BIOLOGICAL CONTROL OF PINEAPPLE ROOT ROT

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A THESIS SUBMITTED IN FULFILLMENT

OF THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY IN PLANT PATHOLOGY

INTERNATIONAL COLLEGE

KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

2010

KMITL-2010-IC-D-009-002

Thesis	Biological Control of Pineapple Root Rot
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Degree	Doctor of Philosophy
Programme	Biotechnology in Plant Pathology
Year	2010
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ABSTRACT

Root rot of pineapple (*Ananas comosus* (L.) Merr.) was found in the fields at Petchaburi, Phatthalung, Prachuap Khiri Khan and Rayong provinces. Soil samples from the disease epidemic planting areas were collected to isolate the pathogen causing root rot of pineapple. Forty-four isolates of pathogen were discovered by baiting technique. All isolates were identified by their morphological characteristics and confirmed by their ITS region of the nuclear rDNA. Fifteen isolates of *Pythium aphanidermatum* and 29 isolates of *P. graminicola* were described. All isolates were proved to be pathogenic to pineapple root by inoculation of pathogen to detached leaves and root. *P. aphanidermatum* RY803 from Rayong provinces gave more virulent than any others while *P. graminicola* was the first reported causing root rot of pineapple in Thailand. Therefore, *P. aphanidermatum* RY803 was used as pathogen for screening antatonistic fungi by bi-culture plate and crude extract test.

Forty-two isolates of soil fungi were isolated by baiting and soil plate techniques for screening antagonists. During a study on antagonistic fungi for controlling root rot disease of pineapple, 2 isolates of *Chaetomium* (MB303 and MB502) could not be identified as any species of *Chaetomium* and were therefore described as new, and nominated as *Chaetomium siamense* sp. nov. In addition, phylogenetic analysis of ITS rDNA sequence data supports that *C. siamense* as a distinct species.

All of the promising antagonists were tested for their abilities to control *P. aphanidermatum* RY803 in bi-culture plate. Bi-culture antagonistic test for antagonism showed that *C. aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocladium cantenulatum* RY102, *G. cantenulatum* RY111, *Trichoderma harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112 gave significantly inhibited the growth and oospore formation of *P. aphanidermatum* RY803 over 80%. The crude ethyl acetate extract of *C. cochliodes* RY301 at the rate of 1,000 µg/ml gave significantly highest inhibition of mycelial growth and oospores formation of *P. aphanidermatum* RY803 by 71.00 and 88.95%, respectively which the ED_{50} value was 64 µg/ml. Moreover, the hyphae, oogonia and oospores of the pathogen formed abnormal protoplasm in cells and demonstrated uncommon shape implies antibiosis. Therefore, *C. cochliodes* RY301 was selected as a potent antagonist to control root rot of pineapple in pot experiment. *C. cochliodes* RY301 was prepared in formulations of oil and powder before applying to pots. The result showed that disease severity index of biofungicide from *C. cochliodes* RY301 in oil and powder formulations were nonsignificantly different from metalaxyl in every month. Moreover, biofungicide in oil and powder formulations gave nonsignificantly different from metalaxyl and fresh weight of pineapple plant applied oil formulation was significantly highest when compared with non-treated control.

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บทคัดย่อ

โรครากเน่าของสับปะรดพบในจังหวัดเพชรบุรี พัทลุง ประจวบคีรีขันธ์ และระยอง ตัวอย่างดินบริเวณรอบๆ และรากของต้นสับปะรดที่แสดงอาการรากเน่าถูกเก็บเพื่อนำมาแยกเชื้อสาเหตุที่ทำให้เกิดโรค โดยวิธีใช้เหยื่อล่อ (baiting technique) เชื้อที่แยกได้ทั้งหมด 44 ไอโซเลท ถูกนำมาจัดจำแนกสปีชีส์โดยการศึกษาลักษณะทางสัณฐานวิทยาและการเปรียบเทียบลำดับเบสบริเวณไอทีเอส (ITS) ของไรโบโซมอลดีเอ็นเอ (rDNA) ซึ่งสามารถจัดจำแนกได้เป็นเชื้อ *Pythium aphanidermatum* 15 ไอโซเลท และเชื้อ *P. graminicola* 29 ไอโซเลท การพิสูจน์ความสามารถในการก่อโรคต่อใบและรากของสับปะรดของเชื้อที่แยกได้ทั้งหมด พบว่าเชื้อ *P. aphanidermatum* RY803 เป็นไอโซเลทที่รุนแรงที่สุดในการก่อให้เกิดโรครากเน่าต่อสับปะรดขณะที่เชื้อ *P. graminicola* นั้นพบว่าเป็นเชื้อที่ก่อให้เกิดโรครากเน่าต่อสับปะรดเป็นครั้งแรกในประเทศไทย ดังนั้นจึงใช้เชื้อ *P. aphanidermatum* RY803 เป็นเชื้อก่อโรครากเน่าต่อสับปะรดในการคัดเลือกเพื่อหาเชื้อราแอนทาโกนิส (antagonist) โดยวิธีไบคัลเจอร์เพลท (bi-culture plate) และทดสอบสารสกัดหยาบ (crude extract) การคัดเลือกเพื่อหาเชื้อราแอนทาโกนิสนั้นใช้เชื้อรา 42 ไอโซเลท ซึ่งแยกได้จากดินโดยเทคนิคเหยื่อล่อและซอยเพลท (soil plate technique) ในระหว่างการศึกษาเชื้อราแอนทาโกนิสเพื่อควบคุมโรครากเน่าของสับปะรดได้พบเชื้อ *Chaetomium* 2 ไอโซเลท ได้แก่ *Chaetomium* MB303 และ MB502 ซึ่งเชื้อทั้ง 2 ไอโซเลทนี้ ไม่สามารถจัดจำแนกได้ว่าเป็นเชื้อ *Chaetomium* สปีชีส์ใด ดั้งนั้นเชื้อทั้ง 2 ไอโซเลทนี้จึงถูกจัดจำแนกเป็นเชื้อ *Chaetomium* สปีชีส์ใหม่โดยใช้ชื่อว่า *Chaetomium siamense* นอกจากนี้การวิเคราะห์ความสัมพันธ์ทางพันธุกรรมจากไฟโลจีนี (phylogeny) โดยอาศัยความแตกต่างของลำดับเบสของไรโบโซมอลดีเอ็นเอบริเวณไอที

เอสและ5.8เอส (ITS 1, 5.8S, ITS2) ยังสนับสนุนผลการจัดจำแนกได้ว่า *C. siamense* เป็นเชื้อที่แตกต่างจากเชื้อ *Chaetomium* สปีชีส์อื่นๆ

ผลการทดสอบความสามารถของเชื้อราที่คาดว่าจะเป็นเชื้อแอนทาโกนิสในการควบคุมเชื้อ *P. aphanidermatum* RY803 ในไบคัลเจอร์เพลทพบว่าเชื้อ *C. aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocladium cantenulatum* RY102, *G. cantenulatum* RY111, *Trichoderma harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112 สามารถยับยั้งการเจริญของเส้นใยและการสร้างโอโอสปอร์ (oospore) ของเชื้อ *P. aphanidermatum* RY803 มากกว่า 80 เปอร์เซ็นต์ และผลการทดสอบสารสกัดหยาบแสดงให้เห็นว่าสารสกัดหยาบซึ่งสกัดได้จากสารทำละลายเอทธิล อะซิเตท (ethyl acetate) ของเชื้อ *C. cochliodes* RY301 ที่ความเข้มข้น 1,000 ไมโครกรัมต่อมิลลิลิตร มีความสามารถในการยับยั้งการเจริญของเส้นใยและการสร้างโอโอสปอร์ของเชื้อ *P. aphanidermatum* RY803 สูงกว่าสารสกัดหยาบอื่นๆ อย่างมีนัยสำคัญทางสถิติที่ 71.00 และ 88.95 เปอร์เซ็นต์ ตามลำดับ และมีค่า ED_{50} ที่ 64 ไมโครกรัมต่อมิลลิลิตร นอกจากนี้ยังพบว่าเส้นใย (hyphe) โอโอโกเนีย (oogonia) และโอโอสปอร์ของเชื้อ *P. aphanidermatum* RY803 มีลักษณะผิดปกติไปจากเดิม ดั้งนั้นการควบคุมโรครากเน่าของสับปะรดในกระถางทดลองจึงใช้เชื้อ *C. cochliodes* RY301 เป็นยาฆ่าเชื้อรา (biofungicide) ในรูปของน้ำมันและผงแป้ง ซึ่งผลการทดลองพบว่า *C. cochliodes* RY301 ในรูปของน้ำมันและผงแป้ง สามารถลดการเกิดโรครากเน่าของสับปะรดได้ไม่แตกต่างกันทางสถิติกับการใช้สารฆ่าเชื้อราเมททาเลกซิล (metalaxyl) และสับปะรดที่ได้รับ *C. cochliodes* RY301 ในรูปของน้ำมันมีน้ำหนักสดสูงสุด ในขณะที่ต้นที่ไม่ได้รับอะไรเลยและต้นที่ได้รับ *C. cochliodes* RY301 ในรูปของผงแป้งไม่มีความแตกต่างกันทางสถิติ

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deep and sincere gratitude to Dr. Kasem Soytong, Associate Professor of Department of Plant Pest Management, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, who has been my advisor since the beginning of my study. He provided me with many helpful suggestions, important advice and constant encouragement during the preparation of this thesis. His guidance helped me in all the time of research and writing of this thesis.

