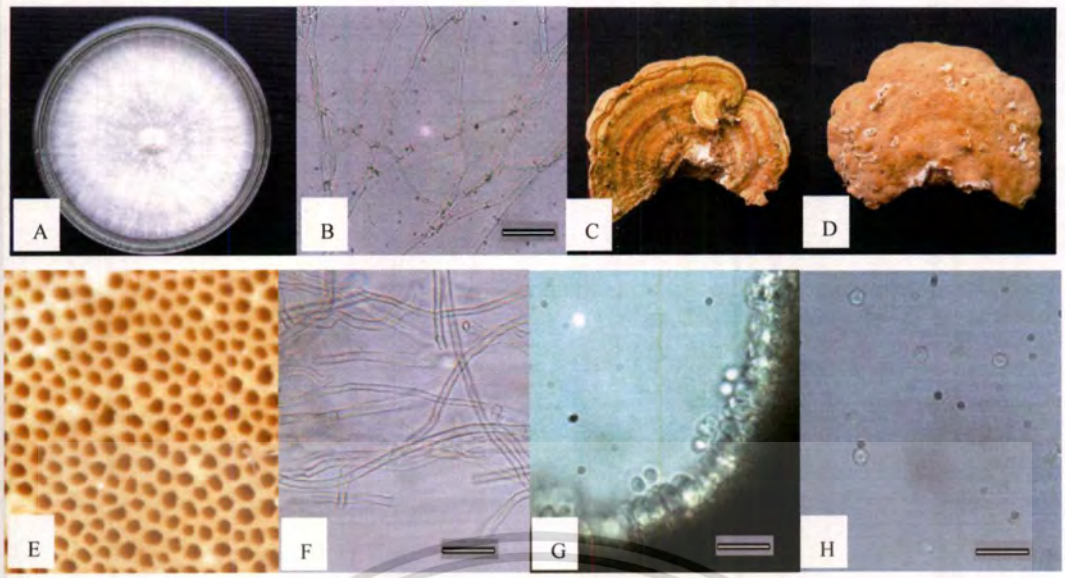


Figure 4.8 The characteristic of *Rigidoporus microporus* SNP06.

A = Colony on PDA at 6 days, B = hypha, C = fruiting body: upper surface, D = lower surface, E = pores at the lower surface, F = monomitic, generative hypha, G = hymenium and H = basidiospores. Bar. B, F, G, H = 10 μm

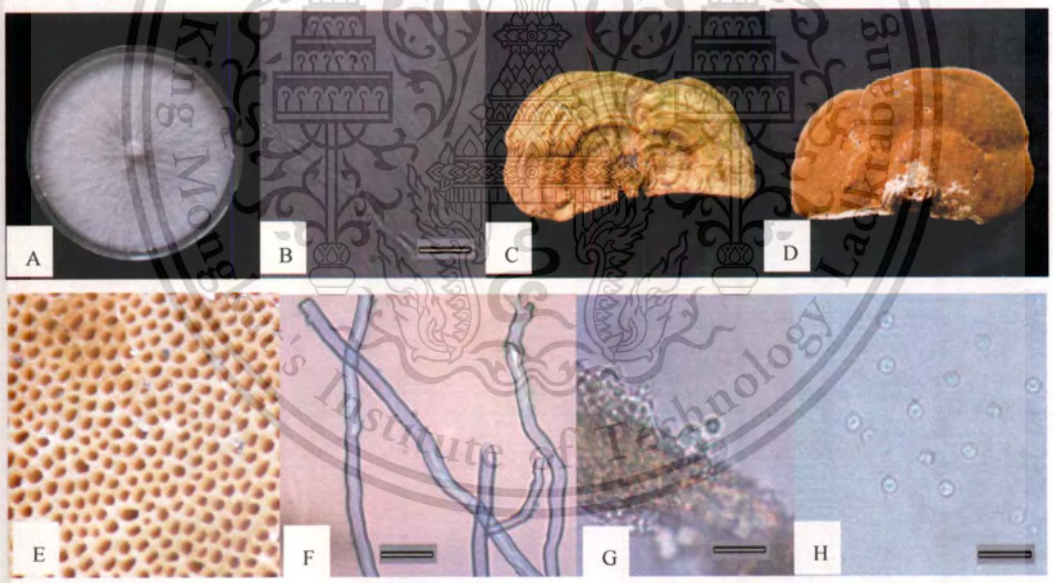


Figure 4.9 The characteristic of *Rigidoporus microporus* SND04.

A = Colony on PDA at 6 days, B = hypha, C = fruiting body: upper surface, D = lower surface, E = pores at the lower surface, F = monomitic, generative hypha, G = hymenium and H = basidiospores. Bar. B, F, G, H = 10 μm

4.2.2 Molecular phylogenetic study

The molecular phylogenetic study was confirmed the taxon of *R. microporus* by DNA sequencing and ISSR technique. This was firstly recorded in Thailand for *R. microporus*.

4.2.2.1 DNA sequencing

The final alignment of the ITS sequences comprised 32 taxa with 607 characters, 280 characters showed constantly data and 31 variable characters were parsimony-uninformative. The number of parsimony-informative characters was 296. The maximum parsimony (MP) heuristic search treating gaps as missing data with no differential weighting of transitions against transversions was studied using random addition sequence and tree-bisection-reconnection (TBR) branch-swapping algorithm resulted in 1 tree. When a weighted parsimony was applied to the same data set, with alignment gaps treated as missing data, the results also yielded 1 tree with consistency index=0.806, retention index=0.948, rescaled consistency index=0.764 and homoplasy index=0.194.

Parsimony analysis of the ITS sequence alignment resulted in one most parsimonious tree. Three major groups were detected. The first group was *Oxyporus* group, the second group was *Rigidoporus* group and the third group was *Laetiporus* group. ITS confirmed that *R. microporus* was distincted from *R. ulmarious* but similar correlated to each other (Figure 4.10).

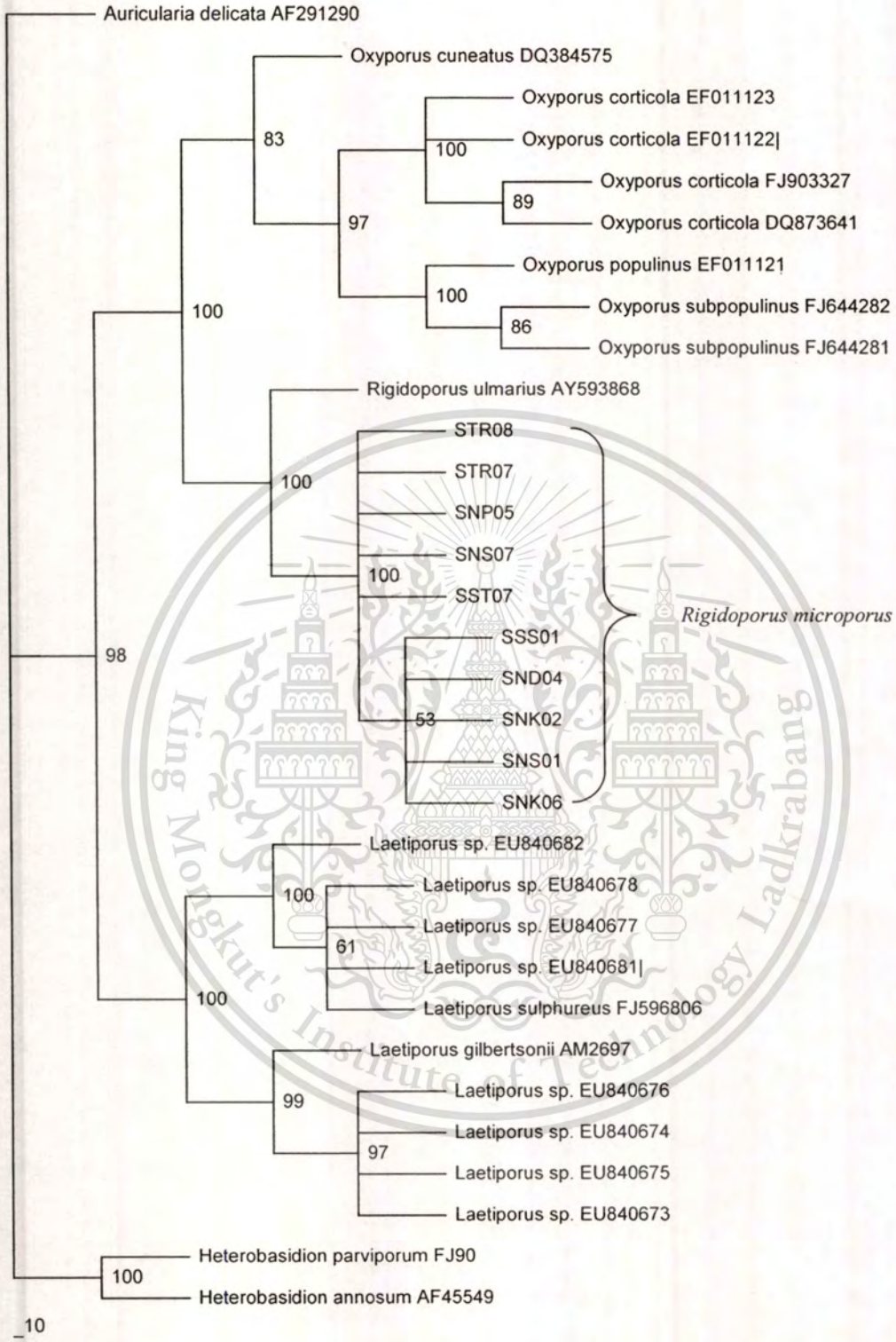


Figure 4.10 Tree derived from maximum parsimony analysis of the sequences of the ITS region. The number at the branches denotes the percentage of bootstrap values after 1000 replications, only bootstraps higher than 50% are shown.

4.2.2.2 ISSR-PCR

Seven ISSR primers showed multi band patterns in each isolate. The primers amplified a total of 34 bands from 41 isolates tested including out group. The average number of bands per primers was 4.6. Band size ranged from 200-2500 bp (Table 4.3). Some DNA banding profiles generated by ISSR-PCR with primer (GA)6GC (A), (GA)8T (B), (TG)8GA (C), (GA)8YG (D), (GT)8YC (E), GGGC(GA)8 (F), and (CGA)5 (G) was shown in Figure 4.11.

An UPGMA analysis based on total ISSR characters difference was carried out to group the 40 isolates of *Rigidoporus microporus* with the outgroup *Ganoderma* spp. GM101. A dendrogram resulting from a cluster analysis showed two main distinct groups, designated as A and B (Figure 4.12) rooting from outgroup *Ganoderma* spp. GM101. All isolates obtained from Surat Thani and Trang province were grouped together as "A" and all of isolates obtained from Narathiwat province were grouped together as "B". The results indicated that isolates from Trang province were grouped together and in the same way as isolates from Surat Thani province were grouped together. There was a high similarity (0.88 – 1.00) within the isolates from Trang province, similarity (0.78 – 1.00) within the isolates from Surat Thani province, and similarity (0.76 – 1.00) within the isolates from Narathiwat province. There also was a high similarity in the range of 0.76 - 0.90 between isolates from three provinces.

Table 4.4 The details of 7 primers used for ISSR analysis.

Primer sequence	Annealing time (°C)	G+C content (%)	Fragment size Range (bp)
5'-AGA GAG AGA GAG AGA GC-3'	52	52.94	250-2500
5'-GAG AGA GAG AGA GAG AT-3'	52	47.06	300-1500
5'-TGT GTG TGT GTG TGT GA-3'	52	47.06	300-1250
5'-GAG AGA GAG AGA GAG AYG -3'	52	52.78	200-1000
5'-GTG TGT GTG TGT GTG TYC -3'	52	52.78	300-1500
5'-GGG CGA GAG AGA GAG AGA GA-3'	52	60.00	300-1500
5'-CGA CGA CGA CGA CGA-3'	52	66.67	250-1000

Y= T or C

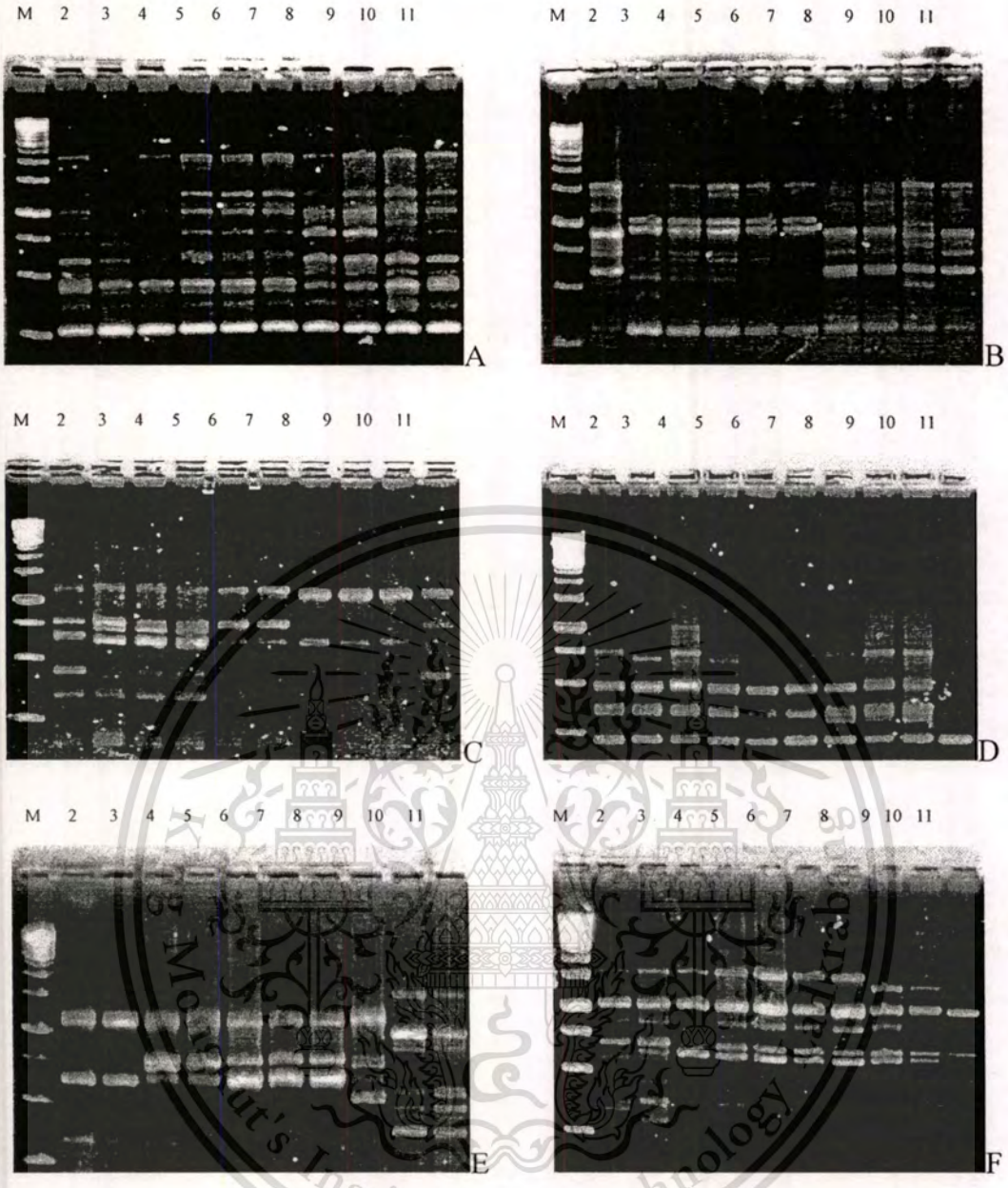


Figure 4.11 DNA banding profiles generated by ISSR-PCR.

The band with primer (GA)6GC (A), (GA)8T (B), (TG)8GA (C), (GA)8YG (D), (GT)8YC (E), GGGC(GA)8 (F), and (CGA)5 (G), Lanes M represent 1 kb DNA ladder and lanes 2-11 represent samples of tested isolates.

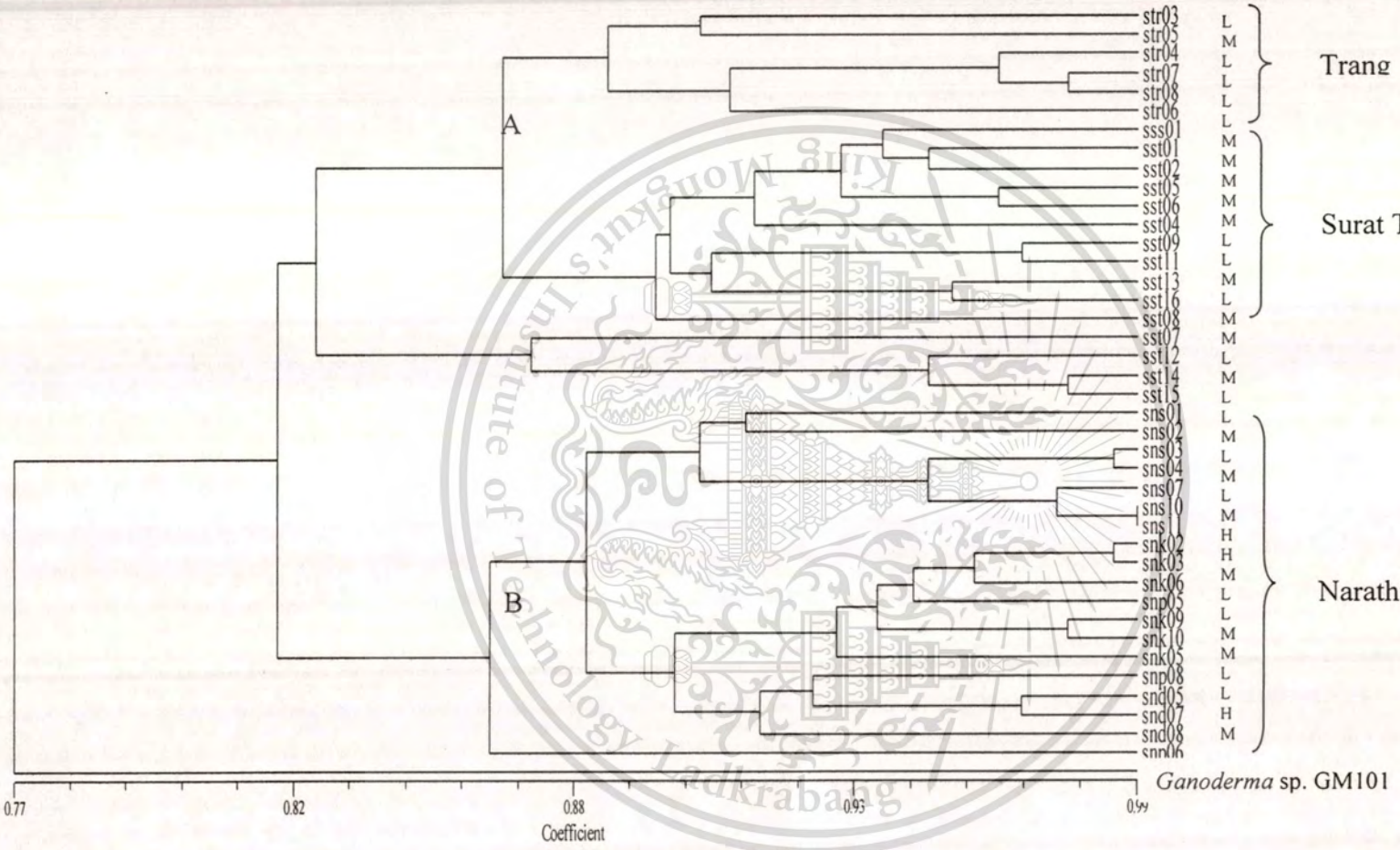


Figure 4.12 Dendrogram constructed with UPGMA-SHAN clustering of band data using 7 ISSR primers in 40 isolates of *Rigidoporus microporus* with outgroup *Ganoderma* spp. GM101, scale in dendrogram shows the genetic similarity coefficient.

4.3 Screening Biological Control Agent Against *Rigidoporus microporus*

4.3.1 Isolation and identification of antagonistic fungi

There were 28 isolates of promising antagonistic fungi were obtained from baiting and soil plate technique that included 9 isolates from Surat Thani province and 19 isolates from Narathiwat province. Identification was mainly referred to Domsch and Gams (1993) and von Arx *et al.* (1986). considered morphological characters by using Compendium of soil fungi by There were identified into 12 species which comprised of 2 isolate of *Acremonium fusidioides* SS01 and SS02, 2 isolates of *Aspergillus niger* SN71 and SN72, 2 isolates of *Chaetomium aureum* BN01 and BN02, 7 isolates of *Ch. bostrychodes* BN08, BN10, BN11, BS01, BS02, BS03 and BS04, 5 isolates of *Ch. fusiforme* BN07, BS31, BS32, BS33, and BS34, 2 isolates of *Ch. indicum* BN05 and BN06, 1 isolate of *Penicillium canescens* SN03, 2 isolates of *Trichoderma hamatum* STN06 and STN07, 2 isolates of *T. harzianum* STN01 and STN02, and 3 isolates of *T. viride* STN03, STN04 and STN05. *Chaetomium cochliodes* RY301 and *Ch. cupreum* RY202 obtained from pineapple plantation in Rayong province were kindly provided by Chaninun Pornsuriya, International College, King Mongkut's Institue of Technology Ladkrabang, Bangkok, Thailand (Table 4.5). The characteristic of promising antagonistic fungi were described below.

Table 4.5 The promising antagonistic fungi.

Fungal species	Isolates[1]	Isolation method	Location
Acremonium fusidioides	SS01, SS02	Soil plate	Surat Thani
Aspergillus niger	SN71, SN72	Soil plate	Narathiwat
Chaetomium aureum	BN01, BN02	Baiting	Narathiwat
Ch. bostrychodes	BN08, BN10, BN11	Baiting	Narathiwat
	BS01, BS02, BS03, BS04	Baiting	Surat Thani
Ch. fusiforme	BN07	Baiting	Narathiwat
	BS31, BS32, BS33, BS34	Baiting	Surat Thani
Ch. indicum	BN05, BN06	Baiting	Narathiwat
Penicillium canescens	SN03	Soil plate	Narathiwat
Trichoderma hamatum	STN06, STN07	Soil plate	Narathiwat
T. harzianum	STN01, STN02	Soil plate	Narathiwat
T. viride	STN03, STN04, STN05	Soil plate	Narathiwat
Ch. cochliodes	RY301	Baiting	Rayong
Ch. cupreum	RY202	Baiting	Rayong

[1]Code of promising antagonistic fungi:- SS = Soil plate and Surat Thani province, SN = Soil plate and Narathiwat province, BS = Baiting and Surat Thani province, BN = Baiting and Narathiwat province, STN = Soil plate and Surat Thani province.

Acremonium fusidioides (Nicot) W. Gams, *Cephalosporium-artige Schimmelpilze* (Stuttgart): 70 (1971) Figures 4.13 - 4.14

Colony reaching 9 cm diameter in 10 days at room temperature (27 – 30 °C) on PDA. Young colonies are usually white by aerial mycelium. Mature colonies become ochraceous brown, powdery. Phialides arising from submerge, smooth, produce spore in chain. Conidia; cylindrical, short with 2 – 4.2 µm.

Specimens examined: Thailand, Surat Thani, isolated from soil under rubber tree plantation, 2 September 2007, Saithong Kaewchai SS01 and SS02. The isolates were deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Aspergillus niger Tiegh., Annls Sci. Nat., Bot., ser. 5 (8): 240 (1867) Figures 4.15-4.16

Colony reaching 9 cm in 10 days at room temperature (27 - 30 °C) on PDA. Colonies are usually dark brown to back, powdery. Hyphae are septate and hyaline. Conidiophores arising from long, broad, thick-walled, brownish. Phialides are born directly on the vesicle. Conidial heads are large, radiating and back. Conidia are globose and roughened with 3.5 – 4.2 µm in diameter.

Specimens examined: Thailand, Narathiwat, isolated from soil under rubber tree plantation, 25 July 2007, Saithong Kaewchai SN71 and SN72. The isolates were deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Chaetomium aureum Chivers, Proc. Amer. Acad. Arts & Sci. 48: 86 (1912) Figures 4.17-4.18

Colony reaches 7-9 cm in 10 days at room temperature (27 - 30 °C) on PDA. Young colonies are usually pale green by aerial mycelium. Mature colonies become red to red brown by red pigment exudates. Ascomata are pale green, ovate in shape, 100.4 – 130.5 x 120.0 – 150.5 µm. Ascomatal hairs arcuate, coiled, septate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are elliptical, hyaline when young, brown when mature, 4.8 – 5.5 x 8.7 – 10. 2 µm with two apical germ pores

Specimens examined: Thailand, Narathiwat, isolated from soil under rubber tree plantation, 8 August 2007, Saithong Kaewchai BN01 and BN02. The isolates were deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Chaetomium bostrychodes Zopf, Abhandl. Botan. Ver. Prov. Brandenburg 19: 173 (1877)

Figures 4.19-4.25

Colony reaches 9 cm in 6 days at room temperature (27 - 30 °C) on PDA. Young colonies are usually pale purple with purple pigment exudates. Mature colonies become green to brown. Ascomata mature within 10-15 days, dark green to brown when mature, ovate in shape, 159.9 – 255.5 x 210.3 – 407.5 µm. Ascomatal hairs usually spirally coiled in the upper part. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are limoniform, hyaline when young, gray when mature, 6.9 – 9.1 x 8.0 – 9.5 µm with an apical germ pore.

Specimens examined: Thailand, Narathiwat, isolated from soil under rubber tree plantation, 23 July 2007, Saithong Kaewchai BN08, BN10 and BN11. Thailand, Surat Thani, isolated from soil under rubber tree plantation, 3 August 2007, Saithong Kaewchai BS01, BS02, BS03 and BS04. The isolates were deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Chaetomium cochliodes Palliser, North American Flora. 3(1): 61 (1910) Figures 4.26

Colony reaches 9 cm in 7 days at room temperature (27 - 30 °C) on PDA. Young colonies are usually white by aerial mycelium with purple pigment exudates. Mature colonies become green to brown with ascomata. Ascomata were olivaceous, mature within 10-14 days, dark green to brown when mature, ovate in shape, 140.3 – 2090.0 x 240.6 – 290.8 µm. Ascomatal hairs usually spirally irregularly sinuous. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are broadly ovate to lemon-shape, hyaline when young, pale green when mature, 6.0 – 7.0 x 7.5 – 8.2 µm with an apical germ pore.

Specimens examined: Thailand, Rayong, isolated from soil under pineaple plantation, 27 September 2007, Chaninun Pornsuriya RY301. The isolate was deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Chaetomium cupreum Ames, Mycologia 41(6): 642 (1949) Figures 4.27

Colony reaches 9 cm in 15 days at room temperature (27 - 30 °C) on PDA. Young colonies are red due to red pigment exudates. Ascomata are red, mature within 10-14 days, ovate in shape, 140.3 – 209.0 x 240.6 – 290.8 µm. Ascomatal hairs arcuate, coiled and septate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are reniform, hyaline when young, brown when mature, 4.5 – 7.0 x 6.6 – 10.6 µm with single apical germ pore.

Specimens examined: Thailand, Rayong, isolated from soil under pineaple plantation, 20 August 2007, Chaninun Pornsuriya RY202. The isolate was deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Chaetomium fusiforme Chivers, Proc. Amer. Acad. Arts & Sci. 48: 87 (1912) Figures 4.28-4.32

Colony reaches 9 cm in 15 days at room temperature (27 - 30 °C) on PDA. Young colonies are white with aerial mycelium, yellow or red pigment exudates. Ascomata are olivacceous, mature within 15-20 days, ovate in shape, 104.9 – 133.3 x 111.3 – 195.0 μm. Ascomatal hairs straight with curl at the top, flexuous and septate. Asci are clavate in shape, short stalk with 8 ascospores per ascus. Ascospores are fusiform, hyaline when young, brown when mature, 5.0 – 6.0 x 12.0 – 17.2 μm with germ pore at both ends.

Specimens examined: Thailand, Narathiwat, isolated from soil under rubber tree plantation, 20 August 2007, Saithong Kaewchai BN07. Surat Thani, isolated from soil under rubber tree plantation, 2 August 2007, Saithong Kaewchai BS31, BS32, BS33 and BS34. The isolates were deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Chaetomium indicum Corda, Icon. Fung. 4: 38 (1840) Figures 4.33- 4.34

Colony reached 9 cm in 15 days at room temperature (27 - 30 °C) on PDA. Young colonies are white with aerial mycelium, an irregular lobed margin, grey yellow pigment exudates. Ascomata are pale grey or olivacceous, mature within 15-20 days, ovate in shape, 180.5 – 225.4 x 168.0 – 206.5 μm. Ascomatal hairs straight with dichotomously branched and septate. Asci are clavate in shape, stalk with 8 ascospores per ascus. Ascospores are ovate, pale green when young, dark brown when mature, 3.0 – 4.2 x 4.0 – 5.4 μm with an apical germ pore.

Specimens examined: Thailand, Narathiwat, isolated from soil under rubber tree plantation, 20 July 2007, Saithong Kaewchai BN05 and BN06. The isolates were deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Penicillium canescens Sopp, Monograph of *Penicillium* 11: 181 (1912) Figure 4.35

Colonies slowly grow, reaching 3 - 4 cm in ten days at room temperature (27 - 30 °C) on PDA. Colonies are slowly grew, grey green, red pigment exudates diffuse in media. Conidiophores conspicuously smooth, divegent. Conidia are globose, dull green, smooth-walled with 2.5 – 3.0 μm.

Specimens examined: Thailand, Narathiwat, isolated from soil under rubber tree plantation, 28 July 2007, Saithong Kaewchai BN03. The isolate was deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Trichoderma hamatum (Bonord.) Bainier, Bull. Soc. Mycol. Fr. 22: 131 (1906) Figures 4.36-4.37

Colony reaches 9 cm in 3 days at room temperature (27 - 30 °C) on PDA. Young colonies are white with aerial mycelium, yellow pigment exudates diffuse in media. Phialides are particularly broad and branched. Conidia are short-cylindical, green, thin-walled, smooth with 1.8 – 2.5 x 2.5 – 4.0 µm.

Specimens examined: Thailand, Narathiwat, isolated from soil under rubber tree plantation, 28 July 2007, Saithong Kaewchai STN06 and STN07. The isolates were deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Trichoderma harzianum Rifai, Mycol. Pap. 116:38 (1969) Figures 4.38-4.39

Colony reaches 9 cm in 3 days at room temperature (27 - 30 °C) on PDA. Young colonies are white with aerial mycelium, dull green when old. Phialides are particularly short and bottle shape. Conidia are globose to sub-globose, green, smooth with 2.2 – 3.0 x 2.4 – 4.0 µm.

Specimens examined: Thailand, Narathiwat, isolated from soil under rubber tree plantation, 28 August 2007, Saithong Kaewchai STN01 and STN02. The isolates were deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

Trichoderma viride Pers., Neues. Mag. Bot. 1: 92 (1794) Figures 4.40 - 4.42

Colony reaches 9 cm in 3 days at room temperature (27 – 30 °C) on PDA. Young colonies are white with aerial mycelium, green when old. Phialides are pyramidically short branches, occurring near the tip. Conidia are globose, green, roughened with 3.2 – 4.6.

Specimens examined: Thailand, Narathiwat, isolated from soil under rubber tree plantation, 5 August 2007, Saithong Kaewchai STN03, STN04 and STN05. The isolates were deposited in the Biocontrol Research Unit and Mycology Section, KMITL.

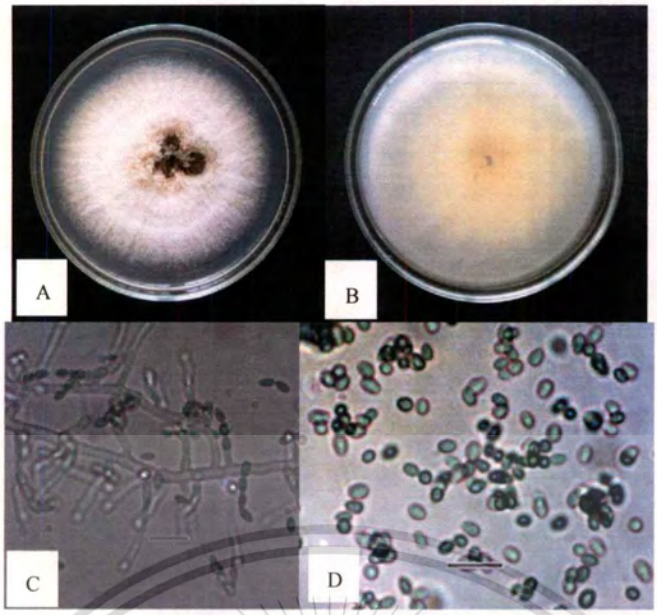


Figure 4.13 *Acremonium fusidioides* SS01. 10-day-old culture on PDA, upper surface (A) and lower surface (B), conidiophore with conidia (C) and conidia (D). Bar. C, D = 10 μm

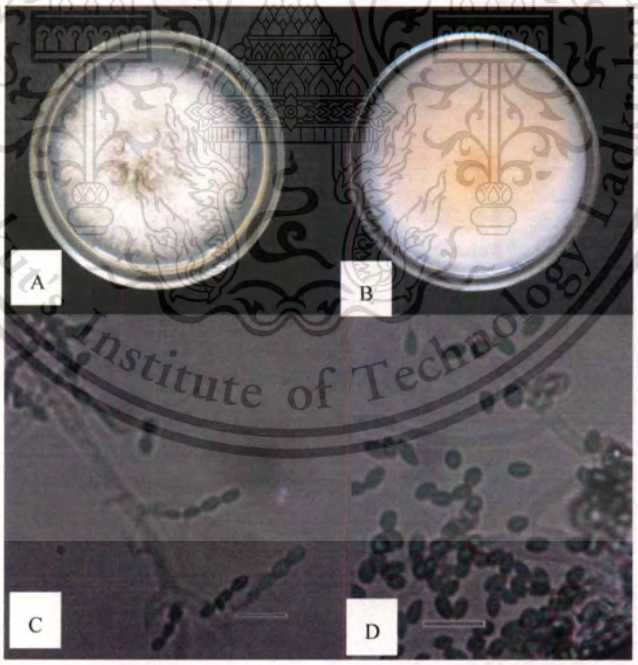


Figure 4.14 *Acremonium fusidioides* SS02. 10-day-old culture on PDA, upper surface (A) and lower surface (B), conidiophore with conidia (C) and conidia (D). Bar. C, D = 10 μm.

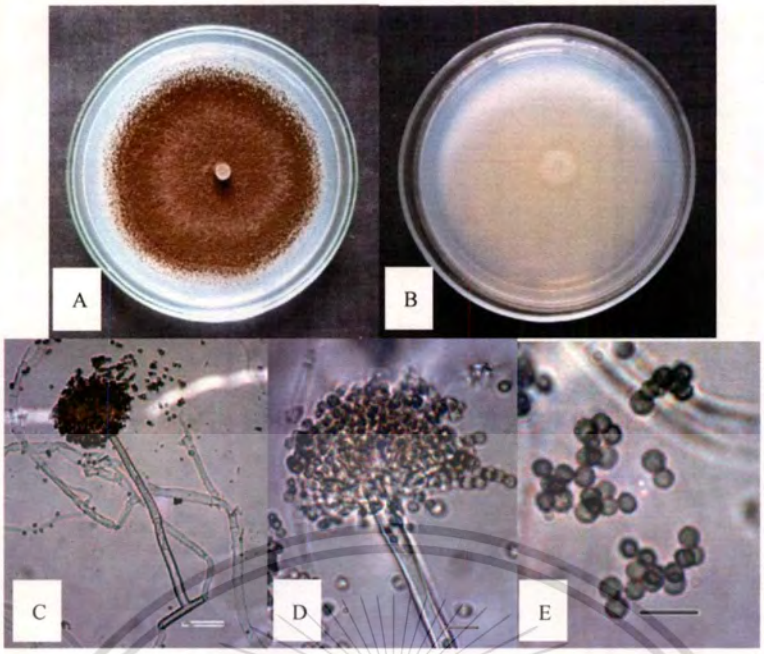


Figure 4.15 *Aspergillus niger* SN71. 10-day-old culture on PDA, upper surface (A) and lower surface (B), conidiophore with conidia (C), conidial head (D), foot cell (E) and conidia (F). Bar. C = 100 μm, D, E = 10 μm.

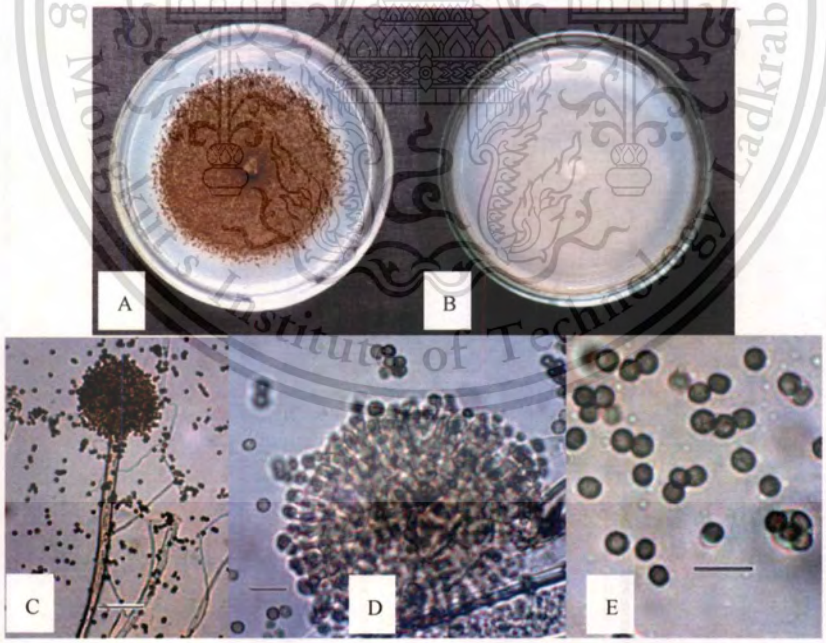


Figure 4.16 *Aspergillus niger* SN72. 10-day-old culture on PDA, upper surface (A) and lower surface (B), conidiophore with conidia (C), conidial head (D), foot cell (E) and conidia (F). Bar. C = 100 μm, D, E = 10 μm.