My sincere gratitude to my co-advisor, Assoc. Prof. Dr. Somdej Kanokmedhakul at Department of Chemistry, Faculty of Science, Khon Kaen University, who made many valuable suggestions and gave constructive advice.

My sincere gratitude also convey to Prof. Dr. Fu-Cheng Lin, my co-advisor at Biotechnology Institute, Zhejiang University, P.R.China, who gave me the opportunity to work in the part of molecular study, and gave me good guidance for experiment.

Besides my advisor, I would like to my sincere gratitude to my thesis committee: Assoc.Prof. Dr. Chaiwat To-anun, Assoc. Prof. Dr. Mayura Soonwera and Assoc. Prof. Dr. Vasun Petcharat for their insightful comments.

I would like to express my gratitude to Assoc. Prof. Dr. Hong Kai Wang at Biotechnology Institute, Zhejiang University, P.R.China for the invaluable tutorials provided on molecular technique.

My sincerely thank expressed to Dr. Supattra Poeaim at Department of Applied Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang for her valuable advice in molecular technique.

I would like to express my thanks to all members at Biotechnology Institute, Zhejiang University, P.R.China, in particular Liu Xiaohong, Liu Tongbao, Hu Boli and Duan Zhibing for their help and friendship.

I would also like to express my thanks to Ruilin Z., Ohmar M.A., Sophea K., Saithong K., Chamaiporn C., Kanchalika R., Supattha J., Chulalak T., Nathacha S., Tanapak I., Phoutthasone S. and Phonsavard S., who are the members of the Biocontrol Unit and Mycological Research Section in Department of Plant Pest Management, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang for their help me directly and indirectly in accomplishing this thesis. Thanks also to Narumon T. at Department of Applied Biology, Faculty

of Science, King Mongkut's Institute of Technology Ladkrabang for sharing her knowledge in molecular biology with me.

I would like to acknowledge the Biocontrol Research Unit and Ph.D. Research Lab for providing necessary facilities in this thesis. Thanks also to Faculty of Agricultural Technology and International College at King Mongkut's Institute of Technology Ladkrabang.

Finally, I would like to express my heartiest thanks to my family for all their love and encouragement. For my parents, Mr.Sammart and Mrs.Chorthip Pornsuriya, who help me for everything and always gives me greatest love, willpower and financial support until this study completion. My sister Ms. Cheewina P. and her buddy, Teddy were thanked for their help and encouragement.

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CHAPTER 1

INTRODUCTION

1.1 Statement and Significance of the Problems

Pineapple (*Ananas comosus* (L.) Merr.) is one of the most important economic plants in Thailand that grown mainly for fresh or canned fruit and juice. It is the only source of bromelain, an enzyme used in pharmaceuticals. Thailand is one of the ten leading exporters of processed pineapples. During the past 5 years, Thailand exported fresh fruits and processed pineapple to Europe, America and Japan over 20 thousand tons per year and the export value over 160 million baht (Bureau of Agricultural Trading Promotion Department of Internal Trade, Thailand. 2009). The growing areas of pineapple in Thailand have been expanded due to the increased demand for pineapple products worldwide and greatly stimulated plantings. Root rot symptoms are commonly found in the fields and become major losses in pineapple plantations. The disease is caused by soil fungi, *Phytophthora cinnamomi*, *Ph. parasitica* and several species of *Pythium*. (Rohrbach and Apt. 1993). Root rot of pineapple in Thailand has been firstly reported by Leelasetthakul (1972) which caused by *Ph. parasitica* and also reported by other researchers (Kueprakone *et al.* 1977; Silayoy. 1987). But in 2008, Department of Agriculture, Ministry of Agriculture and cooperatives, Thailand reported that pineapple root rot caused by *Pythium* but it was not identified into species level. However, *Pythium aphanidermatum, P. arrhenomanes and P. graminicola* were reported to be pathogenic to pineapple root rot in Hawaii (van der Plaats-Niterink. 1981; Gonsalves *et al.* 1994). *Pythium* occurs when soil moisture levels are excessively high and fungal spores present in the soil, come in contact with the susceptible plant. The pathogen invades and infects pineapple plants in the field via zoospores from sporangium which were disperse by soil water, wind or rain splash. Disease control are maimly used chemical fungicides such as metalaxyl, fosetyl-Al and phosphorus acid (Dalldorf. 1993), but usually the pathogen become resistant to chemical fungicides.

Biological control using antagonists for controlling root rot of several plants caused by pathogenic fungi has been extensively studied, and several examples of successful disease control exist. The effective antagonists for controlling root rot have been reported such as bacteria e.g. *Bacillus subtilis*, *Streptomyces rochei*, fungi e.g. *Aspergillus* spp., *Chaetomium cupreum*,

C. globosum, *C. cochliodes*, *Gliocladium virens*, *Penicillium* spp., *Trichoderma harzianum*, *T. viride* (Ezziyyani *et al.* 2007; Abdelzaher. 2003; Soytong *et al.* 2001; Galland and Paul. 2001; Naseby *et al.* 2000; Ahmed *et al.* 1999). Biological control agent is often very specific for a particular pathogen and also less danger to the environment. It is therefore necessary to study on this regard for reducing the application of chemical fungicides and protecting the environment.

This research study was to find out the new antagonistic fungi for controlling pineapple root rot that possibly becomes one of the sound strategies for disease control.

1.2 Goal and Objective

1.2.1 To collect, isolate, identify and test the pathogenicity of pineapple root rot pathogens and to study on molecular phylogenic relationship.

1.2.2 To screen antagonistic fungi for biological control of pineapple root rot and study on control mechanism in laboratory.

1.2.3 To evaluate the new antagonist in oil and powder formulations for controlling pineapple root rot in pot experiment.

1.3 Scope of Limitation of the Study

This research study was to screen the promising antagonistic fungi to control pineapple root rot. The causing agent was isolated from diseased plant parts by baiting technique and tested for pathogenicity. The effective antagonist was screened by bi-culture antagonistic test. Moreover, fungal metabolites (crude extracts) from the promising antagonists were tested to screen the most effective antagonistic fungus and proved for control mechanism. Subsequently, the most effective antagonistic fungus was formulated in oil and powder forms as biological fungicide and tested in pot experiment.

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristics of Pineapple Plants

The pineapple plant (*Ananas comosus* var *comosus*) belongs to the family Bromeliaceae. It is a herbaceous perennial plant, probably native to southern Brazil and Paraguay. Pineapple is grown on almost any type of soil. It can grown up to 1–1.5 m high and 0.5-1 m wide. Bright green leaves have spiny tips and margins. The stem continues to grow and acquires at its apex a compact tuft of stiff, short leaves called the "crown" or "top" (Morton. 1987; Gilman. 1999).

2.2 Varieties of Pineapple Plants

In international trade, pineapple cultivars are grouped into four main classes: 'Smooth Cayenne', 'Red Spanish', 'Queen', and 'Abacaxi', despite much variation in the types within each class (Morton. 1987).

Sripaoraya *et al.* (2001) divided commercial cultivars of pineapple in Thailand into three groups by random amplified polymorphic DNA (RAPD) analysis and morphology. The cultivars 'Tradsithong', 'Phuket', 'Sawee' and 'Tainan', with spiny leaves, form the Queen group. 'Pattavia', 'Nanglae' and 'Petburi no. 2' (Cayenne group), spines are confined to the leaf tips. 'Intrachitdang' is normally placed in the Spanish group, which is morphologically similar to the Queen group, but with inferior quality fruit.

2.3 Diseases of Pineapples

Thailand is the world's largest producer of pineapples and canned pineapples. In the production process of canned pineapples, "Pattavia" variety is generally used as a raw material. This variety is susceptible to pathogen causing root and heart rot disease (Department of Agriculture. 2007). Root and heart rot disease is caused by soilborne fungi which are the most prevalent in prolonged wet area. Heart and root rot of pineapple have been reported that caused by *Phytophthora cinnamomi* (Allen *et al.* 1998), *Ph. nicotiana* var. *parasitica* (Matos. 1995).

Some root rots are caused by *Pythium* species, most commonly *P. arrhenomanes* (Matos. 1995; Van der Plaats-Niterink. 1981).

Pythium root rot occurs when soil moisture levels are excessively high and fungal spores present in the soil, come in contact with the susceptible plant. Symptoms include browning of leaf margins, development of reddish colored leaves, reduction in plant growth and plant decline and death (Crane. 2009).

Pythium root rot is a common crop disease caused by a genus of organisms called "*Pythium*". These are commonly called water moulds (Wikipedia. 2007) or soil-borne fungi (Agrios. 1997). Many *Pythium* species are closely related to *Phytophthora* species, which are plant pathogens of economic importance in agriculture. *Pythium* species tend to be very general and nonspecific in their host range. They can infect a large range of hosts while *Phytophthora* species are generally more specific (Table 2.1) (Wikipedia. 2007).

2.4 The Disease Cycle of *Pythium aphanidermatum*

Pythium aphanidermatum survives in the soil as hyphae, oospores and sporangia. They can be directly infected root tissues by encysted zoospores, oospores, zoospores and sporangia (Fig. 2.1). Encysted zoospores, oospores and sporangia form germ tube directly infect the plant. Otherwise, they may form sporangia which produce the motile zoospores. The zoospores swim to infect plant tissue. After infection, they will release hydrolytic enzymes to destroy the root tissue and absorb nutrients as a food source (Parker. 2009).

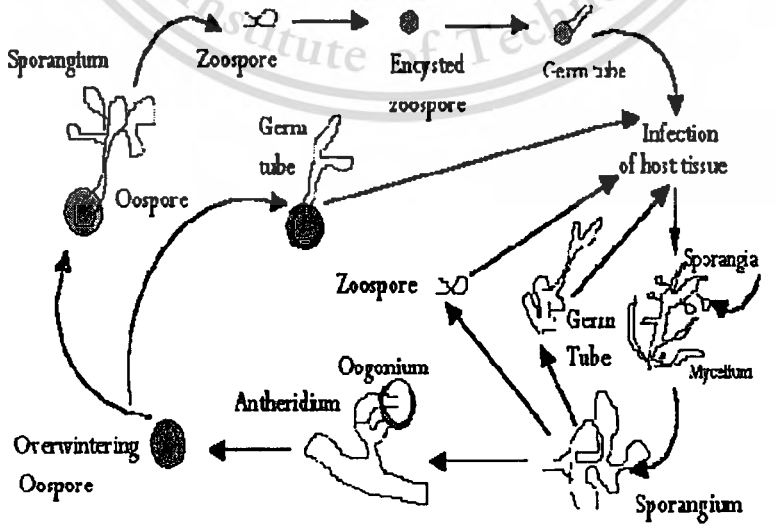

Fig. 2.1 Disease cycle of *Pythium aphanidermatum* (Source: Parker. 2009).

2.5 The Taxonomy of the Genus *Pythium*

Pythium species are ubiquitous in soil and aquatic environments. The genus *Pythium* was firstly established and placed in the family Saprolegniaceae by Pringsheim in 1858 (Martin. 1991). Pythium is now widely accepted as the type genus of the family Pythiaceae in the order Peronosporales of class Oomycetes (Waterhouse, 1974) or order Pythiales of class Peronosporomycetes (Dick, 2001). According to Waterhouse (1974), Pythium species can be identified by the delicate, hyaline, coenocytic, freely branching flexuous hyphae (about 5 μm on average). Non-deciduous sporangia are produced only in water with variable shapes ranging from spherical, subspherical, ovate, obovate, ellipsoidal, pyriform (non-papillate or sometimes papillate without apical thickening) to lobulate and filamentous, terminal or intercalary on undifferentiated, simple, irregular or sympodially branched sporangiophores. Zoospores are not formed within the sporangium. Instead, the sporangial protoplasm passes through an exit tube into a thin membranous globose vesicle within which zoospores are differentiated and dispersed by rupture or dissolution of the vesicle membrane. The sexual reproduction is oogamous, producing spherical female oogonia, and the antheridium is paragynous or occasionally hypogynous. The number, shape and origin of the antheridia vary with the species and the oospore can be aplerotic or plerotic and some species produce more than one oospore per oogonium.

Pythium aphanidermatum (Edson) Fitzp. is a cosmopolitan phytopathogen with a wide host range and an aggressive species of *Pythium*, causing serious root rot and damping-off in various economic plant crops (Parker, 2009). They are characterized by colourless mycelia, its hyphae are hyaline, and the main hyphae are mostly 5-7, occasionally up to 10 μm wide, cross septa are lacking except in old, produce oospores as sexual stage and sporangia as asexual stage. Sporangia are inflacted filamentous that they were branched, forming irregular toruloid complexes. Oogonia are spherical, terminal and intercalary, measuring 20.24-26.46 μm in diameter; antheridia 1-2 per oogonium; oospore aplerotic, spherical, single, smooth-walled, measuring 16.78-22.90 μm in diameter (van der Plaats-Niterink. 1981) (Fig. 2.2 and Fig. 2.3).

Table 2.1 *Pythium* species causing root rot.

Species	Plants	References
Pythium aphanidermatum	Artichokes, pineapple, sweet pepper, watermelon	Stanghellini *et al.* (2000); Gonsalves and Ferreira (1994)
Pythium arrhenomanas	Pineapple	Gonsalves and Ferreira (1994)
Pythium debaryanum	Wheat	Higginbotham *et al* (2004)
Pythium graminicola	Pineapple	van der Plaats-Niterink (1981)
Pythium intermedium	Rose	Metzger *et al* (2007)
Pythium irregulare	Apple	Mazzola *et al.* (2002)
Pythium myriotylum	Cocoyam	Perneel *et al.* (2006)
Pythium phragmitis	Common reed	Nechwatal *et al.* (2005)
Pythium ultimum var. *ultimum*	Cauliflower, Wheat	Abdelzaher (2003);
Pythium sylvaticum	Apple	Mazzola *et al.* (2002)
Pythium spp.	Turfgrasses, ornamentals, sugarcane, wheat	Vincelli (2006); Chase (1999); Office of the cane and sugar board (2009); Milus and Rothrock (1997)

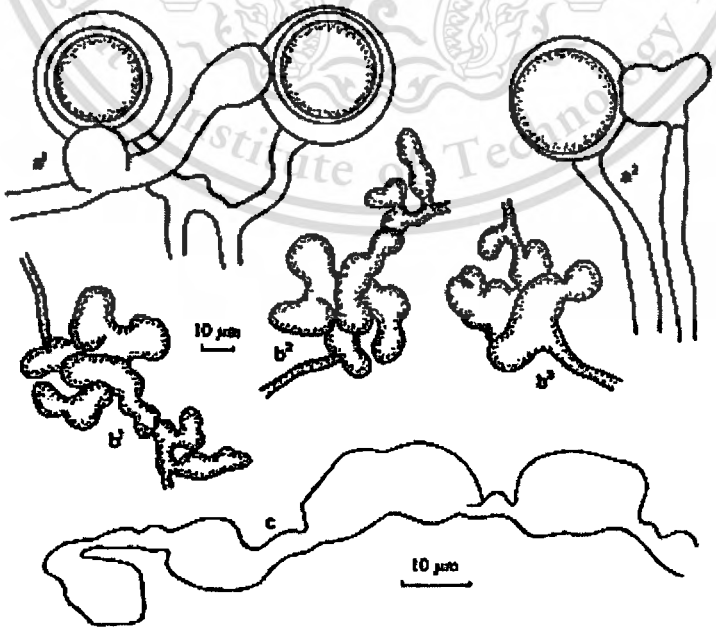

Fig. 2.2. *Pythium aphanidermatum*, CBS 118.80. a. oogonia and antheridia, b. toruloid sporangia, lower magnification, c. Appressoria (Source: van der Plaats-Niterink. 1981).