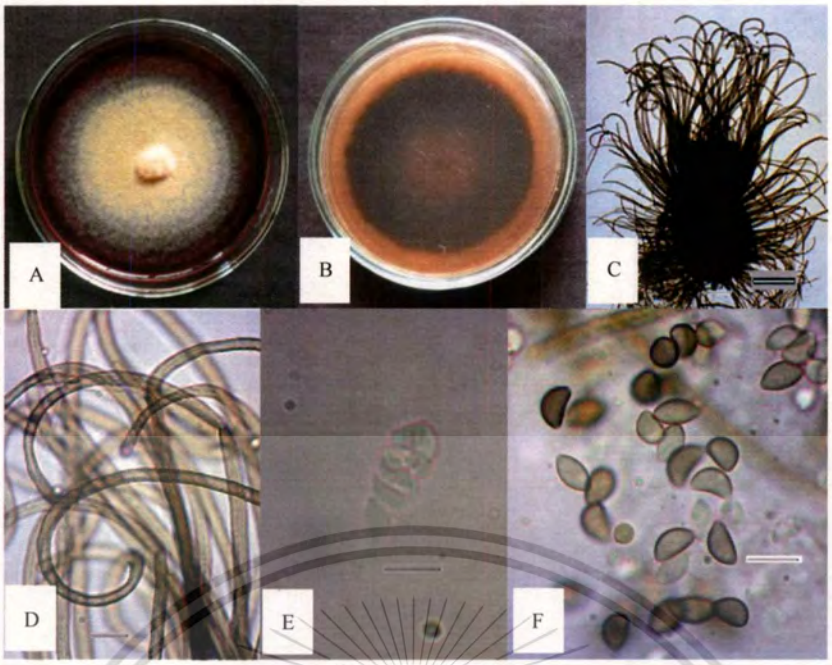


Figure 4.17 *Chaetomium aureum* BN01. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

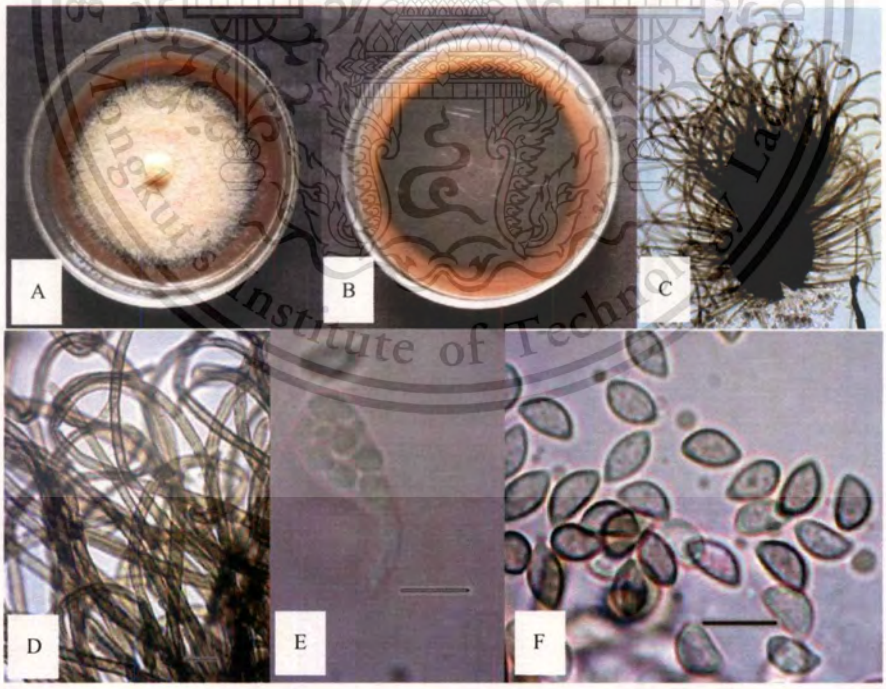


Figure 4.18 *Chaetomium aureum* BN02. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

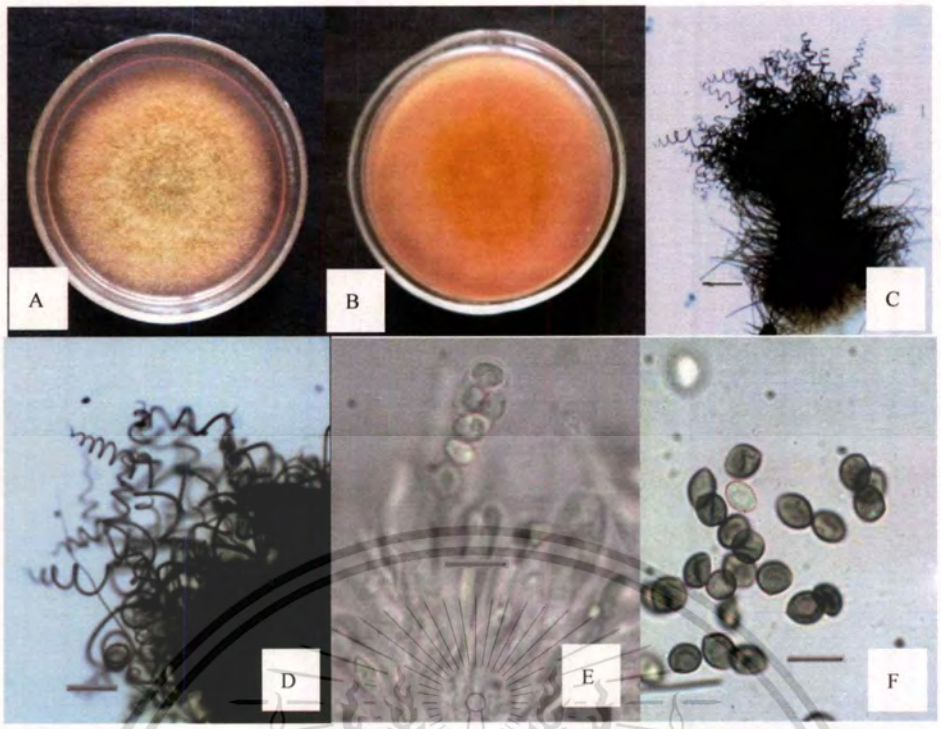


Figure 4.19 *Chaetomium bostrychodes* BN08. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

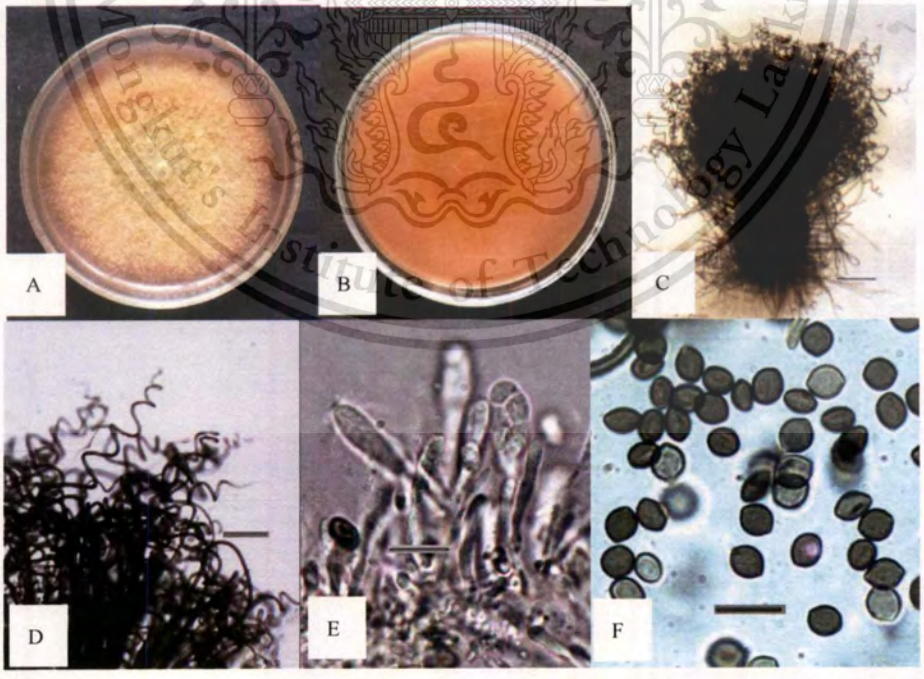


Figure 4.20 *Chaetomium bostrychodes* BN10. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), asci (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

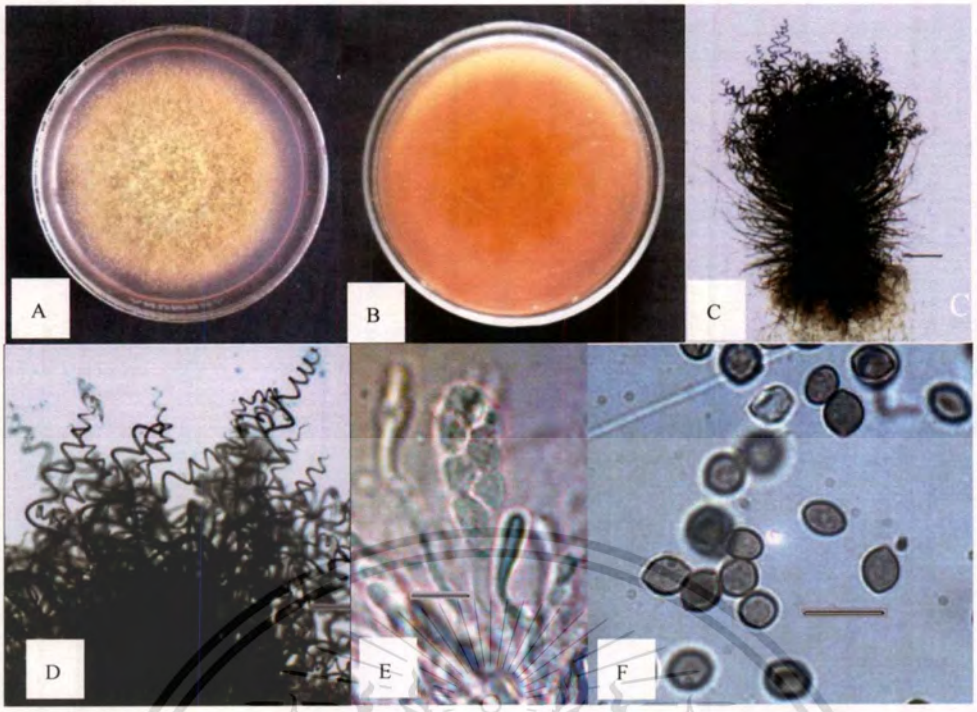


Figure 4.21 *Chaetomium bostrychodes* BN11. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), young asci (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

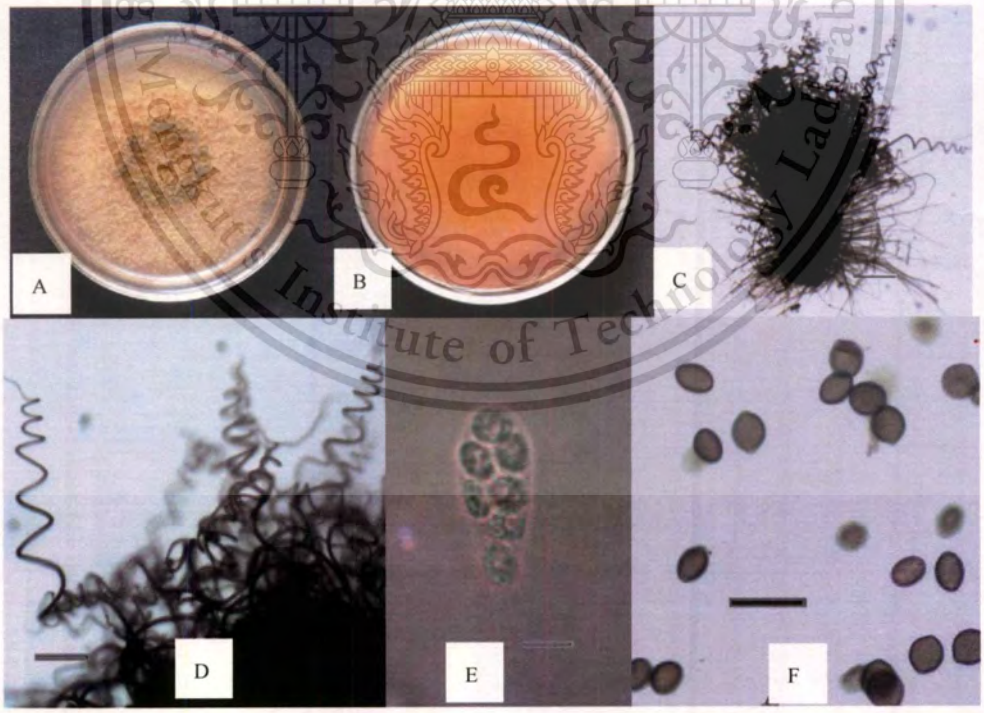


Figure 4.22 *Chaetomium bostrychodes* BS01. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

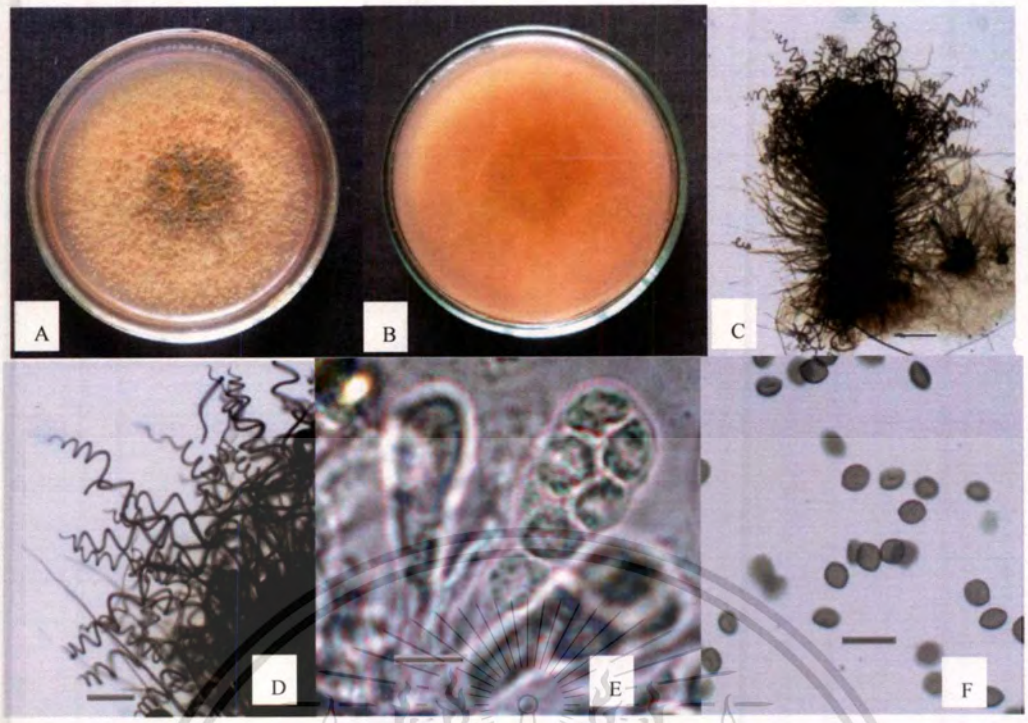


Figure 4.23 *Chaetomium bostrychodes* BS02. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

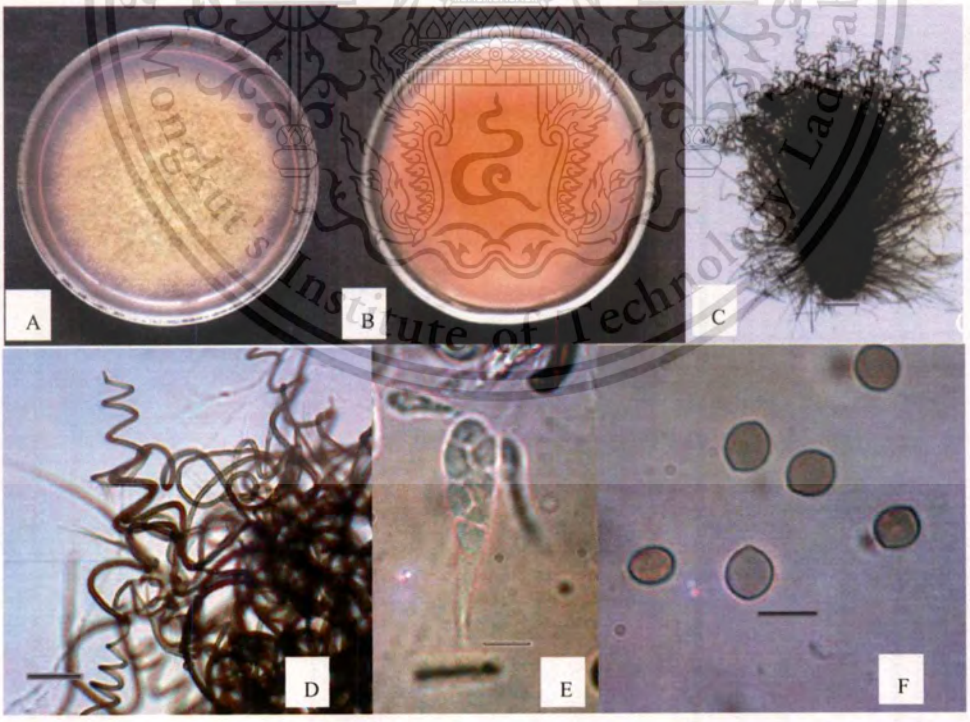


Figure 4.24 *Chaetomium bostrychodes* BS03. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

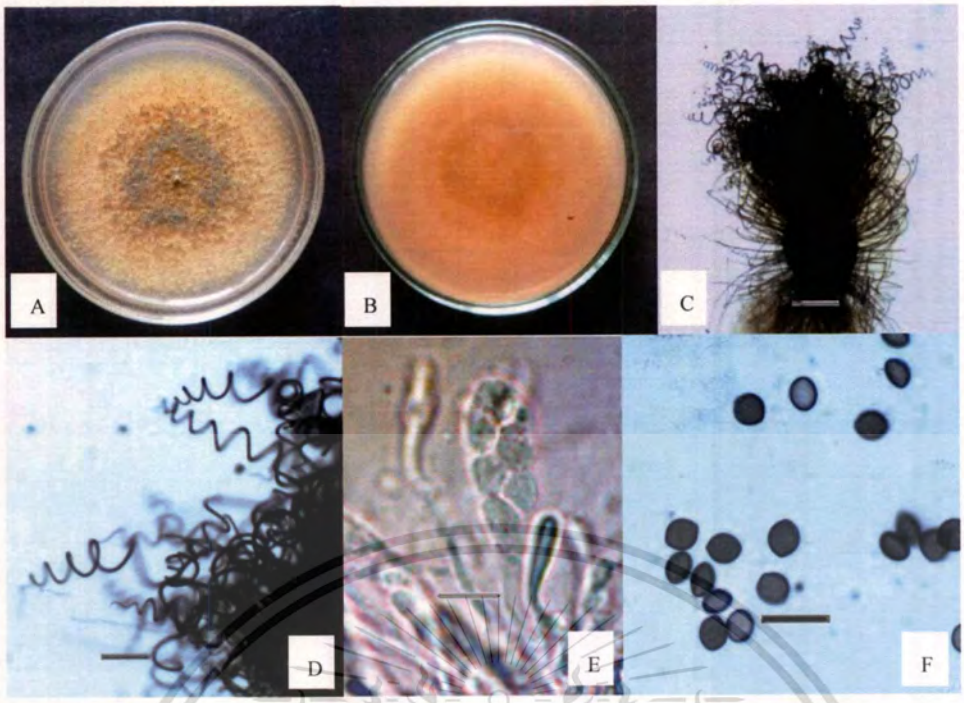


Figure 4.25 *Chaetomium bostrychodes* BS04. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

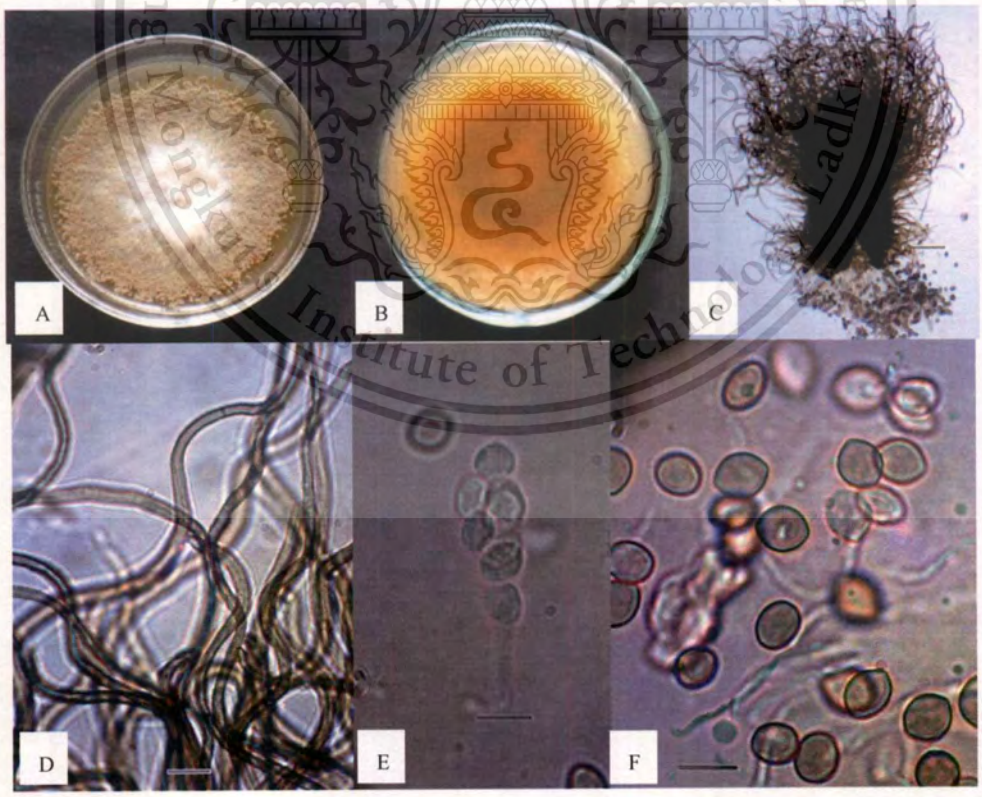


Figure 4.26 *Chaetomium cochliodes* RY301. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

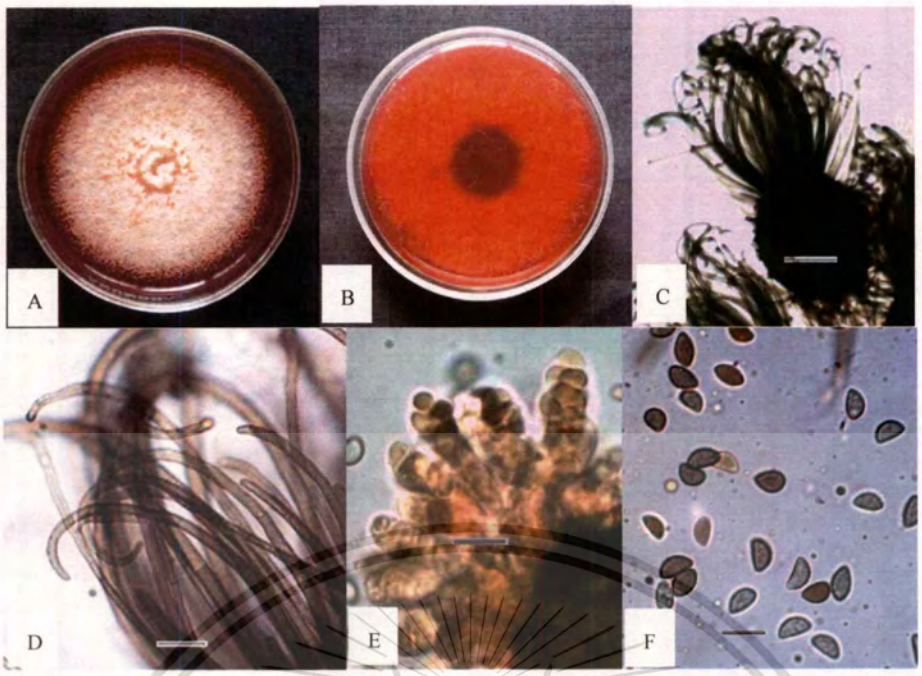


Figure 4.27 *Chaetomium cupreum* RY202. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

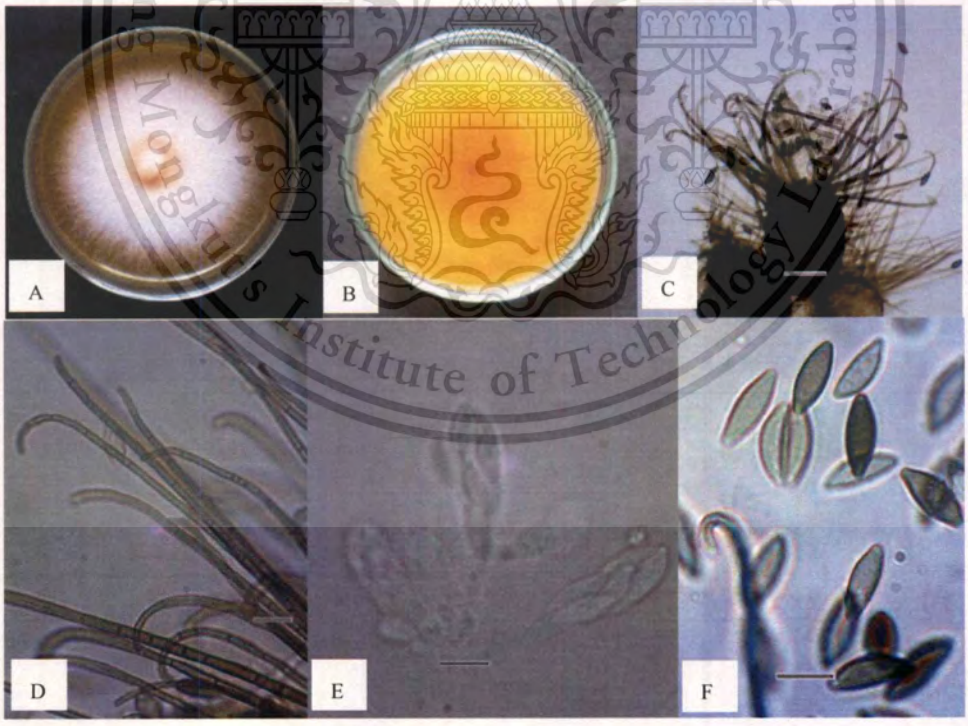


Figure 4.28 *Chaetomium fusiforme* BN07. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

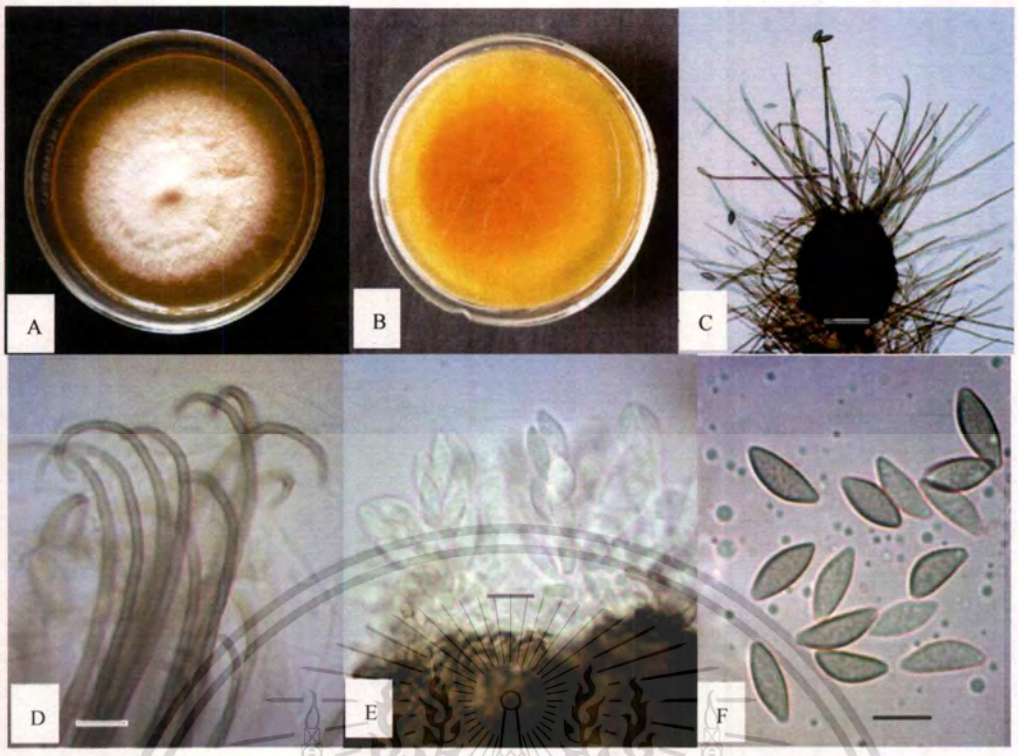


Figure 4.29 *Chaetomium fusiforme* BS31. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), asci (E) and ascospores (F). Bar. C = 100 µm, D, E, F = 10 µm.

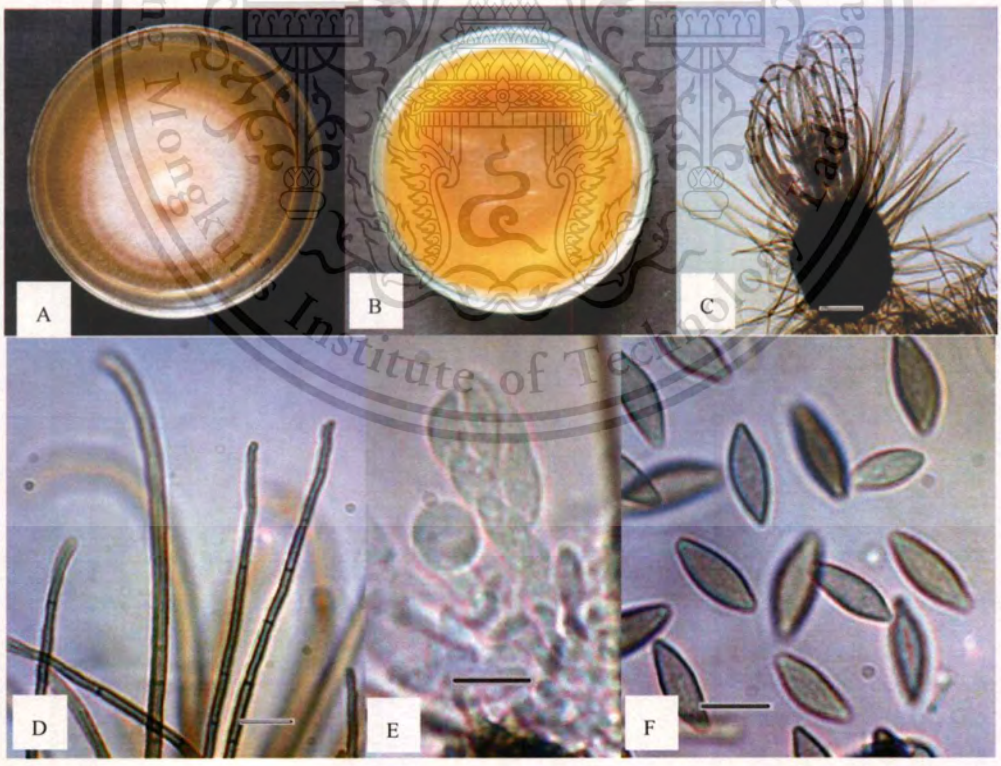


Figure 4.30 *Chaetomium fusiforme* BS32. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 µm, D, E, F = 10 µm.

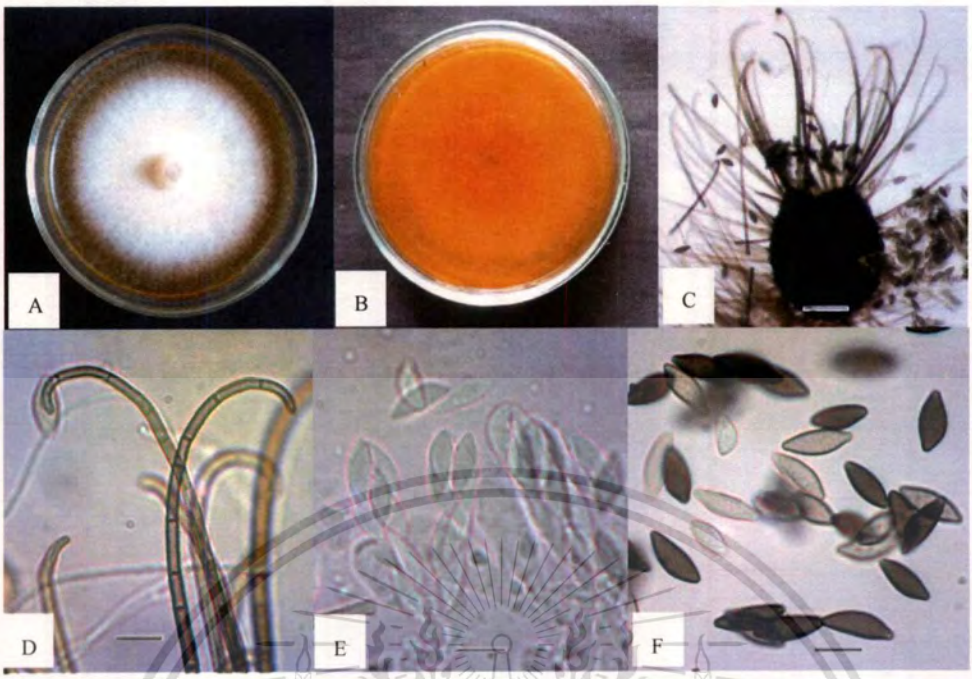


Figure 4.31 *Chaetomium fusiforme* BS33. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), asci (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

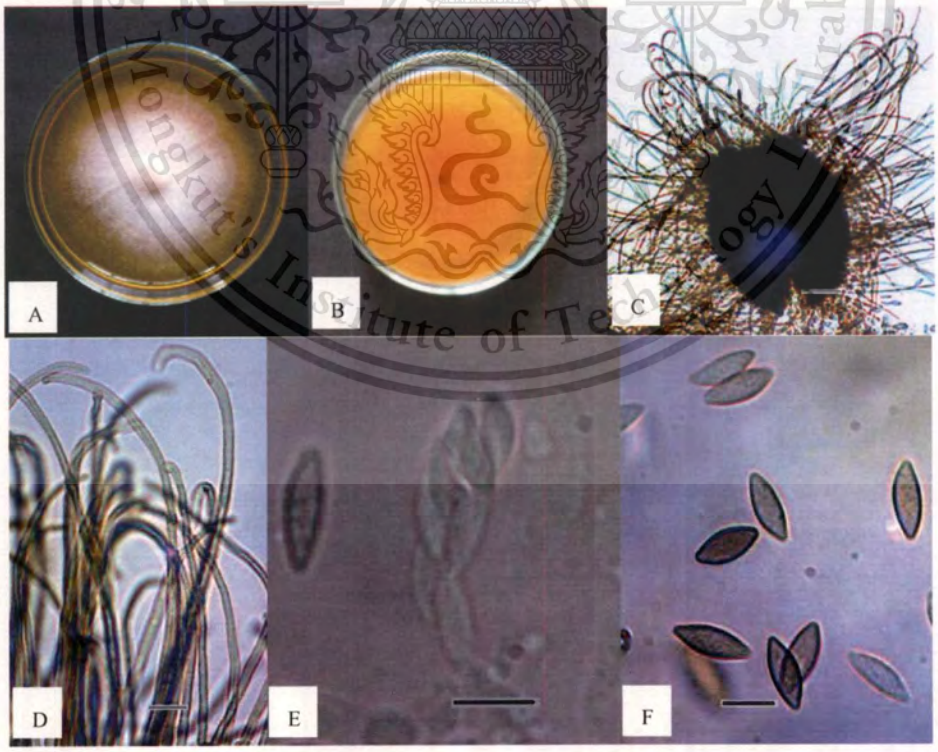


Figure 4.32 *Chaetomium fusiforme* BS34. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

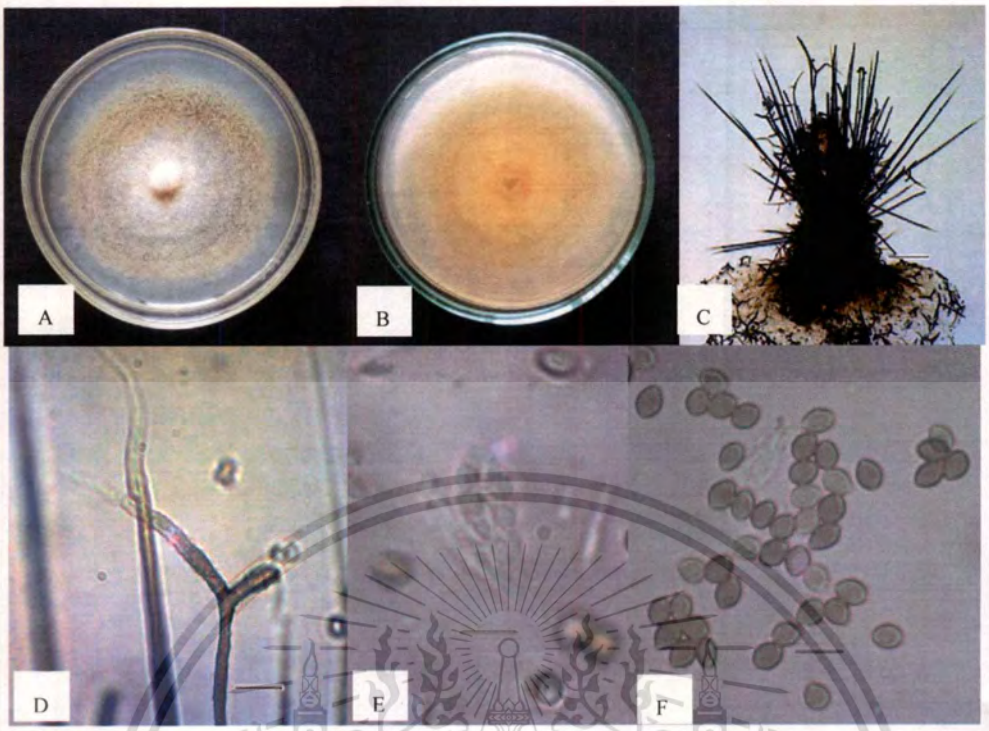


Figure 4.33 *Chaetomium indicum* BN05. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

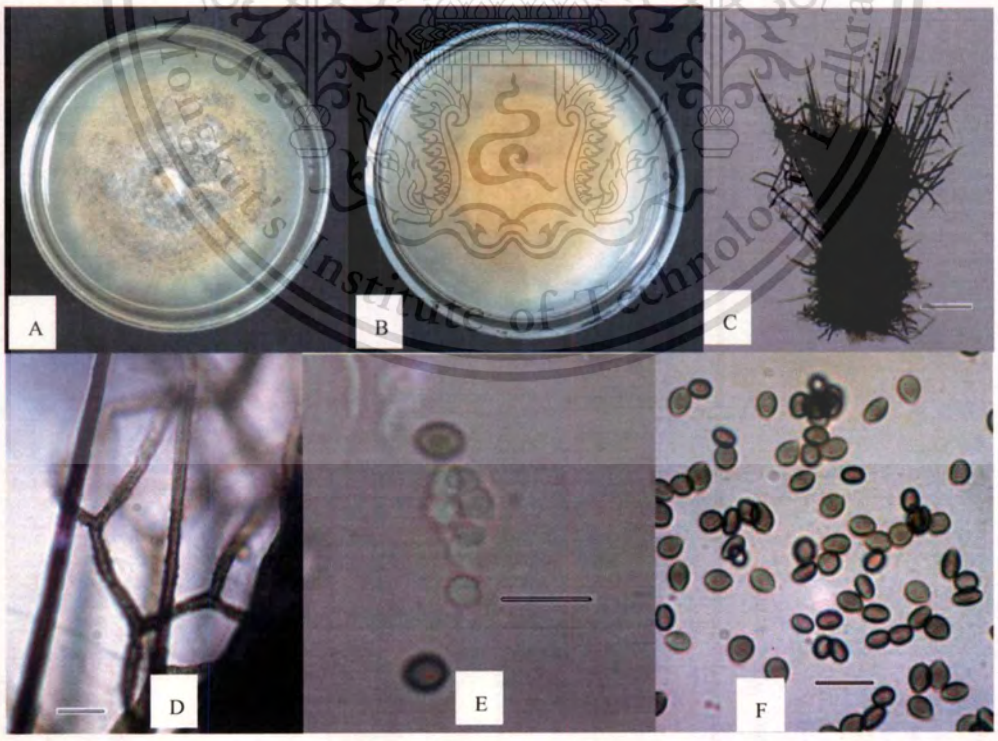


Figure 4.34 *Chaetomium indicum* BN06. 10-day-old culture on PDA, upper surface (A) and lower surface (B), ascomata (C), ascomatal hairs (D), ascus (E) and ascospores (F). Bar. C = 100 μm, D, E, F = 10 μm.

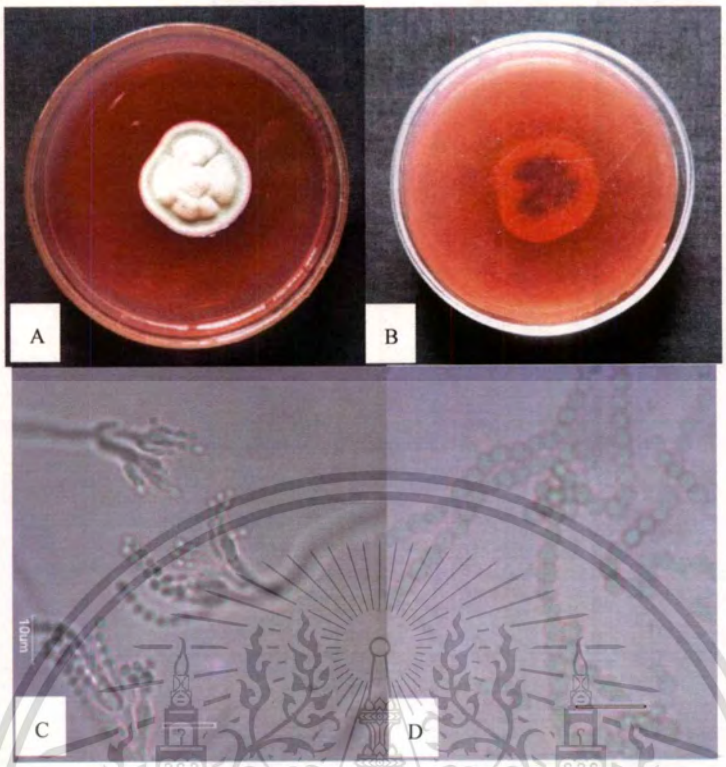


Figure 4.35 *Penicillium canescens* SN03. 10-day-old culture on PDA, upper surface (A) and lower surface (B), conidiophores (C), conidia (D). Bar. C, D = 10 µm.

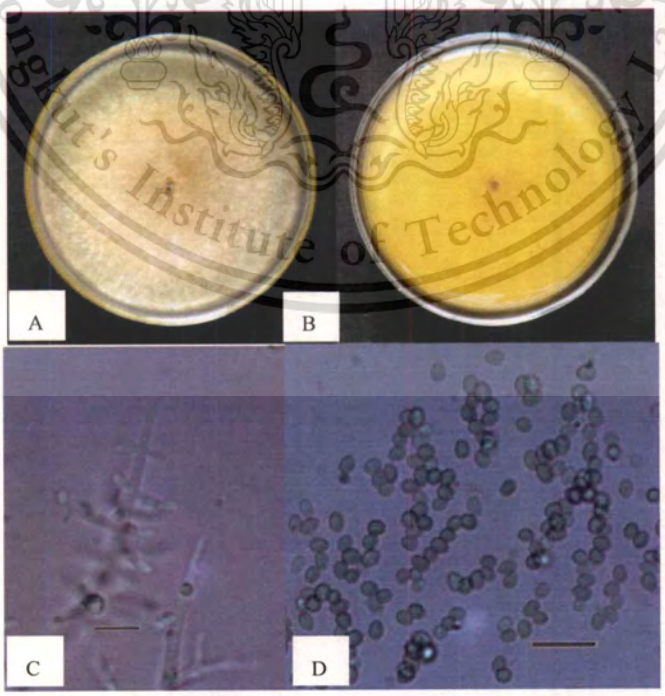


Figure 4.36 *Trichoderma hamatum* STN06. 10-day-old culture on PDA, upper surface (A) and lower surface (B), phialides (C), conidia (D). Bar. C, D = 10 µm

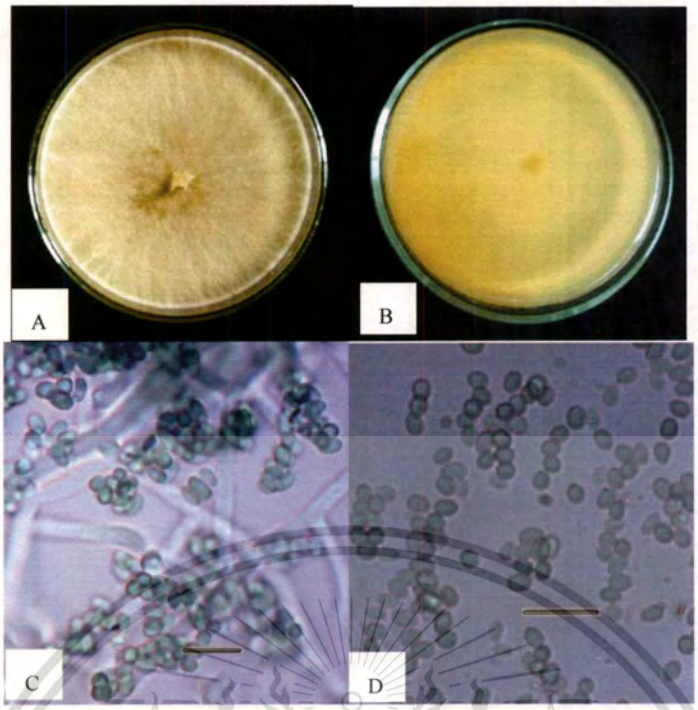


Figure 4.37 *Trichoderma hamatum* STN07. 10-day-old culture on PDA, upper surface (A) and lower surface (B), phialides (C), conidia (D). Bar. C, D = 10 μm

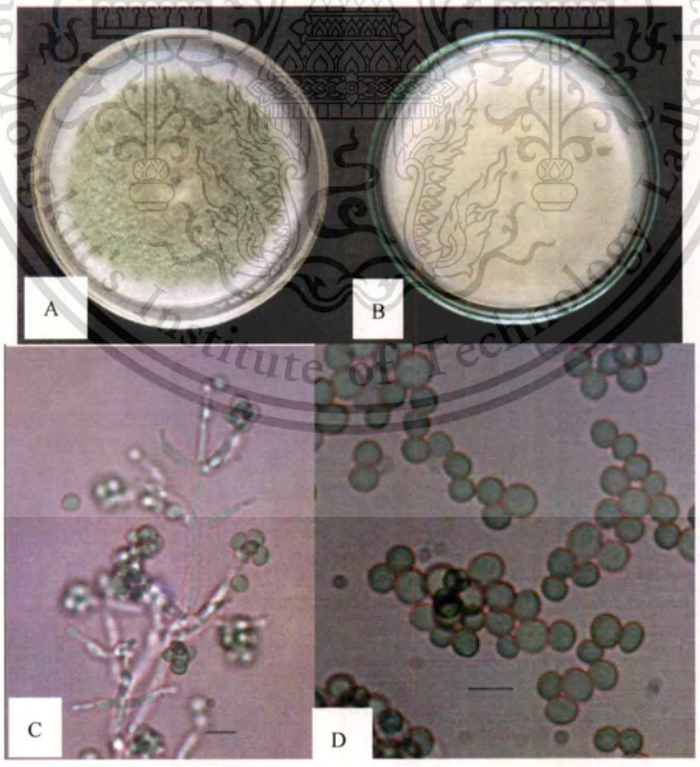


Figure 4.38 *Trichoderma harzianum* STN01. 10-day-old culture on PDA, upper surface (A) and lower surface (B), phialides (C), conidia (D). Bar. C, D = 10 μm

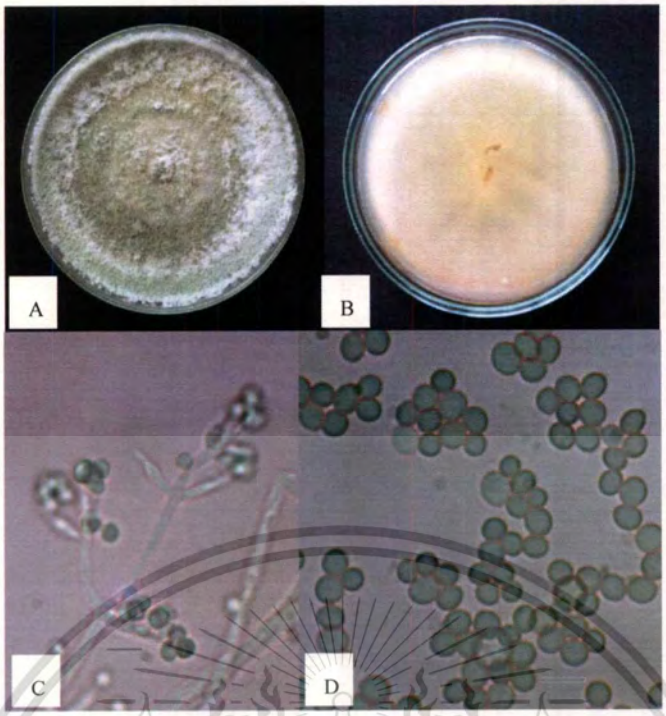


Figure 4.39 *Trichoderma harzianum* STN02. 10-day-old culture on PDA, upper surface (A) and lower surface (B), phialides (C), conidia (D). Bar. C, D = 10 μm

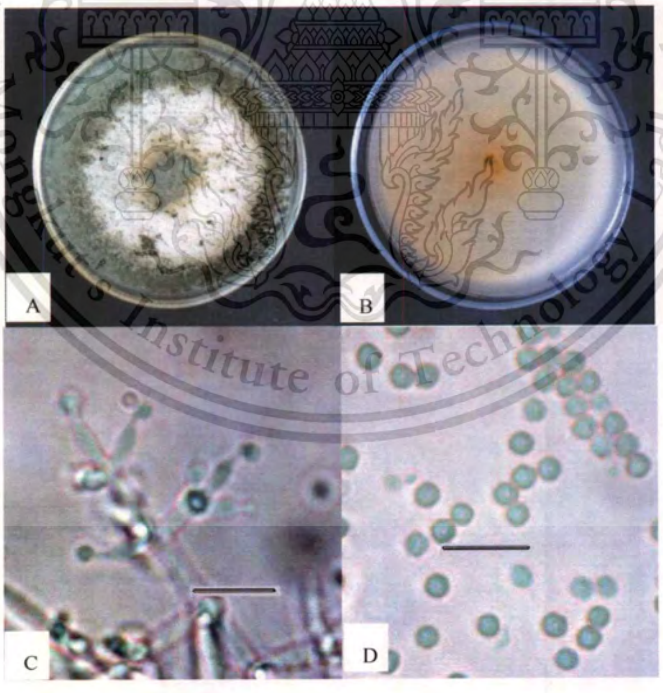


Figure 4.40 *Trichoderma viride* STN03. 10-day-old culture on PDA, upper surface (A) and lower surface (B), phialides (C), conidia (D). Bar. C, D = 10 μm

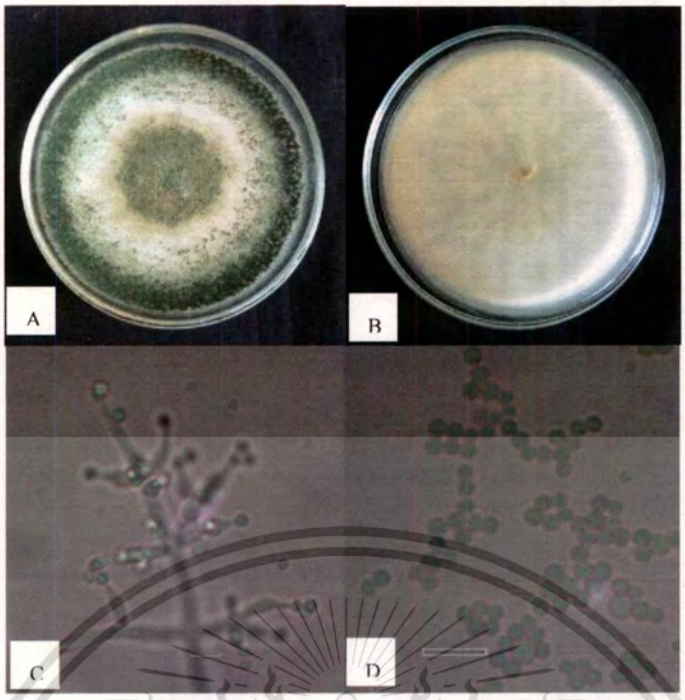


Figure 4.41 *Trichoderma viride* STN04. 10-day-old culture on PDA, upper surface (A) and lower surface (B), phialides (C), conidia (D). Bar. C, D = 10 µm

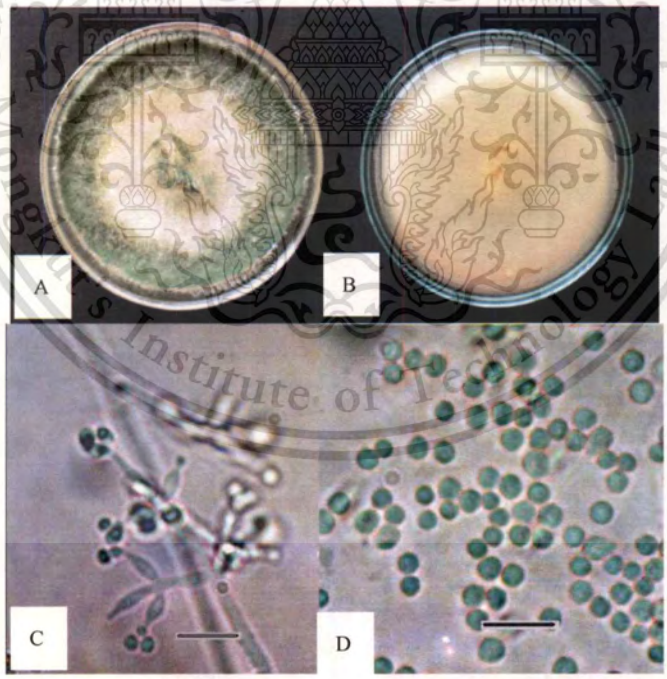


Figure 4.42 *Trichoderma viride* STN05. 10-day-old culture on PDA, upper surface (A) and lower surface (B), phialides (C), conidia (D). Bar. C, D = 10 µm

4.3.2 Dual culture antagonistic test

All promising antagonistic fungi which obtained from 4.3.2 were tested for their antagonistic abilities to control the growth of *R. microporus*. The test was carried out in the laboratory using dual culture technique. The most aggressive isolate, *R. microporus* SNK02 was used in this experiment. The effective antagonistic fungi were screened according to percentage of the growth inhibition (PGI).