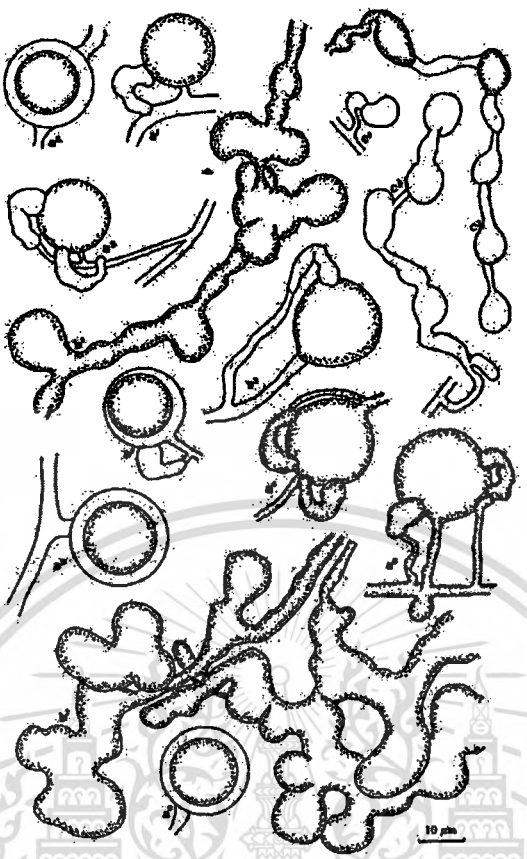

Fig. 2.3. *Pythium graminicola*, CBS 327.62. a. oogonia, some with antheridia, b. toruloid sporangia, c. Appressoria (Source: van der Plaats-Niterink. 1981).

The taxonomy of the genus *Pythium* is mainly based on the morphological descriptions and the keys provided by Middleton (1943), Waterhouse (1968) and Van der Plaats-Niterink (1981), whereas the genus of *Phytophthora* is identified according to the keys of Waterhouse (1963), Erwin *et al.* (1983) and Erwin and Ribeiro (1996). Identification can be made difficult due to characteristic variation within a species. However, morphological observations are now being supplemented with molecular characters becoming a useful tool in fungal taxonomy and currently used to identify different species in mycological studies (Table 2.2).

The region of DNA that codes for the production of ribosome (the ribosomal RNA (rRNA) gene cluster) has been extensively used in studies on evolution and systematic. This region consists of three major genes that code for the large, the small and 5.8S ribosomal subunits. These genes are separated by internal spacer (ITS) regions, and the whole gene cluster is repeated many times along a chromosome. Individual cluster is separated by intergenic spacer (IGS) sequences (Fig. 2.4).

Matsumoto *et al.* (2000) studied intraspecific DNA polymorphisms of *Pythium irregulare*. Forty-seven isolates of *P. irregulare* from different hosts and geographic origins were compared to molecular, morphology and physiology. They were divided into four groups (I-IV) based on ITS-RFLP analysis. Groups I and II included 32 and 8 isolates, respectively, collected from diverse hosts and geographic origins, and groups III and IV comprised 7 isolates derived from sugar beet and sugar beet field soil.

Galland and Paul (2001) identified *Pythium perplexum* which was mistakenly described by Bulter as *P. vexans* by using the internal transcribed spacer1 (ITS1) of rDNA. The morphological character of *P. perplexum* differs from *P. vexans* by its larger oogonia, non-proliferating sporangia, a lack of lobed antheridia and by different temperature requirements. The comparison between the ITS1 of the two fungi indicated that they share only 67.1% identity. This observation supported the morphological and reproductive observations could justify the creation of two taxa as *P. perplexum* and *P. vexans*.

Harvey *et al.* (2001) studied on genetic and pathogenic variation among cereal, medic and sub-clover within 34 isolates of *Pythium irregular* by using restriction fragment length polymorphisms (RFLPs) as genetic markers. Most isolates showed significant differences in their pathogenicity between hosts, indicating that they varied in pathogenic fitness and were better adapted to infect some hosts relative to others.

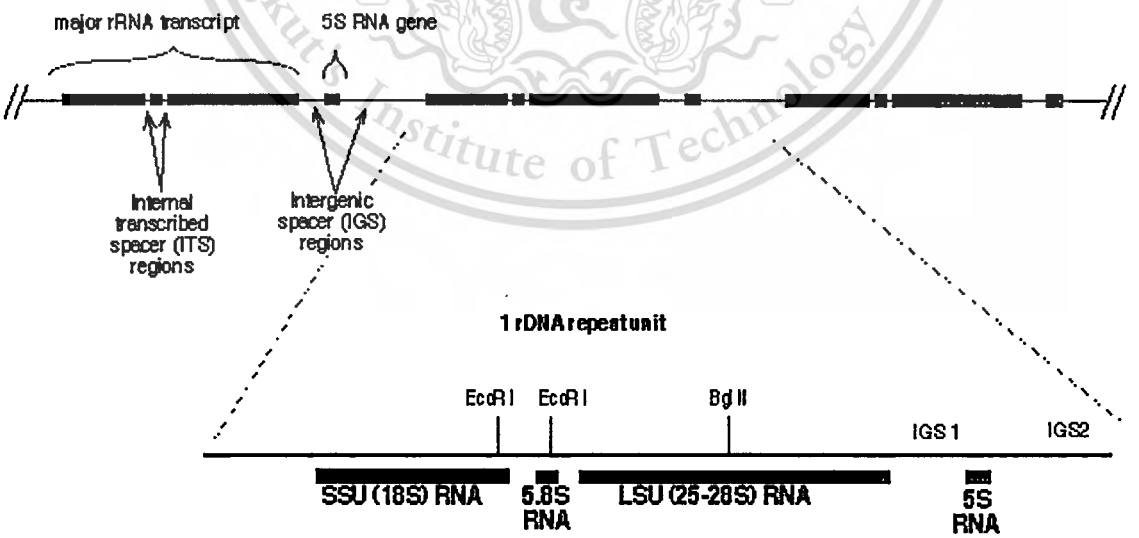

Fig.2.4 Schematic diagram of the ribosomal RNA gene cluster (Source: Vilgalys. Lab. 2009).

Table 2.2 Features of commonly used molecular markers (Bridge. 2002).

Marker	DNA type studied	Taxonomic level resolved	Sample required
Random amplification of polymorphic DNA (RAPD)	Variable, generally nuclear, sometime respective	Individuals, sub-specific groups	Purified DNA
Simple repetitive PCR sequences; micro-and mini-satellite probes and primers	Variable, generally nuclear, repetitive	Individuals, sub-specific groups	Purified DNA
Amplified fragment length polymorphisms (AFLPs)	Subset of total genome	Individuals, sub-specific groups, some closely related species	Purified DNA
Mitochondrial DNA RFLPs	Mitochondrial DNA	sub-specific groups, some closely related species	Purified DNA
ITS/IGS region RFLPs	Nuclear rDNA variable spacers	Closely related species, some sub-specific groups	Organism
ITS region sequences	Nuclear rDNA variable spacers	Some sub-specific groups, closely related species	Organism
rRNA gene sequences	Nuclear rDNA	Species, genera, families, phyla	Organism
Protein genes	Conserved coding regions	Species, genera, families, phyla	Usually purified DNA

Paul (2002) identified *Pythium proliferatum* as a new species by using their morphological features and the sequences of the ITS1 region of rDNA. The comparison of ITS1 with related species supported its morphological features.

Singh *et al.* (2006) reported the molecular analysis could support the morphological variations between the closely related species. *P. rhizosaccharum* (F-1244) was isolated from

soil samples in the rhizosphere of sugarcane (*Saccharum officinarum*) in the north-eastern India. This isolate come closed to species of *P. ultimum*, *P. middletonii*, *P. rostratum* and *P. ramificatum*. However, it was different from the others by its rare antheridial branches that wrapped around the oogonia and its thin-walled, mostly plerotic oospores. These observations matched with the molecular analysis (comparison of the ITS sequences).

Paulitz *et al.* (2003) isolated *Pythium abappressorium* sp.nov. from wheeat and apple roots in eastern Washington. The new species was characterized by abundant appressoria, plerotic oospores and sporangia were formed from the appressoria and remnants of the appressoria remain attached to the base of sporangia at maturity. The sequence of the ITS1 region of the rDNA did not match the sequences from a worldwide collection of over 1,200 isolates.

Schurko *et al.* (2003) used sequence analysis of the ribosomal DNA internal transcribed spacers (ITS) to establish phylogenetic relationships among 23 isolates of *Pythium insidiosum*. The ITS sequence was tended to support the existence of geographic variants by dividing the isolates into three distinct clades that exhibited significant geographic isolation. The obtained sequence information provided an abundance of data for applications in the diagnosis of pythiosis and identification of *P. insidiosum* from clinical samples.

Allain-Boulé *et al.* (2004) identified *Pythium attrantheridium* sp. nov. which was a new species isolated from cavity spot lesions of carrots as well as apple and cherry seedlings by using its morphological and supported the morphological identification by the ITS region of the nuclear rDNA. The ITS region of all strains of *P. attrantheridium* was different from that of all other known as *Pythium* spp.

André Lévesque and De Cock (2004) constucted the phylogeny of 116 species of *Pythium* based on the ITS region of nuclear rDNA. The phylogenetic analyses of ITS regions revealed a divergence of globose and filamentous sporangium types and divided the *Pythium* strains into 11 clades (clades A-K) as sporangia development.

Nechwatal *et al.* (2005) gave a formal description of the taxon as *Pythium phragmitis* sp. nov., providing information on morphology, ecology and pathogenicity in comparison to related species. The new species were proved to be significantly more aggressive towards reed leaves and seedling *in vitro* than related species. It was characterised by filamentous, inflated sporangia and plerotic oospores with usually more than one antheridium. ITS and *cox* II sequence data

indicated this new species shared a common ancestor with *P. arrhenomanas*, but the sequence differences were clearly consistent with a divergence of the two taxa.

Perneel *et al.* (2006) analysed intraspecific variability within 51 isolates of *Pythium myriotylum* from cocoyam and other host crops by using optimum growth temperature, esterase banding patterns, AFLPs, rDNA-ITS sequencing and virulence to cocoyam. They found that molecular evidence of *P. myriotylum* infected cocoyam were distinct from *P. myriotylum* that isolated other crops and developed a certain degree of host adaptation.

Tambong *et al.* (2006) developed a DNA array containing 172 oligonucleotides complementary to specific diagnostic regions of internal transcribed specers (ITS) of more than 100 species for identification and detection of *Pythium* species. They reported that DNA array was a reliable tool for identification and detection of the majority of *Pythium* species in environmental sample.

2.6 Disease Control of Pineapple Root Rot

2.6.1 Chemical Fungicides

Control of heart and root rot of pineapple has been reported using three fungicides as follows: metalaxyl (Ridomil), fosetyl Al (Aliette) (Department of Agriculture. 2008) and phosphorous acid (Dalldorf. 1993). Fungicides were applied either as a preplant dip for propagation material or as a postplant spray.

Singh *et al.* (2003) reported that metalaxyl was a systemic fungicide widely used in citrus production to control *Phytophthora* root and foot rot. But it was found that the commercial formulation of metalaxyl contained various isomers and that these isomers might vary in phytotoxin effects on citrus leaves. In young field-grown citrus trees, bright yellow foliage with some leaf necrosis was observed following metalaxyl treatments.

Jonhson *et al.* (2004) treated phosphorous acid for controlling tuber rots caused by *Phytophthora infestans*, *P. erythroseptica* and *Pythium ultimum* to foliage of potato. They found that applying phosphorous acid at 9.37 kg. a.i./ha could be achieved for controlling late blight and tuber rot within 2 weeks. But control of *P. ultimum* by phosphorous acid was not evident. Total tuber yield at harvest did not differ significantly between the phosphorous acid treatment and the nontreated control.

2.6.2 Biological Control

Biological control aims to the reduction of inoculum and disease producing activity of a pathogen which accomplished by one or more organisms (Jacobsen. 2002). Several species of bacteria and fungi have been reported to be effective as biological control agents (BCAs) for root rot diseases (Table 2.3 and Table 2.4). The principal fungi used as BCAs against soil-borne diseases include the commercially used species, namely, *Gliocladium virens*, *Trichoderma harzianum* (Agrios. 1997) and *Chaetomium* species (Ketomium®) (Soytong *et al.* 2001). The filamentous fungi produce a wonderful diversity of secondary metabolites which include pigments, antibiotics, phytotoxins, toxic compounds, and compounds that promote or inhibit growth (Demain. 1999). Plant pathogens were interfered by antibiosis, competition and paratism (Trigiano *et al.* 2004). Biocontrol of soil-borne pathogens is a direct result of the action of antagonists through one or more of the following mechanisms: Antibiosis which Titus and Pereira (2007) stated that refers to the inhibition or destruction of the pathogen by the metabolic product of the antagonist; Competition that occurs when the antagonist directly complete for the pathogens resources like nutrients, oxygen, space etc; Parasitism, hyperparasitims and mycoparasitism occur when antagonist invades the pathogen by excretion of extracellular enzymes, phenols, chitinases, cellulases and other lytic enzymes.

Table 2.3 Lists of bacteria reported as antagonists.

Antagonists	Pathogens	References
Aquaspirillum autotrophicum	*Helminthosporium solani*	Martinez *et al.* (2006)
Burkholderia cepacia	*Pythium* spp.	Milus and Rothrock(1997)
Cellulomonas fimi	*Helminthosporium solani*	Martinez *et al.* (2006)
Pantoea agglomerans	*Penicillium digitatum*	Poppe *et al.* (2003)
Pseudomonas chlororaphis	*Helminthosporium solani*	Martinez *et al.* (2006)
Pseudomonas fluorescens	*Pythium* spp.	Milus and Rothrock(1997)
Pseudomonas putida	*Helminthosporium solani*	Martinez *et al.* (2006)
Pseudomonas sp.	*Pythium* spp.	Milus and Rothrock(1997)
Rhizobium leguminosarum	*Pythium* spp.	Bardin *et al.* (2004)
Serratia plymuthica	*Pythium perplexum*	Galland and Paul (2001)
Streptomyces griseus	*Helminthosporium solani*	Martinez *et al.* (2006)
Streptomyces rochei	*Phytophthora capsici*	Ezziyyani *et al.* (2007)

Table 2.4 Lists of fungi reported as antagonists.

Antagonists	Pathogens	References
Aspergillus candidus	*Phytophthora cinnamomi*	Duvenhage and Kotze (1993)
Aspergillus spp.	*Phytophthora capsici*	Noveriza and Quimio (2004)
	Fusarium oxysporum f. sp.	
	lycopersici,	
	Sclerotium rolfsii	
Chaetomium globosum	*Cochliobolus sativus*	Aggarwal *et al.* (2004)
Chaetomium cupreum	*Phytophthora palmivora*,	Soytong *et al.* (2001)
	Phytophthora paracitica,	
	Phytophthora cactorum,	
	Fusarium oxysporum f. sp.	
	lycopersici,	
	Sclerotium rolfsii	
Cunninghamella spp.	*Phytophthora capsici*	Noveriza and Quimio (2004)
Gliocladium spp.	*Phytophthora capsici*	Noveriza and Quimio (2004)
Mortierella spp.	*Phytophthora capsici*	Noveriza and Quimio (2004)
Mucor spp.	*Phytophthora capsici*	Noveriza and Quimio (2004)
Paecilomyces lilacinus	*Phytophthora cinnamomi*	Duvenhage and Kotze (1993)
Penicillium funiculosum	*Phytophthora cinnamomi*	Fang and Tsao (1995)
Rhizoctonia spp.	*Pythium ultimun*	Burns and Benson (2000)
Trichoderma hamatum	*Phytophthora cinnamomi*	Duvenhage and Kotze (1993)
Trichoderma harzianum	*Phytophthora capsici*	Ezziyyani *et al.* (2007)
Trichoderma harzianum	*Pythium ultimum*	Naseby *et al.* (2000)
Trichoderma virens	*Phytophthora fragariae*	Wilcox *et al.* (1999)
Trichoderma virens	*Pythium aphanidermatum*,	Howell (2006)
	Pythium ultimum,	
	Rhizopus oryzae	
Trichoderma spp.	*Phytophthora capsici*	Noveriza and Quimio (2004)

2.7 Mechanisms of Antagonism

Antagonists used for biological control of plant pathogens include bacteria, fungi, nematodes and viruses. Biological control of plant disease can occur through different mechanisms, which are generally classified as antibiosis, competition suppression, direct parasitism, induced resistance, hypovirulence and predation (Haggag and Mohamed. 2007).

2.7.1 Antibiosis

Antibiosis is the inhibition of an organism by a metabolite produced by another organism. Antagonists may produce influential growth inhibitory compounds that are effective against numerous microorganisms.