The results showed that eighteen isolates of antagonistic fungi could inhibit the colony of *R. micropors* at 10 days with PGI over 50% as follows:- *T. viride* STN04, STN05 (89.5%), *T. hamatum* STN07 (89.5%), *T. viride* STN03 (88.4%), *T. hamatum* STN02 and *T. hamatum* STN06 (87.3%), *T. harzianum* STN01 (85.3 %), *A. niger* SN72 (75.8%), *A. niger* SN71 (71.4%), *Ch. cochliodes* RY301 (60.3%), *Ch. bostrychodes* BS04 (59.5%), *Ch. bostrychodes* BS02 (59.5%), *Ch. bostrychodes* BS03 (59.2%), *Ch. bostrychodes* BN08 (58.1%), *Ch. cupreum* RY202 (57.5), *Ch. bostrychodes* BN11 (57.0%), *Ch. bostrychodes* BS01 (55.9%) and *Ch. bostrychodes* BN10 (55.6%) (Table 4.6). Among them, the tested *Trichoderma* species gave the highest values of PGI followed by the tested *Aspergillus* species and the tested *Chaetomium* species against *R. microporus* SNK02.

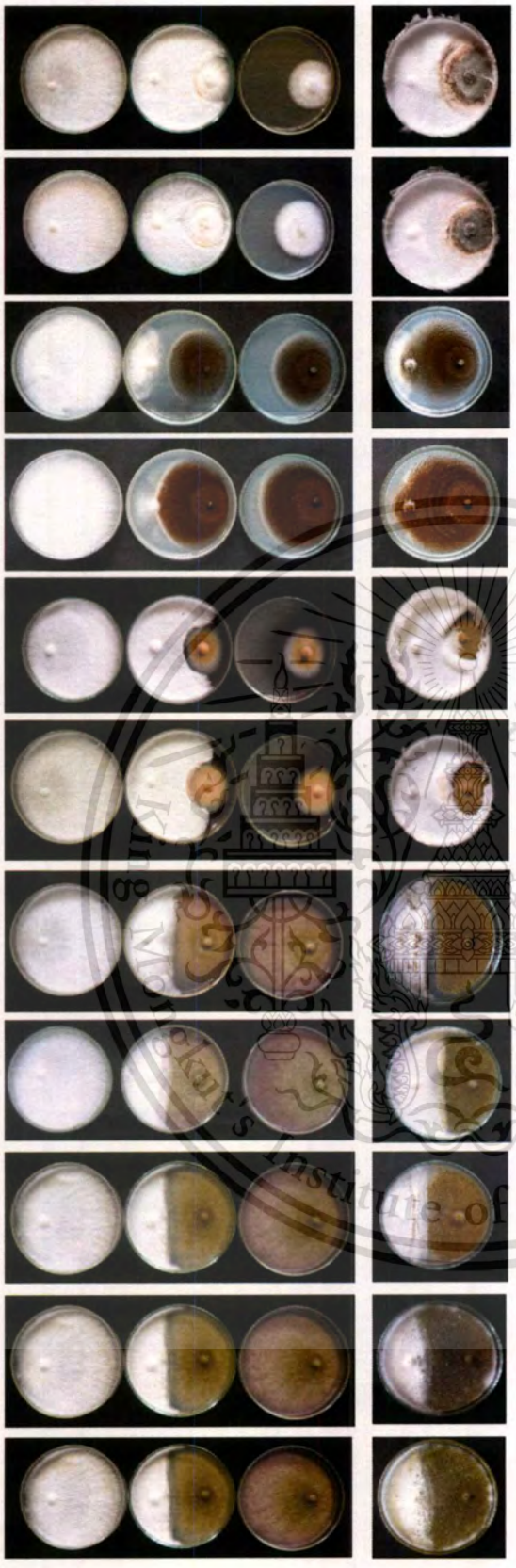
They were observed that *T. hamatum*, *T. harzianum* and *T. viride* were highly antagonistic against *R. microporus* on PDA and all of them rapidly grown over *R. microporus* colony within 10 days. The results also showed that the mycelium of pathogen could not grow when it came to contact with *A. niger*. Dual culture plates were leaved at room temperature (27-30 °C) for 30 days, *A. niger* could grow over the colony of the pathogen. In the same way, *Ch. bostrychodes* and *Ch. cupreum* could produce the fruiting body over the colony of *R. microporus* after leaving them at room temperature (27-30 °C) for 30 days especially *Ch. bostrychodes* BN08 and *Ch. cupreum* RY202. Although *Ch. cochliodes* and *Ch. elatum* gave the PGI at 60.3% and 60.8%, respectively but the mycelium of *R. microporus* could grow over the colony of both fungi after leaved the dual culture plate at room temperature (27-30 °C) for 30 days (Figure 4.43).

It is noticed that dual culture antagonistic test provide a premising result as promising antagonist. The other screening techniques of antagonistic crude extract and bioactive compound tested must be further considered to decide the most effective isolate to develop as biofungicide used to control white root disease.

Table 4.6 Colony diameter of pathogen and PGI of antagonistic fungi at 10 days.

Antagonistic fungi	Colony diameter of pathogen (cm)	PGI
Acremonium fusidioides SS01	6.2f[1]	31.7f[1]
Acremonium fusidioides SS02	6.0f	33.3f
Aspergillus niger SN71	2.6i	71.4c
Aspergillus niger SN72	2.2j	75.8b
Botryothecium sp. BN44	6.8de	24.2gh
Chaetomium aureum BN01	7.0cde	22.2ghi
Chaetomium aureum BN02	6.8de	24.2gh
Chaetomium bostrychodes BN08	3.8gh	58.1de
Chaetomium bostrychodes BN10	4.0g	55.6e
Chaetomium bostrychodes BN11	3.9gh	57.0de
Chaetomium bostrychodes BS01	4.0g	55.9e
Chaetomium bostrychodes BS02	3.7gh	59.5de
Chaetomium bostrychodes BS03	3.7gh	59.5de
Chaetomium bostrychodes BS04	3.7gh	59.5de
Chaetomium cochliodes RY301	3.6gh	60.3de
Chaetomium cupreum RY202	3.8gh	57.5de
Chaetomium fusiforme BN07	7.2bcd	20.3j
Chaetomium fusiforme BS31	7.4b	17.5j
Chaetomium fusiforme BS32	6.8de	24.4gh
Chaetomium fusiforme BS33	6.8de	24.4gh
Chaetomium fusiforme BS34	6.7e	25.6g
Chaetomium indicum BN05	7.3bc	18.9ij
Chaetomium indicum BN06	7.4bc	18.1ij
Penicillium canescens SN03	8.3a	8.3k
Trichoderma hamatum STN06	1.2k	87.3a
Trichoderma hamatum STN07	1.0k	89.5a
Trichoderma harzianum STN01	1.3k	85.3a
Trichoderma harzianum STN02	1.2k	87.3a
Trichoderma viride STN03	1.1k	88.4a
Trichoderma viride STN04	1.0k	89.5a
Trichoderma viride STN05	1.0k	89.5a
CV. (%)	4.7	4.6

[1]Mean of four replications. Mean followed by the same common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.01$.



Acremonium fusidioides SS01

Acremonium fusidioides SS02

Aspergillus niger SN71

Aspergillus niger SN72

Chaetomium aureum BN01

Chaetomium aurieum BN02

Chaetomium bostrychodes BN08

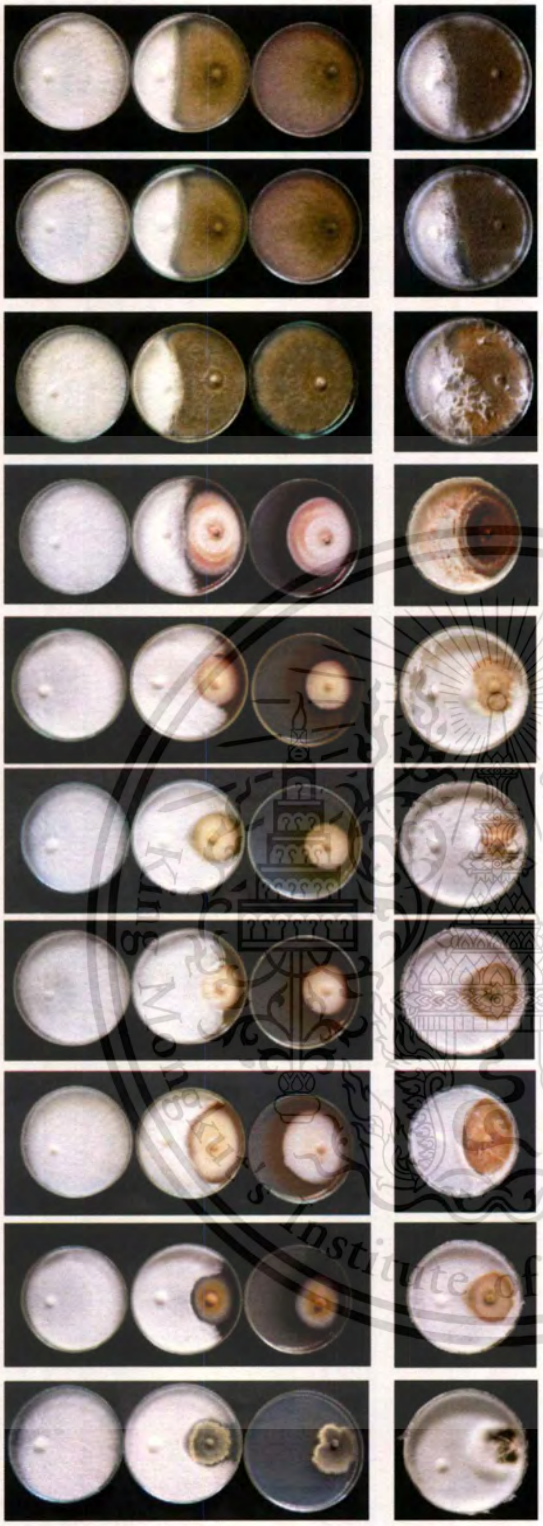
Chaetomium bostrychodes BN10

Chaetomium bostrychodes BN11

Chaetomium bostrychodes BS01

Chaetomium bostrychodes BS02

Figure 4.43 Dual culture of antagonistic fungi. The culture from left to right: pathogen culture at 10 days, dual-culture culture at 10 days, antagonist culture at 10 day and dual-culture at 30 days.



Chaetomium bostrychodes BS03

Chaetomium bostrychodes BS04

Chaetomium cochliodes RY301

Chaetomium cupreum RY202

Chaetomium fusiforme BN07

Chaetomium fusiforme BS31

Chaetomium fusiforme BS32

Chaetomium fusiforme BS33

Chaetomium fusiforme BS34

Chaetomium indicum BN05

Figure 4.43 Dual culture of antagonistic fungi. The culture from left to right: pathogen culture at 10 days, dual-culture culture at 10 days, antagonist culture at 10 day and dual-culture at 30 days (Continue).

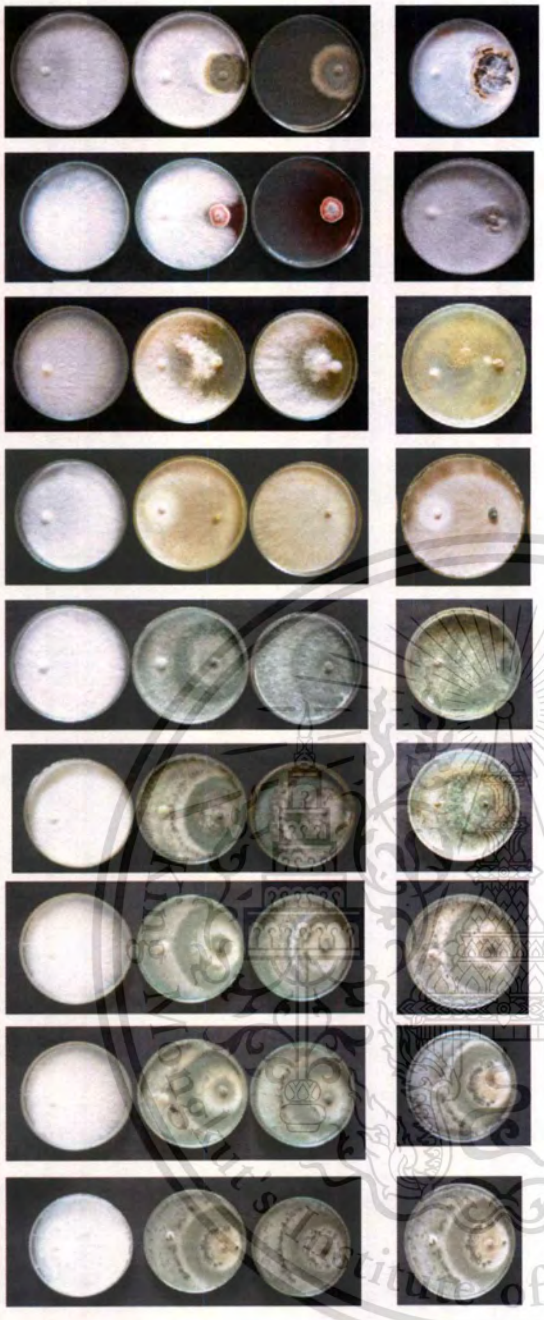


Figure 4.43 Dual culture of antagonistic fungi. The culture from left to right: pathogen culture at 10 days, dual-culture culture at 10 days, antagonist culture at 10 day and dual-culture at 30 days (Continue).

4.3.3 Antagonistic crude extract test

The effective isolates of antagonistic fungi were selected from 4.3.3.1 as follows:- *Aspergillus niger* AN71 and AN72, *Chaetomium bostrychodes* BN08, BN11 and BS01, *Ch. cupreum* RY202, *Trichoderma hamatum* STN07, *T. harzianum* STN01 and STN02 and *T. viride* STN04 to extract their antagonistic substances as crude extracts and tested for their abilities to inhibit the growth *R. microporus* by plate assay. Each isolate was cultured on potato dextrose broth (PDB) for 30 days, yielded mycelial mats, air dried, ground and weighted. The ground mycelium was extracted successively with hexane, ethyl acetate and methanol. The weight of mycelial dry mat, the yield as the weight and percentage of each crude extract were shown in Table 4.7. Crude extracts which extracted with methanol gave the highest percentage of crude extract follow by those which extracted with ethyl acetate and hexane. The crude methanol from *Ch. bostrychodes* BN11 gave the highest yield followed by crude methanol from *T. harzianum* STN02 and crude methanol from *Ch. bostrychodes* BN08. The characteristic of crude extracts were also shown in Figure 4.44. All of them showed oily character and different in color. The crude extracts with methanol gave color darker than those which extracted with ethyl acetate and hexane.

Table 4.7 The yield of crude extracts from antagonistic fungi.

Antagonistic fungi	Mycelium dry weight	Yield of Crude extract					
		Hexane		Ethyl acetate		Methanol	
	(g)	(g)	(%)	(g)	(%)	(g)	(%)
Aspergillus niger AN71	72.3	0.12	0.17	0.18	0.25	0.86	1.19
Aspergillus niger AN72	80.5	0.32	0.40	0.58	0.72	1.76	2.19
Chaetomium bostrychodes BN08	97.1	0.41	0.47	1.67	1.72	7.55	7.78
Chaetomium bostrychodes BN11	87.1	0.46	0.53	1.67	1.92	7.55	8.67
Chaetomium bostrychodes BS01	75.2	0.36	0.48	1.07	1.42	4.55	6.05
Chaetomium cupreum RY202	54.6	0.56	1.03	1.05	1.92	2.24	4.10
Trichoderma hamatum STN07	60.7	0.26	0.45	1.02	1.68	4.42	7.29
Trichoderma harzianum STN01	49.1	2.40	4.89	1.01	2.06	3.51	7.15
Trichoderma harzianum STN02	52.8	2.36	4.47	1.17	2.21	4.15	7.85
Trichoderma viride STN04	60.8	2.29	3.76	2.12	3.48	4.45	7.31

Figure 4.44 The characteristic of crude extracts which extracted from antagonistic fungi.

Crude extracts which extracted from *A. niger*, *Ch. bostrychodes*, *Ch. cupreum*, *T. hamatum*, *T. harzianum* and *T. viride* were tested for their ability to control the growth of *R. microporus*. Table 4.8 showed the effect of crude extracts at the concentration of 1,000 µg/ml to inhibit the growth of *R. microporus* and found here were a significant differ in colony diameter and percentage of colony inhibition at $P = 0.01$. Base on the results, crude hexane, crude ethyl acetate, and crude methanol from *Ch. bostrychodes* BN08, BN11 and BS01 and *A. niger* SN71 and SN72 could not inhibit the growth of *R. microporus*.

Seven kinds of crude extract gave the percentage of colony inhibition over 50% as follows:- crude hexane, crude ethyl acetate, crude methanol from *Ch. cupreum* RY202, crude methanol from *T. hamatum* STN07, *T. harzianum* STN01 and STN02 and *T. viride* STN04. The results also showed that all three kinds of crude which extracted from *Ch. cupreum* RY202 could inhibit the growth of *R. microporus*. Crude hexane from *Ch. cupreum* RY202 gave the highest results to inhibit the growth of pathogen with colony diameter and percentage of colony inhibition of 0.9 cm and 82%, respectively. Among three kinds of crude extract from tested *Trichoderma* species, only crude methanol could inhibit the mycelial growth of *R. microporus*.

Crude hexane extract from *Ch. cupreum* RY202 gave the best result to inhibit the growth of pathogen with ED_{50} value of 170 µg/ml follow by crude methanol extract from *T. hamatum* STN07 and crude ethyl acetate extract from *Ch. cupreum* RY202 with ED_{50} value of 187 and 402 µg/ml, respectively. The effect of crude extracts of these biological control agents are shown in Table 4.8. and Figure 4.45.

From dual culture test, antagonistic crude extract test and bioactive compound test, *Chaetomium cupreum* RY202 was selected to formulate as powder and oil form according to the methods of Assoc. Prof. Dr. Kasem Soytong (unpublished data) for testing their ability to control *R. microporus* in the pot experiment.

Table 4.8 Effect of crude extracts at 1,000 µg/ml to inhibit *Rigidoporus microporus*.

Biological control agents	Crude extracts	Colony diameter (cm)	colony inhibition (%)	ED50 (ug/ml)
Aspergillus niger SN71	hexane	5.0a[1]	0i	NF[2]
	ethyl acetate	3.5ef	30.5de	NF
	methanol	4.8ab	4.0hi	NF
Aspergillus niger SN72	hexane	5.0a	0i	NF
	ethyl acetate	3.4f	33.0d	NF
	methanol	3.7e	25.5e	NF
Chaetoium bostrychodes BN08	hexane	4.5c	10.5g	NF
	ethyl acetate	5.0a	0i	NF
	methanol	5.0a	0i	NF
Chaetoium bostrychodes BN11	hexane	5.0a	0i	NF
	ethyl acetate	5.0a	0i	NF
	methanol	5.0a	0i	NF
Chaetoium bostrychodes BS01	hexane	4.7abc	6.0gh	NF
	ethyl acetate	5.0a	0i	NF
	methanol	5.0a	0i	NF
Chaetomium cupreum RY202	hexane	0.9i	82.0a	170
	ethyl acetate	1.1i	78.0a	402
	methanol	2.5g	50.0c	NF
Trichoderma hamatum STN07	hexane	5.0a	0i	NF
	ethyl acetate	4.1d	17.5f	NF
	methanol	1.0i	80.0a	187
Trichoderma harzianum STN01	hexane	4.7abc	7gh	NF
	ethyl acetate	3.7e	27.5e	NF
	methanol	1.9h	62.5b	556
Trichoderma harzianum STN02	hexane	5.0a	0i	NF
	ethyl acetate	4.7abc	7.0gh	NF
	methanol	1.9h	62.0b	653
Trichoderma viride STN04	hexane	5.0a	0i	NF
	ethyl acetate	5.0a	0i	NF
	methanol	1.9h	61.5b	688
C.V. (%)		3.6	11.3	

[1]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.01$.

NF = no effect

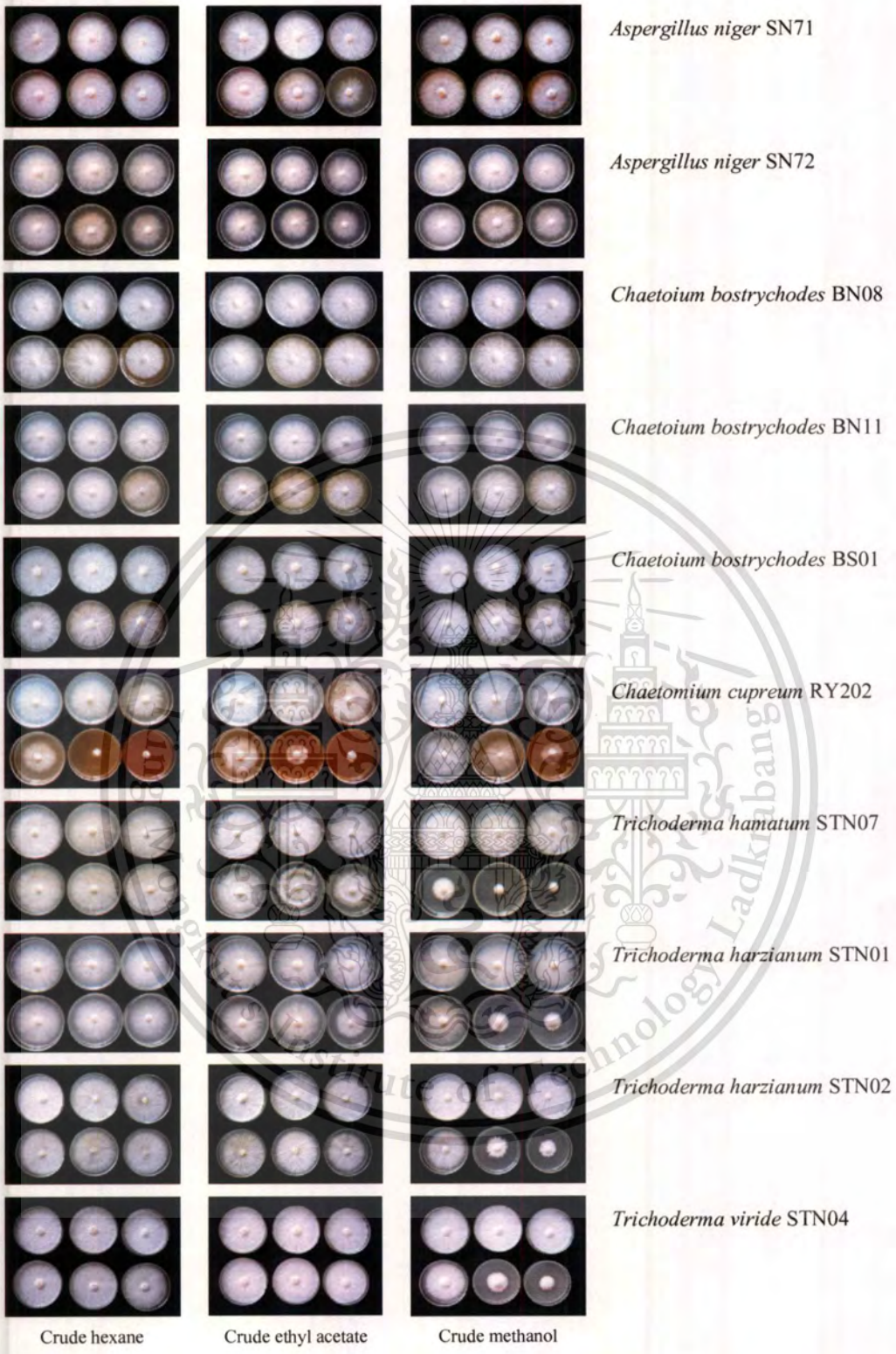


Figure 4.45 Colony of *Rigidoporus microporus* after treated with crude extracts. Each crude extracts test; above row from left to right; concentrations at 0, 10, and 50 µg/l; below row from left to right; concentrations at 100, 500, and 1,000 µg/l.

4.3.4 Bioactive compound test

The characteristic of rotiorinol from *Ch. cupreum* was red and amorphous powder and its structure was showed in Figure 4.46. The results showed that, the effect of rotiorinol on mycelial growth of *R. microporus* was significantly different at $P = 0.01$. The mycelium of *R. microporus* could not grow at concentrations of rotiorinol at 100 and 250 µg/l (Figure 4.47). The fresh weight, dry weight after treated with rotiorinol at 100 and 250 µg/ml were 0.05, 0.03, 0.004 and 0.002 g, respectively. The percent fresh weight and percent dry weight inhibition after treated with rotiorinol at 100 and 250 µg/ml were 97.83, 97.21, 99.32 and 99.05%, respectively. Base on the result, rotiorinol could inhibit the growth of *R. microporus* and ED_{50} was 26 µg/ml.

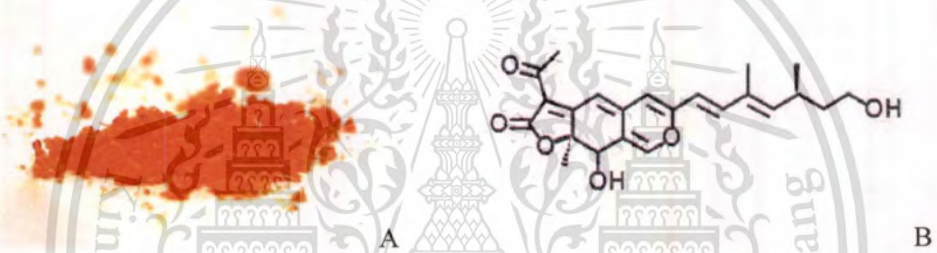


Figure 4.46 Rotiorinol bioactive compound. Red and amorphous powder (A) and chemical structure (B).

Figure 4.47 Mycelial mat of *Rigidoporus microporus* treated with rotiorinol.

Table 4.9 Effect of rotiorinol to inhibit *Rigidoporus microporus*.

Concentrations (µg/ml)	Fresh weight (g)	Fresh weight inhibition (%)	Dry weight (g)	Dry weight inhibition (%)	ED_{50} (µg/ml)
0 (Control)	4.64a[1]	-	0.156a	-	
10	3.26b	29.02c	0.107b	31.37c	
50	2.35c	49.03b	0.091c	42.02b	26.0
100	0.05d	97.83a	0.004d	97.21a	
250	0.03d	99.32a	0.002d	99.05a	
c.v. (%)	15.46	9.72	5.72	3.95	

[1]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.01$.

4.4 Evaluation of Biofungicide to Control White Root Disease in the Pot Experiment

The pot experiment was conducted to test the efficiency of *Chaetomium cupreum* RY202 in the powder and oil form for control *R. microporus*. The experiment was done for five months. Data collection as disease index (DI) was periodically recorded every 4 weeks. Infected root colonization was also be observed and recorded. DI 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.

The results showed that the rhizomorph of the *R. microporus* at the basal stem of rubber tree appeared in the treatment of inoculated with *R. microporus*, whereas there were no any rhizomorph at the basal stem of rubber trees in others treatment at 60 days (Figure 4.48). Disease index at 150 days of non-treated one (T1), inoculated with *R. microporus* (T2), treated with *Ch. cupreum* RY202 in powder form (T3), treated with *Ch. cupreum* RY202 in the oil form (T4), treated with *Ch. cupreum* RY202 in the powder form and *R. microporus* (T5), treated with *Ch. cupreum* RY202 in the oil form and *R. microporus* (T6) and treated with fungicide (sulfur) and *R. microporus* (T7) were 1, 5, 1, 1, 2, 1, and 1, respectively. Base on the results, inoculated with *R. microporus* gave the most severity of symptom followed by treated with *Ch. cupreum* RY202 in the powder form and *R. microporus* with 1-25% yellow leaves (DI = 2). However, there were no significant different in treatments of treated with *Ch. cupreum* RY202 in the powder form and

R. microporus when compared with treatment of fungicide (sulfur) and *R. microporus* (Table 4.10 and Figure 4.49). There was no disease in the treatment of *Ch. cupreum* in the oil form and *R. microporus* (T6). Disease index was reduced when applied biofungicides in the powder and oil form at 60 and 80%, respectively (Table 4.11).

Table 4.10 Disease index of white root disease.

Treatments	Disease Index (DI)[1]				
	30 days	60 days	90 days	120 days	150 days
non-treated (T1)	1	1b[2]	1b	1b	1b
R. microporus (T2)	1	3a	4a	4a	5a
Ch. cupreum in powder form (T3)	1	1a	1b	1b	1b
Ch. cupreum in the oil form (T4)	1	1a	1b	1b	1b
Ch. cupreum in the powder form and *R. microporus* (T5)	1	1a	1b	2b	2b
Ch. cupreum in the oil form and *R. microporus* (T6)	1	1a	1b	1b	1b
Sulfur and *R. microporus* (T7)	1	1a	1b	1b	1b

[1]DI:- 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.

[2]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.01$.

Table 4.11 Disease reduction of white root disease.

Treatments	Disease reduction (%)			
	60 days	90 days	120 days	150 days
Ch. cupreum in powder form	67	75	75	80
Ch. cupreum in the oil form	67	75	75	80
Ch. cupreum in the powder form and *R. microporus*	67	75	50	60
Ch. cupreum in the oil form and *R. microporus*	67	75	75	80
Sulfur and *R. microporus*	67	75	75	80

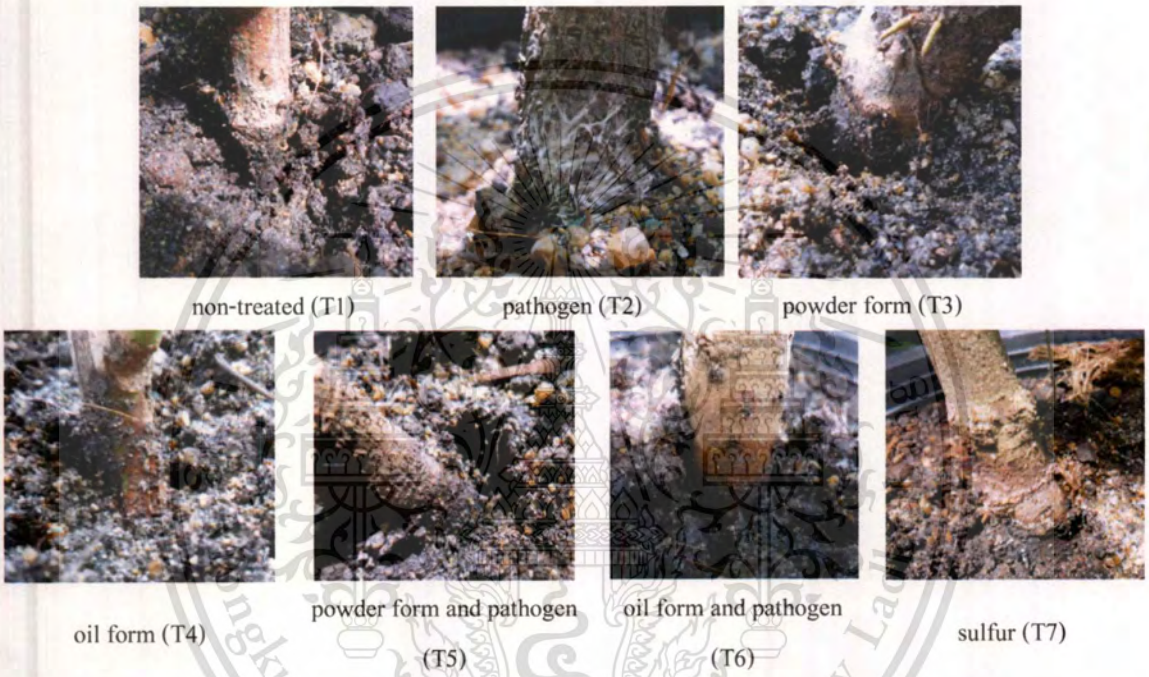


Figure 4.48 Rhyzomorph of the pathogen at the collar of stem after treated with *Chaetomium. cupreum* RY202 in the powder and oil form at 60 days.

T1 = non-treated (control)

T2 = pathogen

T3 = powder form

T4 = oil form

T5 = powder form and pathogen

T6 = oil form and pathogen

T7 = sulfur and pathogen

Figure 4.49 Disease symptom of white root disease in the pot experiment at 120 days.

CHAPTER V

DISCUSSION

White root disease of rubber trees were proved to be seriously occurred in Thailand, especially in the south such as Trang, Narathiwat and Surat Thani provinces as stated by Guyot and Flori (2002) that the disease epidemic was expended to many countries, e.g. India, Indonesia, Maleysia, Sri Lanka, West and Central Africa. The causing agent is *Rigidoporus microporus* causing white root which Hood (2006) stated that *R. microporus* mostly occur in monsoon climate and *Heterobasidion annosum* causing agent of root disease in the temperate zones.

According to white root disease, it is observed that *R. microporus* can infect all stage of plant from seedling to mature trees. The visible symptom of white root disease was clearly shown yellowing leaves on one or a few branches or whole canopy depend on the severity of disease, and finally the tree die. It is also shown that the pathogen can infect the roots by rhizomorph growing from the stumps or infected woody debris remaining in the ground and by contacting with the infected root as also stated by the works of Nandris *et al.* (1987) and Guyot and Flori (2002).

The isolates of *Rigidoporus microporus* were studied the morphology on the PDA medium and studied their fruiting bodies. The colony on PDA showed white and flat. The hypha of this fungus showed hyaline, septum, no clamp connection, and possess many branches. This result was similar to the report of Nandris *et al.* (1987) who stated that the fungus formed many white and flattened mycelium but the colony on malt medium formed superficial, white mycelial felt.

The phylogeny study was still needed for confirmation the species and studying the pathogenic variability among isolates of *R. microporus*. In this study, the phylogenitic tree by ITS revealed that *R. microporus* was in the same branch as *R. ulmaroius*, *Oxyporus*, *Heterobasidion*, and *Laetiporus*, *Melanoporia* defined from out group *Auricularia delicata*. This result is similar to the work of Ryvarden (1991) stated that the *Rigidoporus* is in the same group as *Melanoporia*, *Nigrofomes*, *Heterobasidion*, *Oxyporus*, *Leucophellinus*, *Laetiporus*, *Flavodon* and *Irpex*. However, there is no report on determination of genetic variation among pathogen populations of white root disease pathogen. In this study, 40 isolates of *R. microporus* were tested with ISSR primers to determine the distribution of genetic diversity which represents the difference rubber

tree planting areas Base on the results, the isolates which obtained from three provinces were separated into two groups. It was clearly that there was a relationship between the geographical distributions and clustering of isolates. This result similar to those by Yu *et al.* (2006) stated that ISSR marker of *Melamspora larrici-populina* could divide tested isolates in to northern and western population. On the contrary, Rodrigues *et al* (2004) stated that ISSR-PCR analysis which separated strains of *Guignardia mangiferae* into three groups but not corresponded either to the host or to the geographic origin. It is suggested that ISSR markers could be still a good choice for DNA fingerprint for easy handle and rapid investigation.

All isolated fungi which obtained from soil were tested for their abilities to inhibit the growth of *R. microporus* by dual culture. *Chaetomium cupreum* RY202 was selected to be a good antagonistic fungus against *R. microporus* because it could inhibit the growth of *R. microporus* over 50% in dual culture and grown over the colony of pathogen within 30 days. This is indicated as the first report using *Ch. cupreum* against white root pathogen *in vitro*. The other criteria to select *Ch. cupreum* RY202 was proved by its bioactivity test of crude extract and bioactive compound. Base on the result, crude extracts from *Ch. cupreum* gave the highest results on growth inhibition of *R. microporus* with the effective dose (ED_{50}) of 170 µg/ml. The bioactive compound produced from *Ch. cupreum* named rotiorinol was confirmed its ability to inhibit the growth of *R. microporus* with ED_{50} of 26 µg/ml. This result showed promising biological control potential of *Ch. cupreum* RY202 that could be developed to be biological fungicide to test *in vivo* as stated by Soytong (2005) and Kanokmedhakul *et al.* (2006) that rotiorinol, a bioactive compound from *Ch. cupreum* CC3003 has a potent to inhibit the growth of *Phytophthora parasitica*, *P. palmivora*, *Colletotrichum gloeosporioides*.

In this study, *Ch. cupreum* RY202 was formulated as powder and oil form according to the work of Soytong *et al.* (2001). The biofungicide was tested to inhibit the growth of *R. microporus* and revealed that the disease incidence was not significantly different from sulfur treatment. The results implied that the biofungicide from *Ch. cupreum* RY202 could reduce white root disease of rubber trees. It is suggested that biofungicide produced from *Ch. cupreum* RY202 may have a potential biofungicide to control white root of rubber trees in the field. Further more, this biofungicide will further evaluate in the field trials.

CHAPTER VI

CONCLUSIONS

White root disease caused by *Rigidoporus microporus* (Sw.) Overeem was proved to be seriously disease of rubber plantation in southern part of Thailand e.g. Narathiwat, Trang, Surat Thani provinces.

The phylogeny of *R. microporus* was also confirmed using the technique of DNA sequencing and ISSR-PCR for the species and the pathogenic variability among isolates of *R. microporus*. The phylogenetic tree by ITS showed that *R. microporus* was distincted from *R. ulmarious* but similar correlated to each other. The genetic variation among isolates of *R. microporus* was determined by ISSR. The ISSR technique were distinctly genetic groups into two groups depended on geographical region as Surat Thani and Trang and Narathiwat groups.

Thirty promising antagonistic fungi were tested for their ability to inhibit the growth of *R. microporus* and found *Aspergillus*, *Chaetomium* and *Trichoderma* gave the growth inhibition over 50 %. Among them, crude extracts from *Chaetomium cupreum* RY202 gave the best result to inhibit the growth of *R. microporus*. The crude hexane from *Ch. cupreum* RY202 gave the highest growth inhibition of *R. microporus* over 82.0% and the effective dose (ED_{50}) at 170 µg/l. Whereas, crude ethyl acetate and crude methanol gave the PGI at 78.0 and 50.0% and the effective dose (ED_{50}) at 402 and 1,220 µg/l., respectively. The bioactive compound, rotiorinol from *Ch. cupreum* could inhibit the growth of *R. microporus* with ED_{50} was 26 µg/ml. *Ch. cupreum* RY202 was then formulated as powder and oil forms. These formulations were preliminary proved to control white root disease of rubber trees *in vivo*.

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APPENDIXES

Appendix A: Statistical Analysis

Table 1. The disease index of pathogenicity test.