2.7.2 Competition

Competition is the result of two or more organisms trying to utilize the same food or the same infection site. The competitor successfully excludes the others due to its faster growth or reproductive rate or is more efficient in obtaining nutrients from food sources.

2.7.3 Parasitism

Parasitism is the feeding of an organism on another organism. As a biocontrol mechanism, parasitism can be successfully used to reduce inoculums of many pathogens.

Ahmed *et al.* (1999) studied the ability of *Trichoderma harzianum* to control the rot rot of pepper (*Capsicum annuum*) caused by *Phytophthora capsici.* Analysis of the fungal populations in the plant growth substrate showed that *T. harzianum* constantly reduced the population of *P. capsici* over time.

Naseby *et al.* (2000) reported five strains of *Trichoderma* (4 strains of *T. harziznum* and 1 strain of *T. pseudokoningii*) for their biocontrol activities to *Pythium ultimum.* They found that all *Trichoderma* strains reduced the number of lesions caused by *P. ultimum* and increased the number of lateral roots. *Trichoderma harzianum* strains T4 and N47 were the greatest beneficial characteristics. They improved plant growth in the absence of *P. ultimum* and reduced plant damage in the presence of *P. ultimum.*

Galland and Paul (2001) reported the antagonism between *Pythium perplexum* F-926 and *Serratia plymuthica* B-781 *in vitro* on the Potato Carrot Agar plates inoculated with both organisms. The fungus grew rapidly to a certain extent and then suddenly stopped, producing a clear zone of inhibition around the bacterial inoculum.

Soytong *et al.* (2001) reported that biological product formulated from 22 strains of *Chaetomium cupreum* (isolates CC01-CC10) and *Chaetomium globosum* (isolates CG01-CG12) in the form of pellet and powder formulation has been successfully reduced disease incidence which applied to infested field-soils in China, Philippines, Russia, Thailand and Vietnam for the long-term protection of durian and black pepper caused by *Phytophthora palmivora*, tangerine caused by *P. parasitica* and strawberry caused by *P. cactorum*, wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* and basal rot of corn caused by *Sclerotium rolfsii*.

Mazzola *et al.* (2002) reported that three nonpathogenic isolates of *Pythium* MM1, *Pythium* MM3 and *Pythium* MM5 could be used as biological control of root rot caused by *P. sylvaticum* and *P. ultimum*.

Xiao *et al.* (2002) collected 53 antibiotic-producing *Streptomyces* isolated from soils in Minnesota, Nebraska and Washington. Eight isolates were the greatest pathogen-inhibitory capabilities for their ability to control Phytophthora root rot on alfalfa and soybean in sterilized vermiculite and naturally infested field soil. All 8 isolates significantly reduced root rot severity in alfalfa and soybean caused by *P. medicaginis* and *P. sojae*.

Poppe *et al.* (2003) studied mechanisms of *Pantoea agglomerans* CPA-2 to control postharvest pathogens, *Penicillium digitatum* and *P. italicum* on citrus fruits. The mechanism was antibiosis, induced resistance, competition and production of chitinolytic enzymes. The results indicated that competition for nutrients was one of the mechanisms of *P. agglomerans* CPA-2, but the physical contact between pathogen and antagonist was important for effective control.

Noveriza and Quimio (2004) tested antagonistic activity of mycoflora against *Phytophthora capsici* using bi-culture technique. The results showed that antagonistic mechanism of *Mucor* (isolate 1001), *Trichoderma* (isolates 125, 170, 171, 179, 180 and 181), *Gliocladium* (isolate 109), *Cunninghamella* (isolates 165 and 168), *Mortierella* (isolate 177) and *Aspergillus* (isolate 106) were act as competitor (competition for nutrient) due to they rapidly grew over the pathogen, and *Trichoderma* (isolates 125, 170, 171, 179, 180 and 181) were able to penetrate the hyphae of the pathogen. *Aspergillus* (isolates 67, 79, 81, 83, 108 and 202) inhibited the pathogen apparently by producing antibiotic.

Xue (2002) reported that a strain of *Clonostachys rosea* (syn. *Gliocladium roseum*), ACM941 was identified as mycoparasite against pea root rot pathogens (*Alternaria alternata*,

Aphanomyces euteiches, *Fusarium oxysporum* f. sp. *pisi*, *F. solani* f. sp. *pisi*, *Mycosphaerella pinodes*, *Pythium* spp., *Rhizoctonia solani* and *Sclerotinia sclerotiorum*).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Collection, Isolation, Pathogenicity Tests and Identification

3.1.1 Collection Samples

Soil, root and leaf samples of pineapples were taken from the disease epidemic planting areas at Petchaburi, Phatthalung, Prachuap Khiri Khan and Rayong provinces by collecting soil around rhizosphere, root rot and leaves of pineapple var. Pattavia. All collected samples were kept in individual clean plastic bag and brought to laboratory at Biocontrol Research Unit and Mycology Section, Department of Plant Pest Management Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

3.1.2 Isolation of Pathogens

Soil, root and leaf samples were isolated root rot pathogen by using baiting technique. Baiting technique was carried out as described by Dhingra and Sinclair (1987) using pineapple leaf blades as baits. Each sample (*ca* 10 g) was put in sterilized Petri dish and flooded with sterile distilled water, while pineapple leaf blades were cut into segments (*ca* 0.5x0.5 cm) and surface-sterilized with 10% sodium hypochlorite for 1 min and followed by 1 min in sterile distilled water. The segmented baits were placed over the water surface. After 2-3 days of incubation at room temperature (28-30°C), the colonized baits showing discoloration were rinsed with sterile distilled water, blotted dry on sterilized paper towels, plated onto water agar (WA, 15 g agar, 1,000 ml distilled water) and incubated at room temperature. Developing mycelia were observed under compound microscope and transferred to potato dextrose agar (PDA, 200 g fresh potato, 20 g agar, 20 g glucose, 1,000 ml distilled water) for purification the cultures.

3.1.3 Identification

Morphological study was done to identify the species from which their descriptions were recorded for all isolates. Moreover, molecular phylogeny was performed in order to comparison of different diversity of fungi.

3.1.3.1 Morphological Identification

All isolates were identified according to the keys of van der Plaats-Niterink (1981), Erwin *et al.* (1983), Erwin and Ribeiro (1996) and Domsch *et al.* (1993). The morphological characters of hyphae, oogonia, antheridia, oospores and sporangia were examined for each isolate as follows: for the assessment of hypha, oogonia, antheridia and oospores. An agar plug (0.5-mm in diameter) of each isolate was cut from the edge of actively growing colony, placed on PDA, V8A (16 g agar, 3 g $CaCO_3$, 100 ml Campbell's V8 juice, 900 ml distilled water) and PCA (van der Plaats-Niterink, 1981) in the center of Petri dishes (9-cm diameter), and incubated at room temperature. Seven days later 50 main hyphae, 30 mature oogonia and oospores chosen at random were measured at 400 magnifications with the compound microscope. Investigation on sporangial development was made on discs (5 mm diameter) cut from the edge of colony actively growing on PDA, and floated in sterile water and observed for 1-7 days at room temperature. Dimensions and characteristic features of 30 fully-mature sporangia chosen at random were measured for each isolate at 400 magnifications with the compound microscope.

3.1.3.2 Molecular Identification

3.1.3.2.1 DNA Extraction

DNA was extracted from mycelia growing from single oospore by using a hexadecyltrimethyl-ammonium bromide (CTAB) procedure (Lee and Taylor. 1990). Each isolate was grown in 50 ml test tube containing 20 ml PDB (Potato dextrose broth: 200 g fresh potatoes, 20 g glucose, 1,000 ml distilled water) at 28°C on an orbital shaker (180 rpm) for 3-10 days. The mycelia were harvested by filtration, excess water was removed from mycelia by pressing in paper towels. A mycelial mat was placed in a prechilled mortar, frozen with liquid nitrogen, and ground to fine powder. Mycelial powder was suspended in 600 µl CTAB buffer, vortexed and incubated at 65°C for 30 min, and 600 µl CIA (chloroform: isoamyl alcohol, 24:1 (v/v)) was added. The solution was gently shaken for 25 min on a shaking platform and centrifuged at 7,000 rpm for 5 min at 4°C. The aqueous phase (top) was transferred to a new microcentrifuge tube and repeated CIA extraction. After the second CIA was washed, 300 µl isopropanal was added and mixed by inverting the tube several times. The supernatant was stored at room temperature, and then was centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and drained on a paper towel for 30 min. DNA pellets were resuspended with 50 ml sterile double distilled water. The genomic DNA was examined by electrophoresis in 1.0% agarose gel (0.5 agarose, 50 ml

TAE buffer). The gel was stained with ethidium bromide (0.5 μg/ml) and photographed under ultraviolet light.

3.1.3.2.2 PCR Amplification of ITS rDNA

PCR was conducted in a 50-μl reaction volume by the procedure of White *et al.* (1990). PCR amplification of ITS regions was performed with primer pair ITS1 and ITS4. Reaction mixtures contained of: 38.2 μl ddH_2O, 5 μl 10xPCR buffer, 3 μl Mg^{2+}, 1 μl dNTPs, 1 μl genomic DNA, 0.4 μl each of primer and 1.0 μl *Taq* DNA polymerase, and the amplification were conducted using the thermocycler with the following temperature cycling parameters: denaturation at 94 °C for 3 min for the first cycle and 1 min each for subsequent cycles, annealing for 30 sec at 55°C and elongation for 1 min at 72°C with a total of 35 cycles followed by a final extension for 10 min at 72°C. To assess the efficiency of amplification, 5 μl aliquots of PCR products were electrophoresed in 1.0% agarose gel in 1xTAE buffer, stained with ethidium bromide and photographed under ultraviolet light. The remaining volumes of the PCR amplicons were used for DNA sequencing.

3.1.3.2.3 DNA Purification

It is necessary to purify the PCR product from all contaminants before sequencing. In this process, the PCR products were purified by the U-gene gel Extraction Kit II (U-gene Biotechnology Co, P.R China) according to the manufacturer instructions. Each band of PCR products was cut out of the gel, transferred to a microcentrifuge tube. One hundred fifty μl of NJ buffer (per 0.01 g gel) were added and incubated at 60°C until the gel melted. The supernatant was removed to a column tube and centrifuged at 10,000 rpm for 1 min. The supernatant was decanted and 700 μl SPW buffer added to get rid of NJ buffer. After incubation at room temperature for 2-3 min, the tube was centrifuged at 10,000 rpm for 1 min and then the column tube was changed. Thirty μl elution buffer was added directly into the center of the column tube. The column tube was incubated at 60°C for 8 min and then centrifuged at 11,000 rpm for 1 min. Two μl of purified DNA were examined by electrophoresis in 1.0% agarose gel. Gel was stained with ethidium bromide (0.5 μg/ml) and photographed under ultraviolet light. The purified DNA was kept at -20°C for further sequence DNA.

3.1.3.2.4 Molecular Phylogeny

The phylogenetic tree was constructed by following the protocol of Harrison and Langdale (2006). The phylogenetic relationship of the pathogens was performed and data compared to those of related species. These sequences were either generated during this study or

retrieved from GenBank. All sequences were edited by using BioEdit, version 7.0.1 and aligned using ClustalX. Phylogenetic analyses were performed using PAUP* 4.0b8 (Swofford. 2001). Tree was viewed in Treeview.

3.1.4 Pathogenicity Tests

All isolates were tested for their pathogenicities by Koch's Postulate on pineapple leaves and pineapple roots to screen the most aggressive isolate for further use. Pathogenicity test was done by using detached leaf and root inoculation as follows:

3.1.4.1 Pathogenicity Tests on Detached Leaves

The pathogenicity test was carried out as described by Soytong *et al.* (2005) with some modifications. Pineapple leaves from 6-month-old pot-grown pineapples were used for the assessment of the pathogenicity. Pineapple leaves of approximately the same age (the same position on the plant) were collected, clipped on base and apex (length *ca* 10 cm), and surface-sterilized by soaking in 10% ethanol for 3 min and then each pineapple leaf was wounded with needle-pricking method (0.5 cm in length and 1 min in depth from the surface). Each wounded leaf was inoculated with a plug (3 mm diameter) of tested isolate which was taken from the margin of an actively growing colony. The control treatment was treated with a plug consisted of PDA medium alone. The inoculated leaves were kept in sealed plastic boxes containing moist paper towels at room temperature. The lesion diameter was recorded and confirmed by re-isolation after 3 days of incubation. The experiment was designed as completely randomized design (CRD) with four replicates and data were subjected to statistical analysis, and the variances of lesion diameter were computed, then treatment means were compared using the Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

3.1.4.2 Pathogenicity Tests on Root Inoculation

The pathogenicity test was designed as completely randomized design (CRD) with four replicates. The experiment was carried out using the slips of pineapples ('Pattavia' variety). All isolates were grown on PDA in 9-cm-diameter Petri dishes. The inocula were prepared by the methods of Biles *et al.* (1995), Shang *et al.* (1999) and Babadoost and Islam (2002) with modifications. Mycelia were separately scraped out from 7-day-old cultures of each pathogen and put in sterilized Petri dish and added with 20 ml sterile distilled water, incubated at room temperature until sporangia were formed (5-7 days). The suspension was homogenized then sporangia suspension was adjusted to $1x10^6$ sporangia/ml (Chern *et al.* 1998). Pineapple slips

were stripped off the lower leaves and placed in 8 cm diameter of clear glass containing 100 ml distilled water. After 2 weeks, the plants were removed from plastic cup, and 10 roots end per plant were cut (3 mm) before inoculation. The root systems of each tested plant were placed in sterile distilled water inoculated with 100 ml sporangia suspension ($1x10^6$ sporangia/ml). Control plants were placed with 100 ml sterile distilled water without inoculum. All tested plants were maintained indoor near a sunny window until root rot occurs (about 1 week). All plants were removed from plastic cup and then disease severity index (DSI) were recorded by modified following scale of Ahmed *et al.* (1999) as 1 = no root rot, 2= 1-25% root rot, 3=26-50% root rot, 4=51-75% root rot and 5= 76-100% root rot. All isolates were screened and the most aggressive isolate were selected for further experiment.

Data were subjected to statistical analysis, and the disease severity index was computed, then treatment means were compared using the Duncan's Multiple Range Test (DMRT) test at $P \leq 0.05$.

3.2 Screening for Antagonistic Fungi and Antagonistic Substances Against the Pathogen Causing Pineapple Root Rot

3.2.1 Isolation and Identification of Antagonists from Soil

Soil samples were separately collected from rhizosphere of pineapple plants showing virulent root rot symptom, non-virulent root rot symptom and healthy pineapple plants. The soil samples were kept in sealed plastic bags and brought to laboratory at King Mongkut's Institute of Technology Ladkrabang, Bangkok. The antagonistic fungi were originally isolated by soil plate and baiting technique according to the method described by Petcharatand Soytong (1991).

3.2.1.1 Soil Plate Technique

Soil samples were dried and ground to fine particles; 0.005 g of each soil sample, placed to sterilized Petri dishes and then overlaid with glucose-ammonium nitrate agar (GANA) medium (10 g glucose, 1 g NH_4NO_3, 1 g Difco bacto yeast extract, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 20 g agar, 0.06 g rose bengal, 0.03 g streptomycin, 1,000 ml distilled water). After 2-7 days incubation at room temperature in the dark, fungal colonies showing clear zone, pigments and well growth were collected and isolated into pure culture for further use.

3.2.1.2 Baiting Technique

Each soil sample (*ca* 10 g) was placed to sterilized Petri dishes and moistened with sterile distilled water before being baited with small pieces of sterilized rice straws, filter paper, tissue paper and pineapple leaves. The fungal colonies or fruiting structures on baits were observed by stereomicroscope and then a small piece of mycelia or fruiting structure was taken and placed on WA in a 9-cm-diameter Petri dish, incubated at room temperature. The hyphal tips were transferred to PDA plates and isolated into pure culture.

All isolates taken from soil plate and baiting techniques were screened for biological control activity against the most selected aggressive isolate of pathogen causing root rot of pineapple variety Pattavia.

3.2.2 Screening of Antagonistic Fungi

The promising antagonistic fungi (from 3.2.1) were screened by bi-culture test which arranged in a CRD with four replications. The interaction in bi-culture was studied on 9-cm-diameter Petri dish according to the method of Ahmed, *et al.* (1999). The mycelial disc (3 mm Ø) of the pathogen was cut out from the edge of a growing colony, and placed on PDA about 1 cm from the edge of the Petri dish, a similar sized disc from the promising antagonistic fungus was placed on the opposite site, about 1 inch from the edge of the Petri dish while the control was performed either the pathogen or the promising antagonistic fungus alone, then incubated at room temperature for 20 days. Colony diameters and the number of oospore production were recorded, and the data were transformed into a percent inhibition of mycelial growth or oospore production of pathogen according to the following formula: % inhibition = [(colony diameter or oospore of pathogen in control plate - colony diameter or oospore of pathogen in bi-culture plate)/ colony diameter or oospore of pathogen in control plate]x100. Colony diameter and oospore production were statistically computed analysis of variance, then treatment means were compared using the DMRT at $P \leq 0.05$. The promising antagonistic fungi showing the percentage of inhibition over 80% were selected for further study.