Isolate	Disease Index (DI)				Total	Mean
	1	2	3	4		
SSS01	1	1	1	2	5	1.3
SST01	5	4	1	2	12	3.0
SST02	5	1	5	1	12	3.0
SST 04	1	1	2	5	9	2.3
SST05	5	5	1	5	16	4.0
SST06	3	1	1	5	10	2.5
SST07	1	3	1	4	9	2.3
SST08	2	1	1	1	5	1.3
SST09	5	1	1	1	8	2.0
SST11	1	2	1	1	5	1.3
SST12	3	5	1	1	10	2.5
SST13	5	3	2	1	11	2.8
SST14	1	1	1	5	8	2.0
SST15	5	5	1	2	13	3.3
SST16	5	5	5	1	16	4.0
SND04	5	5	4	1	15	3.8
SND05	5	1	1	1	8	2.0
SND07	5	1	1	1	8	2.0
SND08	5	3	5	5	18	4.5
SND10	5	4	1	1	11	2.8
SNP02	5	5	1	1	12	3.0
SNP05	5	1	1	1	8	2.0
SNP06	5	5	1	1	12	3.0
SNP08	5	5	3	1	14	3.5
SNK02	5	5	5	5	20	5.0
SNK03	5	5	5	5	20	5.0
SNK04	5	1	1	1	8	2.0
SNK05	5	5	5	1	16	4.0
SNK06	5	5	4	2	16	4.0

Table 1. The disease index of pathogenicity test (Continue).

Isolate	Disease Index (DI)				Total	Mean
	1	2	3	4		
SNK09	5	1	1	1	8	2.0
SNK10	5	1	1	1	8	2.0
SNS01	1	2	1	1	5	1.3
SNS02	3	2	1	2	8	2.0
SNS03	1	5	2	2	10	2.5
SNS04	2	1	1	1	5	1.3
SNS05	1	5	1	1	8	2.0
SNS06	1	1	1	1	4	1.0
SNS07	1	5	2	2	10	2.5
SNS08	5	1	1	1	8	2.0
SNS09	5	1	1	1	8	2.0
SNS10	2	2	1	1	6	1.5
SNS11	2	1	5	5	13	3.3
STR01	1	1	1	1	4	1.0
STR02	5	5	1	1	12	3.0
STR03	1	1	1	1	4	1.0
STR04	5	4	1	1	11	2.8
STR05	3	2	1	1	7	1.8
STR06	1	1	1	1	4	1.0
STR07	4	1	1	1	7	1.8
STR08	2	1	1	1	5	1.3

Table 2. Analysis of variance of disease index of pathogenicity test.

Source	df	SS	MS	F	F.05	F.01
Treatment	49	216.50	4.42	1.63*	1.50	1.76
Ex.Error	50	407.00	2.71			
Total	99	623.50	3.13			
Grand mean	2.45					
CV (%)	67.23					

LSD.05 = 2.31

LSD.01 = 3.05

*Significant difference at P = 0.05

Table 3. Colony diameter of pathogen in dual culture.

Promising antagonist	Colony diameter (cm)				Total	Mean
	1	2	3	4		
Acremonium fusidioides SS01	6	6.5	6.3	5.8	24.6	6.2
A. fusidioides SS02	6	6	6.3	5.8	24.1	6.0
Aspergillus niger SN71	2.2	2.5	2.6	3	10.3	2.6
A. niger SN72	2.4	2	2.3	2	8.7	2.2
Chaetomium aureum BN01	7	6.8	7.2	7	28	7.0
Ch. aureum BN02	6.6	7	6.6	7	27.2	6.8
Ch. bostrychodes BN08	3.9	3.5	3.7	4	15.1	3.8
Ch. bostrychodes BN10	4.2	3.8	3.9	4.1	16	4.0
Ch. bostrychodes BN11	4	3.8	3.7	4	15.5	3.9
Ch. bostrychodes BS01	3.9	4	4	4	15.9	4.0
Ch. bostrychodes BS02	3.5	3.8	3.5	3.8	14.6	3.7
Ch. bostrychodes BS03	3.8	3.8	3.6	3.5	14.7	3.7
Ch. bostrychodes BS04	3.5	3.6	3.5	4	14.6	3.7
Ch. cochliodes RY301	3.5	3.7	3.6	3.5	14.3	3.6
Ch. cupreum RY202	3.9	3.8	4	3.6	15.3	3.8
Ch. fusiforme BN07	7.4	7.3	7	7	28.7	7.2
Ch. fusiforme BS31	7.4	7.3	7.5	7.5	29.7	7.4
Ch. fusiforme BS32	7	6.7	6.7	6.9	27.3	6.8
Ch. fusiforme BS33	7	7	6.4	6.8	27.2	6.8
Ch. fusiforme BS34	6.5	6.7	6.7	6.9	26.8	6.7
Ch. indicum BN05	7	7.5	7.2	7.5	29.2	7.3
Ch. indicum BN06	7	7.5	7.5	7.5	29.5	7.4
Penicillium canescens SN03	8	8	9	8	33	8.3
Trichoderma hamatum STN06	1	1.2	1.5	1.2	4.9	1.2
T. hamatum STN07	1	1	1	1	4	1.0
T. harzianum STN01	1	1.3	1.5	1.5	5.3	1.3
T. harzianum STN02	1	1.3	1.1	1.2	4.6	1.2
T. viride STN03	1	1.2	1	1	4.2	1.1
T. viride STN04	1	0.9	1	0.9	3.8	1.0
T. viride STN05	1	0.9	1	0.9	3.8	1.0

Table 4. Analysis of variance of colony diameter of pathogen in dual culture.

Source	df	SS	MS	F	F.05	F.01
Treatment	29	690.45	23.81	544.91**	1.65	2.03
Ex.Error	90	3.93	0.04			
Total	19	694.41	5.84			
Grand mean	4.34					
CV (%)	4.82					

LSD .05 = 0.29

LSD .01 = 0.39

*Significant difference at P = 0.01

Table 5. Percent colony inhibition of pathogen in dual culture.

Promising antagonist	Colony inhibition (%)				Total	Mean
	1	2	3	4		
Acremonium fusidioides SS01	33.3	27.8	30.0	35.6	126.7	31.7
A. fusidioides SS02	33.3	33.3	30.0	35.6	132.2	33.1
Aspergillus niger SN71	75.6	72.2	71.1	66.7	285.6	71.4
A. niger SN72	73.3	77.8	74.4	77.8	303.3	75.8
Chaetomium aureum BN01	22.2	24.4	20.0	22.2	88.9	22.2
Ch. aureum BN02	26.7	22.2	26.7	22.2	97.8	24.4
Ch. bostrychodes BN08	56.7	61.1	58.9	55.6	232.2	58.1
Ch. bostrychodes BN10	53.3	57.8	56.7	54.4	222.2	55.6
Ch. bostrychodes BN11	55.6	57.8	58.9	55.6	227.8	56.9
Ch. bostrychodes BS01	56.7	55.6	55.6	55.6	223.3	55.8
Ch. bostrychodes BS02	61.1	57.8	61.1	57.8	237.8	59.4
Ch. bostrychodes BS03	57.8	57.8	60.0	61.1	236.7	59.2
Ch. bostrychodes BS04	61.1	60.0	61.1	55.6	237.8	59.4
Ch. cochliodes RY301	61.1	58.9	60.0	61.1	241.1	60.3
Ch. cupreum RY202	56.7	57.8	55.6	60.0	230.0	57.5
Ch. fusiforme BN07	17.8	18.9	22.2	22.2	81.1	20.3
Ch. fusiforme BS31	17.8	18.9	16.7	16.7	70.0	17.5
Ch. fusiforme BS32	22.2	25.6	25.6	23.3	96.7	24.2
Ch. fusiforme BS33	22.2	22.2	28.9	24.4	97.8	24.4

Table 5. Percent colony inhibition of pathogen in dual culture (Continue).

Promising antagonist	Colony inhibition (%)				Total	Mean
	1	2	3	4		
Ch. fusiforme BS34	27.8	25.6	25.6	23.3	102.2	25.6
Ch. indicum BN05	22.2	16.7	20.0	16.7	75.6	18.9
Ch. indicum BN06	22.2	16.7	16.7	16.7	72.2	18.1
Penicillium canescens SN03	11.1	11.1	0.0	11.1	33.3	8.3
Trichoderma hamatum STN06	88.9	86.7	83.3	86.7	345.6	86.4
T. hamatum STN07	88.9	88.9	88.9	88.9	355.6	88.9
T. harzianum STN01	88.9	85.6	83.3	83.3	341.1	85.3
T. harzianum STN02	88.9	85.6	87.8	86.7	348.9	87.2
T. viride STN03	88.9	86.7	88.9	88.9	353.3	88.3
T. viride STN04	88.9	90.0	88.9	90.0	357.8	89.4
T. viride STN05	88.9	90.0	88.9	90.0	357.8	89.4

Table 6. Analysis of variance percent colony inhibition of pathogen in dual culture.

Source	df	SS	MS	F	F.05	F.01
Treatment	29	85262.03	2940.07	545.32**	1.65	2.03
Ex.Error	90	485.23	5.39			
Total	19	85747.26	720.57			
Grand mean	51.77					
CV (%)	4.49					

LSD .05 = 3.25

LSD .01 = 4.30

*Significant difference at P = 0.01

Table 7. Effect of crude extracts at 1000 µg/ml to *Rigidoporus microporus*.

Antagonistic fungi	Crude extract	Colony diameter (cm)				Total	Mean
		1	2	3	4		
Aspergillus niger SN71	hexane	5	5	5	5	20	5
	Ethyl acetate	3.7	3.5	3.2	3.5	13.9	3.475
	Methanol	4.8	5	4.7	4.7	19.2	4.8
A. niger SN72	hexane	5	5	5	5	20	5
	Ethyl acetate	3.5	3.2	3.3	3.4	13.4	3.35
	Methanol	3.6	3.8	3.9	3.6	14.9	3.725
Chaetomium bostrychodes BN08	hexane	4.3	4.6	4.5	4.5	17.9	4.475
	Ethyl acetate	5	5	5	5	20	5
	Methanol	5	5	5	5	20	5
Ch. bostrychodes BN11	hexane	5	5	5	5	20	5
	Ethyl acetate	5	5	5	5	20	5
	Methanol	5	5	5	5	20	5
Ch. bostrychodes BS04	hexane	4.8	4.7	4.7	4.6	18.8	4.7
	Ethyl acetate	5	5	5	5	20	5
	Methanol	5	5	5	5	20	5
Ch. cupreum RY202	hexane	0.9	1	0.8	0.9	3.6	0.9
	Ethyl acetate	1.5	1.1	0.9	0.9	4.4	1.1
	Methanol	2.5	2.5	2.5	2.5	10	2.5
Trichoderma hamatum STN07	hexane	5	5	5	5	20	5
	Ethyl acetate	4	4.1	4.2	4.2	16.5	4.125
	Methanol	0.9	1	1.1	1	4	1
T. harzianum STN01	hexane	4.8	4.8	4	5	18.6	4.65
	Ethyl acetate	3.5	3.5	3.9	3.7	14.6	3.65
	Methanol	1.8	2.1	1.8	1.8	7.5	1.875
T. harzianum STN02	hexane	5	5	5	5	20	5
	Ethyl acetate	5	5	5	5	20	5
	Methanol	2	1.5	2.1	2	7.6	1.9
T. viride STN04	hexane	5	5	5	5	20	5
	Ethyl acetate	4.8	4.6	4.6	4.8	18.8	4.7
	Methanol	2	1.8	1.9	2	7.7	1.925

Table 8. Analysis of variance of effect of crude extracts at 1000 µg/ml to *Rigidoporus microporus*.

Source	df	SS	MS	F	F.05	F.01
Treatment	29	234.11	8.07	418.76**	1.65	2.03
Ex.Error	90	1.74	0.02			
Total	19	235.84	1.98			
Grand mean	3.93					
CV (%)	3.53					

LSD .05 = 0.19

LSD .01 = 0.25

*Significant difference at P = 0.01

Table 9. Percentage of effect of crude extracts at 1000 µg/ml to *Rigidoporus microporus*.

Antagonistic fungi	Crude extracts	Colony inhibition (%)				Total	Mean
		1	2	3	4		
Aspergillus niger SN71	hexane	0	0	0	0	0	0
	Ethyl acetate	26	30	36	30	122	30.5
	Methanol	4	0	6	6	16	4
A. niger SN72	hexane	0	0	0	0	0	0
	Ethyl acetate	30	36	34	32	132	33
	Methanol	28	24	22	28	102	25.5
Chaetomium bostrychodes BN08	hexane	14	8	10	10	42	10.5
	Ethyl acetate	0	0	0	0	0	0
	Methanol	0	0	0	0	0	0
Ch. bostrychodes BN11	hexane	0	0	0	0	0	0
	Ethyl acetate	0	0	0	0	0	0
	Methanol	0	0	0	0	0	0
Ch. bostrychodes BS04	hexane	4	6	6	8	24	6
	Ethyl acetate	0	0	0	0	0	0
	Methanol	0	0	0	0	0	0

Table 9. Percentage of effect of crude extracts at 1000 µg/ml to *Rigidoporus microporus* (Continue).

Antagonistic fungi	Crude extracts	Colony inhibition (%)				Total	Mean
		1	2	3	4		
Ch. cupreum RY202	hexane	82	80	84	82	328	82
	Ethyl acetate	70	78	82	82	312	78
	Methanol	50	50	50	50	200	50
Trichoderma hamatum STN07	hexane	0	0	0	0	0	0
	Ethyl acetate	20	18	16	16	70	17.5
	Methanol	82	80	78	80	320	80
T. harzianum STN01	hexane	4	4	20	0	28	7
	Ethyl acetate	30	30	22	26	108	27
	Methanol	64	58	64	64	250	62.5
T. harzianum STN02	hexane	0	0	0	0	0	0
	Ethyl acetate	0	0	0	0	0	0
	Methanol	60	70	58	60	248	62
T. viride STN04	hexane	0	0	0	0	0	0
	Ethyl acetate	4	8	8	4	24	6
	Methanol	60	64	62	60	246	61.5

Table 10. Analysis of variance of percentage of effect of crude extracts at 1000 µg/ml to *Rigidoporus microporus*.

Source	df	SS	MS	F	F.05	F.01
Treatment	29	93643.47	3229.09	418.76**	1.65	2.03
Ex.Error	90	694.00	7.71			
Total	19	94337.47	792.75			
Grand mean	21.43					
CV (%)	12.96					

LSD .05 = 3.89

LSD .01 = 5.14

*Significant difference at P = 0.01

Table 11. Fresh weight of *Rigidoporus microporus* after treated with rotiorinol.

Concentration	Fresh weight (g)				Total	Mean
(µg/ml)	1	2	3	4		
0	5.39	4.52	4.27	4.37	18.55	4.64
10	3.33	3.20	3.267	3.27	13.07	3.23
50	2.25	3.04	1.86	2.25	9.39	2.35
100	0.06	0.05	0.06	0.05	0.23	0.06
250	0.04	0.02	0.04	0.025	0.13	0.03

Table 12 Analysis of variance of fresh weight of *Rigidoporus microporus* after treated with rotiorinol.

Source	df	SS	MS	F	F.05	F.01
Treatment	4	65.20	16.30	159.42**	3.06	4.89
Ex.Error	15	1.53	0.10			
Total	19	66.73	3.51			
Grand mean	2.07					
CV (%)	15.46					

LSD.05 = 0.48

LSD.01 = 0.67

*Significant difference at P = 0.01

Table 13. Dry weight of *Rigidoporus microporus* after treated with rotiorinol.

Concentration (µg/ml)	Dry weight (g)				Total	Mean
	1	2	3	4		
0	0.1649	0.1490	0.1524	0.1584	0.6247	0.1562
10	0.1090	0.1109	0.1085	0.0995	0.4279	0.1070
50	0.0950	0.0880	0.0895	0.0895	0.3620	0.0905
100	0.0042	0.0050	0.0039	0.0043	0.0174	0.0044
250	0.0016	0.0018	0.0011	0.0014	0.0059	0.0015

Table 14. Analysis of variance of dry weight of *Rigidoporus microporus* after treated with rotiorinol.

Source	df	SS	MS	F	F.05	F.01
Treatment	4	0.0728	0.0182	1075.16**	3.06	4.89
Ex.Error	15	0.0003	0.0000			
Total	19	0.0731	0.0038			
Grand mean	7.19					
CV (%)	5.72					

LSD .05 = 6.20

LSD .01 = 8.57

*Significant difference at P = 0.01

Table 15. Percentage fresh weight inhibition after treated with rotiorinol.

Concentration	Fresh weight inhibition (%)				Total	Mean
(µg/ml)	1	2	3	4		
0						
10	38.14	29.24	23.51	25.20	116.10	29.024
50	58.32	32.79	56.45	48.54	196.10	49.025
100	98.85	98.26	96.59	97.64	391.33	97.83
250	99.28	99.46	99.09	99.44	397.26	99.32

Table 16. Analysis of variance of percentage fresh weight inhibition after treated with rotiorinol.

Source	df	SS	MS	F	F.05	F.01
Treatment	3	14988.86	4996.29	111.80**	5.95	3.49
Ex.Error	12	536.28	44.69			
Total	15	15525.14	1035.01			
Grand mean	68.80					
CV (%)	9.72					

LSD.05 = 10.30

LSD.01 = 14.44

*Significant difference at P = 0.01

Table 17. Percentage dry weight inhibition after treated with rotiorinol.

Concentration (µg/ml)	Dry weight inhibition (%)				Total	Mean
	1	2	3	4		
0	0	0	0	0		
10	33.90	25.57	28.81	37.18	125.46	31.36
50	42.39	40.94	41.27	43.50	168.10	42.02
100	97.45	96.64	97.44	97.29	388.82	97.21
250	99.03	98.79	99.28	99.12	396.22	99.05

Table 18. Analysis of variance of percentage dry weight inhibition after treated with rotiorinol.

Source	df	SS	MS	F	F.05	F.01
Treatment	3	15331.15	5110.38	721.30**	3.49	5.95
Ex.Error	12	85.02	7.09			
Total	15	15416.17	1027.75			
Grand mean	67.41					
CV (%)	3.95					

LSD .05 = 4.10

LSD .01 = 5.75

*Significant difference at P = 0.01

Table 19. Effect of biofungicide in the pot experiment at 60 days.

Treatments	Disease index				Total	Mean
	1	2	3	4		
T1	1	1	1	1	4	1
T2	1	5	5	1	12	3
T3	1	1	1	1	4	1
T4	1	1	1	1	4	1
T5	1	1	1	1	4	1
T6	1	1	1	1	4	1
T7	1	1	1	1	4	1

Table 20. Analysis of variance of effect of biofungicide in the pot experiment at 60 days.

Source	**df**	**SS**	**MS**	**F**	**F.05**	**F.01**
Treatment	6	13.71	2.29	3.00*	2.57	3.81
Ex.Error	21	16.00	0.76			
Total	27	29.71	1.10			
Grand mean	1.29					
CV (%)	67.89					

LSD .05 = 1.28

LSD .01 = 1.75

*Significant difference at P = 0.05

Table 21. Effect of biofungicide in the pot experiment at 90 days.

Treatments	Disease index				Total	Mean
	1	2	3	4		
T1	1	1	1	1	4	1
T2	1	5	5	5	16	4
T3	1	1	1	1	4	1
T4	1	1	1	1	4	1
T5	1	1	1	1	4	1
T6	1	1	1	1	4	1
T7	1	1	1	1	4	1

Table 22. Analysis of variance of effect of biofungicide in the pot experiment at 90 days.

Source	df	SS	MS	F	F.05	F.01
Treatment	6	30.86	5.14	9.00**	2.57	3.81
Ex.Error	21	12.00	0.57			
Total	27	42.86	1.59			
Grand mean	1.43					
CV (%)	52.92					

LSD .05 = 1.11

LSD .01 = 1.51

*Significant difference at P = 0.01

Table 23. Effect of biofungicide in the pot experiment at 120 days.

Treatment	Disease index				Total	Mean
	1	2	3	4		
T1	1	1	1	1	4	1
T2	1	5	5	5	16	4
T3	1	1	1	1	4	1
T4	1	1	1	1	4	1
T5	1	1	5	1	8	2
T6	1	1	1	1	4	1
T7	1	1	1	1	4	1

Table 24. Analysis of variance of effect of biofungicide in the pot experiment at 120 days.

Source	df	SS	MS	F	F.05	F.01
Treatment	6	30.86	5.143	4.50**	2.57	3.81
Ex.Error	21	24.00	1.143			
Total	27	54.86	2.03			
Grand mean	1.571					
CV (%)	68.03					

LSD .05 = 1.57

LSD .01 = 2.14

*Significant difference at P = 0.05

Table 25. Effect of biofungicide in the pot experiment at 150 days.

Treatment	Disease index				Total	Mean
	1	2	3	4		
T1	1	1	1	1	4	1
T2	5	5	5	5	20	5
T3	1	1	1	1	4	1
T4	1	1	1	1	4	1
T5	1	1	5	1	8	2
T6	1	1	1	1	4	1
T7	1	1	1	1	4	1

Table 26. Analysis of variance of effect of biofungicide in the pot experiment at 150 days.

Source	df	SS	MS	F	F.05	F.01
Treatment	6	53.71	8.95	15.67**	2.57	3.81
Ex.Error	21	12.00	0.57			
Total	27	65.71	2.43			
Grand mean	1.71					
CV (%)	44.09					

LSD .05 = 1.11

LSD .01 = 1.51

*Significant difference at P = 0.01

Table 27. Colony inhibition of *Rigidoporus microporus* after treated with crude extracts.

Biological control agents	Crude extracts	Colony diameter (cm)				
		10	50	100	500	1000
Aspergillus niger SN71	hexane	5[a*]	5[a]	5[a]	5[a]	5[a]
	ethyl acetate	5[a]	5[a]	5[a]	4.7[b]	3.5[f]
	methanol	5[a]	5[a]	5[a]	5[a]	4.8[ab]
Aspergillus niger SN72	hexane	5[a]	5[a]	5[a]	5[a]	5[a]
	ethyl acetate	5[a]	4.0[d]	3.8c	3.6[g]	3.4[f]
	methanol	5[a]	5[a]	5[a]	4.0[e]	3.7[e]
Chaetomium bostrychodes BN08	hexane	5[a]	5[a]	5[a]	5[a]	4.5[c]
	ethyl acetate	5[a]	5[a]	5[a]	5[a]	5[a]
	methanol	5[a]	5[a]	5[a]	5[a]	5[a]
Chaetomium bostrychodes BN11	hexane	5[a]	5[a]	5[a]	5[a]	5[a]
	ethyl acetate	5[a]	5[a]	5[a]	5[a]	5[a]
	methanol	5[a]	5[a]	5[a]	5[a]	5[a]
Chaetomium bostrychodes BS01	hexane	5[a]	5[a]	5[a]	5[a]	4.7[bc]
	ethyl acetate	5[a]	5[a]	5[a]	5[a]	5[a]
	methanol	5[a]	5[a]	5[a]	5[a]	5[a]
Chaetomium cupreum RY202	hexane	4.4[c]	4.0[d]	4.0b	1.0[k]	0.9[i]
	ethyl acetate	4.6[b]	4.4[c]	4.0b	2.9[h]	1.1[i]
	methanol	5[a]	4.7[b]	4.1b	3.8[f]	2.5[g]
Trichoderma hamatum STN07	hexane	5[a]	5[a]	5[a]	5[a]	5[a]
	ethyl acetate	5[a]	5[a]	5[a]	4.3[d]	4.1[d]
	methanol	5[a]	5[a]	2.4d	1.0[k]	1.0[i]
Trichoderma harzianum STN01	hexane	5[a]	5[a]	5[a]	4.9[a]	4.7[bc]
	ethyl acetate	5[a]	5[a]	5[a]	4.5[c]	3.7[c]
	methanol	5[a]	5[a]	5[a]	2.1[j]	1.9[h]
Trichoderma harzianum STN02	hexane	5[a]	5[a]	5[a]	5[a]	5[a]
	ethyl acetate	5[a]	5[a]	5[a]	5[a]	4.7[bc]
	methanol	5[a]	5[a]	5[a]	2.7[i]	1.9[h]
Trichoderma viride STN04	hexane	5[a]	5[a]	5[a]	5[a]	5[a]
	ethyl acetate	5[a]	5[a]	5[a]	5[a]	5[a]
	methanol	5[a]	5[a]	5[a]	2.7[i]	1.9[h]
C.V. (%)		0.8	2.6	1.1	1.6	3.6

*Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.01$.

Table 28. Percentage colony inhibition of *Rigidoporus microporus* after treated with crude extracts.

Biological control agents	Crude extracts	colony inhibition (%)				
		10	50	100	500	1,000
Aspergillus niger SN71	hexane	0^{c*}	0^{d}	0^{e}	0^{k}	0^{j}
	ethyl acetate	0^{c}	0^{d}	0^{e}	6.0^{j}	30.0^{f}
	methanol	0^{c}	0^{d}	0^{e}	0^{k}	4.0^{ij}
Aspergillus niger SN72	hexane	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
	ethyl acetate	0^{c}	19.5^{a}	24.0^{b}	28.5^{c}	33.0^{e}
	methanol	0^{c}	0^{d}	0^{e}	20.0^{g}	25.5^{f}
Chaetomium bostrychodes BN08	hexane	0^{c}	0^{d}	0^{e}	0^{k}	9.0^{h}
	ethyl acetate	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
	methanol	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
Chaetomium bostrychodes BN11	hexane	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
	ethyl acetate	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
	methanol	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
Chaetomium bostrychodes BS01	hexane	0^{c}	0^{d}	0^{e}	0^{k}	5.5^{hi}
	ethyl acetate	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
	methanol	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
Chaetomium cupreum RY202	hexane	11.0^{a}	13.0^{b}	21.0^{c}	80.5^{a}	82.0^{a}
	ethyl acetate	9.5^{b}	13.5^{b}	20.5^{c}	42.5^{d}	78.0^{a}
	methanol	0^{c}	9.0^{c}	19.0^{d}	24.5^{f}	50.0^{c}
Trichoderma hamatum STN07	hexane	0^{c}	0^{d}	0^{e}	0^{k}	4.5^{i}
	ethyl acetate	0^{c}	0^{d}	0^{e}	15.0^{h}	17.5^{g}
	methanol	0^{c}	0^{d}	53.0^{a}	80.0^{a}	80.0^{a}
Trichoderma harzianum STN01	hexane	0^{c}	0^{d}	0^{e}	0.0^{k}	0^{j}
	ethyl acetate	0^{c}	0^{d}	0^{e}	10.0^{i}	27.5^{f}
	methanol	0^{c}	0^{d}	0^{e}	59.0^{b}	61.5^{b}
Trichoderma harzianum STN02	hexane	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
	ethyl acetate	0^{c}	0^{d}	0^{e}	0^{k}	6.0^{hi}
	methanol	0^{c}	0^{d}	0^{e}	46.0^{c}	63.5^{b}
Trichoderma viride STN04	hexane	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
	ethyl acetate	0^{c}	0^{d}	0^{e}	0^{k}	0^{j}
	methanol	0^{c}	0^{d}	0^{e}	47.0^{c}	61.5^{b}
C.V. (%)		62.7	37.2	15.1	8.7	11.3

*Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.01$.

Table 29. Average genetic similarity of *Rigidoporus microporus* isolates, calculated using DICE similarities coefficient in SIMQUAL program of NTSYSpc Package by ISSR markers.

	STR03	STR04	STR05	STR06	STR07	STR08	SSS01	SST01	SST02	SST04	SST05	SST06	SST07	SST08	SST09	SST11	SST12	SST13	SST14	SSt15	SSt16
STR03	1.00																				
STR04	0.91	1.00																			
STR05	0.90	0.89	1.00																		
STR06	0.89	0.91	0.88	1.00																	
STR07	0.88	0.97	0.88	0.92	1.00																
STR08	0.88	0.95	0.88	0.90	0.97	1.00															
SSS01	0.87	0.91	0.85	0.88	0.90	0.92	1.00														
SSt01	0.86	0.88	0.84	0.87	0.90	0.92	0.94	1.00													
SSt02	0.87	0.88	0.83	0.86	0.88	0.87	0.93	0.95	1.00												
SSt04	0.86	0.84	0.83	0.90	0.84	0.84	0.89	0.92	0.92	1.00											
SSt05	0.87	0.87	0.85	0.84	0.87	0.87	0.92	0.94	0.95	0.92	1.00										
SSt06	0.87	0.88	0.83	0.85	0.89	0.89	0.91	0.92	0.94	0.92	0.96	1.00									
SSt07	0.87	0.85	0.85	0.88	0.87	0.85	0.86	0.89	0.88	0.90	0.87	0.87	1.00								
SSt08	0.86	0.83	0.87	0.85	0.85	0.85	0.89	0.89	0.92	0.87	0.92	0.89	0.85	1.00							
SSt09	0.84	0.83	0.83	0.84	0.85	0.86	0.89	0.90	0.89	0.90	0.91	0.90	0.84	0.89	1.00						
SSt11	0.85	0.84	0.84	0.85	0.85	0.86	0.89	0.90	0.90	0.90	0.92	0.90	0.86	0.89	0.96	1.00					
SSt12	0.82	0.81	0.87	0.84	0.80	0.79	0.80	0.82	0.83	0.82	0.82	0.81	0.89	0.87	0.77	0.79	1.00				
SSt13	0.86	0.88	0.86	0.88	0.88	0.89	0.92	0.93	0.93	0.90	0.90	0.90	0.89	0.90	0.88	0.91	0.85	1.00			
SSt14	0.83	0.79	0.86	0.81	0.78	0.78	0.79	0.81	0.81	0.79	0.82	0.80	0.85	0.87	0.78	0.80	0.94	0.82	1.00		
SSt15	0.83	0.79	0.86	0.82	0.78	0.78	0.79	0.81	0.81	0.79	0.82	0.81	0.86	0.87	0.79	0.80	0.95	0.82	0.97	1.00	
SSt16	0.89	0.85	0.88	0.87	0.86	0.87	0.88	0.88	0.88	0.87	0.89	0.87	0.86	0.88	0.90	0.92	0.84	0.95	0.84	0.84	1.00
SNS01	0.85	0.85	0.83	0.89	0.84	0.84	0.86	0.89	0.82	0.85	0.79	0.80	0.83	0.83	0.78	0.80	0.83	0.89	0.81	0.83	0.87
SNS02	0.83	0.83	0.82	0.84	0.83	0.83	0.88	0.87	0.87	0.86	0.84	0.84	0.83	0.84	0.82	0.82	0.82	0.88	0.77	0.79	0.84
SNS03	0.82	0.83	0.85	0.86	0.82	0.83	0.84	0.85	0.84	0.87	0.82	0.82	0.83	0.84	0.79	0.80	0.83	0.86	0.81	0.82	0.82
SNS04	0.83	0.83	0.84	0.86	0.82	0.83	0.84	0.85	0.84	0.87	0.82	0.82	0.83	0.84	0.79	0.80	0.83	0.86	0.79	0.81	0.82
SNS07	0.82	0.83	0.82	0.85	0.81	0.82	0.82	0.83	0.83	0.85	0.80	0.79	0.81	0.82	0.78	0.79	0.81	0.83	0.78	0.79	0.79
SNS10	0.83	0.83	0.83	0.87	0.82	0.83	0.82	0.82	0.82	0.85	0.79	0.80	0.83	0.83	0.78	0.80	0.83	0.85	0.80	0.81	0.81
SNS11	0.82	0.83	0.83	0.87	0.82	0.82	0.81	0.82	0.82	0.84	0.79	0.79	0.82	0.83	0.77	0.80	0.82	0.84	0.79	0.80	0.79
SNK02	0.85	0.84	0.88	0.85	0.82	0.83	0.82	0.82	0.82	0.84	0.82	0.83	0.81	0.82	0.79	0.82	0.81	0.83	0.80	0.81	0.80
SNK03	0.84	0.83	0.85	0.84	0.81	0.82	0.81	0.81	0.81	0.83	0.81	0.82	0.80	0.81	0.78	0.81	0.81	0.82	0.80	0.81	0.80
SNK05	0.85	0.82	0.87	0.85	0.80	0.81	0.81	0.81	0.80	0.83	0.81	0.81	0.82	0.83	0.79	0.81	0.84	0.83	0.83	0.85	0.83
SNK06	0.84	0.83	0.84	0.84	0.81	0.82	0.80	0.81	0.80	0.83	0.82	0.82	0.80	0.80	0.80	0.83	0.80	0.82	0.79	0.81	0.82
SNK09	0.81	0.82	0.82	0.83	0.80	0.81	0.81	0.81	0.82	0.83	0.80	0.80	0.82	0.81	0.77	0.80	0.81	0.83	0.79	0.81	0.80
SNK10	0.83	0.83	0.84	0.83	0.81	0.82	0.82	0.83	0.83	0.83	0.81	0.81	0.83	0.82	0.78	0.81	0.82	0.83	0.80	0.81	0.80
SNP05	0.82	0.81	0.84	0.83	0.80	0.81	0.82	0.82	0.82	0.84	0.82	0.81	0.81	0.81	0.79	0.81	0.81	0.82	0.78	0.80	0.79
SNP06	0.87	0.83	0.88	0.86	0.81	0.83	0.86	0.85	0.85	0.87	0.85	0.85	0.84	0.88	0.83	0.83	0.87	0.85	0.84	0.86	0.85
SNP08	0.84	0.80	0.82	0.83	0.79	0.79	0.81	0.80	0.79	0.8	0.80	0.79	0.79	0.81	0.81	0.82	0.79	0.80	0.77	0.77	0.81
SND05	0.85	0.81	0.83	0.83	0.79	0.79	0.80	0.80	0.79	0.83	0.81	0.80	0.79	0.80	0.79	0.82	0.81	0.82	0.80	0.80	0.82
SND07	0.83	0.80	0.81	0.82	0.78	0.79	0.79	0.79	0.77	0.81	0.77	0.79	0.79	0.78	0.76	0.78	0.79	0.81	0.77	0.78	0.79
SND08	0.82	0.78	0.81	0.81	0.78	0.79	0.79	0.80	0.78	0.81	0.80	0.81	0.79	0.78	0.77	0.80	0.78	0.81	0.77	0.78	0.80
GM101	0.75	0.77	0.78	0.79	0.75	0.75	0.75	0.75	0.76	0.75	0.76	0.78	0.76	0.81	0.75	0.75	0.79	0.76	0.780	0.81	0.75

Table 29. Average genetic similarity of *Rigidoporus microporus* isolates, calculated using DICE similarities coefficient in SIMQUAL program of NTSYSpc Package by ISSR markers (Continue).

	SNS01	SNS02	SNS03	SNS04	SNS07	SNS10	SNS11	SNK02	SNK03	SNK05	SNK06	SNK09	SNK10	SNP05	SNP06	SNP08	SND05	SND07	SND08	GM101
SNS01	1.00																			
SNS02	0.91	1.00																		
SNS03	0.92	0.91	1.00																	
SNS04	0.92	0.93	0.98	1.00																
SNS07	0.90	0.91	0.95	0.97	1.00															
SNS10	0.88	0.89	0.97	0.96	0.98	1.00														
SNS11	0.88	0.88	0.93	0.95	0.97	0.99	1.00													
SNK02	0.86	0.85	0.88	0.89	0.88	0.91	0.92	1.00												
SNK03	0.86	0.85	0.88	0.89	0.89	0.92	0.91	0.98	1.00											
SNK05	0.88	0.87	0.90	0.91	0.90	0.92	0.92	0.93	0.94	1.00										
SNK06	0.87	0.86	0.87	0.89	0.90	0.91	0.90	0.95	0.96	0.93	1.00									
SNK09	0.86	0.87	0.88	0.90	0.90	0.92	0.92	0.94	0.95	0.93	0.93	1.00								
SNK10	0.86	0.87	0.87	0.89	0.88	0.91	0.91	0.95	0.94	0.92	0.91	0.97	1.00							
SNP05	0.86	0.87	0.88	0.89	0.90	0.91	0.91	0.95	0.95	0.92	0.94	0.95	0.94	1.00						
SNP06	0.86	0.83	0.85	0.86	0.85	0.86	0.87	0.88	0.87	0.90	0.84	0.88	0.89	0.86	1.00					
SNP08	0.84	0.85	0.85	0.87	0.88	0.88	0.88	0.88	0.89	0.88	0.88	0.89	0.88	0.91	0.87	1.00				
SND05	0.84	0.86	0.86	0.88	0.89	0.91	0.90	0.92	0.93	0.92	0.93	0.93	0.92	0.94	0.87	0.94	1.00			
SND07	0.83	0.85	0.85	0.87	0.88	0.89	0.88	0.90	0.91	0.89	0.90	0.91	0.90	0.91	0.83	0.91	0.96	1.00		
SND08	0.83	0.84	0.84	0.85	0.86	0.86	0.85	0.86	0.88	0.88	0.89	0.88	0.87	0.88	0.82	0.89	0.91	0.94	1.00	
GM101	0.76	0.78	0.78	0.76	0.76	0.77	0.76	0.77	0.77	0.79	0.76	0.77	0.75	0.75	0.78	0.75	0.77	0.79	0.80	1.00

Appendix B: Publications

Publications

Kaewchai, S., Soytong, K. and Hyde, K.D. (2009). Mycofungicides and fungal biofertilizers. Fungal Diversity 38: 25-50.

Kaewchai, S., Lin, F.C., Wang, H.K., Hyde, K.D. and Soytong, K. 2009. Genetic variation among isolates of *Rigidoporus microporus* causing white root disease of rubber trees in Southern Thailand revealed by ISSR markers and pathogenicity. African Journal of Microbiology Research Vol. 3(10): 641-648.

Kaewchai, S. and Soytong, K. (2010). Application of biofungicides against *Rigidoporus microporus* causing white root disease of rubber trees. The Journal of Agricultural Technology 6(2): 349-363.

Kaewchai, S., Lin, F.C., Wang, H.K. and Soytong, K. (2010). Characterization of *Rigidoporus microporus* isolated from rubber trees based on morphology and ITS sequencing. The Journal of Agricultural Technology 6(2): 289-298.

Oral presentations

Kaewchai, S., Lin, F.C., Hyde, K.D. and Soytong, K. 2007. Effects of media and pH levels on the growth of *Rigidoporus lignosus* (Koltzsch) Imaz. p. 16. In Proceeding of the 2nd Annual Meeting of Thai Mycological Association and Mycology Conference in Thailand, Building Up Mycology in Thailand. 23 June, Chiang Mai University, Chiang Mai, Thailand.

Kaewchai, S., Lin, F.C., Wang, H.K., Hyde, K.D. and Soytong, K. 2008. Genetic variation among isolates of *Rigidoporus lignosus* causing agent of white root disease of rubber tree in Thailand using ISSR markers. p. 8. In Proceeding of the 3th Annual Meeting of Thai Mycological Association and Mycology Conference in Thailand, Building Up Mycology in Thailand. 11 October, Khon Kaen University, Khon Kaen, Thailand.

Kaewchai, S., Lin, F.C., Hyde, K.D. and Soytong, K. 2009. Biological control of *Rigidoporus microporus* causing white root disease of rubber trees. p. 33. In Proceeding of the Fourth Annual Meeting of Thai Mycological Association and Mycology Conference in Thailand, Building Up Mycology in Thailand. 24 October, Maejo University, Chiang Mai, Thailand.

Kaewchai, S., Lin, F.C., Hyde, K.D. and Soytong, K. 2007. Effects of media and pH levels on the growth of *Rigidoporus lignosus* (Koltzsch) Imaz. p. 236-239. In Proceeding of the International Conference on Integration of Science & Technology for Sustainable Development (ICIST) "Biological Diversity, Food and Agricultural Technology". 26-27 April, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Kaewchai, S., Lin, F.C., Hyde, K.D. and Soytong, K. 2007. White Root Disease: Morphology, Media and Growth pH Levels. 713-716. In Proceeding of the International Conference on Engineering, Applied Sciences, and Technology, 21-23 November, The SwissOtel Le Concorde, Bangkok, Thailand.

Poster presentation

Kaewchai, S. and Soytong, K. 2006. White root Disease of Rubber tree. p. 30. In Proceeding of the First Annual Meeting of Thai Mycological Association and Mycology Conference in Thailand, Building Up Mycology in Thailand. 28-29 October, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Fungal Diversity

Reviews, Critiques and New Ideas

Mycofungicides and fungal biofertilizers

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Kaewchai, S., Soytong, K. and Hyde, K.D. (2009). Mycofungicides and fungal biofertilizers. Fungal Diversity 38: 25-50.

Mycofungicides and fungal biofertilizers have been promoted for agricultural use because of their ability to control plant diseases and their ability to increase crop production in an environmentally friendly manner. In recent years several mycofungicides have been patented and registered for plant disease control, while fungal biofertilizers have also been registered for application in crop production. Several effective mycofungicides and fungal biofertilizers have been formulated for commercial production. Formulation of mycofungicides includes wettable powders and granules; these being applied to seeds, seedlings and mature plants. Examples are Ketomium®, formulated from *Chaetomium globosum* and *Ch. cupreum*, Promote® formulated from *Trichoderma harzianum* and *T. viride*, SoilGard® formulated from *Gliocladium virens*, and Trichodex® from *T. harzianum*. Fungal biofertilizers include plant growth stimulating fungi e.g. *Trichoderma*, mycorrhizal fungi (ectomycorrhiza e.g. *Pisolithus tinctonus* and arbuscular mycorrhizae e.g. *Glomus intraradices* which form mutualistic associations with plants), enzymatic producing fungi for compost production and P-solubilizing fungi and K-solubilizing fungi. Fungal biofertilizers play an important role in promoting plant growth, health, productivity and improving soil fertility.

Key words: mycofungicide, fungal biofertilizer, biological control agent

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Introduction

Fungi are ubiquitous; some having beneficial effects on plants, while others may be detrimental (Anderson and Cairney, 2004; Ipsilantis and Sylvia, 2007). A decrease in crop yield as a result of a plant disease caused by a pathogen is a negative effect. Some fungi are the main pathogens responsible for plant disease and they may cause high yield losses (Park *et al.*, 2005; Pereira *et al.*, 2007; Shenoy *et al.*, 2007; Soares and Barreto, 2008; Than *et al.*, 2008a,b). There are many ways to reduce yield losses caused by fungal disease, with the application of chemical fungicides presently being the most common method (Rosslenbroich and Stuebler, 2000; Than *et al.*, 2008a). Chemical fungicides however, have a negative effect on human health and on the environment (Voorrips *et al.*, 2004; Soytong *et al.*, 2005; Gavrilescua and Chisti, 2005; Calhelha *et al.*, 2006; Haggag and Mohamed, 2007). The application of chemical fungicides over a long period may result in plant pathogenic fungi developing resistance (Benitez *et al.*, 2004, Agrios, 2005; Kim and Hwang, 2007). When this happens the chemical fungicides become ineffective and other fungicides must be used for effective disease control. The use of microorganisms as biological control agents to control plant disease is a potentially powerful alternative method (Kulkarni *et al.*, 2007). Because of their rich diversity, complexity of interactions and numerous metabolic pathways,

microbes are an amazing resource for biological activity (Emmert and Handelsman, 1999; Alabouvette *et al.*, 2006; Tejesvi *et al.*, 2007; Mitchell *et al.*, 2008; Raghukumar, 2008). Over the past 30 years, microorganisms have been described, characterized, and tested for their use as biocontrol agents against diseases caused by soil borne plant pathogens. Biocontrol agents and especially antagonistic fungi have been used to control plant diseases with 90% of applications being formulated using different strains of *Trichoderma* e.g. *T. harzianum*, *T. virens*, *T. viride* (Benítez *et al.*, 2004). Many species of *Chaetomium* e.g. *Chaetomium globosum*, *C. cochlioides*, *C. cupreum* can also be antagonistic against various soil microorganisms (Soytong *et al.*, 2001; Kanokmedhakul *et al.*, 2002, 2006). A wide range of biological control agents have been developed as commercial mycofungicide products (Benítez *et al.*, 2004; Kim and Hwang, 2004; Fravel, 2005). The initial stage of mycofungicide development is the collection of fungal isolates and screening for the effective strains against target plant pathogens, both in the laboratory, greenhouse, and in the field (Khetan, 2001). One of the most important considerations in mass production is compatibility of the product with both formulation and application techniques (Jenkins *et al.*, 1998; Khetan, 2001). The USA and France are the main biofungicide users, although other countries are promoting biological control of agents because of bans on synthetic chemical pesticide residues on the agricultural products (Ricard and Ricard, 1997; Ecobichon, 2001; Spadaro and Gullino, 2005; Wesseling *et al.*, 2005).

An alternative way to increase the crop yield besides using chemical fertilizers is biofertilizers. Biofertilizers promote increased absorption of nutrients in plants (Vessey, 2003; Hart and Trevors, 2005; Chen, 2006). Biofertilizers include materials derived from living organisms and microbial sources (Rola, 2000; Chen, 2006). Biofertilizers have various benefits, such as increased access to nutrients, providing growth-promoting factors for plants, and composting and effective recycling of solid wastes (Gaur and Adholeya, 2004; Das *et al.*, 2007). Biofertilizers, commonly known as microbial inoculants are produced from cultures of certain soil organisms that can improve soil fertility and crop productivity such as mycorrhizae (Malik *et al.*, 2005; Marin, 2006). Mycorrhizae are fungi which form mutualistic relationships with roots of 90% of plants (Gaur and Adholeya, 2004; Das *et al.*, 2007; Rinaldi *et al.*, 2008). Mycorrhizae promote absorption of nutrients and water, control plant diseases, and improve soil structure (Rola, 2000; Zhao *et al.*, 2003; Chandanie *et al.*, 2006; Rinaldi *et al.*, 2008). Plants colonized by mycorrhizae grow better than those without them (Yeasmin *et al.*, 2007, Singh *et al.*, 2008) and are beneficial in natural and agricultural systems (Adholeya *et al.*, 2005; Marin, 2006).

In this review, we focus on the advantages of using mycofungicides for plant disease control and fungal biofertilizers to increase crop production.

Mycofungicides

Microbial antagonists can suppress plant diseases and organisms that suppress pathogens can be referred to as biological control agents (BCA) (Alabouvette *et al.*, 2006; Pal and Gardener, 2006). Various fungal species can be used as biological control agents and may provide effective activity against various pathogenic microorganisms. Examples are *Trichoderma harzianum* - a species with biocontrol potential against *Botrytis cineria*, *Fusarium*, *Pythium* and *Rhizoctonia* (Khetan, 2001); *Ampelomyces quisqualis*, - a hyperparasite of powdery mildew (Liang *et al.*, 2007; Viterbo *et al.*, 2007); *Chaetomium globosum* and *C. cupreum*, - having biocontrol activity against root rot disease caused by *Fusarium*, *Phytophthora* and *Pythium* (Soytong *et al.*, 2001); *Gliocladium virens* - effective biocontrol of soil born pathogens (Viterbo *et al.*, 2007); *Coniothyrium minitans* - a mycoparasite of *Sclerotinia* (Whipps *et al.*, 2008); and *Fusarium oxysporum* (nonpathogenic species) - having biocontrol potential against *Fusarium oxysporum* (Fravel, 2003).

An effective biological control agent should be genetically stable, effective at low concentrations, easy to mass produce in culture on inexpensive media, and be effective against a wide range of pathogens (Wraight *et al.*, 2001; Irtwange, 2006). The fungal biological control agent should also occur in an easily distributed

form, be non-toxic to humans, have resistance to pesticides, be compatible with other treatments, and be non-pathogenic against the host plant (Fravel, 2005; Irtwange, 2006). Several fungal taxa have been reported to be antagonist against plant pathogens and have been successfully formulated as mycofungicides or biological control products e.g. *Ampelomyces quisqualis*, *Aspergillus niger*, *Candida oleophila*, *Chaetomium cupreum*, *Ch. globosum*, *Coniohyrium minitans*, *Cryptococcus albidus*, *Gliocladium virens*, *G. catenulatum*, *Fusarium oxysporum*, *Phlebiosis gigantean*, *Pythium oligandrum*, *Rhodotorula glutinis*, *Trichoderma harzianum*, *T. polysporum*, *T. viride*, (Boyetchko *et al.*, 1999; Butt *et al.*, 1999; But, 2000; Hofstein and Chapple, 1999; Khetan, 2001; Soytong *et al.*, 2001; Ghisalberti, 2002; Fravel, 2005; Ezziyyani *et al.*, 2007) as seen in Table 1.

In this review, we highlight some of the important biological control agents used as mycofungicides.

Ampelomyces

Ampelomyces quisqualis is the mycoparasitic anamorphic ascomycete that reduces the growth and kills powdery mildews. It can affect the pathogen through anti-biosis and parasitism (Kiss, 2003; Viterbo *et al.*, 2007). The fungus *A. quisqualis* was the first organism reported to be a hyperparasite of powdery mildew and it can be easily found associated with powdery mildew colonies (Paulitz and Belanger, 2001). Hyphae of *Ampelomyces* penetrate the hyphae of powdery mildews and grow internally then kill all the parasitized cells (Kiss, 2003). *Ampelomyces quisqualis* isolate M-10 has been formulated as AQ10 Biofungicide, developed by Ecogen, Inc. USA. This mycofungicide contains conidia of *A. quisqualis* and formulated as water-dispersible granules for the control of powdery mildew of carrot, cucumber and mango (Khetan, 2001; Paulitz and Belanger, 2001; Shishkoff and McGrath, 2002; Kiss, 2003; Viterbo *et al.*, 2007).

Chaetomium

Chaetomium species are normally found in soil and organic compost (Soytong *et al.*, 2001). The genus *Chaetomium* was first established in 1817 by Gustav Kunze (Soytong and Quimio, 1989a). The application of *Chaetomim* as a biological control agent to control plant pathogens first commenced in about 1954 when Martin Tviet and M.B. Moor found *Ch. globosum* and *Ch. cochliodes* occurring on oat seeds and that these taxa provided some control of *Helminthosporium victoriae* (Tviet and Moor, 1954). *Chaetomium* species have been reported to be potential antagonists of various plant pathogens, especially soil-borne and seedborne pathogens (Soytong and Quimio, 1989b; Dhingra *et al.*, 2003; Aggarwal *et al.*, 2004; Park *et al.*, 2005). Many species of *Chaetomium* with potential to be biological control agents suppress the growth of bacteria and fungi through competition (for substrate and nutrients), mycoparasitism, anti-biosis, or various combinations of these (Marwah *et al.*, 2007; Zhang and Yang, 2007). *Chaetomium globosum* and *Ch. cupreum* in particular have been extensively studied and successfully used to control root rot disease of citrus, black pepper, strawberry and have been shown to reduce damping off disease of sugar beet (Soytong *et al.*, 2001; Tomilova and Shternshis, 2006). These taxa have been formulated in the form of powder and pellets as Ketomium®, a broad spectrum mycofungicide. Ketomium® has been also registered as a biological biofertilizer for degrading organic matter and for inducing plant immunity and stimulating plant growth (Soytong *et al.*, 2001). The mycofungicide Ketomium® which comprises a *Chaetomium* spore suspension has been evaluated for its effect on Siberian isolates of the phytopathogenic fungi *Botrytis cinerea*, *Didymella applanata*, *Fusarium oxysporum* and *Rhizoctonia solani*. It was found that Ketomium-mycofungicide was most efficient in suppressing raspberry spur blight caused by *Didymella applanata* and could also reduce potato disease caused by *R. solani*, increasing potato yield (Shternshis *et al.*, 2005). After 2-years in storage, this mycofungicide was still capable of inhibiting the growth of phytopathogens but at higher doses (Tomilova and Shternshis, 2006). Other species of *Chaetomium* which can act as biological control agents include *Ch. globosum* isolate CgA-1 which can reduce soybean stem canker disease caused by *Diaporthe phaseolorum* f. sp.

meridionalis (Dhingra *et al.*, 2003) and *Ch. cochliodes* CTh05 and VTh01 which has activity against *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt, while isolate CTh05 showed activity against *Phytophthora parasitica* causing citrus root rot (Phonkerd *et al.*, 2008). *Chaetomium* species are reported as a broad spectrum mycofungicide that is not only used for protection but also for curative effect as well (Soytong, 2001). Moreover, a new strain of *Ch. cupreum* RY202 has preliminary proved to be antagonistic against *Rigidoporus microporus* which causes white root disease of rubber trees variety RRIM600. This promising strain is being investigated as a potential biological control agent against *R. microporus* (Saithong, pers comm.).

Gliocladium

Gliocladium species are common soil saprobes and several species have been reported to be parasites of many plant pathogens (Viterbo *et al.*, 2007), for example, *Gliocladium catenulatum* parasities *Sporidesmium sclerotiorum* and *Fusarium* spp. It destroys the fungal host by direct hyphal contact and forms pseudoappressoria (Punja and Utkhede, 2004; Viterbo *et al.*, 2007). *Gliocladium catenulatum* (Strain J1446) has also been used as a wettable powder named Primastop® by Kemira Agro Oy, Finland. This product can be applied to soils, roots, and foliage to reduce the incidence of damping-off disease caused by *Pythium ultimum* and *Rhizoctonia solani* in the greenhouse (Paulitz and Belanger, 2001; Punja and Utkhede, 2004). *Gliocladium virens* has been used as a biological control agent against a wide range of soil borne pathogens such as, *Pythium* and *Rhizoctonia* under greenhouse and field conditions (Hebbar and Lumsden, 1999; Viterbo *et al.*, 2007). *Gliocladium virens* isolate GL-21 was formulated as an alginate prill named GlioGard® by W.R. Grace Co. and a granular formulation with the trade name SoilGard® produced by the Thermo Triology Corp., Columbia, MD. SoilGard® was developed for greenhouse application (Paulitz and Belanger, 2001; Punja and Utkhede, 2004). *Gliocladium virens* produces anti-biotic metabolites such as gliotoxin which have anti-bacterial, anti-fungal, anti-viral and anti-tumor activities. Recently, molecular evidence indicates that *G. virens* is more closely related to *Trichoderma* than those *G. virens*. This supports suggestions that this taxon should be referred to as *Trichoderma virens* (Hebber and Lumsden, 1999; Paulitz and Belanger, 2001; Punja and Utkhede, 2004).

Trichoderma

Trichoderma species are common in soil and root ecosystems and are ubiquitous saprobes (Harman *et al.*, 2004; Thormann and Rice, 2007; Vinale *et al.*, 2008; Kodsueb *et al.*, 2008) and they are easily isolated from soil, decaying wood, and other organic material (Howell, 2003; Zeilinger and Omann, 2007). There are several reports on the use of *Trichoderma* species as biological agents against plant pathogens (Harman *et al.*, 2004; Zeilinger and Omann, 2007). *Trichoderma* species have been used as biological control agents against a wide range of pathogenic fungi e.g. *Rhizoctonia* spp., *Pythium* spp., *Botrytis cinerea*, and *Fusarium* spp. *Phytophthora palmivora*, *P. parasitica* and different species can be used, e.g. *T. harzianum*, *T. viride*, *T. virens* (Benítez *et al.*, 2004; Sunantapongsuk *et al.*, 2006; Zeilinger and Omann, 2007). Among them, *Trichoderma harzianum* is reported to be most widely used as an effective biological control agent (El-Katathy *et al.*, 2001; Szekeres *et al.*, 2004; Abdel-Fattah *et al.*, 2007). *Trichoderma harzianum* strain T-22 was produced by protoplast fusion between *T. harzianum* T-95 and T-12 and this strain was formulated as granular named RootSield® and powder named PlantShield® by Biworks, Geneva, NY. *Trichoderma harzianum* T-22 has efficacy against a wide range of plant pathogenic fungi including, *Botrytis cinerea*, *Fusarium*, *Pythium*, *Rhizoctonia* in many crops such as, corn, soybean, potato, tomato, beans, cotton, peanut, and various trees (Khetan, 2001; Paulitz and Belanger, 2001). *Trichoderma harzianum* strain T-39 is marketed as TRICHODEX, 20P by Makhteshim Ltd. for control of pink rot and stem rot of tomato caused by *Phytophthora erythroseptica* (Etebarian *et al.*, 2000) and control of blight disease caused by *Botrytis cinerea* (Paulitz and Belanger, 2001).

The biocontrol mechanism in *Trichoderma* is a combination of mechanisms (Howell, 2003; Benitez *et al.*, 2004; Zeilinger and

Omann, 2007). The main mechanism is mycoparasitism and anti-biosis (Howell, 2003; Vinale *et al.*, 2008). Mycoparasitism relies on the recognition, binding and enzymatic disruption of the host fungus cell wall (Woo and Lorito, 2007). *Trichoderma* species have been very successfully used as mycofungicides because they are fast growing, have high reproductive capacity, inhibit a broad spectrum of fungal diseases, have a diversity of control mechanisms, are excellent competitors in the rhizosphere, have a capacity to modify the rhizosphere, are tolerant or resistance to soil fungicides, have the ability to survive under unfavorable conditions, are efficient in utilizing soil nutrients, have strong aggressiveness against phytopathogenic fungi, and also promote plant growth (Tang *et al.*, 2001; Benítez *et al.*, 2004; Vinale *et al.*, 2006). Their ability to colonize and grow in association with plant roots is known as rhizosphere competence. The taxonomy of *Trichoderma* species is very complex and has been the subject of many recent taxonomic studies (Tang *et al.*, 2001; Woo *et al.*, 2006; Samuels, 2006). They also have a high level of genetic diversity (Harman *et al.*, 2004; Harman, 2006). Thus it is likely that only a few of the species available have been utilized as mycofungicides. However, *Trichoderma* species are the most common fungal biocontrol control agents and are commercially formulated as biofungicides, biofertilizers, and soil amendments (Harman *et al.*, 2004, Vinale *et al.*, 2006; Harman, 2006).

Other fungi as mycofungicides

Coniothyrium minitans is an anamorphic coelomycete (Gong *et al.*, 2007) which has been reported to be a mycoparasite of *Sclerotinia* species such as *Sclerotinia minor*, *S. sclerotiorum*, *S. trifoliorum* and *S. cepivorum* (Yang *et al.*, 2007; Viterbo *et al.*, 2007; Whipps *et al.*, 2008). It has been applied successfully to control disease in many crops including lettuce (Jones *et al.*, 2004), oil oilseed rape (Li *et al.*, 2006), peanut (Partridge *et al.*, 2006) and alfalfa (Li *et al.*, 2005). The conidia of *Coniothyrium minitans* has been formulated as Contans® by Prophyta Biologischer Pflanzengchutz GmbH, Germany (Paulitz and Belanger, 2001; Gavrilescu and Chisti, 2005; Yang *et al.*, 2007; Whipps *et al.*, 2008) and has been registered for disease control in Germany, Switzerland, Norway and USA (Partridge *et al.*, 2006;Yang *et al.*, 2007). The main biological control mechanism of *C. minitans* is mycoparasitism which uses sclerotia of *S. sclerotiorum* as the source of food for survival (Yang *et al.*, 2007; Whipps *et al.*, 2008). The products of *C. minitans* can be applied to soil or can be sprayed on foliage (Shi *et al.*, 2004; Li *et al.*, 2005) and they can survive in soil for several years (Jones and Whipps 2002; Whipps *et al.*, 2008). The efficiency of *C. minitans* can be improved by combinations with *Trichoderma* species (Li *et al.*, 2005; Whipps *et al.*, 2008).

The genus *Fusarium* includes both plant-pathogenic and non-pathogenic races (Larkin and Fravel, 1999). The non-pathogenic species are known to have effective biocontrol activity (Whipps; 2001; Harman *et al.*, 2004; Kvas *et al.*, 2009). Mechanisms of action include competition and induction of host defenses (Paulitz and Belanger, 2001; Whipps, 2001; Fravel *et al.*, 2003; Agrios, 2005). The use of non-pathogenic strains of *Fusarium oxysporum* to control Fusarium wilt has been reported for many crops, but there has been little commercial production, because of lack of understanding of their genetics, biology and ecology (Fravel *et al.*, 2003; Kvas *et al.*, 2009). Non-pathogenic *F. oxysporum* strain Fo47 was marketed as liquid formulation named as Fusaclean® by Natural Plant Products, Nogueres, France for soil less culture (Khetan, 2001; Paulitz and Belanger, 2001). Similarly the genus *Rhizoctonia* contains both plant-pathogenic and non-pathogenic species and the latter can act as biocontrol agents (Harman *et al.*, 2004).

Pythium oligandrum has shown ability to control soil-borne pathogens both in the laboratory and in the field. *Pythium oligandrum* oospores have been applied as seed treatments which reduce damping-off disease caused by *P. ultimum* in sugarbeet (Lewis *et al.*, 1989; Khetan, 2001). *Pythium oligandrum* has been formulated as a granular or powder product named as Polygangron® by Vyskumny Ustav of Slovak Republic (Khetan, 2001). This fungus has indirect effects by controlling pathogens in the rhizosphere and/or direct

effects by inducing plant resistance. It also induces plants to respond more rapidly and efficiently to pathogen infections and increase phosphorus uptake (Le Floch *et al.*, 2003). *Pythium nunn* is also an antagonistic fungus being a mycoparasite of pathogens such as *Rhizoctonia solani*, *Phytophthora cinnamomi*, *P. parasitica*, *Pythium aphanidermatum*, *P. ultimum* and *P. vexans*. The hyphae coil around the host, forming appressoria-like structures and then penetrate and parasitize the "host" hyphae (Khetan, 2001; Viterbo *et al.*, 2007).

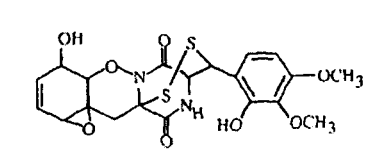
Other fungi that can be used as mycofungicides are *Aspergillus* and *Penicillium* species. *Aspergillus* species are effective against the white-rot basidiomycetes (Bruce and Highley, 1991). The fungal antagonists *Aureobasidium pullulans*, and *Ulocladium atrum* have also been tested for the control of *Botrytis aclada* which causes onion neck rot (Köhl *et al.*, 1997). *Clonostachys rosea* is also reported as a biological control agent. The application of *Clonostachys rosea* as a single strain and mixture of strains against *Moniliophthora roreri* in cocoa crops has been tested by using motorized mist blowers and a directional hydraulic spray technique. Both mycofungicides reduced sporulation of the pathogen and the motorized mist blower technique gave better results than those by the directional hydraulic spray technique (Hidalgo *et al.*, 2003).

Mechanisms of biological control

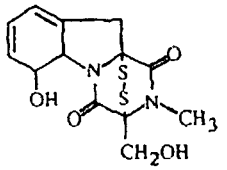
Biological control may result from direct or indirect interactions between the beneficial microorganisms and the pathogen (Alabouvette and Lemanceau, 1999; Benítez *et al.*, 2004; Viterbo *et al.*, 2007). A direct interaction may involve physical contact and synthesis of hydrolytic enzymes, toxic compounds or antibiotics as well as competition. An indirect interaction may result from induced resistance in the host plant, the use of organic soil amendments to improve the activity of antagonists against the pathogens (Benítez *et al.*, 2004; Pal and Gardener, 2006; Viterbo *et al.*, 2007). The mechanisms of biocontrol agents and reaction with the pathogen are many and complex. Mechanisms are influenced by soil type, temperature, pH and moisture of the plant and soil environment and also by the presence of other microorganisms (Howell, 2003). There are four principle mechanisms of biological control anti-biosis, competition, mycoparasitism or lysis and induced resistance (Renwick and Poole, 1989; Chet *et al.*, 1990; Fravel *et al.*, 2003; Irtwange, 2006; Viterbo *et al.*, 2007). These are detailed below.

Antibiosis: Antibiosis is defined as the inhibition or destruction of the microorganism by substances such as specific or nonspecific metabolites or by the production of anti-biotics that inhibit the growth of another microorganism (Benítez *et al.*, 2004; Irtwange, 2006; Viterbo *et al.*, 2007; Haggag and Mohamed, 2007). Many biological control agents produce several types of anti-biotics (Lewis *et al.*, 1989; Handelsman and Stabb, 1996). Some anti-biotics have been shown to play role in disease suppression (Lewis *et al.*, 1989; Handelsman and Stabb, 1996) either impede spore germination (fungistasis), or kill the cells (anti-biosis) (Benítez *et al.*, 2004; Haggag and Mohamed, 2007).

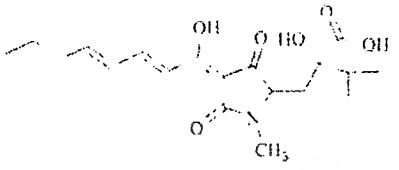
Gliocladium and *Trichoderma* species are well known biological control agents which produce a wide range of anti-biotics and suppress disease by diverse mechanisms (Handelsman and Stabb, 1996; Whipps, 2001; Harman *et al.*, 2004). Gliovirin (Fig. 1A) produced by *Gliocladium virens* can kill *Pythium ultimum* by causing coagulation of the protoplasm (Whipps, 2001; Howell, 2003; Viterbo *et al.*, 2007). Many anti-biotics are produced by *Trichoderma* species. These include gliotoxin (Fig. 1B), harzianic acid (Fig. 1C), trichoviridin (Fig. 1D), viridin (Fig. 1E), viridiol (Fig. 1F), and alamethicins. These anti-biotics are synergistic when combined with various cell wall degrading enzymes thus producing a strong inhibitory effect on many plant pathogens (Benítez *et al.*, 2004; Woo and Lorito, 2007; Vinale *et al.*, 2008). Trichotoxin A50 (Fig. 1K) produced by *T. harzianum* PC01 can inhibit the mycelial growth and sporangial production of *Phytophthora palmivora* (Suwan *et al.*, 2000). *Chaetomium globosum* produces the anti-biotic chaetoglobusin C (Fig. 1G) which suppresses the growth of plant pathogens such as *Colletotrichum gloeosporioides*, *C. dematium*, *Fusarium oxysporum*, *Phytophthora palmivora*, *P. parasitica*, *P. cactorum*, *Pyricularia oryzae*, *Rhizoctonia solani* and



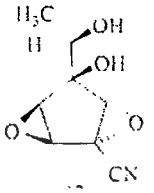
A: gliovirin



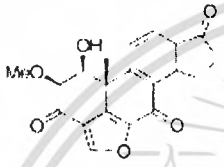
B: gliotoxin



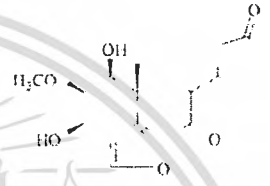
C: harzianic acid



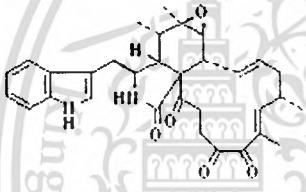
D: trichoviridin



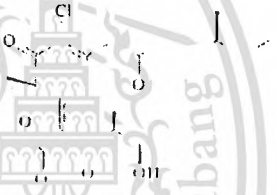
E: viridin



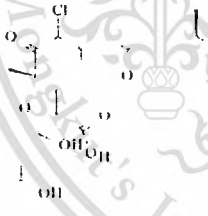
F: viridiol



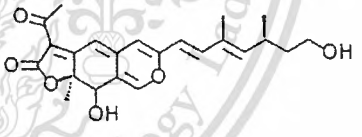
G: chaetoglobusin C



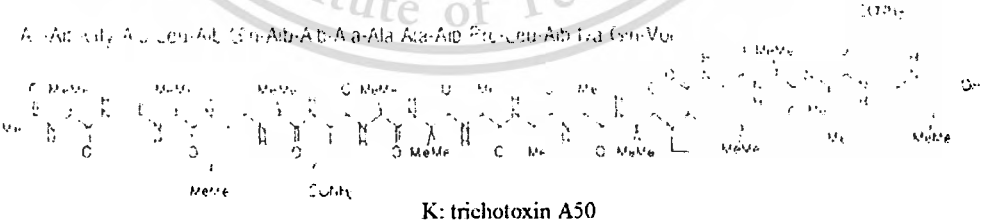
H: chaetoviridins A



I: chaetoviridins B



J: rotiorinol



K: trichotoxin A50

Fig. 1. Structure of some antibiotics produced by fungal biocontrol agents; gliovirin (A) produced by *Gliocladium virens*, gliotoxin (B), harzianic acid (C), trichoviridin (D), viridin (E) and viridiol (F) produced by *Trichoderma* species, chaetoglobusin C (G), chaetoviridins A (H), and chaetoiridins B (I) produced by *Chaetomium globosum*, rotorinol (J) produced by *Chaetomium cupreum* and trichotoxin A50 (K) produced by *Trichoderma harzianum* PC01.

Sclerotium rolfsii (Soytong *et al.*, 2001) and chaetoviridins A (Fig. 1H) and B (Fig. 1I) which inhibit the mycelial growth of various plant pathogenic fungi such as, *Pyricularia oryzae*, *Magnaporthe grisea*, *Pythium ultimum* (Park *et al.*, 2005). *Chaetomium cupream* produces rotiorinol (Fig. 1J) which can inhibit the growth of pathogens (Kanokmedhakul *et al.*, 2006).

Competition: Competition occurs between microorganisms when space and nutrients are a limiting factor (Lewis *et al.*, 1989; Howell, 2003; Benítez *et al.*, 2004; Viterbo *et al.*, 2007). The rhizosphere is a major concern where competition for space and nutrient occurs (Whipps; 2001; Howell, 2003; Viterbo *et al.*, 2007). Competition can be divided into saprobic competition for nutrients in the soil and rhizosphere, and competition for infection sites on and in the root (Fravel *et al.*, 2003). Competition between the biocontrol agent and the pathogen can result in displacement of the pathogen. Biological control agents can compete with other fungi for food and essential elements in the soil and around the rhizosphere (Chet *et al.*, 1990; Irtwange, 2006) and can complete the space or modify the rhizosphere by acidifying the soil, so that pathogens cannot grow (Benítez *et al.*, 2004). For example, *Trichoderma harzianum* T-35 control of *Fusarium* species on various crops occurs via competition for nutrients and rhizosphere colonization (Viterbo *et al.*, 2007).

Mycoparasitism: In addition to antibiosis and competition, biological control agents also reduce plant disease by mycoparasitism (Benítez *et al.*, 2004; Irtwange, 2006). Mycoparasitism involves the complex process that includes the following steps: (1) the chemothophic growth of the antagonist to the host; (2) recognition of the host by mycoparasite; (3) attachment; (4) excretion of extracellular enzymes; (5) lysis and exploitation of the host (Whipps, 2001; Benítez *et al.*, 2004; Viterbo *et al.*, 2007). Biological control agents are able to lyse hyphae of pathogens by release the lytic enzymes and this is an important and powerful tool for control of plant disease (Chet *et al.*, 1990; Flores *et al.*, 1997; Viterbo *et al.*, 2007) such as chitinases, proteases, and β-1, 3 glucanases (Whipps, 2001). These enzymes lyse pathogen hyphal cell walls during mycoparasitic activity (Cruz *et al.*, 1992; Schirmbock *et al.*, 1994; El-Katathy *et al.*, 2001; Khetan, 2001). β-1, 3 glucanases have properties for degrading cell walls, inhibiting mycelium growth and spore germination of plant pathogenic fungi (Benítez *et al.*, 2004; Lin *et al.*, 2007). For example, β-1, 3 glucanases produced from *Chaetomium* sp. can degrade cell walls of plant pathogens including *Rhizoctonia solani*, *Gibberella zeae*, *Fusarium* sp. *Colletotrichum gloeosporioides*, and *Phoma* sp. (Sun *et al.*, 2006) and β-1, 3 glucanases produced from *Periconia byssoides* can degrade cell walls, inhibit mycelium growth and spore germination in *Fusarium* sp. and *Rhizoctonia solani* (Lin *et al.*, 2007). Chitinases play important roles in the degradation of chitin, the main cell wall structure component of fungi (Cruz *et al.*, 1992; Whipps, 2001). Proteases produced by *Trichoderma harzianum* T-39 are involved in the degradation of pathogen hyphal membranes and cell walls. They can deactivate the hydrolytic enzymes, endo-polygalacturonase and exo-polygalacturonase produced by *Botrytis cinerea* causing agent of grey mold, which results in reduction of disease severity (Elad and Kapat, 1999).

Induced resistance: Induced resistance occurs in most plants in response to infestation by pathogens (Harman *et al.*, 2004). Induced resistance of host plants can be localized and/or systematic, depending on the type, source, and amount of stimuli (Pal and Gardener, 2006). Induced resistance by biocontrol agents involves the same suite of genes and gene products involved in plant response known as systematic acquired resistance (SAR) (Handelsman and Stabb, 1996; Whipps, 2001). *Trichoderma* strains are capable of establishing interaction induced metabolic changes in plants that increase resistance to a wide range of plant-pathogenic fungi (Harman *et al.*, 2004). Strains of *Trichoderma* added to the rhizosphere protect plants against many pathogens including viruses, bacteria, and fungi, because of the induction of resistance mechanisms similar to the hypersensitive response (HR), systematic acquired resistance (SAR), and induced systematic resistance (ISR) in plants (Harman *et al.*, 2004; Benitez *et al.*, 2004; Haggag, 2008). This concept is supported by Yedidia *et al.* (1999) who treated cucumber seedlings in a

hydroponic system with *T. harzianum* T-203 and found that plant defense responses had occurred in roots and leaves and the plant response was marked by an increase in peroxidase activity and chitinase activity. Howell (2003) also reported that peroxidase activity was induced by *T. virens* in cotton seedlings more than in the control experiment. Other fungal taxa can also induce resistant responses in plants, for example; *Chaetomium globosum* produces chaetoglobosin C and can induce a localized and sub-systemic oxidative burst in carrots, potatoes, sweet potatoes, tomatoes, and tobacco and this substance can act to induce plant immunity for disease resistance (Soytong *et al.*, 2001; Kanokmedhakul *et al*, 2002). A non-pathogenic strain of *Fusarium*, *Pythium ultimum*, and *Rhizoctonia* could induce plant resistance to pathogenic stains (Harman *et al.*, 2004).

Resistance may be the result in an increase in the concentration of metabolites and enzymes related to defense mechanisms, such as phenyl-alanine ammonio-lyase (PAL) and chalcone synthase (CHS) (Viterbo *et al.*, 2007). These enzymes are involved in the biosynthesis of phytoalexins, chitinases and glucanases (Benitez *et al.*, 2004; Viterbo *et al.*, 2007). The metabolites produced by *Trichoderma* may act as elicitors of plant resistance (Benitez *et al.*, 2004). There are at least three groups of substances that elicit plant defense responses and these include proteins, peptides, and low-molecular-weight compounds (Harman *et al.*, 2004; Viterbo *et al.*, 2007).

Production of mycofungicides

The use of fungal biological control agents to control plant pathogens has been investigated for more than 70 years, however research in this area has increased dramatically only in the past 20 years. Over 40 biological control products have been introduced into the market within the past ten years (Table 1), but these are used on a very small scale as compared to chemical fungicides (Paulitz and Belanger, 2001; Kim and Hwang, 2007). There is a little investment into research and development of biological control agents as compared with chemical fungicides because mycofungicides usually have narrow host ranges and mycofungicides have tended to provide inconsistent or poor control in the field (Kim and Hwang, 2004, 2007). Therefore, research into mycofungicides has emphasized on fungi with broad spectrum effects and on improvements in their associated production, formulation and application (Butt, 2000). The others reasons for the limited commercial formula is the high cost of production which may be due to high cost of substrate, low biomass productivity, or limited economics of scale (Spadaro and Gullino, 2005; Fravel, 2005) however, the starch industry wastewater may be used for antagonist production (Verma, 2007).

The commercial development of mycofungicides has increased significantly in recent years because of the progress in isolation and characterization of antagonistic fungal strains (Hofstein and Chapple, 1999; Spadaro and Gullino, 2005). Mycofungicides have shown potential for disease control in the laboratory, greenhouse, and field studies and they can be cultured for mass production by standard fermentation (Lumsden and Lewis, 1989; Hofstein and Chapple, 1999; Khetan, 2001; Spadaro and Gullino, 2005). Two common methods used for producing inocula of biological control agents are liquid and solid fermentation (Tang *et al.*, 2001; Spadaro and Gullino, 2005). Low cost and capacity to control disease following processing and storage are also important considerations (Alabouvette and Lemanceau, 1999; Spadaro and Gullino, 2005).

The development of the high-quality mycofungicides relies on the biological properties of the isolates. The factors that need to be considered when selecting isolates for potential biological control agents are as follows: laboratory virulence, field performance, genetic stability, productivity, stability of conidia in storage, stability in formulation, field persistence (tolerance to environmental factors such as UV, temperature, extremes and desiccation), mammalian safety, low environmental impact, and capacity to persist in the environment (Jenkins *et al.*, 1998; Spadaro and Gullino, 2005). The important characteristics of a successful commercial product are good market potential, simplicity in production and application, adequate product and stability, shelf life during transport and storage, efficacy over a long term, guaranteed propagule viability,

Table 1. Some of the biological control products available in the market.

Products	Fungus	Target pathogen	Formulation	Producer
AQ10 Biofungicide	*Ampelomyces quisqualis* M-10	Powdery mildew	Water-dispersible granule	Ecogen, Inc. Langhorne, PA www.groworganic.com
Binab T WG Bineb T Pellet Bineb T Vector Bineb TF WP	*Trichoderma harzianum* *Trichoderma polysporum*	Fungi causing wilt, root rot wood decay	Wettable powder Granules pellets	Bio-Innovation, Sweden www.algonet.se/~binab/index2.html
Bioderma	*Trichoderma viride*	*Sclerotinia*, *Rhizoctonia*	Wettable powder	Biotech International Ltd., India www.biotech-int.com
Bioderma-H	*Trichoderma harzianum*	*Phytophthora*, *Fusarium* *Pythium* spp., *Cercospora*, , *Colletotrichum*, *Alternaria*, *Ascochyta*, *Macrophomina*, *Myrothecium*, *Ralstonia*	Wettable powder	Biotech International Ltd., India www.biotech-int.com
Biofox C	*Fusarium oxysporum* (nonpathogenic)	*Fusarium oxysporum* *Fusarium miniliforme*		SIAPA, Italy
Biofungus	*Trichoderma* spp.	*Sclerotinia*, *Phytophthora*, *Rhizoctonia solani*, *Pythium* spp., *Fusarium*, *Verticillium*	Granule Wettable powder	Grondortsmettingen deCuester n. v., Belgium
Cotans WG	*Coniothyrium minitans*	*Sclerotinia* spp.	granules	Prophyta, Germany www.prophyta.de
Fungi-Killer	*Trichoderma harzianum*	*Phytophthora*. *Fusarium*	Powder	Bangkok Organic Compost Ltd. Thailand
Fusaclean	*Fusarium oxysporum* Fo47 (nonpathogenic)	*Fusarium oxysporum*	Spores, microgranule	Natural plant Protection, France
Ketocin	*Chaetomium cupreum*	*Fusarium oxysporum f.sp. lycopersici*	Powder	Neoworld Ltd., Thailand
Ketomium	*Chaetomium globosum* *Chaetomium cupreum*	*Phytophthora palmivora* *Phytophthora parasitica* *Colletotrichum gloeospoiodes* *Fusarium oxysporum f.sp. lycopersici* *Fusarium moniliform* *Pyricularia oryzae* *Sclerotium rolfsii* *Drechsiera maydis*	Pellets, Powder	Guangxi Guilin Green Harvest Fertilizer Factory, China Nova Science, Thailand
Koni	*Coniothyrium minitans*	*Sclerotinia* spp.		BIOVED Ltd., Hungary www.bioved.hu
Novacide	*Chaetomium cupreum*	*Fusarium oxysporum f.sp. lycopersici*	Powder	Nova Science, Thailand
Polygandron Polyversum	*Pythium oligandrum*	*Pythium ultimum*	Granule or powder	Plant Protection Institute, Slovak Republic

Table 1 (continued). Some of the biological control products available in the market.

Products	Fungus	Target pathogen	Formulation	Producer
Prestop Primastop	*Gliocladium catenulatum* Strain J1446	*Pythium* spp. *Rhizoctonia solani* *Botrytis* spp. *Didymella* spp.	Wettable powder	Kemira Agro Oy, Fingland
Promote	*Trichoderma harzianum* *Trichoderma viride*	*Pythium* *Rhizoctonia* *Fusarium*		JH Biotech Inc., Ventura, CA, USA www.jhbiotech.com
RootShield PlantShield	*Trichoderma harzianum* Strain T-22	*Pythium, Rhizoctonia, Fusarium, Sclerotina*	Granules and Wettable powder	Bioworks, Inc. NY. USA www.bioworksbiocontrol.com
Sentinel®	*Trichoderma* spp. Strain LC52	*Botrytis cinera*	Wettable powder	Agrimms Technologies Ltd, www.vinevax.com
SoilGard (GlioGard)	*Gliocladium virens* GL21	Several plant diseases Damping-off and root pathogens	Granules Alginate prill	Thermo Triology, USA.
Trichodex	*Trichoderma harzianum* T-39	Fungal diseases e.g. *Botrytis cinerea* *Colletotrichum,* *Monilinia laxa,* *Plasmospara viticola* *Rhizopus stolonifer*	Wettable powder	Makhteshim-Agan, DeCeuster, Belgium
Vinevax™	*Trichoderma* spp.	Wood-infecting fungal pathogens of vineyard, orchard, ornamental trees, and vines	Wettable powder	Agrimms Technologies Ltd, www.vinevax.com

economic, suitable and appropriate action, and compatibility with agronomic practices and equipment (Boyetchko *et al.*, 1999; Spadaro and Gullino, 2005).

An efficient formulation is essential to transfer the biological control agent from laboratory to the field. The formulation must be compatible to preserve the biological control agent activities. The living organisms must remain inactive whilst in storage, but rapidly become active when applied in the field (Butt *et al.*, 1999). To achieve this, a drying process is necessary, for example air drying, freeze drying, atomization, bed-fluid drying or lyophilization (Butt *et al.*, 1999; Spadaro and Gullino, 2005).

There are many types of formulation for fungal antagonists, for example, alginate prill formulation, fluid-bed granulation including dextrin as a binder, liquid formulation, water dispersible granule formulation, wettable powder formulation, dusts, granular or powder products (Khetan, 2001; Soytong *et al.*, 2001). Some formulation types of commercial mycofungicides are shown in Fig. 2. The formulations can be applied to seeds, tubers, cuttings, seedlings, transplants, mature plants and soil (Boyetchko *et al.*, 1999). However, liquid formulation is preferred with drip irrigation, granular formulations are more appropriate for combining with potting mix, while a wettable powder is more appropriate for root dips or sprays (Spadaro and Gullino, 2005). Biological control agents should be capable of application through standard hydraulic nozzles or application equipment with few special application requirements (Butt, 2000).

Mycofungicides comprise many ingredients such as active ingredients (microorganism or spores), adjuvants, dilution agents, bulking additives, membrane stabilizers, growth and contaminant suppressants, buffers, binders, dispersants, lubricants, activators, food sources, and coatings. These ingredients are added for various proposes such as follows: maintenance viability of biological control agents, manipulating bulk for handing and delivery, promoting the activity of biological control agents, protecting the inocula from unfavorable environmental conditions, and

Fig. 2 Some formulation types of mycofungicides. A Ketocin® in powder formulation, B Ketomium®in pellet formulation, C Fungi Killer® in pellet formulation and D Novacide® in powder formulation.

impressive growth of contaminants (Hynes and Boyetchko, 2005; Spadaro and Gullino, 2005). Presently, there are many mycofungicides worldwide in the market as show in Table 1 and Figs 2 and 3.

Fungal biofertilizers

Biofertilizers comprise microbial inocula or assemblages of living microorganisms which exert direct or indirect benefits on plant growth and crop yield through different mechanisms (Fuentes-Ramirez and Caballero-Mellado, 2005) These microorganisms are able to fix atmospheric nitrogen or solubilize phosphorus, decompose organic material, or oxidize sulfur in the soil properties (Marin, 2006) that are beneficial to agricultural production in terms of nutrient supply (Malik *et al.*, 2005). One type of biofertilizer are the arbuscular mycorrhizal fungi, which are probably the most abundant fungi in agricultural soil (Marin, 2006; Khan, 2006). The inocula improve crop yield because of increased availability or uptake or absorption of nutrients, stimulation of plant growth by hormone action or antibiosis and by decomposition of organic residues (Wani and Lee, 2002). Selected fungal species which are used as biofertilizers are mentioned below.

***Mycorrhizal fungi* used as biofertilizers**

Mycorrhizae form mutualistic symbiotic relationships with plant roots of more than 80% of land plants including many important crops and forest tree species (Smith and Zhu, 2001; Gentili and Jumpponen, 2006; Rinaldi *et al.*, 2008). There are seven types of mycorrhiza: arbutoid mycorrhiza, ectomycorrhiza, endomycorrhiza or arbuscular mycorrhiza, ect-endomycorrhiza, ericoid mycorrhiza, monotropoid mycorrhiza, and orchidoid mycorrhiza (Raina *et al.*, 2000; Gentili and Jumpponen, 2006; Das *et al.*, 2007; Tao *et al.*, 2008; Zhu *et al.*, 2008). The two dominant types of mycorrhizae are ectomycorrhizae (ECM) and arbuscular mycorrhizae (AM) which can improve water and nutrient uptake and provide protection from pathogens but only a few families of plants are able to form functional associations with both AM and ECM fungi (Haskins and Gehring, 2005; Siddiqui and Pichtel, 2008). However, AM fungi are most commonly found in the rhizosphere roots of a wide range of herbaceous and woody plants (Das *et al.*, 2007; Rinaldi *et al.*, 2008). In this review, we focus

Fig. 3. Some of mycofungicides; (A) Fungi Killer, (B) Ketomium, (C) NovacideE, and (D) KONI® WG.

on ectomycorrhizal fungi and arbuscular mycorrhizal fungi because they are the most widespread and economically important types of mycorrhizal fungi. Ectomycorrhizal (ECM) fungi form mutualistic symbioses with many tree species (Anderson and Cairney, 2007). Most ECMfungi do not penetrate the living cells in the roots, but only surround them (Raina, 2000; Gupta *et al*., 2000; Das *et al*., 2007). ECM fungi occur naturally in association with many forest trees, for example, pine, spruce, larch, hemlock, willow, poplar, oak birch and eucalyptus (Dahm, 2006; Raja, 2006; Rinaldi *et al*., 2008). Most ECM fungi that are associated with forest trees are basidiomycetes, such as *Amanita* sp., *Lactarius* sp., *Pisolithus* sp., and *Rhizopogon* sp. and many of these are edible (Le *et al*., 2007; Buyck *et al*., 2008; Rinaldi *et al*., 2008) Some ascomycetes also form mycorrhizae such as, *Cenococcum* sp., *Elaphomyces* sp., and *Tuber* sp. (Dahm, 2006; Das *et al*., 2007; Rinaldi *et al*., 2008). The importance of ECM fungi to trees is in their ability to increase the tree growth due to better nutrient acquisition (Gentili and Jumpponen, 2006). ECM fungi help the growth and development of trees because the roots colonized with ectomycorrhiza are able to absorb and accumulate nitrogen, phosphorus, potassium, and calcium more rapidly and over a longer period than nonmycorrhizal roots. ECM fungi help to break down the complex minerals and organic substances in the soil and transfer nutrients to the tree. ECM fungi also appear to increase the tolerance of trees to drought, high soil temperatures, soil toxins, and extremes of soil pH. ECM fungi can also protect roots of trees from pathogens (Dahm, 2006). The most commonly widespread ectomycorrhizal product is inoculum of *Pisolithus tinctorius* (Schwartz *et al*., 2006; Gentili and Jumpponen, 2006). *Pisolithus tinctorius* has a wide host range and their inoculum can be produced and applied as vegetative mycelium in a peat vermiculite carrier. These fungus inocula are applied to nursery or forestry plantations (Gentili and Jumpponen, 2006). *Piriformospora indica* (Hymenomycetes, Basidiomycota) is another ECM fungus used as a biofertilizer. This taxon can promote plant growth and biomass production and help plant tolerance to herbivory, heat, salt, disease, drought, and increased below- and above-ground biomass (Waller *et al*., 2005; Tejesvi *et al*., 2007)

Endomycorrhizae from mutually symbiotic relationships between fungi and plant roots (Ipsilantis and Sylvia, 2007). The plant roots provide substances for the fungi and the fungi transfer nutrients and water to the plant roots (Adholeya *et al*., 2005; Chen, 2006). Endomycorrhizal fungi are intercellular and penetrate the root cortical cells and form structures called arbuscular vesicles and known as vesicular arbuscular mycorrhiza (VAM) but in some cases no vesicles are formed and they known as arbuscular mycorrhiza (AM) (Gupta *et al*., 2000). The agriculturally produced crop plants that form endomycorrhizae of the vesicular-arbuscular mycorrhiza type are now called arbuscular mycorrhizal (AM) fungi (Raja, 2006). AM fungi belong to nine genera: *Acaulospora*, *Archaeospora*, *Enterophospora*, *Gerdemannia*, *Geosiphon*, *Gigaspora*, *Glomus*, *Paraglomus*, and *Scutellospora* (Das *et al*., 2007). AM fungi are a widespread group and are found from the arctic to tropics and are present in most agricultural and natural ecosystems. They play an important role in plant growth, health, and productivity (Douds,

2005; Marin, 2006). AM fungi help plants to absorb nutrients, especially the less available mineral nutrients such as copper, molybdenum, phosphorus and zinc (Yeasmin *et al.*, 2007). They increase seedling tolerance to drought, high temperatures, toxic heavy metals, high or low pH and even extreme soil acidity (Gupta *et al.*, 2000; Kannaiyan, 2002; Chen, 2006). AM fungi can also affect plant growth indirectly by improving the soil structure, providing antagonist effects against pathogens and altered water relationships (Smith and Zhu, 2001). AM fungi can reduce the severity of soil-borne pathogens and enhance resistance in roots against root rot disease (Azcon-Aguilar and Barea, 1996; Kannaiyan, 2002; Chen, 2006; Akhtar and Siddiqui, 2008a,b). This results because of competition for colonization sites or nutrients in the same root tissues and production of fungistatic compounds (Johansson *et al.*, 2004; Marin, 2006). AM fungi have been shown to have benefits to host plants including increasing herbivore tolerance, increasing pollination, increasing soil stability, and heavy metal tolerance (Hart and Trevors, 2005). The use of AM fungi as biofertilizers is not new, they have been produced for use in agriculture, horticulture, landscape restoration, and soil remediation for almost two decades (Hart and Trevors, 2005). Mass production of AM fungi has been achieved with several species such as *Acaulospora laevis, Glomus. clarum, G. etunicatum, G. intraradices, G. mosseae, Gigaspora ramisporophora and Gigaspora rosea* (Schwartz *et al.*, 2006) but *Glomus intraradices* is the most common inoculum of endomycorrhizae products (Adholeya *et al.*, 2005; Wu *et al.*, 2005; Schwartz *et al.*, 2006; Akhtar and Siddiqui, 2008b). Effective management of AM fungi involves increasing populations of propagules such as spores, colonized root fragments and hyphae using host plants and also by adoption soil management techniques (Smith and Zhu, 2001; Tiwari *et al.*, 2004; Kapoor *et al.*, 2008).

Production of mycorrhizal fungi as fungal biofertilizers

AM fungi are obligate symbiotic microorganisms since they cannot be grown without the plant host on synthetic media (Hart and Trevors, 2005). Therefore AM fungal inocula must be produced in association with the host plant and therefore there are many constraints to large scale commercial production. Mass production is by pot culture either in the greenhouse or in growth chambers is the most commonly used production method (Bagyaraj *et al.*, 2002; Raja, 2006; Gentili and Jumpponen, 2006; Marin, 2006; Kapoor *et al.*, 2008). AM fungal inocula have to be prepared by multiplication of the selected fungi in roots of susceptible host plants growing in the sterilized soil or substrates for example perlite, vermiculite, peat, sand, or mixture of them (Naqvi and Mukerji, 2000). The inocula of AM fungi can be applied as spores, or fragments of colonized roots. The spores and hyphae can be isolated from the soil rhizosphere and mixed with carrier substrates (Gentili and Jumpponen, 2006). Spore inocula are the most resistant and can survive unfavorable environmental conditions for a long period, but they colonize new root systems more slowly than other preparations. Therefore both types of inocula, e.g. spores and fragments of colonized roots should be combined in commercial products (Marin, 2006).

Root-based bulk inoculum production technology utilizes mass produced seedlings grown in sterilized soil infected with selected AM fungi using spores from fruiting bodies from cultivated plants. This technology results in seedlings with infected root systems and the roots and adhering soil are chopped up and used as the starter inoculum for scale up production. The inocula are produced in bulk by infecting fresh seedling of selected plants (Singh and Tilak, 2002; Gentili and Jumpponen, 2006). The root inocula are kept in polythene bags and used for pelleting seeds or in the preparation of granules for seed bed inoculation (Singh and Tilak, 2002). The others methods such as soil-free aeroponic, nutrient film, and root organ culture system have been used for production of AM, but these methods are costly and preclude commercial mass production (Gentili and Jumpponen, 2006). It may be possible to mass produce plants in tissue culture in sterile agar media and induce mycorrhizal associations using spores from fruiting bodies of selected mycorrhizal fungi. The dried root tissues and fungal mycelia could then be developed into mycorhizal seeding products (Hyde, *pers. comm.*).

Some steps are essential for development of a commercial fungal biofertilizers. They include selection, large scale production, carrier selection and preparation, mixing and curing, maintenance of appropriate numbers of inocula, and strong quality control (Malik *et al.*, 2005). The criteria for selecting AM fungi will depend on details of the local environment, soil conditions, and host plants. The AM fungi must 1) colonize roots rapidly after inoculation, 2) absorb phosphate from the soil, 3) transfer phosphorus to the plant, 4) increase plant growth, 5) persist in soil and reestablish mycorrhizal symbiosis during the following seasons, and 6) form propagules that remain viable during and after inoculum production (Tanu *et al.*, 2006).

The success of a formulation depends on whether it 1) is economically viable to produce, 2) does not alter the viability and function of the inoculum, 3) is easy to carry and enhance dispersal during application. The inoculum formulation may comprise one or more AM fungi and other organisms which together enhance the ability of the inoculum to form mycorrhizal associations with the target plant. The formulations are available in the form of powder, tablets/pellets or granules, gel beads and balls (Adholeya *et al.*, 2005; Tiwari and Adholeya, 2005). There are many ways to apply the AM inocula (Adholeya *et al.*, 2005; Schwartz *et al.*, 2006) including; scattering by hand, in-furrow application, seed coating, root dipping, and seedling inoculation. The efficacy of the application of AM inocula depends on the product, environmental condition, delivery method, and other variables. The success of AM fungi inoculation depends on crop species, size and effectiveness of indigenous AM fungi populations, fertility of the soil, and cultural practices (Adholeya *et al.*, 2005; Tiwari and Adholeya, 2005).

The production of commercial mycorrhizal inoculum has evolved considerably in recent years (Douds *et al.*, 2000). There are various types of microbial cultures and inoculants available on the market today and these have rapidly increased because of the advances in technology (Raja, 2006). There are more than 30 companies worldwide marketing mycorrhiza products (some of them shown in Table 2 and Fig. 4) comprising one or multiple mycorrhizal fungal inocula. These products are plant growth promoters and to be used in horticulture, agriculture, restoration and forestry (Schwartz *et al.*, 2006).

Other fungi used as biofertilizers

Other fungal biofertilizers which have been used to improve plant growth are *Penicillium* species. They are phosphate solubilizing microorganisms that improve phosphorus absorption in plants and stimulate plant growth (Wakelin *et al.*, 2004; Pradhan and Sukla, 2005). *Penicillium bilaiae* has been formulated as a commercial product named Jumpstart® and was released to the market as a wettable powder in 1999 (Burton and Knight, 2005). *Penicillium bilaiae* is applied to increase dry matter, phosphorus (P) uptake and seed yield in canola (*Brassica napus*) (Grant *et al.*, 2002; Burton and Knight, 2005). *Penicillium radicum* and *P. italicum* are also phosphate-solubilizing taxa (Whitelaw *et al.*, 1999; Wakelin *et al.*, 2004; El-Azouni, 2008). *Penicillium radicum* isolated from the rhizosphere of wheat roots, has shown a good promise in plant growth promotion (Whitelaw *et al.*, 1999) while *P. italicum* isolated from the rhizosphere soil was tested for its ability to solubilize tri-calcium phosphate (TCP) and could promote the growth of soybean (El-Azouni, 2008).

Several species of *Aspergillus* have been reported to be involved in the solubilization of inorganic phosphates such as *A. flavus*, *A. niger* and *A. terreus*, (Akintokun *et al.*, 2007). These fungi are able to solubilize of inorganic phosphate through the production of acids for example citric, gluconic, glycolic, oxalic acids, and succinic acid (Barroso *et al.*, 2006). *Aspergillus fumigatus* which isolated from compost *has been reported to be* potassium releasing fungus (Lian *et al.*, 2008).

The product of *Chaetomium* species can be fungal biofertilizers for example Ketomium® which is formulated from *Ch. globosum* and *Ch. cupreum* is not only a mycofungicide but also plant growth stimulant because tomato, corn, rice, pepper, citrus, durian, bird's of paradise and carnation treated with Ketomium® have a greater plant growth and high yields than non-treated plants (Soytong *et al.*, 2001).

Trichoderma species can not only reduce the occurrence of disease and inhibit pathogen growth when used as mycofungicides, but they also increase the growth and yield of plants (Elad *et al.*, 1981; Harman *et al.*, 2004; Vinale *et al.*, 2008). They are also increase the survival of seedlings, plant height, leaf area and dry weight (Kleifeld and Chet, 1992). *Trichoderma* species improve mineral uptake, release minerals from soil and organic matter, enhance plant hormone production, induce systematic resistance mechanisms, and induced root systems in hydroponics (Yedidia *et al.*, 1999). For these reasons *Trichoderma* species are known as plant growth promoting fungi (Hyakumachi and Kubota, 2004; Herrera-Estrella and Chet, 2004) or are increasing plant growth (biofertilization) (Benitez *et al.*, 2004). *Trichoderma* species have therefore, successfully been used as biofungicides and biofertilizers in greenhouse and field plant production (Harman *et al.*, 2004; Vinale *et al.*, 2008). There are many *Trichoderma* products as fungal biofertilizers available in the market (some of them shown in Table 2 and Fig. 4). Their applications are however related to their ability to control plant diseases and promote plant growth and development (Harman *et al.*, 2004; Vinale *et al.*, 2006). *Trichoderma* also has various applications and important sources of antibiotics, enzymes, decomposers and plant growth promoters (Daniel and Filho, 2007).

Future trends in mycofungicides and fungal biofertilizers

The use of fungi as fungicides and biofertilizers is not new although most have been developed in the last two decades. There are numerous reports stating the success in the ability of fungi to control plant diseases and promote plant growth as biofertilizers. Mycofungicides and fungal biofertilizers help to minimize the use of synthetic chemical fungicides and chemical fertilizers. This is beneficial since synthetic chemical compounds have probable detrimental affects on humans and the environment (Calhelha *et al.*, 2006; Haggag and Mohamed, 2007).

Mycofungicides and fungal biofertilizers are presently used on a very small scale as compared to chemical compounds (Fravel, 2005). There has been little investment in the research and development of fungal products because they may have poor effect in the field (Tang *et al.*, 2001). There is still a wide gap between the considerable, often unpublished research carried out in laboratories as compared to development for use in the field. Future research should therefore develop fungal products which have significant effects in field applications and that are also stable when stored. Aspects which should be considered include 1) which strains of beneficial fungi should be used; 2) whether they are reliable and cheap to produce on a large scale; 3) whether they are detrimental to the environment; 4) whether they are safe to humans and to the environment, and 5) whether patentability of the formulation is possible. Greater communication is needed between researchers and industry in the early stages of development. Integration or combination of inocula or combinations with other beneficial fungi should be considered since combinations may be more effective than a single inoculum. The production of mycofungicides and fungal biofertilizers should be directed to a new focus that will search for commercial properties through the use of biotechnologies of the inoculation of fungi and the benefits should clearly be demonstrated to the growers, both through extension and proven field trials. Commercial interest will then increase.

Although there are many biocontrol products (Table 1), there are still many problems to overcome to achieve successful commercialization of other potential biocontrol products. Some biocontrol agents work well in the laboratory but do not work well in the field (Tang *et al.*, 2001). Biological control of plant diseases by fungal antagonists remains a challenge for future research and development (Spadaro and Gullino, 2005). Several private companies have been involved in the development of mycofungicides. There are many species of fungal antagonists that have been formulated and registered as commercial products. Unfortunately, these products have been used on a small scale due to their capacity to control plant diseases in the field which is often not as good (or perceived to be less effective) than synthetic fungicides (Paulitz and Belanger, 2001; Tang *et al.*, 2001).

Table 2. Some fungal biofertilizers available globally.

Products	Fungi	Companies
AgBio-Endos	Ectomycorrhizal fungi	AgBio Inc, Westminter, USA
AgBio-Ectos	Endomycorrhizal fungi	Agbio-inc.com
AM120	Mycorrhizal fungi	Reforestation Technologies International, USA www.reforest.com
Bioorganic Plus	*Trichoderma harzianum* *Trichoderma hamatum*	NovaScience Co. Ltd, Thailand.
BioVam	Mycorrhizal fungi *Trichoderma* spp.	T&J Enterprises, USA www.tandjenterprises.com
BuRize	AM fungi	BioScientific Inc, Arizona, USA www.biosci.com
Diehard™ mycorrhizal inoculant	Mycorrhizal fungi *Trichoderma* spp.	Horticultural Alliance, Inc, Fl, USA www. horticulturalalliance.com
Endomycorrhizal inoculant (BEI), inoculant micronized (BEIM), Mycorrhizal root dip	Endomycorrhizal fungi	Bio-Organics, Oregan, USA www.bio-organics.com
MycoApply® Endo	Ectomycorrhizal fungi	Mycorrhizal application Inc, Oregon, USA
MycoApply® Endo/Ecto	Endomycorrhizal fungi	www.mycorrhizae.com
MycoApply® Maxx		
Plant Success ™		
Mycogrow™	Ectomycorrhizal fungi Endomycorrhizal fungi	Fungi perfecti, LLC, WA., USA www.fungi.com
Mycomax	AM fungi (*Glomous intraradices*)	JHBiotech Inc. California, USA www.jhbiotech.com
Myke	Mycorrhizal fungi	Premier Tech Biotechnologies, Canada
Myke® Pro		www.premiertech.com
Mycorise®		
PLantmate®	*Trichoderma* spp.	Agrimms Technologies Ltd. www.vinevax.com
Promote®	Ectomycorrhizal fungi (*Pisolithus tinctorius*)	JHBiotech Inc. California, USA www.jhbiotech.com
Rhizanova	Mycorrhizal fungi	Becker-Underwood Inc., USA www.beckerunderwood.com
Rootgrow, Rootgrow Professional	Mycorrhizal fungi	PlantWorks Ltd., United Kingdom www.plantworksuk.co.uk
SoilMoist™	Ectomycorrhizal fungi Endomycorrhizal fungi	JRM chemical, Inc. Ohio, USA www.soilmoist.com
Superzyme	*Trichoderma* spp.	JH Biotech, Inc., Ventura, CA. USA www.jhbiotech.com
Tricho®	*Trichoderma* spp.	Agrimms Technologies Ltd, www.vinevax.com

Many biological control agents produce secondary metabolites which have properties to control plant diseases. The inoculum therefore not only can be used as mycofungicides, but also the secondary metabolites can be developed as mycofungicides. The secondary metabolites, however, should be tested and studied and must be harmless to humans and the environment. Recent advances in the study of molecular genetics of biological control agent strains have provided a powerful tool that will help to improve the effectiveness of biocontrol activity and exploitation of the genetic potential of fungal antagonists (Irtwange, 2006; Paterson, 2006; Haggag and Mohamed, 2007). There should be further research on the application of fungal biofertilizers to the soil because they help to increase crop yield and improve soil quality (Tanu *et al.*, 2006). Fungal biofertilizers help to enhance crop yield and promote sustainable agricultural production and are safe to the environment (Smith and Zhu, 2001). Fungal biofertilizers have advantages in terms of nutrient supply, soil quality and crop growth and yield. Development in the effectiveness of fungal species for formulation as biofertilizers should be considered. New strains of fungi should 1) improve nutrient uptake, 2)

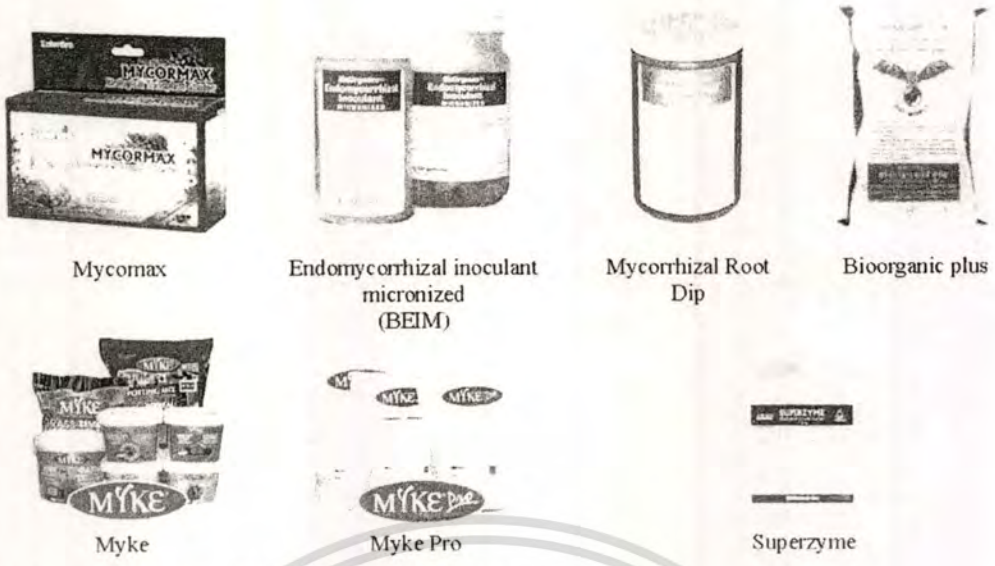


Fig. 4. Some of fungal biofertilizers available in the market.

be resistance for biotic and abiotic stresses, 3) be fast growing and 4) have high productivity. The large scale inoculum production cost should be low. A future prospect in fungal biocontrol agents may be obtained by using transgenic fungi (Marin, 2006).

Research into other ecological fungi should be pursued to find novel biofungicides and biofertilizers. For example, endophytic fungi which are symptomless colonizers of plants (Oses *et al*., 2008; Hyde and Soytong, 2008) and some, especially grass endophytes are symbionts (Sánchez Márquez *et al*., 2007; Tejesvi *et al*., 2007; Wei *et al*., 2007; Sánchez Márquez *et al*., 2008) could be exploited. Endophytes play an important role in ecosystem processes such as decomposition and nutrient cycling, and thus may be utilized as biofertilzers. Endophytes also have beneficial symbiotic relationships with the seeds and roots of many plants, such as orchids (Tao *et al*., 2008; Zhu *et al*., 2008) and could be use to improve orchid seed germination and orchid growth. Endophytic fungi may also have roles in plant growth and survival (Mitchell *et al*., 2008) by enhancing nutrient uptake and producing growth promoting metabolites such as gibberellins and auxins which are plant hormones (Khan *et al*., 2008). They have been shown to benefit the host plant, including tolerance to herbivory, heat, salt, disease, drought, and increased below- and above-ground biomass (Waller *et al*., 2005; Tejesvi *et al*., 2007). Moreover, they may have potential biocontrol properties to inhibit pathogen infection within the host via antibiosis, competition, mycoparasitism, inducing resistance to the host plant (Mejia *et al*., 2008), or by producing bioactive secondary metabolites (Evans *et al*., 2003). Rungjindamai *et al*. (2008) are searching for endophytes that can reduce white rot decay of *Elaeis guineensis*. Endophytic fungi are also known to be a rich source of bioactive metabolites (Tejesvi *et al*., 2007; Pongcharoen *et al*., 2008; Raghukumar, 2008; Rungjindamai *et al*., 2008).

The use of mycorrhizal fungi as biofertilizers is often limited due the fact that they will not grow in artificial culture. Ways should be sort by which we can grow these fungi in culture and produce inocula. As mentioned above plate cultivation of these fungi with tissue culture plants may be a solution. *Phlebopus portentosus*, the black bolete, is purportedly mycorrhizal and forms associations with several fruit trees (e.g coffee, mango, jack fruit). Lumyong *et al*. (2009) have successfully grown this species on artificial media may be good for *in vitro* cultivation. This fungus is a perfect target for a biofertilizer since it should enhance tree growth but also produce an annual crop of the expensive Black Bolete which is a sort after fungus which demands a good price. *Phlebopus portentosus* is an unusual bolete in that it has clamp connections and therefore its close relatives should also be examined to

establish whether they can be utilized in a similar way (Ji *et al.*, 2007).

The moves towards safe food production and organic food should increase biofungicide and biofertilizer use and thus result in environmental and ecosystem savings. Reduction in the use of chemical fungicides and fertilizers is necessary to maintain ecosystem function and develop sustainable agriculture. Research and development on mycofungicides and fungal biofertilizers should therefore emphasize on improving effective stable strains for disease control or for promoting plant growth though traditional and molecular techniques.

Conclusion

The benefits of using fungi as mycofungicides and biofertilizers include decreasing the occurrence of plant diseases by inhibiting the growth of pathogens, suppressing the amount of inocula of pathogens, increasing in uptake of nutrient from the soil or atmosphere, and producing bioactive compounds, hormones and enzymes which stimulate plant growth. These benefits maintain and increase the crop production. There are many commercial mycofungicides and fungal biofertilizers available worldwide. Using mycofungicides and fungal biofertilizers offer more environmentally friendly alternatives than chemical fungicides and chemical fertilizers. There are however, some limitations in using these products. Their success can be affected by environment conditions, while application difficulties, limited shelf life, and slow action as compared to chemical products may discourage farmers to utilize them. Research on the development of mycofungicides and fungal biofertilizers needs to be carried out so that more effective products are produced.

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Full Length Research Paper

Genetic variation among isolates of *Rigidoporus microporus* causing white root disease of rubber trees in Southern Thailand revealed by ISSR markers and pathogenicity

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The genetic variation among 32 isolates of *Rigidoporus microporus* was determined using pathogenicity tests and molecular marker (intersimple sequence repeats; ISSR). Isolates were collected from six sites of two provinces in the south of Thailand. Based on the pathogenicity results, all isolates could divide into three groups depended on their virulence to rubber trees as follows: low virulent isolates, moderately virulent isolates and high virulent isolates. Cluster analysis based on ISSR characters grouped the isolates according to geographical origins. A dendrogram resulting from a cluster analysis showed two main distinct groups, designated as A and B rooting from outgroup. Group A contained isolates collected from Surat Thani province and group B those collected from Narathiwat province. There was no clear relationship between degrees of disease and geographical regions.

Key words: ISSR, pathogenicity, *Rigidoporus microporus*, white root disease.

INTRODUCTION

Rubber tree (*Hevea brasilliensis* (Wild.) Muell.-Arg) is an economically important crop in Thailand which their products are exported worldwide and produces significant revenue for the country. The planting areas are mostly in the south which climate suitable for rubber plantation and also suitable for pathogen growth, especially white root disease (Anonymous. 2009). This disease is the most destructive root in rubber plantation in many countries. The causal agent of this disease is *Rigidoporus microporus* (Fr.) Overeem (Jayasuriya and Thennakoon 2007). This fungus belongs to Basidiomycete. It persists on dead or live root debris for a long time. It forms many white, flattened myce lialstrands with 1 - 2 mm thick which grows and extends rapidly through the soil in the absence of any woody substrate (Nandris et al.. 1987). Thus, the root of healthy rubber tree can be infected by contact with a disease source, such as rhizomorphs, infected root, dead stump, or wood debris (Guyot and Flori, 2002). The fruiting body of this fungus form mainly at the collar of the dead infected tree which can produce a large number of basidiospores seems to have a limited role in dissemination of this disease (Nandris et al., 1987).

The molecular techniques were accepted to be made in genomics, and in many areas of applied mycology. The molecular or DNA data has led a major study in development of the systematic, biochemistry and ecology of fungi. (Bridge, 2002). The development of molecular technology has provided approach which is already beingused to identification of unknown species, genetic variability, characterization and relatedness of fungal isolates and species and pathogen detection (Bridge, 2002; Wang et al., 2005; Glen, 2006). Moreover, molecular

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methods are now starting to give function of fungi in the environment, where they are exists, and how they interact with other organisms (Bridge, 2002). During the last years different methods of DNA such as microsatellites (simple sequence repeats; SSR), inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) have become available to detect genetic variation within populations and among individuals. These methods detect genetic variation directly at the DNA level (McDonald, 1997; Hassel and Gunnarsson, 2003) and require different genetic markers due to important features, such as genomic abundance, level of polymorphism, locus-specificity, quantities of DNA required, reproducibility, technical requirements and operation costs. Each molecular method has its own advantages and disadvantages (Spooner et al., 2005). The ISSR method was first developed by Zietkiewicz et al. (1994) and developed from SSR technique (Yu et al., 2006). Although SSR analysis technique provides a codominant and high reproductivity but development needs time-consume and laborious (Chadha and Gopalakrishna, 2007). ISSR technique which involves polymerase chain reaction (PCR) amplification of DNA segments using a primer composed of a microsatellite region (Ratnaparkhe et al., 1998). ISSR do not require genome sequence information but provide multi-loci am plification and quickly and easy operates as same as RAPD but give more high reproductivity than those with RAPD (Bornet and Branchard 2001; Spooner et al., 2005). The ISSR is detects a higher level of polymorphism than those detected with RFLP or RAPD (Godwin et al., 1997). ISSR method was first reported as a technique for analysis the genetic variation in plant and animals and later used to obtain DNA markers in fungi (Bornet and Branchard, 2001; Bayraktar et al., 2008). It is power tool for investigating genetic variation within closely related species (Yu et al., 2008) and also for studying of genetic population on fungi (Menzies et al., 2003; Chadha and Gopalakrishna, 2007). This technique is reliable and suitable for population genetics analysis of intraspecies (Yu et al., 2006) and study on intraspecific genetic variation of fungi pathogenic provide useful information for controlling disease (Takatsuka, 2007). ISSR was successfully to study the diversity and molecular relationships of fungi such as *Colletotrichum* isolates from the Iwokrama forest in Guyana (Lu et al. 2004) and study detection of genetic variation and population structure among isolates of *Fusarium oxyaporum* f.sp. *ciceris* on Chickpae in Turkey (Bayraktar et al., 2008).

The genetic diversity and gene flow within or between pathogen populations will lead to an understanding of how the pathogen is likely to adapt or evolve in the environmental change such as exposure to abiotic stresses, fungicides and plant resistant (McDonald et al., 2002). Thus, understanding the genetic variation in populations of the causal agent of disease would be useful in the improvement of disease management system or help to develop cultivars with tolerance and/or resistance to disease (Stenglein and Balatti, 2006; Bayraktar et al., 2008). Although white root disease of rubber trees has been an economically important disease in Thailand, but it lacks of information about the genetic variation of pathogen population associated with rubber trees by using molecular technique. The objective of the study was to assess the genetic variability within populations of white root disease pathogen by using ISSR technique and pathogenicity.

MATERAILS AND METHODS

Fungal isolates

All isolates used in this study were obtained from 6 sites in 2 provinces (Surat Thani and Narathiwat) in the south of Thailand (Figure 1). The cultures were maintained in potato dextrose agar (PDA) medium and deposited at Biocontrol Research Unit, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Pathogenicity tests

Inoculum preparation: All tested isolates were separately cultured in the inoculum medium of 200 g mixed substrates in plastic bag. The inoculum medium was consisted of 100 g sawdust, 3 g rice bran, 2 g sugar, and 5 ml water. The inoculum mixed substrates were sterilized in autoclave at 121°C, 15 lbs/inch^2 for 30 min. Then each isolate which cultured on sterile sorghum seed at the age of 7 days was separately transferred onto sterilized inoculum and incubated at room temperature (27 - 30°C) for 30 days. After that these inoculum were taken to test for their pathogenicity.

Inoculation technique: The experiment was done by Completely Randomized Design (CRD) with four replications. The 5 months rubber trees variety RRIM600 were planted in pots containing sterilized mixed soil (soil : sand : compost; 8:8:2). The 30 day-old inoculum mixed substrates was placed into the soil planting pot next to the root system of the rubber tree. Control plants were grown in a sterilized mixed soil without inoculum. The inoculated rubber trees were maintained and recorded for disease incidence at 90 days. The Disease Index (DI) were determined 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. The virulent group of the isolates was determined according to DI. The root rot of inoculated trees were reisolated to confirm the pathogenic isolate. Treatment means were compared with Duncan's Multiple Range Test (DMRT) at P = 0.05.

ISSR-PCR analysis

DNA extraction: Mycelium of each isolate was grown on Petri dishes (90 mm diameter), containing 20 ml potato dextrose agar (200 g potato, 20 g dextrose, 20 g agar and 1,000 ml distilled water) and incubated for approximately 10 days at 25°C and aerial mycelium were harvested from each isolate under sterile conditions. Genomic DNA was extracted by CTAB buffer as described by Ratanacherdchai et al. (2007) by grinding mycelium of each isolate with liquid nitrogen and transferred to 1.5 ml Eppendorf tube. The 600 µl Cetyltrimethylammonium bromide (CTAB) was added and incubated at 65°C for 30 min and vortexed every 10 min. The tube was cooled for a few minutes after that 600 µl

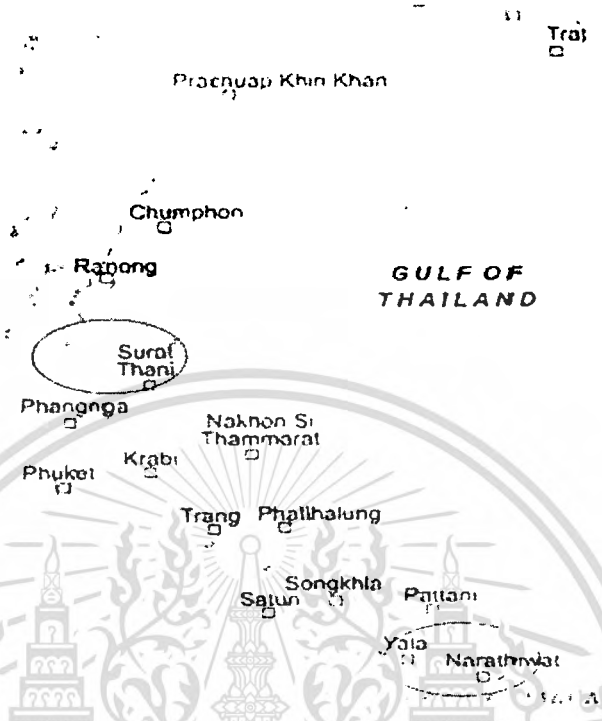

Figure 1 Collection area in the south of Thailand, Surat Thani and Narathiwat province.

Table 1. Sequence, the amount of $MgCl_2$ and dNTP, annealing time and G+C content for ISSR analysis.

Primer sequence	$MgCl_2$ (µl)	dNTP (µl)	Annealing time (°C)	G+C content (%)
(AG)8C	4	1	52	47.06
(GA)8T	4	1	52	47.06
(TG)8A	3	1	52	44.06
(GA)8YG	3	1	52	52.78
(GT)8YC	4	1	52	52.78
GGGC(GA)8	3	1	52	60.00
(CGA)5	3	1	52	66.67

Y = Pyrimidine.

chloroform : isoamyl alcohol (CIA; 24:1, v/v) was added, gently mixed and centrifuged at 7000 rpm for 5 min at 4°C. The aqueous layer was removed to new tubes and extracted again with CIA. Finally DNA was precipitated by adding 300 µl isopropanol, mixed well and incubated at room temperature for 30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was decanted. The DNA pellet was suspended in 40 µl ddH_2O.

Amplification conditions: Ten primers were screening for ISSR analysis and sevens primers yielded polymorphic banding patterns and were selected to analyzed diversity among isolates of *Rigidoporus microporus* as follows: (AG)8C, (GA)8T, (TG)8A, (GA)8YG, (GT)8YC, GGGC(GA)8, and (CGA)5. To optimize reaction conditions, the concentration of $MgCl_2$ and the amount of 10 mM dNTP were examined. The annealing temperature was optimized to obtain clear and reproducible patterns. The optimum reaction condition and G+C content of seven primers were shown in Table 1. The amplifications were performed in reaction volumes of 50 µl containing 5 µl of 10x PCR buffer, 2 µl of 10 µM primer, 3 µl of 25 mM $MgCl_2$ (for (TG)8A, (GA)8YG, GGGC(GA)8 and (CGA)5) and 4 µl of 25 mM

Table 2. Fungal isolates used in this study and their pathogenicity results.

Provinces	Collection sites	Isolates	Disease index (DI)*	Virulent group**
Surat Thani	Muang	Sss 01	1.3^{c***}	L
	Tachana	Sst 01	3.0^{abc}	M
		Sst 02	3.0^{abc}	M
		Sst 04	2.3^{abc}	M
		Sst 05	4.0^{abc}	M
		Sst 06	2.5^{abc}	M
		Sst 07	2.3^{abc}	M
		Sst 08	1.3^{c}	L
		Sst 09	2.0^{bc}	L
		Sst 11	1.3^{c}	L
		Sst 12	2.5^{abc}	M
		Sst 13	2.8^{abc}	M
		Sst 14	2.0^{bc}	L
		Sst 15	3.3^{abc}	M
		Sst 16	4.0^{abc}	M
Narathiwat	Sungai Padi	Sns 01	1.3^{c}	L
		Sns 02	2.0^{bc}	L
		Sns 03	2.5^{abc}	M
		Sns 04	1.3^{c}	L
		Sns 07	2.5^{abc}	M
		Sns 10	1.5^{c}	L
		Sns 11	3.3^{abc}	M
	Kokparimeng	Snk 02	5.0^{a}	H
		Snk 03	5.0^{a}	H
		Snk 05	4.0^{abc}	M
		Snk 06	4.0^{abc}	M
	Parulu	Snp 05	2.0^{bc}	L
		Snp 06	3.0^{abc}	M
		Snp 08	3.5^{abc}	M
	Todeng	Snd 05	2.0^{bc}	L
		Snd 07	2.0^{bc}	L
		Snd 08	4.5^{ab}	H

*DI; 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.
**L= low virulence, M= moderately virulence, H = high virulence.
***Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at P = 0.05.

$MgCl_2$ (for (AG)8C, (GA)8T and (GT)8YC), 1 µl of 10 mM dNTP and 0.3 µl of 1.5 U Taq polymerase, 1 µl of 10 ng genomic DNA, and 37.7 µl of ddH_2O. PCR was carried out in a MyGene™ Series Peltier Thermal Cycler (Model MG96G) under the following conditions: 2 min initial denaturation at 94°C followed by 35 cycles of 1 min denaturation at 94°C, annealing for 1 min at 52°C, extension for 1.5 min at 72°C, and final extension for 6 min at 72°C. PCR products were separated by 1.5% agarose gel strained with ethidium bromide which included in the agarose and visualized under UV fluorescence.

Data analysis: The ISSR DNA bands obtained for each isolate were scored based on their presence (1) or absence (0). Only reliable and reproducible bands were considered for score. A similarity matrix was generated from the binary data using DICE similarities coefficient in SIMQUAL program of NTSYS-PC Package (Rohlf, 2000). Dendrograms were constructed by cluster analysis based upon the unweighed pair group method with arithemetical mean (UPGMA).

RESULTS

Fungal isolates

All tested isolated of *Rigidoporus microporus* used in this study which obtained from six sites from two provinces in the south of Thailand were shown in Table 2. Their mycelium on potato dextrose agar (PDA) medium at room

Figure 2. Morphology of *Rigidoporus microporus*: mycelium on PDA medium at 6 days (a), hypha (40x) (b) and basidiospore (40x) (c).

temperature appeared white and flattened mycelium. The hypha of the fungal isolate showed hyaline, septate, and possession many branches but no clamp connection. The width of the hypha varies from 2.8 - 7.2 um. Basidiospores showed globose, colorless, thin-walled, smooth and 3.7 - 4.1 um in size (Figure 2). It also showed that all isolates were similar in morphology.

Pathogenicity test

Pathogenicity tests of all isolates on rubber trees variety RRIM600 showed symptom of yellowing leaves that was significantly different in disease incidence at $P = 0.05$. Base on the results, all isolates were grouped into three categories depended on DI as follow: high virulent isolates (DI = 4.1 - 5), moderately virulent isolates (DI = 2.1 - 4) and low virulent isolates (DI = 1 - 2). There were 17 isolates which grouped into moderately virulence, 12 isolates found low virulence and only 3 isolates showed high virulence and these three high virulent isolates were obtained from Narathiwat Province (Table 2).

ISSR-PCR analysis

Inter-simple sequence repeat (ISSR) markers were used to investigate genetic diversity among 32 isolates of *Rigidoporus microporus* isolates from different geographical origin and one isolate outgroup of *Ganoderma* spp GM101. Among ten primers, seven ISSR primers showed multi band patterns in each isolate. The primers amplified a total of 32 bands from 33 isolates tested. The average number of bands per primers was 4.6. Band size ranged from 250 - 2000 bp. In this study the G+C content of the primers ranged from 47.06 - 66.67% and annealing temperature was 52°C for all primers. The bands obtained from all isolates were shown in Figure 3.

UPGMA analysis based on total ISSR characters differences was carried out to group the 32 isolates of *R. microporus* with the outgroup *Ganoderma* spp GM101. A dendrogram resulting from a cluster analysis showed two main distinct groups, designated as A and B rooting from outgroup. All of the isolates obtained from Surat Thani province were grouped together as "A" and all of the isolates obtained from Narathiwat province were grouped together as "B". The results also indicated that there was an association between *R. microporus* isolates and their geographical origin. Within group B, isolates from different site could be separated from each other. The high virulence isolates were found to obtain from Narathiwat province. However, there was no clear relationship between degrees of disease and geographical region (Figure 4).

DISCUSSION

Rigidoporus mciroporus, the causal agent of white root disease of rubber tree, is an important fungal pathogen causing economically important crop loses in rubber plantation in Thailand and many countries. White root disease is being controlled by an integration of cultural and chemical methods such as removal and burning of the infected root, applying the chemical fungicides but sometimes it is too late to control disease. The important control strategies are to reduce the source of inoculum and to inhibit disease spread (Guyot and Flori, 2002). The understanding the genetic variation in populations of the causal agent of disease would be useful in the improvement of disease management system (Bayraktar et al., 2008) or help to develop cultivars with tolerance and/or resistance to disease (Stenglein and Balatti, 2006). However, there is no report on determination of genetic variation among pathogen populations of white root disease pathogen in Thailand. In this study, 32 isolates of *R. microporus* were tested with 7 ISSR primers to determinate the distribution of genetic diversity among isolates which represents the difference rubber tree planting areas. Base on the results, *R microporus* isolates obtained from different sites of two provinces from the south of Thailand were examined with pathogenicity test and ISSR analysis. The isolates which obtained from two provinces were separated into two groups. All of the isolates obtained from Surat Thani province were grouped together and were found to be a moderately virulent and low virulent isolates. All isolates which obtained from Narathiwat province were also grouped together and found three kinds of virulence isolate. It was clearly that there was a relationship betweenthe geographical distributions and clustering of isolates. These may be depending on the two areas of sample collection; Surat Thani which located in the middle of the south and Narathiwat which located in the southernmost of Thailand (Figure 1). However, there was no clear relationship between the pathogenicity and clustering of the isolates in this study. This result similar to those by Yu *et al* (2006) studied ISSR marker of *Melamspora larrici-populina* and found that ISSR analysis could divide tested isolates in to Northern population and Western

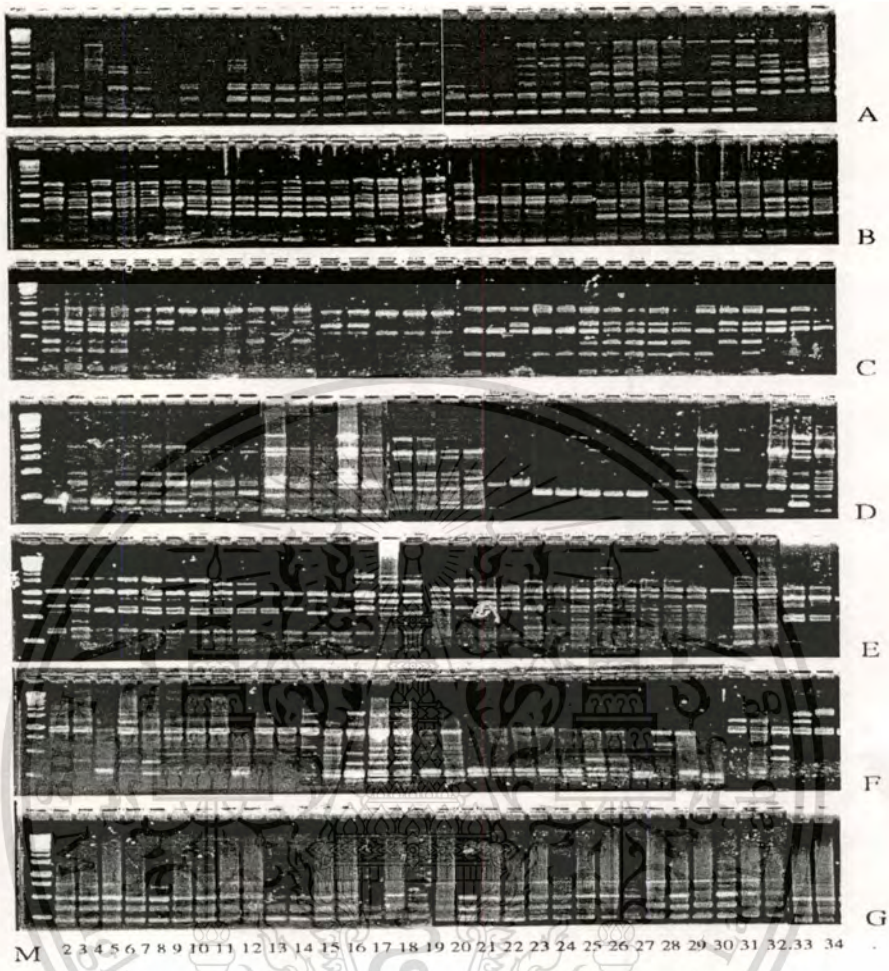

Figure 3. DNA banding profiles generated by ISSR-PCR with the (AG)8C (A), (GA)8T (B), (TG)8A (C), (GA)8YG (D), (GT)8YC (E), GGGC(GA)8 (F), and (CGA)5 (G) primer, M lanes represent 1 kb DNA ladder and lanes 2-33 represent tested isolates as shown in Table 2 and lanes 34 represent Gm101.

population. On the contrary, Rodrigues et al. (2004) found that ISSR-PCR analysis which separated strains of *Guignardia mangiferae* into three groups but not corresponded either to the host or to the geographic origin and Bayraktar *et al* (2008) also found that the genetic variation among isolates of *Fusarium oxysporum* f.sp. *ciceris* had no correlation with the clustering of isolates from different geographical regions. Moreover, the isolates of wood-decay fungus *Fomitopsis rosea* from the different geographical area did not separate in clusters (Kauserud and Schumacher, 2003). However, ISSR-PCR technique is suitable and reliable tool for population structure studies and discrimination among individual fungal isolates (Rodrigues et al., 2004). ISSR markers also are a good choice for DNA fingerprinting because they are quick and easy to handle (Bornet and Branchard, 2001).

In conclusion, the genetic variation among isolates of *Rigidoporus microporus* was determined by pathogenicity test and ISSR. The distinct genetic groups depended on geographical region but no clear relationship in disease virulence and pathogenicity. Moreover, this work provided new information on the ISSR analysis among isolates of *R. microporus* causing white root disease of rubber trees in Thailand.

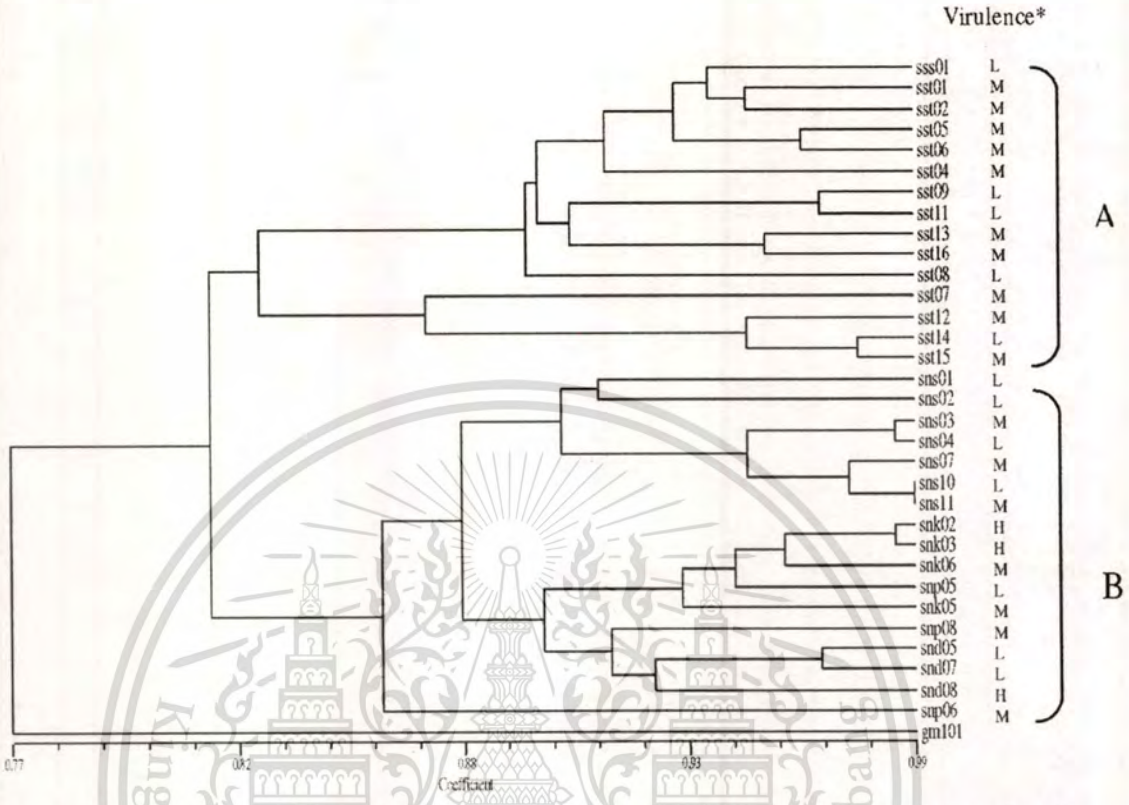

Figure 4. Dendrogram constructed with UPGMA-SHAN clustering of band data using 7 ISSR primers in 32 *isolates of Rigidoporus microporus* with outgroup *Ganoderma* spp GM101 and A = Surat Thani group and B = Narathiwat group.
*L = low virulent isolates, M = moderately virulent isolates, and H = high virulent isolates.

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Application of biofungicides against *Rigidoporus microporus* causing white root disease of rubber trees

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Ten species of fungi were tested for their abilities to control the growth of *Rigidoporus microporus* causing white root disease of rubber trees. Among them, five species were screened as follows:- *Aspergillus niger* SN72, *Chaetomium bostrychodes* BN08, *Ch. cupreum* RY202, *Trichoderma hamatum* STN07, and *T. harzianum* STN01 to inhibit the growth of pathogen over 50%. *Trichoderma hamatum* STN07 and *T. harzianum* STN01 were rapidly grown over the colony of pathogen whereas *Ch. bostrychodes* BN08 and *Ch. cupreum* RY202 could grow over the colony of pathogen within 30 days. The crude extracts with hexane, ethyl acetate, and methanol from these antagonistic fungi were determined by plate assay and found that crude extracts from *Ch. cupreum* RY202 gave the best results to inhibit the growth of *R. microporus* with ED_{50} value of 170, 402, and 1,220 µg/l, respectively. Moreover, rotiorinol; a bioactive compound produced from *Ch. cupreum* could inhibit the growth of pathogen with ED_{50} value of 26 µg/l that implies, a control mechanism. The formulation of *Ch. cupreum* RY202 in the powder and oil form could significantly inhibit the pathogen to infect the root of the rubber trees.

Introduction

Rigidoporus microporus the causing agent of white root disease is well known destructive agent to several crops and fruit trees especially rubber trees (*Hevea brasiliensis)* (Jayasuriya and Thennakoon, 2007). It is considered to be one of the main pathogen in rubber plantation. White root disease is found in all rubber planting areas throughout the world, for example India, Indonesia, Malaysia, Sri Lanka, Thailand, West and Central Africa. In some countries, it causes greater losses than those caused by all other diseases and pests. It can result in substantial death of trees and sometimes losses of a whole stand (Guyot and Flori, 2002). Normally, this disease is controlled by using an

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integration of cultural methods and chemical fungicides but chemical fungicides have been known to have a negative effect on human health, cause environmental pollution and leave residues in the agricultural soil (Soytong *et al.*, 2005; Haggag and Moamed, 2007). Moreover, several plant pathogenic fungi have developed resistance to chemical fungicides (Benítez *et al.*, 2004, Kim and Hwang, 2007). To avoid the negative or harmful effect of chemical use, biological control would therefore be an alternative method to save and sound measure for controlling disease by reducing the inoculum sources, as well as inhibiting the disease spread.

Numerous kinds of fungal species have been found in soil and reported to be biological control agent against plant diseases, e.g. *Aspergillus niger*, *Chaetomium globosum*, *Ch. cochlioides*, *Ch. cupreum*, *Gliocladium catenulatum*, *Trichoderma hamatum*, *T. harzianum*, *T. virens*, *T. viride* (Butt *et al.*, 1999; Soytong *et al.*, 2001; Fravel, 2005). *Aspergillus* species are effective against the white-rot basidiomycetes (Bruce and Highley, 1991). *Chaetomium globosum*, *Ch. cochliodes*, *Ch. cupreum* are reported to be antagonistic fungi which are able to suppress plant pathogens such as *Curvularia lunata*, *Pyricularia oryzae*, *Rhizoctonia oryzae*, *Fusarium oxysporum* f.sp. *lycopersici* (Soytong *et al.*, 2001). *G. catenulatum* has been reported to reduce the incidence of damping-off disease caused by *Pythium ultimum* and *Rhizoctonia slolani* (Panja and Utkhede, 2004). *Trichoderma* species have been used as biological control agent against a wide range of plant pathogenic fungi including; *Botrytis cineria*, *Fusarium*, *Pythium*, *Rhizoctonia* in many crops such as; corn, soybeans, potatoes, tomatoes, beans, cotton, peanuts, and trees, (Khetan, 2001; Paulitz and Belanger, 2001). Some *Trichoderma* species were reported to control white root disease for example *T. harzianum*, *T. virens* and *T. viride* (Bruce and Highley, 1991; Hightley, 1997; Jayasuriya and Thennakoon, 2007). *Trichoderma harzianum* is reported to be most widely used as an effective biological control agent (Vizcaino *et al.*, 2005; Abdel-Fattah *et al.*, 2007).

Biological control may result from direct or indirect interactions between biological control agent and pathogen (Viterbo *et al.*, 2007) such as physical contact and synthesis of hydrolytic enzyme, toxic compound or antibiotic, competition, and induce resistance in plant host (Benítez *et al.*, 2004; Pal and Gardener, 2006). Many antagonistic fungi produce toxic compound or antibiotic. Some antibiotics have been shown to play role in impede spore germination or kill the cells (Handelsman and Stabb, 1996; Benítez *et al.*, 2004; Haggag and Mohamed, 2007). The interest in the secondary metabolites produced by biological control agents has been substantial studying. An increasing number of metabolites from biological control agents have been

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discovered due to the application of biochemical assays that are used to identify metabolites (Vizcaino *et al.*, 2005).

In this study, the effective antagonistic fungi were investigated to control the growth of *R. microporus* causing agent of white root disease of rubber trees.

Materials and methods

Pathogen and pathogenicity test

Rigidoporus microporus was isolated from infected root of rubber trees by tissue transplanting technique. The culture was maintained in potato dextrose agar (PDA) medium and deposited at Biocontrol Research Unit, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The isolate was also proved for pathogenicity by modified technique from Rodesuchit (1998). The isolate was cultured on sterilized inoculum medium (100 g sawdust, 3 g rice bran, and 2 g sugar, moisten with water) contained in plastic bag and incubated at room temperature (28-30 ^{o}C) for 30 days. Then the 5 months rubber trees were planted in pots containing sterilized mixed soil (soil : sand : compost; 8 : 8 : 2) and inoculum was placed into planting pot next to the root system as seen in Fig. 1. The inoculated rubber trees were maintained in nursery and observed for disease incidence.

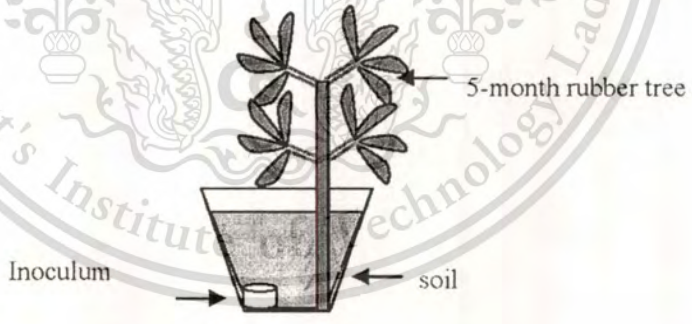

Fig. 1 Inoculation technique for pathogenicity test.

Biological control agents

The fungal isolates used in this study were isolated from rhizosphere soil from rubber plantation by soil plate and baiting techniques as described by Soytong and Quimio (1989). All fungal isolates were tested for their abilities to control *R. microporus* by dual culture. Then the fungal isolates which could inhibit the colony of *R. microporus* over 50% were taken to study their crude extracts to inhibit the colony of *R. microporus* by plate essay.

Effect of biological control agents by dual culture

The fungal isolates were separately evaluated for their abilities to inhibit the mycelial growth of *R. microporus* causing white root disease of rubber trees by dual culture technique. The experiment was conducted by Completely Randomized Design (CRD) with four replications. Biological control agents were separately grown on PDA for 6 days at room temperature (28-30 °C). The agar plug from the growing edges of colony of biological control agents was removed with a sterile cork borer (3 mm. diameter) and placed on one side of Petri dishes (9-cm diameter). The agar plug of *R. microporus* was taken at the age of 6 days colony and placed on the opposite sides of Petri dish. The dual plates were incubated at room temperature (28-30 °C) for 10-30 days. Data were recorded as colony diameter (cm) at 10 days. Percent inhibition of mycelial growth (PI) was computed as [(Dc-Dd)/Dc] x 100, where Dc is growth diameter of pathogen in control plates and Dd is growth diameter of pathogen in the dual culture plates. The experiment was repeated twice.

Effect of crude extracts of biological control agents

***Crude extraction method*:** The crude extracts were extracted from mycelial mats of biological control agents using solvent as described by Kanokmedahkul *et al.* (2006) The biological control agents were separately cultured in potato dextrose broth (PDB) for 30 days, filtered to yield mycelial mats. Air dried mycelial mats were ground and extracted successively with hexane, ethyl acetate, and methanol. The solvents were evaporated using rotary vacuum evaporator to yield crude hexane, ethyl acetate, and methanol extracts, respectively. Each crude extract was kept in the refrigerator until used.

***Testing method*:** The experiment was conducted by 3 x 6 factorials in CRD with 4 replications. Factor A was crude extracts from antagonistic fungus as follows:-crude hexane, crude ethyl acetate, and crude methanol extracts. Factor B was the concentrations of crude extracts, varies from 0 (control), 10,

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50, 100, 500, and 1,000 µg/l. Each crude extract in each concentration was dissolved in dimethyl sulfoxide (DMSO), and then mixed in PDA before sterilization. The agar plug from growing edges of colony of pathogen were placed at the centre of petri dish containing PDA incorporating with each concentration of crude extract and incubated at room temperature (28-30 °C) for 6 days. The mycelial growth of pathogen was measured as colony diameter (cm). Effective dose (ED_{50}) of each crude extract was also computed by probit analysis. Treatment means were compared using Duncan's Multiple Range Test (DMRT) at $P = 0.01$. The experiment was repeated twice.

Effect of rotiorinol, a bioactive compound from *Chaetomium cupreum*

The bioactive compound named rotiorinol produced by *C. cupreum* was provided by Somdej Kanokmedhakul, Faculty of Chemistry, Khon Kaen University, Khon Kaen, Thailand. It was tested for ability to inhibit mycelial growth of *R. microporus*. The experiment was conducted by CRD with 5 treatments and 4 replications. The treatments were different concentrations of rotiorinol as follows:- 0 (control), 10, 50, 100 and 250 µg/l. Rotiorinol was dissolved in dimethyl sulfoxide (DMSO) after that mixed with PDB in different concentrations and sterile. The agar plug of *R. microporus* at 6 days was transferred to the flask containing PDB which mixed with different concentrations of rotiorinol and shaked at 100 rpm for 10 days. The fresh weight and dry weight of mycelium of pathogen were recorded and computed as percent of fresh weight and dry weight inhibition. The effective dose (ED_{50}) was also computed. The experiment was repeated twice.

Effect of *Chaetomium cupreum* RY202 formulated as biofungicide to control pathogen *in vivo*

The experiment was set up to study the effect of *Ch. cupreum* RY202 as biofungicide in greenhouse condition. The experiment was conducted by CRD with 7 treatments and 4 replications. The treatments were as follows:- non-treated one, inoculated with pathogen, treated with antagonistic fungus in powder form, treated with antagonistic fungus in the oil form, treated with antagonistic fungus in the powder form and pathogen, treated with antagonistic fungus in the oil form and pathogen and treated with fungicide (sulfur). *Chaetomium cupreum* RY202 was culture in PDB for a month and after that formulated according to the methods of Dr. Kasem Soytong, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand (unpublished data), in the powder and oil form at the standard concentrations of 1

x 10^6 cfu/g and 1 x 10^6 cfu/ml, respectively. The most virulent isolate of *R. microporus* was cultured as pathogen inoculum in medium mixed substrate as described above. The 5 months rubber trees (variety RRIM600) was planted in the pot containing sterilized mixed soil (soil : sand : compost; 8 : 2 : 2). Then the pathogen inoculum was inoculated in the pot near the root of rubber trees. The biofungicide was mixed with the planting sterilized soil according into each treatment as stated at the rate of 100 g or 100 ml/plot. After that each biofungicide was applied by spraying every 2 weeks in the treatments at the rate of 1 g or 1 ml/tree. Data collection as disease index (DI) was recorded at 120 days after treatment. The disease index 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. Infected root colonized was observed and recorded. The percentage of disease reduction was also calculated. The experiment was repeated twice.

Results

Pathogen and pathogenicity test

The characteristic of *R. microporus* which isolated from infected root of rubber trees by tissue transplanting technique were showed in Fig. 2. The colony on PDA at 6 days showed white and flattened mycelium. The hypha showed hyaline, septate, and possession many branches but no clamp connection. The fruiting body showed broad, thin, and orange-red. Basidiospores showed globose, colorless, thin-walled, and smooth.

Pathogenicity tests of *R. microporus* on rubber trees variety RRIM600 showed symptom of yellowing leaves at 70 days. The root of the dead tree was possessed with rhizomorph of the pathogen and it produced fruiting body at the collar of the dead stem (Fig. 3).

Biological control agents

The fungal isolates used in this study are listed in Table 1. They were isolated from Narathiwat and Surat Thani provinces which located in the south of Thailand. Four species of *Chaetomium* were isolated by baiting technique. One species of *Aspergillus*, one species of *Penicillium*, and two species of *Trichoderma* were isolated by soil plate technique. *Chaetomium cupreum* RY202 and *Ch. cocchliodes* RY301 were kindly provided by Dr. Chaninan Pronsuriya, International College, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

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Table 1 The fungal isolates used in this study.

Isolate code	Species	Isolation method	Geographical origin
BN01	*Chaetomium aureum*	Baiting technique	Narathiwat province
BN08	*Chaetomium bostrychodes*	Baiting technique	Surat Thani province
BS31	*Chaetomium fusiforme*	Baiting technique	Surat Thani province
BN05	*Chaetomium indicum*	Baiting technique	Narathiwat province
SN72	*Aspergillus niger*	Soil plate technique	Narathiwat province
SN03	*Penicillium canescens*	Soil plate technique	Narathiwat province
STN07	*Trichoderma hamatum*	Soil plate technique	Surat Thani province
STN01	*Trichoderma harzianum*	Soil plate technique	Surat Thani province
RY301	*Chaetomium cochliodes*	Baiting technique	Rayong province
RY202	*Chaetomium cupreum*	Baiting technique	Rayong province

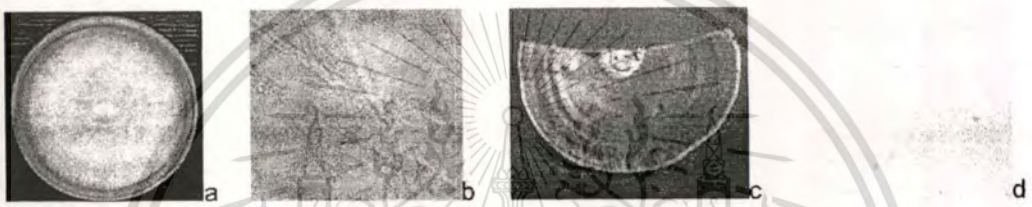

Fig. 2 Characteristic of *Rigidoporus microporus*: colony on PDA at 6 days (a), hypha (b) fruiting body (c), and basidiospores (d).

Fig. 3 The symptom of white root disease of rubber trees; yellowing leaves (a), rhizomorph at the root (b) and fruiting body at the collar of the dead stem (c)

Effect of biological control agents by dual culture

Based on the results, five tested fungal isolates could inhibit the mycelial growth of *R. microporus* over 50% as follows:- *A. niger* SN72, *Ch. bostrychodes* BN08 *Ch. cupreum* RY202, *T. hamatum* STN07 and *T. harzianum* STN01. *Aspergillus niger* SN72, *Ch. bostrychodes* BN08 and *Ch. cupreum* RY202 which inhibited the mycelial growth for 10 days as 75.8, 58.1, and 57.5%, respectively. The mycelium of pathogen could not grow when it came to contact with *A. niger* SN72. Moreover, *Ch. bostrychodes* BN08 and *Ch. cupreum* RY202 could produce the fruiting bodies over the colony of *R. microporus* after leaving them at room temperature (28-30 °C) for 30 days. The

results showed that *T. hamatum* STN07 and *T. harzianum* STN01 were highly antagonistic against *R. microporus* on agar plate with inhibition of mycelial growth at 89.5% and 85.3%, respectively and both of them rapidly grown over *R. microporus* colony.

Effect of crude extracts of biological control agents

Five tested biological control agents inhibited the mycelial growth of *R. microporus* over 50% as follows:- *A. niger* SN72, *Ch. bostrychodes* BN08 *Ch. cupreum* RY202, *T. hamatum* STN07 and *T. harzianum* STN01 as previous experiment. They were then cultured and extracted for their metabolites. Results showed significant differed in percentage of colony inhibition at the concentration of 10, 50, 100, 500, and 1,000 µg/l. All crude extract at the concentration of 1,000 µg/l, which extracted from *Ch. cupreum* RY202 inhibited colony of *R. microporus* as 82.0, 78.0, and 50.0%, respectively. Whereas, the methanol crude extract which extracted from *T. hamatum* STN07 and *T. harzianum* STN01 inhibited colony of pathogen at 1,000 µg/l as 80.0 and 61.5%, respectively. All crude extracts which extracted from *Ch. bostrychodes* BN08 and *A. niger* SN72 could not inhibit the mycelial growth of *R. mocroporus* (Table 2).

The effective dose (ED_{50}) of each crude extract which extracted from biological control agents was showed in Table 3. Crude hexane extract from *Ch. cupreum* RY202 gave the best inhibition to mycelial growth of *R. microporus* with ED_{50} of 170 µg/l follow by crude methanol extract from *T. hamatum* STN07 and crude ethyl acetate extract from *Ch. cupreum* RY202 with ED_{50} of 187 and 402 µg/l, respectively.

Effect of rotiorinol, a bioactive compound from *Chaetomium cupreum*

Rotiorinol is charaterized as red and amorphous powder and its structure was showed in Fig. 4. Rotiorinol gave significantly inhibit the growth of *R. microporus*. The percent of fresh weight inhibition of mycelium after treated with rotiorinol at concentrations of 10, 50, 100 and 250 µg/l were 29.0, 49.0, 97.8 and 99.3%, respectively and percent of dry weight inhibition were 31.4, 42.0, 97.2 and 99.1%, respectively (Table 4). The effective dose (ED_{50}) of this bioactive compound was 26 µg/l. Based on the results, the mycelium of *R. microporus* could not grow at concentrations 100 and 250 µg/l (Fig. 5). This research finding is implies antibiosis, a control mechanism of *Ch. Cupreum* against *R. microporus*.

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Table 2. Percent growth inhibition after treated with crude extracts for 6 days.

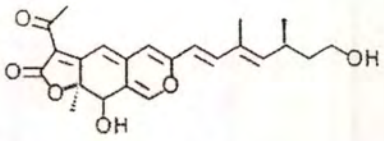
Biological control agents	Crude extracts	% growth inhibition				
		10	50	100	500	1,000
Aspergillus niger SN72	hexane	0.0c	0.0d	0.0e	0.0i	0.0h
	ethyl acetate	0.0c	19.5a	24.0b	28.5d	33.0d
	methanol	0.0c	0.0d	0.0e	20.0f	25.5e
Chaetoium bostrychodes BN08	hexane	0.0c	0.0d	0.0e	0.0i	9.0g
	ethyl acetate	0.0c	0.0d	0.0e	0.0i	0.0h
	methanol	0.0c	0.0d	0.0e	0.0i	0.0h
Chaetomium cupreum RY202	hexane	11.0a	13.0b	21.0c	80.5a	82.0a
	ethyl acetate	9.5b	13.5b	20.5c	42.5c	78.0a
	methanol	0.0c	9.0c	19.0d	24.5e	50.0c
Trichoderma hamatum STN07	hexane	0.0c	0.0d	0.0e	0.0i	4.5gh
	ethyl acetate	0.0c	0.0d	0.0e	15.0g	17.5f
	methanol	0.0c	0.0d	53.0a	80.0a	80.0a
Trichoderma harzianum STN01	hexane	0.0c	0.0d	0.0e	0.0i	0.0h
	ethyl acetate	0.0c	0.0d	0.0e	10.0h	27.5e
	methanol	0.0c	0.0d	0.0e	59.0b	61.5b
C.V. (%)		42.2	25.1	10.2	6.2	8.4

*Mean of four replications. Mean followed by a common letter are not significantly different when compared by Duncan's Multiple Range Test (DMRT) at $P = 0.01$.

Table 3. Effective dose (ED_{50}) of crude extracts from antagonistic fungi.

Biological control agents	Crude extracts	ED_{50} (µg/l)
Aspergillus niger BN72	hexane	NF
	Ethyl acetate	1,981
	Methanol	2,949
Chaetomium bostrychodes BN08	hexane	NF
	Ethyl acetate	NF
	Methanol	NF
Chaetomium cupreum RY202	hexane	170
	Ethyl acetate	402
	Methanol	1,220
Trichoderma hamatum STN07	hexane	NF
	Ethyl acetate	2,495
	Methanol	187
Trichoderma harzianum STN01	hexane	NF
	Ethyl acetate	1,635
	Methanol	556

NF = No effect



Source: Kanokmedhakul *et al.* (2006)

Fig. 4 Chemical structure of rotiorinol.

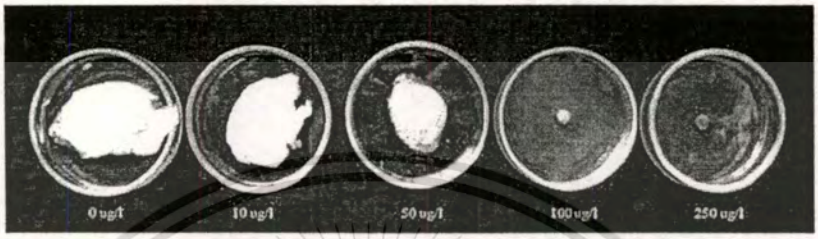


Fig. 5 Mycelial growth of *Rigidoporus microporus* after treated with rotiorinol at different concentrations.

Table 4. Fresh and dry weights of mycelia of *Rigidoporus microporus* after treated with rotiorinol.

Concentrations (µg/l)	% Fresh weight inhibition	% Dry weight inhibition
0	0.0d*	0.0d
10	29.0c	31.4c
50	49.0b	42.0b
100	97.8a	97.2a
250	99.3a	99.1a
cv. (%)	10.34	5.14

*Mean of four replications. Mean followed by a common letter are not significantly different when compared by Duncan's Multiple Range Test (DMRT) at $P = 0.01$.

Effect of *Chaetomium cupreum* RY202 formulated as biofungicide to control pathogen *in vivo*

The results showed that the disease symptom occurred in the inoculated with *R. microporus* (DI = 4) and inoculated with *Ch. cupreum* in the powder form and *R. microporus* (DI=2). The percentage of disease reduction in the treatment of inoculated with *R. microporus* and *Ch. cupreum* RY202 in the powder form, oil form and sulfur were 50, 75, and 75%, respectively (Table 5).

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Table 5. Disease index (DI) and disease reduction (DR) after treated with *Rigidoporus microporus* and biofungicide for 120 days.

Treatments	DI*	DR** (%)
Non-treated	1***	-
R. microporus	4	-
Ch. Cupreum in powder form	1	75
Ch. Cupreum in the oil form	1	75
Ch. Cupreum in the powder form and *R. microporus*	2	50
Ch. Cupreum in the oil form and *R. microporus*	1	75
Sulfur and *R. microporus*	1	75

*Disease index 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.
** Disease reduction
***Mean of four replications.

Discussion

Rigidoporus mciroporus was isolated from infected root of rubber tree and causes white root disease. This disease is an important disease of rubber trees which causing economically important loses in rubber plantation in Thailand and many countries. Nandris *et al.* (1987) reported that this fungus infects the roots by free rhizomorphs growing from the stumps or infected woody debris remaining in the ground and by contacting with the infected root. The visible symptom is changed in color of the leaves from green to yellow (Guyot and Flori. 2002). The decrement or inhibition the source of inoculum by using effective biological control agents is the best way to control this disease. This research tried to find out the biological control agents that possess the antagonistic properties to control *R. microporus* by dual culture and their bioactive compound. The results of dual culture showed that *A. niger*, *Ch. bostrychodes*, *Ch. cupreum*, *T. hamatum* and *T. harzianum* could inhibit the mycelial growth of *R. microporus* over 50%, especially *T. hamatum* STN07 and *T. harzianum* STN01 which rapidly grown and could grow over the colony of pathogen within a few days. These results were similar to those reported by Jayasuriya and Thennakoon (2007) who reported that *T. harzianum* was highly antagonistic against *R. microporus* and rapidly overgrown on *R. microporus* colonies in vitro. Bruce (1991) also reported that *Trichoderma* strains quickly overgrown and killed Basidiomyctes causing wood decay fungi. The other species of *Trichoderma* which reported to be an effective antagonist for pretreatment to protect white rot fungi was *T. virens* (Hightley, 1997; Bruce 1991). Nowadays, there are several reports on the use of *T. hamatum* and *T. harzianum* as biological agents against plant pathogens, especially *T.*

harzianum (Harman *et al.*, 2004). This taxa used as biocontrol in a wide range of pathogenic fungi such as *Botrytis cinerea, Fusarium* spp., *Phytopthora palmivora, P. parasitica, Pythium* spp. and *Rhizoctonia* spp. (Soytong *et al.*, 2001; Benítez *et al.*, 2004). Some strain of *T. harzianum* was formulated as mycofungicide such as *T. harzianum* T-22 and strain T-39 (Etebarian *et al.*, 2000; Khetan, 2001; Paulitz and Belanger, 2001). *Trichoderma* species successfully used as an biological control agent because they are fast growing, high productivity, diversity of control mechanisms, excellent competitors in the rhizosphere, tolerant or resistance to fungicides, strong aggressiveness against phytopathogenic fungi, and promote plant growth (Tang *et al.*, 2001; Benítez *et al.*, 2004; Szekeres *et al.*, 2004). *Chaetomium bostrychodes* BN08 and *Ch. cupreum* RY202 were isolated from soil and inhibited the mycelial growth of *R. microporus* over 50% in dual culture and produced the fruiting bodies on the colonies. This is the first report using *Ch. bostrychodes* and *Ch. cupreum* to control the colony of white root pathogen *in vitro*. These fungi may be one of the antagonists which can be applied to decrease the source of inoculum or inhibit the spread of disease. In this study, *Ch. cupreum* RY202 was selected to be used as the most effective antagonist against R. microporus due to the results from crude extract test and rotiorinol an antibiotic substance from Ch. Cupreum. Then, *Ch. cupreum* RY202 was formulated as powder and oil forms. These formulations were applied to inhibit *R. microporus* to protect the root of the rubber trees *in vivo*. The results showed that formulated biofungicide produced from *Ch. cupreum* RY202 could inhibit the pathogen to infect the root of the rubber trees. However, several species of *Chaetomium* can act as an antagonists such as *Ch. globosum* and *Ch. cupreum* (Soytong *et al.*, 2001, Soytong, 2003; Tomilova and Shternshis, 2006), *Ch. spirale* (Xin and Shang, 2005). *Ch. cupreum* can antagonizes a wide range of plant pathogens such as *Curvularia lunata, Pyricularia oryzae* and *Rhizoctonia solani* (Soytong, 2003). Moreover, *Ch. cupreum* and *Ch. globosum* were formulated as mycofungicide to control several diseases such as Fusarium wilt of tomato and Phytophthora root rot (Soytong *et al.*, 2001).

Based on this research, *A. niger* could inhibit the growth of *R. microporus* in dual culture. The result was similar to the report of Bruce and Hightley (1991) who found that *Aspergillus* species were effective against the white-rot basidiomycetes. Moreover, Bruce (1991) reported that *Aspergillus* could lysis the mycelium of white rot fungi at the point of contact but did not overgrow the colony of pathogen.

Our reports provide evidence of control mechanism as antibiosis from *Ch. cupreum* that produces antifungal metabolites for inhibition of growth of *R. microporus*. The crude extracts and rotiorinol from *Ch. cupreum* could inhibit

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the growth of *R. microporus*. There are many reports using crude extracts produced from *Ch. cupreum* to control the plant pathogens such as *Colletotrichum gloeosporioides* causing anthracnose disease and *Phytophthora parasitica* causing root rot *Pythium* sp. causing Pummelo root rot (Soytong *et al.*, 2005; Meepeung and Soytong, 2004). Moreover, Kanokmedhakul *et al.* (2006) extracted bioactive compound from *Ch. cupreum* CC3003 named rotiorinol and tested their antagonistic properties against pathogenic fungi and found that this compound could inhibit the growth of *P. parasitica*, *P. palmivora*, *C. gloeosporioides*.

In conclusion *Ch. cupreum* and its metabolite could inhibit the growth of *R. microporus*. The formulation of *Ch. cupreum* in the powder and oil form could inhibit *R. microporus* to infect the root of the rubber trees *in vivo*. This study was the first report using *Chaetomium* species and their metabolites to control *R. microporus*. However, these biological control agents need to prove their ability to control white root disease in the field by using their formulations or metabolites.

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Characterization of *Rigidoporus microporus* isolated from rubber trees based on morphology and ITS sequencing

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Kaewchai, S., Lin, F.C., Wang, H.K. and Soytong, K. (2010). Characterization of *Rigidoporus microporus* isolated from rubber trees based on morphology and ITS sequencing. The Journal of Agricultural Technology 6(2): 289-298.

White root disease of rubber trees caused by *Rigidoporus microporus* was studied the morphological and molecular phylogeny. The colony on PDA was white and flattening. The hypha showed hyaline, septum, no clamp connection. The fruiting bodies were broad shape, leathery, orange–red–brown in color. The hyphal system was monomitic, hyaline, thick walled with septate without clamp connection. Basidiospores were globose, thin-walled, colourless, and smooth. The *Rigidoporus microporus* is characterized by simple septate generative hypha, globose to subglobose smooth spores, mono to dimitic hyphal system, and causing white rot. Parsimony analysis of the ITS sequence confirmed the *Rigidoporus microporus* is distincted from *Rigidoporus ulmarious* but similar correlated to each other. According to molecular study, this is the first recorded studying R. microporus based on ITS sequencing in Thailand.

Introduction

White root disease of rubber trees caused by *Rigidoporus microporus* (Sw.) Overeem is the most well known destructive disease in rubber plantation in many countries for example India, Indonesia, Malaysia, Sri Lanka, Thailand, West and Central Africa (Jayasuriya and Thennakoon, 2007). *Rigidoporus microporus* persists on dead or live root debris for a long time. It forms many white, flattened mycelial strands which grows and extends rapidly through the soil in the absence of any woody substrate (Nandris *et al.*, 1987). The root of healthy rubber tree can be infected by contact with a disease source, such as rhizomorphs, infected root, dead stump, or wood debris (Nandris *et al.*, 1987; Guyot and Flori, 2002). It can result in substantial death of trees and sometimes

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losses of a whole stand (Guyot and Flori, 2002). The fruiting bodies of this fungus form at the collar of the dead stem which produce a large number of basidiospores but seems to have a limited role in dissemination of this disease (Nandris *et al.*, 1987).

The identification of wood decay fungi has been based on morphology, substrate utilization, and reproductive structures. This method is complicated because the fruiting bodies of wood decay fungi are frequently absent or difficult to detect in culture and also often show wide ranges of variability in physiologic characteristics, appearances and abilities (Hibbett and Donoghue, 2001). The morphological descriptions are used to identify cultivars; however, this approach lacks objectivity and reliability (Moukhamedov *et al.*, 1994). The advances in molecular technology appear to offer a rapid method to identify fungi base on more objective evaluations (Diehl *et al.*, 2004). The molecular techniques are already being used to identify of unknown species, genetic variability, characterization and relatedness of fungal isolates and species and pathogen detection (Wang, *et al.*, 2005; Glen, 2006). The internal transcribed spacers (ITS) have been used widely in study on the relationships among species within a single genus or among infraspecific populations and study characterization in fungi (Hibbett and Donoghue, 2001). The ITS region is useful study for characterization in fungi for four reasons as follows:-1) the ITS region is relatively short (500-800 bp) and can be easily amplified by PCR using universal single primer (White *et al.*, 1990), 2) the multicopy of the rDNA repeat makes the ITS region easy to amplify from small or dilute DNA samples, 3) the ITS region may be highly variable among morphologically distinct species, and 4) PCR-generated ITS species-specific probes can be produced quickly (Bridge and Arora 1998). ITS region have been used for the characterization, identification and detection of *Verticillium albo-atrum* and *V. dabliae* (Nazar *et al.*, 1991) and used to differentiate *V. tricorpus* from the other species of *Verticillium* (Moukhamedov *et al.*, 1994).

Although white root disease of rubber trees has been recorded as an economically important disease in Thailand, but it lacks of information on the genetic data. The objective of the study was to characterize of *R. microporus* by morphology and ITS sequencing.

Materials and methods

Fungal isolates and morphological characterization

Rigidoporus microporus, causing agent of white root disease of rubber tree was isolated by tissue transplanting technique from infected roots and fruiting bodies which obtained from the southern part of Thailand. The cultures

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were maintained in potato dextrose agar (PDA) medium and deposited at Biocontrol Research Unit, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The characteristic of the fungus on PDA medium was studied such as growth rate, colony colour, the characteristic of hypha and other specific structures. The fruiting body of this fungus was also studied by culture in plastic bag containing sterile inoculum mixed substrate which consisted of 100 g sawdust, 3 g rice bran, 2 g sugar for 30 days. The plastic bag was removed and covered the inoculum mixed substrate with soil, watered every day until fruiting bodies were produced. The morphology of fruiting bodies such as shape, color, and basidiospores was studied.

DNA sequencing

DNA extraction

Hyphal tip isolation was grown on potato dextrose agar (PDA) and incubated at 25 °C for 10 days. The mycelium was scraped from the surface of PDA by aseptic technique. DNA was extracted with CTAB buffer as described by Kaewchai *et al.* (2009) by grinding mycelium with liquid nitrogen to fine powder. The mycelial powder was suspended in 600 µl Cetyltrimethylammonium bromide (CTAB), incubated at 65°C for 30 min. During incubated period, the tube was vortexed every 10 min. After that the tube was cooled for a few minutes then 600 µl chloroform : isoamyl alcohol (CIA; 24:1, v/v) was added, gently mixed and centrifuged at 7,000 rpm for 5 min at 4 °C. The aqueous layer was transferred to new tubes and extracted again with CIA. Finally, DNA was precipitated by adding 300 µl isopropanol, mixed well and incubated at room temperature for 30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was decanted. The DNA pellet was suspended in 40 µl ddH2O.

PCR amplification

The ITS regions were amplified using the universal primers ITS 1 (5′TCC GTA GGT GAA CCT GCG G 3′) and ITS 4 (5′TCC TCC GCT TAT TGA TAT GC 3′) (White *et al.*, 1990). The amplifications were performed in reaction volumes of 50 µl containing 5 µl of 10x PCR buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 0.3 µl of 1.5 U *Taq* DNA polymerase, 1 µl of each primer, 1 µl genomic DNA and 37.7 µl of ddH_2O. PCR was carried out in a MyGene™ Series Peltier Thermal Cycler (Model MG96G) using the following program: 2 min initial denaturation at 94 °C followed by 35 cycles of

50 sec denaturation at 94 °C, 1 min annealing at 55 °C, 1 min extension at 72 °C, and final extension for 5 min at 72 °C. Amplification products were separated by 1% agarose gel in 1xTAE buffer strained with ethidium bromide which included in the agarose gel and visualized under UV fluorescence. PCR products were purified using the AxyPrep™ DNA Gel Extraction Kit (Axygen Scientific, Inc. USA) according to the manufacturer's instructions. DNA was sequenced by Shanghai Invitrogen Biolotech Co., Ltd. (Shanghai, P.R China).

Data analysis

The ITS sequence from an isolate and related species retrieved from GenBank (Table 1) were included in the analysed. *Auricularia delicata* was set as out group. All sequences were edited and initial aligned by BioEdit, version 7.0.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit. html). Multiple sequence alignments were determined with the ClustalX version 1.83 program. Gaps were set as missing data. Phylogenetic analyses were performed from aligned sequences of data sets using PAUP 4.0 version 4.0 Beta 10 (Swofford, 2001). Optimality criterion was set to parsimony. Bootstrap analyses were based on 1000 replications.

Results

Morphological study

The isolates of *Rigidoporus microporus* were studied on the morphology on the PDA medium and their fruiting bodies. The colony on PDA at room temperature (27-30 °C) was circular in shape, white and flattening, grew full the PDA plate within 6 days with growth rate of 1.3 cm per day (Fig. 1a). The hypha showed hyaline, septum, no clamp connection, and possess many branches. The width of the hypha was vary from 2.8- 7.2 um (Fig. 1b). The fruiting bodies were broad shape, leathery, and no stalk. The upper surface was orange–red–brown, smooth and the lower surface orange–brown (Fig. 1c and d), fine pores (Fig.1e). In section, the hyphal system was monomitic, hyaline, thick walled with septate without clamp connection (Fig. 1f), hymenium with cystidioles (Fig. 1g). Basidiospores were globose, thin-walled, colourless, and smooth. The spore size was 3.6 - 4.1 μm (Fig. 1h).

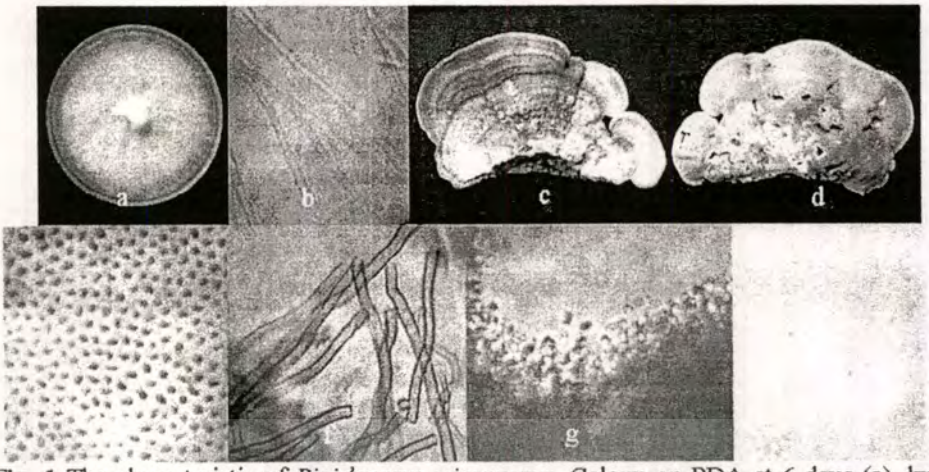

Fig. 1 The characteristic of *Rigidoporus microporus*. Colony on PDA at 6 days (a), hypha (b), fruiting body: upper surface (c) and lower surface (d), pores at the lower surface (e), monomitic, generative hypha (f), hymenium (g) and basidiospores (h).

DNA sequencing

The final alignment of the ITS sequences comprised 32 taxa with 607 characters, 280 characters were constant expressed and 31 variable characters were parsimony-uninformative. The number of parsimony-informative characters was 296. The maximum parsimony (MP) heuristic search treating gaps as missing data with no differential weighting of transitions against transversions was studied using random addition sequence and tree-bisection-reconnection (TBR) branch-swapping algorithm resulted in 1 tree. When a weighted parsimony was applied to the same data set, with alignment gaps treated as missing data, the results also yielded 1 tree with consistency index=0.806, retention index=0.948, rescaled consistency index=0.764 and homoplasy index=0.194.

Parsimony analysis of the ITS sequence alignment resulted in one of the most parsimonious tree. Tree major groups were detected. The first group consisted of isolate of *Oxyporus* species. The second group consisted of *Rigidoporus microporus* and *R. ulmarious*. The third group was consisted of *Laetoporus* species (Fig. 2).

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Table 1. Sequences from Genbank.

Taxon	Strain N0.	Origin	Genbank accession
Auricularia delicata		Costa	AF291290
Heterobasidion annosum	wb276	Austria	AF455496
Heterobasidion parviporum	E1	Latvia	FJ903330
Laetiporus gilbertsonii		USA	AM269785
Laetiporus sp.	6676	Uruguay	EU840673
Laetiporus sp.	6677	Uruguay	EU840674
Laetiporus sp.	6688	Uruguay	EU840675
Laetiporus sp.	6689	Uruguay	EU840676
Laetiporus sp.	6692	Uruguay	EU840677
Laetiporus sp.	6693	Uruguay	EU840678
Laetiporus sp.	6730	Uruguay	EU840681
Laetiporus sp.	5179	Uruguay	EU840682
Laetiporus sulphureus	TENN61397	USA	FJ596806
Oxyporus corticola	R-3714	USA	EF011122
Oxyporus corticola	R-3713	USA	EF011123
Oxyporus corticola	5385b	Estonia	DQ873641
Oxyporus corticola	C70	Latvia	FJ903327
Oxyporus cuneatus		Canada	DQ384575
Oxyporus latemarginatus	1239	Latvia	GU062267
Oxyporus latemarginatus	CTM10133	Tunisia	DQ000295
Oxyporus populinus	R-3716	USA	EF011121
Oxyporus subpopulinus	2313	China	FJ644281
Oxyporus subpopulinus	2251	China	FJ644282
Rigidoporus ulmarius		England	AY593868

Discussion

The colony *of Rigidoporus microporus* on PDA showed white and flat. The hypha of this fungus showed hyaline, septum, no clamp connection, and possess many branches. This result was similar to the report of Nandris *et al.* (1987) who stated that the fungus formed many white and flattened mycelium but the colony on malt medium formed superficial, white mycelial felt. In the soil, the mycelial strands or rhizomorphs grow rapidly and may extend several meters in the absence of any woody substrate and can infect the root of the healthy trees. Moreover, the root of the healthy trees are infected by rhizomorph growing from stumps, infected wood debris buried in the ground as well as by roots contacting those of a diseased neighbouring tree (Nandris *et al.*, 1987; Guyot and Flori, 2002).

The fruiting bodies of *Rigidoporus microporus* were broad shape, leathery, and no stalk. The upper surface was orange–red–brown, smooth and the lower surface orange–brown, fine pores. In section, the hyphal system was monomitic, hyaline, thick walled with septate without clamp connection, hymenium with cystidioles. Basidiospores were globose, thin-walled, colorless, and smooth. The spore size was 3.6 - 4.1 µm. These characters were similar to those which reported by Hood (2006) who reported that fruiting bodies a broad (to 20 cm wide), relatively thin, annual to less frequency perennial, leathery, broadly attached shelf, clustered, often imbricate; upper surface, concentrically furrowed, initially orange-red-brown, faintly, velvety, later smooth, faded; lower surface bright orange-brown, eventually paling, pores fine (6-9 per mm). In section, context pale colored. Monomitic, generative hyphae thin- or thick-walled, with cross walls (septa), with out clamps, hyaline. Hymenium with cystidioles, basidiospores sub-globose to globose (3.5-4.5 x 3.5-4 um), thin-walled, colorless, smooth.

The phylogeny study is still needed for confirmation the specie of *R. microporus*. The phylogenitic tree by ITS showed that *R. microporus* was similarly correlated to *R. ulmaroius*. The others groups were *Oxyporus*, *Heterobasidion* and *Laetoporus* group. Ryvarden (1991) reported that the *Rigidoporus* is in the group as same as *Melanoporia*, *Nigrofomes*, *Heterobasidion*, *Oxyporus*, *Leucophellinus*, *Laetiporus*, *Flavodon* and *Irpex*. This group is characterized by simple septate generative hypha, globose to subglobose smooth spores, mono to dimitic hyphal system, binding hyphae absent, and causing white rot but this research finding is confirmed that *R. microporus* is distinct from *R. ulmarius*.

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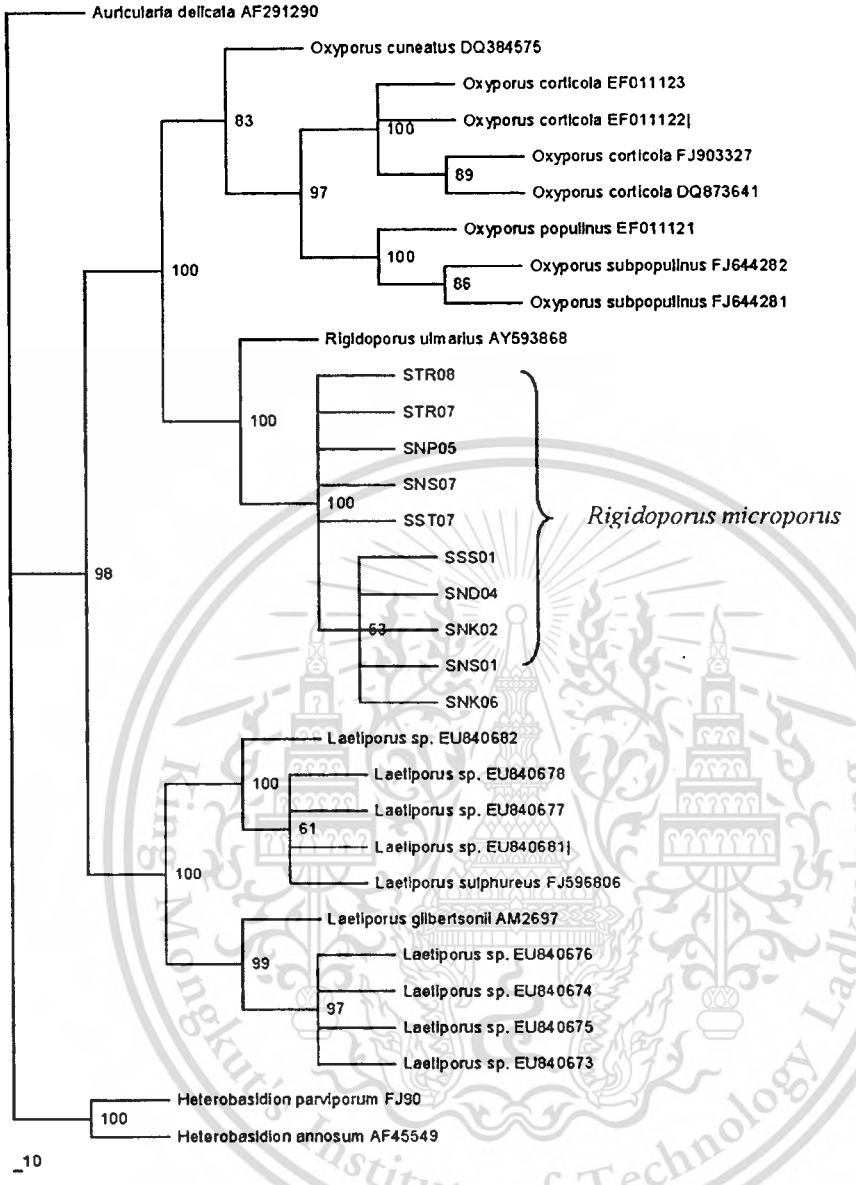

Fig. 2 Tree derived from maximum parsimony analysis of the sequences of the ITS region. The number at the branches denotes the percentage of bootstrap values after 1,000 replications and bootstraps higher than 50%.

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Effects of Media and pH Levels on the Growth of *Rigidoporus lignosus* (Klotzsch) Imaz.

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Abstract: Four tested isolates (SST01, SND07, SNP06, and SNK10) of *Rigidoporus lignosus* (Klotzsch) Imaz. were isolated from the infected root of rubber tree from the South of Thailand. Four tested isolates were culture in different media:- PDB (potato dextrose broth), GYEB (Glucose yeast extract broth), PDYEB (potato dextrose yeast extract broth), and PDPYEB (potato dextrose peptone yeast extract broth) to select the most suitable medium for growth. The mycelial growth of all isolates were significantly affected by culture media. PDPYEB was the most favorable for the growth of mycelium of all tested isolates. The fresh weight and dry weight of all isolates gave significantly higher in PDPYEB, than in PDYEB, PDB, and GYEB media. The mycelial growth of all isolates were tested in the pH range of 3-9. The pH value had significant affected the mycelial growth of all tested isolates. The optimum growth occurred at pH 6-8, whereas s a highly acidic pH reduced the mycelial growth.

Key words: *Rigidoporus lignosus*, pH level, media, mycelial growth

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Genetic Variation among Isolates of *Rigidoporus lignosus* causing agent of white root disease of Rubber tree in Thailand by using ISSR Markers

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Abstract: *Rigidoporus lignosus*, the causal agent of white root disease, is an important fungal pathogen causing economically important crop losses in rubber plantations. Thirty-two isolates of *R. lignosus* were obtained from infected roots of rubber trees which were collected from planting areas from the south of Thailand. They were tested for pathogenicity and twenty eight isolates could infect the root of rubber trees and showed symptoms of yellowing leaves, but were significantly different in disease levels.

Inter simple sequence repeat (ISSR) markers were used to determine the genetic variation among 32 isolates of *R. lignosus* obtained from different locations. Sevens primers contained different simple repeat were chosen for their reproducibility. UPGMA analysis based on total ISSR characters was carried out to group the 32 isolates of *R. lignosus*. A dendrogram resulting from a cluster analysis was showed two main distinct groups. All tested isolates could be divided into two groups according to geographical origin as a group from Surat Thani Province and another group from Narathiwat Province. The results indicate that there is a certain association between *R. lignosus* isolates and their geographical origin but there was no clear correlation between those isolates and their pathogenicity.

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Biological control of *Rigidoporus microporus* causing white root disease of rubber trees

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Abstract: Biological control agents: *Aspergillus*, *Chaetomium* and *Trichoderma* were tested for their ability to control the mycelial frowth of *Rigidoporus microporus* causing white root disease of rubber trees by dual culture and by using their crude extracts. All tested isolates were able to inhibit the mycelial growth of pathogen over 50%. *Trichoderma hamatum* TSN01 and *T. harzianum* TSN02 were rapidly grown over the colony of pathogen whereas *Chaetomium bostrychodes* BS01 and *Ch. cupreum* RY202 could grow over the colony of pathogen after 30 days. *Aspergillus niger* SN71 could also inhibit the mycelial growth of pathogen. The effects of crude extracts which extracted with hexane, ethyl acetate, and methanol from these antagonistic fungi were determined by plate assay. It was found that all three kinds of crude extracts from *Ch. cupreum* RY202 gave the best results to inhibit the mycelial growth of *R. microporus* which the ED_{50} value of 257, 296, and 788 µg/ml, respectively.

Key words: biocontrol, *Rigidoporus microporus*, crude extract, rubber tree

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Effects of media and pH levels on the growth of *Rigidoporus lignosus* (Klotzsch) Imaz.

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ABSTRACT

Four isolates (SST01, SND07, SNP06, and SNK10) of *Rigidoporus lignosus* (Klotzsch) Imaz. were isolated from the infected root of rubber tree from the South of Thailand. Four different media:- PDB (potato dextrose broth), GYEB (Glucose yeast extract broth), PDYEB (potato dextrose yeast extract broth), and PDPYEB (potato dextrose peptone yeast extract broth) were tested for the growth of fungi, and were employed to select the suitable medium for the mycelial growth of *R. lignosus*. The mycelial growth of all isolates were significantly (P=0.05) affected by culture media. In general, PDPYEB was the most favorable for the growth of mycelium of all tested isolates. The fresh weight and dry weight of all isolates gave significantly higher in PDPYEB medium than in PDYEB, PDB, and GYEB media. The mycelial growth of all isolates was tested in the pH range of 3-9. Result showed that pH value had significant effected on the mycelial growth of all tested isolates. The optimum growth of tested isolates occurred at pH 6, 7, and 8, whereas more acidic pH reduced the mycelial growth.

Key words

Rigidoporus lignosus, pH level, media, mycelial growth

1. INTRODUCTIONS

Root diseases are a major constraint in rubber cultivation. If untreated, they can result in heavy death of trees and sometimes in loss of a whole stand. *Rigidoporus lignosus* (Klotzeki) Imaz., which causes white root rot, is the most harmful and found in all rubber growing areas. The consequences of white root disease varies considerably depending on the countries, and even between close estates but its economic importance is major in Malaysia, Indonesia, Thailand, India, Sri Lanka, West and Central Africa[1]. *R. lignosus* has a wide host range which includes many jungle and horticultural plants[2]. The main source of inoculum is the remnants of infected wood, either pieces of roots or stumps left buried in the soil. Roots of young rubber plants are infected when they come into contact with the source of inoculum and the disease spreads to the tree collar and out to the other lateral roots through the fungal rhizomorphs which grow ahead of the rot. As the trees mature and their lateral roots intermingle, white root disease is transmitted from tree to tree especially along the planting rows. Spores produced by the fungal fructifications also spread the disease. Spores deposited onto wounds on the roots or on cut surfaces of stumps initiate new source of infection[3]. Despite description of the fungus, little information is available on its biology, including environmental requirement for growth. This information was valuable to further mycological and pathological research on the fungus and disease, including disease management. The objective of this study was to provide information on effects of culture media and pH levels on mycelial growth of *R. lignosus*.

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2. MATERIALS AND METHODS

Fungal species

Four isolates (SST01, SND07, SNP06, and SNK10) of *R. lignosus* were used in this study. The diseased samples were collected from infected root of Heavea tree in the South of Thailand. Isolate SST01 was obtained from infected root of rubber tree in Surat Thani province. Isolates SND 07, SNP06, and SNK10 were obtained from infected root of rubber tree in Narathiwat province. They were isolated by tissue transplanting technique which the infected tissue was surface disinfected for 1 minute in 10% Clorox, followed by washing in sterile water and transferred onto isolating media (water agar, WA). The mycelia growing out of the plant tissue were transferred onto PDA (potato dextrose agar) medium, isolated to pure culture and maintained at room temperature.

Effect of culture media and pH on mycelium growth

Isolates were grown on PDA at room temperature for 5 days. Four millimeters of diameter agar plugs were removed with a sterile cork borer from the leading edges of colonies, and one plug was placed in flask containing 20 ml of medium. Mycelial growth of four isolates were evaluated on four media as followed:- PDB (potato dextrose broth) containing (g/l): potato 200; dextrose 20, GYEB (glucose yeast extract broth) containing (g/l): glucose 10; yeast extract 2, PDYEB (potato dextrose yeast extract broth) containing (g/l): potato 200; dextrose 20; yeast extract 0.5, and PDPYEB (potato dextrose peptone yeast extract broth) containing (g/l): potato 200; dextrose 20, peptone 2; yeast extract 0.5. The media were adjusted pH with NaOH or HCl to value ranging from 3 to 9 (3, 4, 5, 6, 7, 8, and 9). The isolates were cultured at room temperature and shaken at 100 rpm for 9 days. The fresh and dry weight of the mycelia were measured by collecting the mycelial mat in each conical flask, then, filtered and air dried (28-32 °C) for 72 h and weighted.

Statistical analysis

The experiment was employed by factorial experiment in Completely Randomized Design. The data were analyzed for variance by Duncan's Multiple Range Test (DMRT) at $P = 0.05$. The experiment was repeated four times.

3. RESULTS AND DISCUSSION

Resulted showed that the best fresh weight of isolate SNP06 was observed in PDYEB at pH 8 (7.97 g) followed by PDPYEB at pH 7 (7.67g), pH 8 (6.86 g), and pH 9 (6.85 g), and the best dry weight was also observed in PDYEB at pH 8 (0.19 g) followed by PDB at pH 8 (0.15 g), PDPYEB at pH 7 (0.14 g) and pH 8 (0.14 g) (Table 1). For the isolate SND07, the mycelia grown in PDPYEB medium at pH 7 gave the best fresh and dry weight (10.64 and 0.19 g, respectively) (Table 2). The fresh and dry weight of isolate SST01 were higher in PDPYEB medium in all pH levels than in PDYEB, PDB, and GYEB media. This isolate grew better at optimum pH of 6, 7, and 8 (Table 3). Whereas GYEB medium of pH 8, 9, and 7 (4.14, 4.12, and 4.04 g, respectively) gave more fresh weight than PDPYEB, PDYEB, and PDB media. At pH 6 (0.13 g) gave the best dry weight for isolate SNK10 (Table 4). It was observed that the mycelial growth of all tested isolates were reduced at pH 4 – 5, while there were no mycelial growth at pH 3 as seen in Figure 1.

The mycelial growth of all tested isolates were significantly ($P = 0.05$) affected by culture media and pH levels. In general, PDPYEB media was the most favorable for the growth of mycelium of all isolates tested followed by PDYEB, PDB, and GYEB medium. The mycelial growth was obtained in the pH range 3 - 9. The Optimum growth occurred at pH 6, 7, and 8, whereas at pH 4 and 5 reduced the mycelial growth and no growth was observed at pH 3. These results implied that all isolates preferred neutrality pH values toward alkalinity. These results were similar to those obtained by Rodesuchit[4], whose reported that the optimum mycelial growth of *R. lignosus* occurred at pH between 6-7. Richard and Botton[2] found that pH 8 gave the best mycelium growth of *R. lignosus*, whereas more acidic pH reduced the growth of mycelium of *R. lignosus*. Fasola *et al.*[5] reported that, at very strong acidic or alkaline pH, cell wall may corrode and selective permeability function of the cell membrane may be impaired. This may be given the reason why there were no growth at pH 3.

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Table 1 Fresh and dry weight of *Rigidoporus. lignosus* isolate SNP06 in various media and pH levels.

Media[1/]	pH 3	4	5	6	7	8	9
	Fresh weight (g)						
PDB	0.11i[2/]	1.38h	4.16defg	4.44def	4.57de	4.87cd	4.37def
GYEB	0.03i	0.98hi	3.32fg	3.73defg	3.06g	3.49efg	3.68defg
PDYEB	0.18i	1.95h	4.65d	4.64d	5.72c	7.97a	5.84c
PDPYEB	0.17i	1.48h	4.80cd	5.80c	7.67ab	6.86b	6.85b
C.V. = 18.18%							
	Dry weight (g)						
PDB	0.02j	0.04ijk	0.08efg	0.09def	0.13bc	0.15b	0.09def
GYEB	0.01jk	0.02jk	0.07efghi	0.08efg	0.06fghi	0.06fghi	0.08efg
PDYEB	0.01jk	0.04hij	0.12bcd	0.12bcd	0.14b	0.19a	0.13bc
PDPYEB	0.01jk	0.06fghi	0.10cde	0.13bc	0.14b	0.14b	0.13bc
C.V. = 24.18%							

[1/]PDB = potato dextrose broth; GYEB = glucose yeast extract broth; PDYEB = potato dextrose yeast extract broth; PDPYEB = potato dextrose peptone yeast extract broth.
[2/]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

Table 2 Fresh and dry weight of *Rigidoporus. lignosus* isolate SND07 in various media and pH levels.

Media[1/]	pH 3	4	5	6	7	8	9
	Fresh weight (g)						
PDB	0.08j[2/]	1.96i	5.55ef	4.76efg	3.48gh	6.29cde	6.30cde
GYEB	0.08j	1.75i	3.94gh	4.50fgh	3.72gh	4.83efg	2.96hi
PDYEB	0.04j	1.98i	5.77ef	5.92def	5.74ef	4.57fg	7.37cd
PDPYEB	0.02j	3.90gh	7.68c	9.12b	10.64a	6.37cde	10.55a
C.V. = 21.20%							
	Dry weight (g)						
PDB	0.01j	0.07gh	0.12cd	0.13bc	0.07gh	0.11cde	0.12cd
GYEB	0.01j	0.03ij	0.07gh	0.07gh	0.06gh	0.07gh	0.04hi
PDYEB	0.01j	0.05hi	0.09fg	0.15a	0.10def	0.09fg	0.12cd
PDPYEB	0.01j	0.10cde	0.15a	0.13bc	0.19a	0.10cde	0.13bc
C.V. = 21.97%							

[1/]PDB = potato dextrose broth; GYEB = glucose yeast extract broth; PDYEB = potato dextrose yeast extract broth; PDPYEB = potato dextrose peptone yeast extract broth.
[2/]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

Table 3 Fresh and dry weight of *Rigidoporus lignosus* isolate SST01 in different media and pH levels.

Media[1/]	pH 3	4	5	6	7	8	9
	Fresh weight (g)						
PDB	0.05j[2/]	1.37ij	4.79gh	7.42def	7.49cdef	8.07cde	7.41def
GYEB	0.03j	1.85i	3.95h	5.19gh	5.73fg	5.94fg	4.83gh
PDYEB	0.05j	1.21ij	6.31efg	8.10cde	9.92b	9.22bc	9.80b
PDPYEB	0.13ij	1.82i	7.19def	12.68a	12.72a	12.59a	8.53bcd
C.V. = 18.99%							
	Dry weight (g)						
PDB	0.01m	0.04kl	0.07hij	0.14def	0.14def	0.14def	0.08hij
GYEB	0.01m	0.02lm	0.05jk	0.09ghi	0.11g	0.11fg	0.10gh
PDYEB	0.01m	0.05jk	0.15cde	0.17cd	0.20b	0.16cde	0.18c
PDPYEB	0.01m	0.06ijk	0.21b	0.27a	0.26a	0.26a	0.21b
C.V. = 15.29%							

[1/]PDB = potato dextrose broth; GYEB = glucose yeast extract broth; PDYEB = potato dextrose yeast extract broth; PDPYEB = potato dextrose peptone yeast extract broth.
[2/]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

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Table 4 Fresh and dry weight of *Rigidoporus. lignosus* isolate SNK10 in various media and pH levels.

Media[1/]	Fresh weight (g)						
	pH 3	4	5	6	7	8	9
PDB	0.14hi[2/]	1.25g	2.53ef	2.69def	3.02cde	2.87cde	2.05f
GYEB	0.02i	0.79gh	2.09f	2.46ef	4.04a	4.14a	4.12a
PDYEB	0.08hi	1.18g	2.73def	2.74def	2.88cde	2.58def	2.53ef
PDPYEB	0.13hi	0.90g	2.68def	3.51abc	3.85ab	3.73ab	3.30bcd
C.V. = 21.74%							
	Dry weight (g)						
PDB	0.01i	0.04fgh	0.05defgh	0.06cdef	0.07bcd	0.06cdef	0.05defgh
GYEB	0.01i	0.03gh	0.05defgh	0.05defgh	0.08bc	0.06cdef	0.06cdef
PDYEB	0.01i	0.04fgh	0.07bcd	0.06cdef	0.07bcd	0.07bcd	0.07bcd
PDPYEB	0.01i	0.03gh	0.06cdef	0.13a	0.12a	0.08bc	0.06cdef
C.V. = 22.19%							

[1/]PDB = potato dextrose broth; GYEB = glucose yeast extract broth; PDYEB = potato dextrose yeast extract broth; PDPYEB = potato dextrose peptone yeast extract broth.

[2/]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

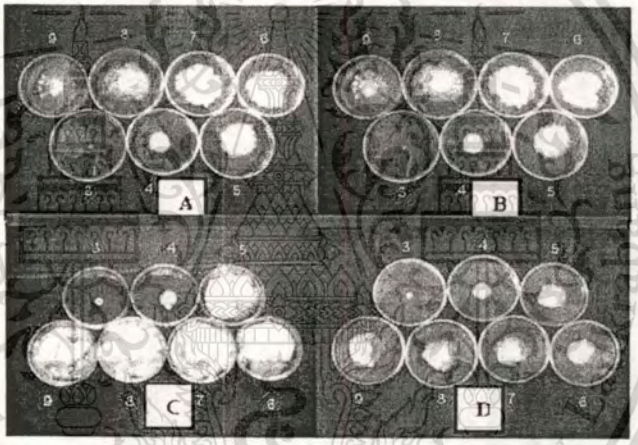

Figure 1 The mycelial mat of *Rigidoporus lignosus* isolate SNP06 (A), SND07 (B), SST01 (C), and SNK10 (D) in PDPYEB media and pH varies from 3 to 9.

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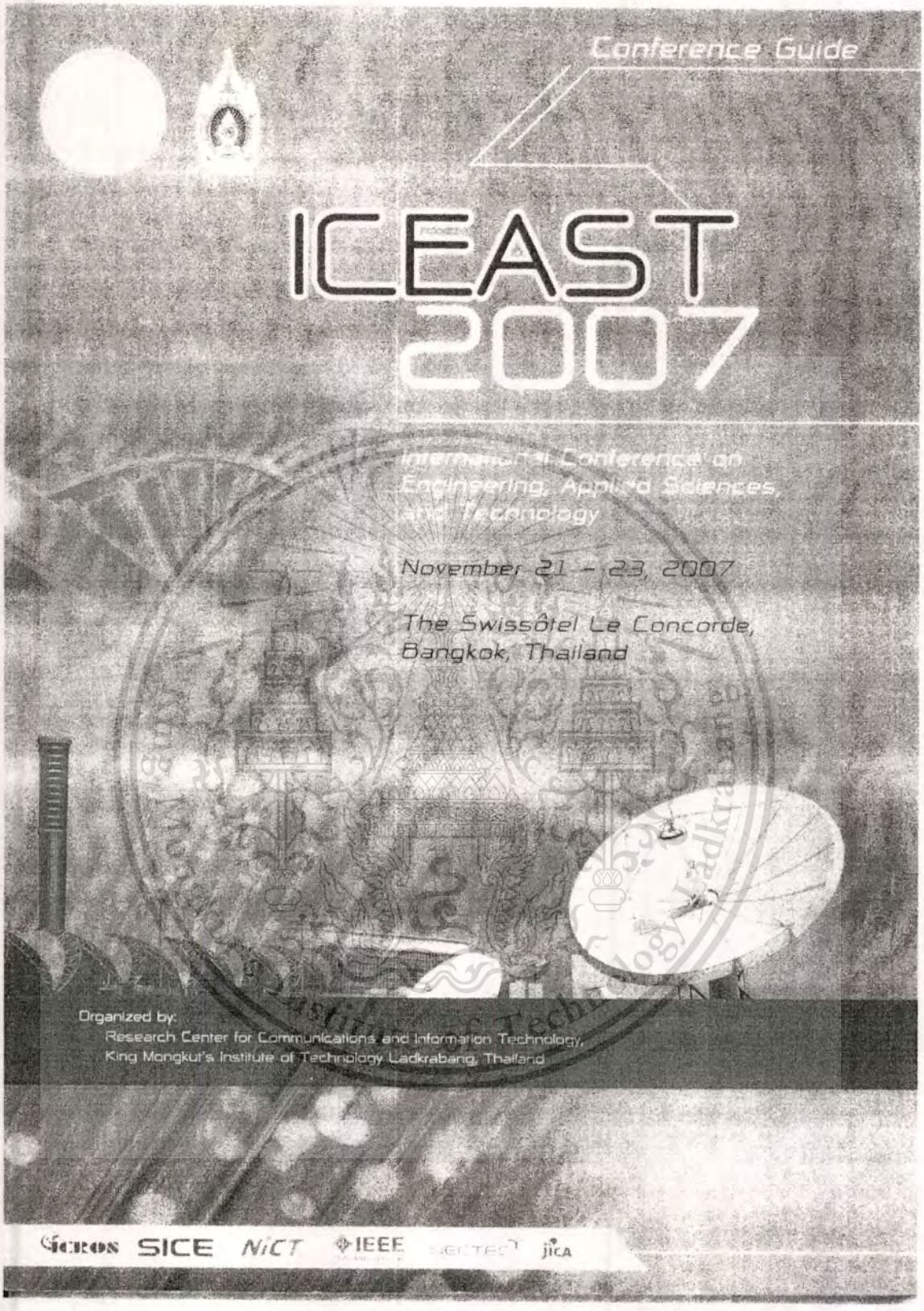

White Root Disease: Morphology, Media and Growth pH Levels

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Abstract— **Four isolates (SST01, SND07, SNP06, and SNK10) of *Rigidoporus lignosus* were isolated from the infected root of rubber trees from the South of Thailand. The hypha of all isolates varied from 2.8-7.2 µm wdie and were hyaline, septate, with many branches, but lacking clamp connections. Isolates grew on the PDA (potato dextrose agar) at room temperature and comprised white, flattened mycelium. Four tested isolates were cultured in different media:- PDB (potato dextrose broth), GYEB (Glucose yeast extract broth), PDYEB (potato dextrose yeast extract broth), and PDPYEB (potato dextrose peptone yeast extract broth) to select the most suitable medium for growth. PDPYEB was most favorable medium for the growth of our *Rigidoporus lignosus* isolates. The fresh weight and dry weight of all isolates were significantly higher in PDPYEB, than in PDYEB, PDB, and GYEB media. The mycelial growth of all isolates were tested in the pH range of 3-9. The pH value significant affected the mycelial growth of all tested isolates. The optimum growth occurred at a pH level of 6-8, whereas a highly acidic pH reduced the mycelial growth.**

Keywords—**Rigidoporus lignosus, pH level, media, mycelial growth**

I. INTRODUCTION

Root diseases are a major constraint in rubber cultivation. If untreated, they can result in heavy death of trees and sometimes in loss of a whole stand. *Rigidoporus lignosus* (Klotzeki) Imaz., which causes white root rot, is the most harmful and found in all rubber growing areas. The consequences of white root disease varies considerably depending on the country, and even between adjacent rubber plantations, but its economic importance is foremost in Malaysia, Indonesia, Thailand, India, Sri Lanka, West and Central Africa [3]. *Rigidoporus lignosus* has a wide host range which includes many forest and horticultural plants [5]. The main source of inoculum is the remnants of infected wood, particularly pieces of roots or stumps left buried in the soil. Roots of young rubber plants are infected when they come into contact with the source of inoculum and the disease spreads to the tree collar and out to the other lateral roots through the fungal rhizomorphs which grow ahead of the rot. As the trees mature and their lateral roots intermingle, white root disease is transmitted from tree to tree especially along the planting rows. Spores produced by the fungal fructifications also spread the disease. Spores deposited onto wounds on the roots or on cut surfaces of stumps initiate new sources of infection [4]. Little information is available on the morphology and biology of the causative fungus, including environmental requirements for growth. This information is valuable to mycological and pathological research on the fungus and disease, including disease management. The objective of this study is to provide information on effects of culture media and pH levels on mycelial growth of *R. lignosus*.

II. METHODOLOGY

Fungal species and its morphology

Four isolates (SST01, SND07, SNP06, and SNK10) of *R. lignosus* were used in this study. The diseased samples were collected from infected roots of the Heavea tree in the South of Thailand. Isolate SST01 was obtained from an infected root of a rubber tree in Surat Thani Province. Isolates SND 07, SNP06, and SNK10 were obtained from infected roots of rubber trees in Narathiwat Province. They were isolated by a tissue transplanting technique with the infected tissue being surface disinfected for 1 minute in 10% Clorox, followed by washing in sterile water and then transferred onto isolating media (water agar, WA). The mycelia growing out of the plant tissue were transferred onto PDA (potato dextrose agar) medium, isolated to pure culture and maintained at room temperature. The morphology of all tested isolates was studied under binocular compound microscope.

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Effect of culture media and pH on mycelium growth

Isolates were grown on PDA at room temperature for 5 days. Four mm diameter agar plugs were removed with a sterile cork borer from the growing edges of the colonies, and one plug was placed in a flask containing 20 ml of medium. Mycelial growth of four isolates were evaluated on four media as followed:- PDB (potato dextrose broth) containing (g/l): potato 200; dextrose 20, GYEB (glucose yeast extract broth) containing (g/l): glucose 10; yeast extract 2, PDYEB (potato dextrose yeast extract broth) containing (g/l): potato 200; dextrose 20; yeast extract 0.5, and PDPYEB (potato dextrose peptone yeast extract broth) containing (g/l): potato 200; dextrose 20, peptone 2; yeast extract 0.5. The media were adjusted pH with NaOH or HCl to values ranging from 3 to 9 (3, 4, 5, 6, 7, 8, and 9). The isolates were cultured at room temperature and shaken at 100 rpm for 9 days. The fresh and dry weight of the mycelia were measured by collecting the mycelial mat in each conical flask, filtered, air dried (28-32 °C) for 72 h and weighed (g).

Statistical analysis

The experiment was conducted by factorial experiment using a Completely Randomized Design. Means were compared by Duncan's Multiple Range Test (DMRT) at $P = 0.05$. The experiment was repeated four times

III. RESULTS AND DISCUSSION

Morphology

The 4 isolates of *R. lignosus* were isolated from infected roots by a tissue transplanting technique. All hyphae of all isolates were hyaline, septate, with many branches, but lacked clamp connections. The hyphal width varied from 2.8-7.2 µm (Fig. 1). Isolates on the PDA (potato dextrose agar) at room temperature comprised white and flattened mycelium (Fig. 2). All isolates were similar in morphology and could not be distinguished without using molecular techniques.

Effect of culture media and pH on mycelium growth

The highest fresh weight of isolate SNP06 was observed in PDYEB at pH 8 (7.97 g) followed by PDPYEB at pH 7 (7.67g), pH 8 (6.86 g), and pH 9 (6.85 g), and the highest dry weight was also observed in PDYEB at pH 8 (0.19 g) followed by PDB at pH 8 (0.15 g), PDPYEB at pH 7 (0.14 g) and pH 8 (0.14 g) (Table 1). Isolate SND07 grown in PDPYEB medium at pH 7 gave the highest fresh and dry weight (10.64 and 0.19 g, respectively) (Table 2). The fresh and dry weight of isolate SST01 was higher in PDPYEB medium at all pH levels, than in PDYEB, PDB, and GYEB media. This isolate grew better at optimum pH of 6, 7, and 8 (Table 3). GYEB medium at pH 8, 9, and 7 (4.14, 4.12, and 4.04 g, respectively) on the other hand, yielded a higher fresh weight than PDPYEB, PDYEB, and PDB media. At pH 6 (0.13 g) the highest dry weight for isolate SNK10 (Table 4) was obtained. It was observed that the mycelial growth of all tested isolates was low at pH 4 – 5, while there were no mycelial growth at pH 3 (Fig. 3).

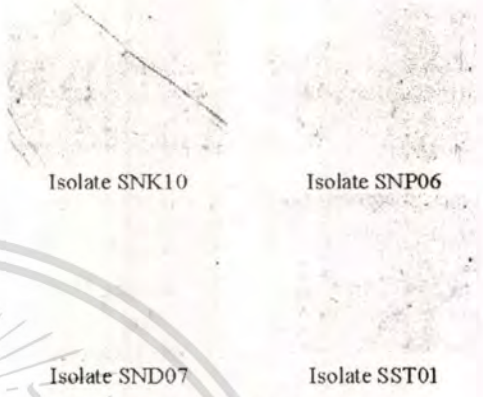

Fig. 1 The hypha of the fungus

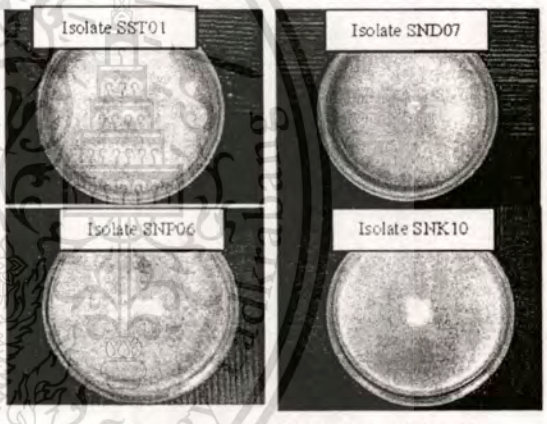

Fig. 2 The mycelium on the PDA medium at 5 day-old

The mycelial growth of all tested isolates were significantly ($P = 0.05$) affected by culture media and pH levels. In general, PDPYEB media was the most favorable for the growth of mycelium of all isolates tested followed by PDYEB, PDB, and GYEB medium. The mycelial growth was obtained in the pH range 3 - 9. The Optimum growth occurred at pH 6, 7, and 8, whereas at pH 4 and 5 reduced the mycelial growth and no growth was observed at pH 3. These results suggest that all isolates preferred neutrality pH values toward alkalinity. These results were similar to those obtained by Arom [1], who reported that the optimum mycelial growth of *R. lignosus* occurred at pH between 6-7. Richard and Botton [5] found that pH 8 gave the best

mycelium growth of *R. lignosus*, whereas more acidic pH reduced the growth of mycelium of *R. lignosus*. Fasola *et al.* [2] reported that, at very strong acidic or alkaline pH, cell walls may corrode and selective permeability function of the cell membrane may be impaired. This may be the reason why there were no growth at pH 3.

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TABLE I

FRESH AND DRY WEIGHT OF *RIGIDOPORU LIGNOSUS* ISOLATE SNP06 IN VARIOUS MEDIA AND pH LEVELS

Media[1/]	Fresh weight (g)						
	pH 3	4	5	6	7	8	9
PDB	0.11i[2/]	1.38h	4.16defg	4.44def	4.57de	4.87cd	4.37def
GYEB	0.03i	0.98hi	3.32fg	3.73defg	3.06g	3.49efg	3.68defg
PDYEB	0.18i	1.95h	4.65d	4.64d	5.72c	7.97a	5.84c
PDPYEB	0.17i	1.48h	4.80cd	5.80c	7.67ab	6.86b	6.85b
C.V. = 18.18%							
	Dry weight (g)						
PDB	0.02j	0.04ijk	0.08efg	0.09def	0.13bc	0.15b	0.09def
GYEB	0.01jk	0.02jk	0.07efghi	0.08efg	0.06fghi	0.06fghi	0.08efg
PDYEB	0.01jk	0.04hij	0.12bcd	0.12bcd	0.14b	0.19a	0.13bc
PDPYEB	0.01jk	0.06fghi	0.10cde	0.13bc	0.14b	0.14b	0.13bc
C.V. = 24.18%							

[1/]PDB = potato dextrose broth; GYEB = glucose yeast extract broth; PDYEB = potato dextrose yeast extract broth; PDPYEB = potato dextrose peptone yeast extract broth.

[2/]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

TABLE II

FRESH AND DRY WEIGHT OF *RIGIDOPORU LIGNOSUS* ISOLATE SND07 IN VARIOUS MEDIA AND pH LEVELS

Media[1/]	Fresh weight (g)						
	pH 3	4	5	6	7	8	9
PDB	0.08j[2/]	1.96i	5.55ef	4.76efg	3.48gh	6.29cde	6.30cde
GYEB	0.08j	1.75i	3.94gh	4.50fgh	3.72gh	4.83efg	2.96hi
PDYEB	0.04j	1.98i	5.77ef	5.92def	5.74ef	4.57fg	7.37cd
PDPYEB	0.02j	3.90gh	7.68c	9.12b	10.64a	6.37cde	10.55a
C.V. = 21.20%							
	Dry weight (g)						
PDB	0.01j	0.07gh	0.12cd	0.13bc	0.07gh	0.11cde	0.12cd
GYEB	0.01j	0.03ij	0.07gh	0.07gh	0.06gh	0.07gh	0.04hi
PDYEB	0.01j	0.05hi	0.09fg	0.15a	0.10def	0.09fg	0.12cd
PDPYEB	0.01j	0.10cde	0.15a	0.13bc	0.19a	0.10cde	0.13bc
C.V. = 21.97%							

[1/]PDB = potato dextrose broth; GYEB = glucose yeast extract broth; PDYEB = potato dextrose yeast extract broth; PDPYEB = potato dextrose peptone yeast extract broth.

[2/]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

TABLE III
FRESH AND DRY WEIGHT OF *RIGIDOPORU LIGNOSUS* ISOLATE SST01 IN VARIOUS MEDIA AND pH LEVELS

Media[1/]	pH 3	4	5	6	7	8	9
	Fresh weight (g)						
PDB	0.05j[2/]	1.37ij	4.79gh	7.42def	49cdef	8.07cde	7.41def
GYEB	0.03j	1.85i	3.95h	5.19gh	5.73fg	5.94fg	4.83gh
PDYEB	0.05j	1.21ij	6.31efg	8.10cde	9.92b	9.22bc	9.80b
PDPYEB	0.13ij	1.82i	7.19def	12.68a	12.72a	12.59a	8.53bcd
C.V. = 18.99%							
	Dry weight (g)						
PDB	0.01m	0.04kl	0.07hij	0.14def	0.14def	0.14def	0.08hij
GYEB	0.01m	0.02lm	0.05jk	0.09ghi	0.11g	0.11fg	0.10gh
PDYEB	0.01m	0.05jk	0.15cde	0.17cd	0.20b	0.16cde	0.18c
PDPYEB	0.01m	0.06ijk	0.21b	0.27a	0.26a	0.26a	0.21b
C.V. = 15.29%							

[1/]PDB = potato dextrose broth, GYEB = glucose yeast extract broth, PDYEB = potato dextrose yeast extract broth, PDPYEB = potato dextrose peptone yeast extract broth.
[2/]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

TABLE IV
FRESH AND DRY WEIGHT OF *RIGIDOPORU LIGNOSUS* ISOLATE SST01 IN VARIOUS MEDIA AND pH LEVELS

Media[1/]	pH 3	4	5	6	7	8	9
	Fresh weight (g)						
PDB	0.14hi[2/]	1.25g	2.53ef	2.69def	3.02cde	2.87cde	2.05f
GYEB	0.02i	0.79gh	2.09f	2.46ef	4.04a	4.14a	4.12a
PDYEB	0.08hi	1.18g	2.73def	2.74def	2.88cde	2.58def	2.53ef
PDPYEB	0.13hi	0.90g	2.68def	3.51abc	3.85ab	3.73ab	3.30bcd
C.V. = 21.74%							
	Dry weight (g)						
PDB	0.01i	0.04fgh	0.05defgh	0.06cdef	0.07bcd	0.06cdef	0.05defgh
GYEB	0.01i	0.03gh	0.05defgh	0.05defgh	0.08bc	0.06cdef	0.06cdef
PDYEB	0.01i	0.04fgh	0.07bcd	0.06cdef	0.07bcd	0.07bcd	0.07bcd
PDPYEB	0.01i	0.03gh	0.06cdef	0.13a	0.12a	0.08bc	0.06cdef
C.V. = 22.15%							

[1/]PDB = potato dextrose broth, GYEB = glucose yeast extract broth, PDYEB = potato dextrose yeast extract broth, PDPYEB = potato dextrose peptone yeast extract broth.
[2/]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.05$.

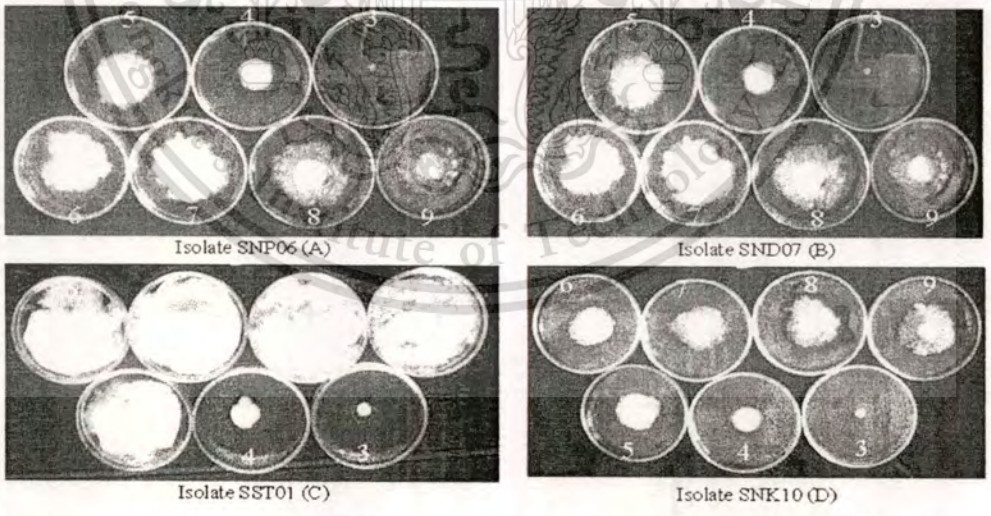

Fig. 3 The mycelial mat of *Rigidoporus lignosus* isolate SNP06 (A), SND07 (B), SST01 (C), and SNK10 (D) in PDPYEB media and pH varies from 3 to 9

TMA_P_09

Gene Expression During Oosporogenesis in Heterothallic and Homothallic Phytophthora

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A large-scale screen for genes induced during sexual development was performed in the heterothallic oomycete *Phytophthora infestans*, the potato blight agent. Of 15,644 unigenes on an Affymetrix chip, 87 were induced >10-fold during mating, with 28 induced >100-fold. This was validated in independent matings using RNA blots and RTPCR. Only 44 genes resembled sequences in GenBank. These encoded regulators such as protein kinases, protein phosphatases, and transcription factors, plus enzymes with metabolic, transport, and cell-cycle activities. Several genes were induced during mating and asexual sporogenesis, suggesting crosstalk between those pathways. In the homothallic species *P. phaseoli*, 80% of genes were expressed at similar levels in both conditions conducive to and blocking oosporogenesis, with 20% up-regulated during sexual development. Many of the 87 genes were expressed much higher in *P. phaseoli* than in any non-mating culture of *P. infestans*, suggesting that part of the sexual pathway is constitutively active in homothallics.

Key words: heterothallism, homothallism, microarray, oomycete, *Phytophthora*, sexual development

TMA_P_10

White Root Disease of Rubber Tree

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White root disease of rubber trees (*Hevea brasiliensis*) caused by *Rigidoporus lignosus* (Klotzsch) Imazeki was studied. This fungus belongs to Basidiomycetes, *R. lignosus* infects and destroys the roots of the rubber tree, and the first visible symptom on the foliage is the change of leave color to off-green and yellowish. The infected roots of the rubber tree were collected from Amphur Rangai, Amphur Sungai-padee, Narathiwat province, and Amphur Tachana, Nakhonsithumarat province. Twenty two isolates of the fungus were isolated by tissue transplanting technique. The growth of this fungus on the PDA (potato dextrose agar) plate at room temperature appeared white and flattened mycelium on the medium plate. The hypha shows hyaline, septate, and possession many branches but no clamp connection. These isolates will be done for pathogenicity, molecular phylogeny and for further studies.

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