3.2.3 Testing Antagonistic Substances in Laboratory

The screened antagonistic fungi from previous experiment were tested for their mechanism of action for inhibition to the most aggressive isolate of root rot pathogen by testing their antagonistic releasing substances.

The mycelial disc of the antagonist were cut out with 3-mm-diameter cork borer from the edge of a growing colony, and transferred into PDB, incubated in static state at room temperature for 4 weeks. Fungal mycelia were removed from liquid by cheesecloth filtration and dried over night at 28-32°C. The fresh weight and dry weight of mycelial mat were recorded.

Subsequently, the extraction of the antagonistic fungi was performed by the method described by Kanokmedhakul *et al.* (2006). Each air-dried mycelial mat was ground with electrical blender and dissolved with hexane (H) at 1:1 (vol/vol) and incubated by shaking for 24 hrs at room temperature. The solvent was separated out of the marc by filtration through filter paper (Whatman No.4). The marc from hexane extraction was further extracted with ethyl acetate (EtOAc) and followed with methanol (MeOH) using the same procedure as hexane. The extracted solutions from hexane, ethyl acetate and methanol were concentrated to crude hexane, ethyl acetate and methanol extracts by using a rotary vacuum evaporator. Each crude extract was weighed, and then kept in refrigerator (4°C) until use for testing antagonistic substances against pineapple root rot pathogen as shown in diagram (Fig. 3.1).

Each concentration of crude extract was dissolved with 2% dimethylsulfoxide (DMSO), and then mixed with PDA before autoclaving at 121°C, 15 lbs/inch^2 for 20 min. Agar plug (3 mm diameter) of the most aggressive isolate of root rot pathogen was cut from the margin of the 3-d-old colony and transferred to the middle of PDA containing each concentration of crude extract, incubated at room temperature for 2-10 days.

The experiment was carried out using a completely randomized design (CRD) with four replications. Data were recorded as colony diameter (cm) and number of oospore, and then transformed to inhibition percentage. Data were subjected to statistical analysis of variance. Treatment means were compared using the Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. The effective dose (ED_{50}) of each crude extract was computed by using probit analysis. The effect of each crude extract to the mycelia and oospore characteristics was observed under compound microscope.

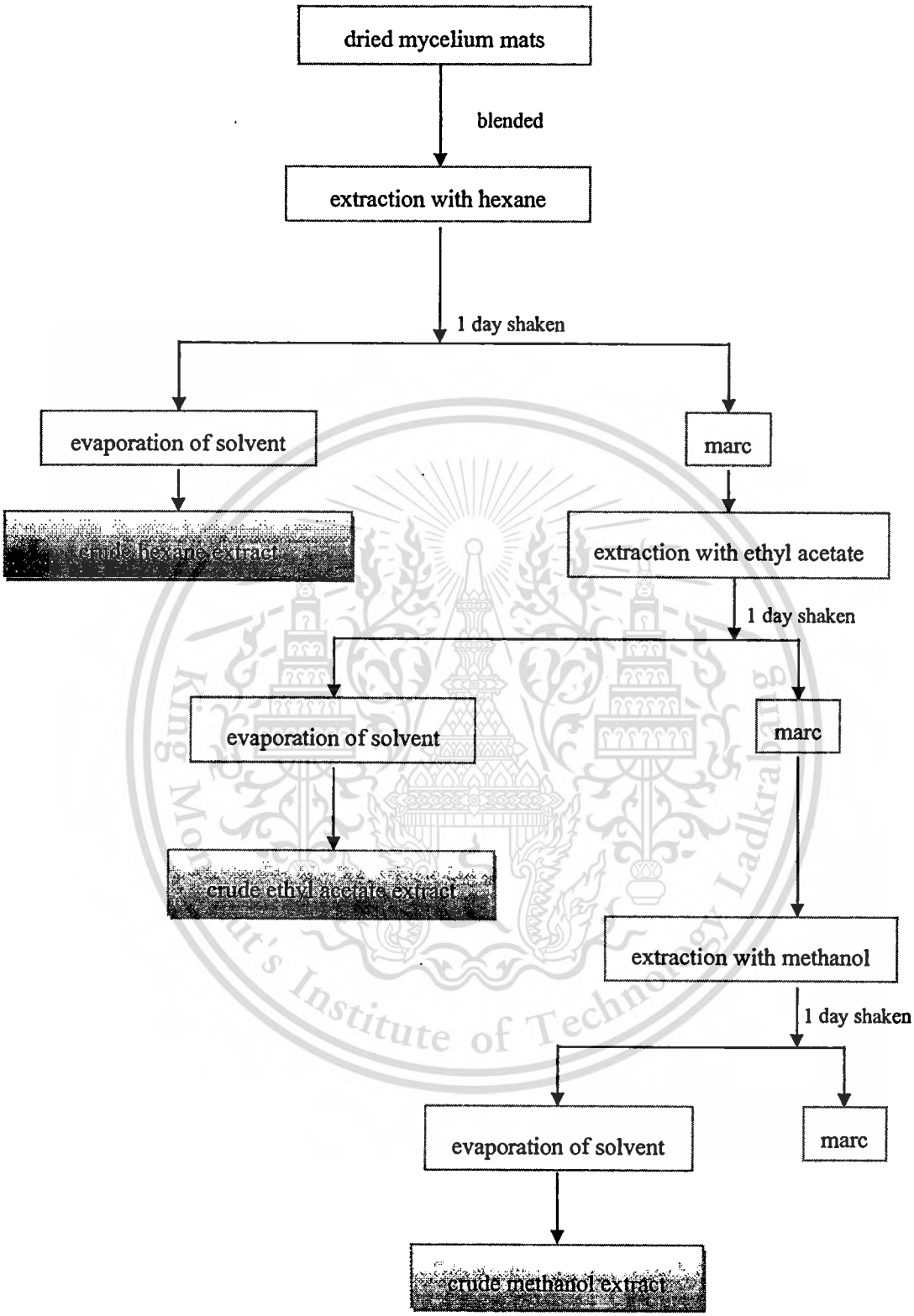

Fig. 3.1 Diagram of microbial crude extraction.

3.3 Controlling Pineapple Root Rot Using Biofungicides in Pot Experiment

Results from previous experiment, the most effective isolate of antagonist was tested for biological control property against the most aggressive isolate of root rot pathogen of pineapple 'Pattavia' variety in pot experiment.

The inoculum was prepared by the methods of Biles *et al.* (1995), Shang *et al.* (1999) and Babadoost and Islam (2002) with modifications. Seven-to ten-day-old culture on PDA was removed with a flame sterilized glass spreader to rub the colony surface and flooded with 20 ml sterile distilled water for 48 hrs incubated at 25°C. Sporangial suspension was collected and adjusted to $1x10^6$ sporangia/ml using haemacytometer.

The most effective antagonist was cultured on PDA for 4 weeks. antagonist was harvested for preparing in the formulation of oil and powder forms according to the method of Soytong (2001). Both formulations periodically checked for shelf-life by dilution plate method.

Preparation for formulation of oil form, spores were collected, mixed with sterilized bio-oil and adjusted to $1x10^6$ CFU/ml.

Preparation for formulation of powder form, air dried mycelia and spores were ground to fine. Their spores were counted and adjusted to $1x10^6$ CFU/g. before mixing with sterilized 10g. charcoal, 1 kg. talcum powder.

Planting soil was prepared in the soil mixture as follows: soil : sand : compost at a ratio of 8:2:2 (vol/vol/vol) before autoclaving at 121°C, 15 lbs/ $inch^2$ for 2 hrs. Eight kilograms of sterilized soil were placed into experimental plastic pots.

The lower leaves of pineapple slips were stripped off and the slips were placed in 8 cm diameter cleaned plastic pots containing 100 ml distilled water, then kept on the shelf at room temperature for growing out of young roots. The pineapple slips with new young roots were taken out from plastic pots after 2 weeks. The 10-root tips of each pineapple slip were cut off (3 mm) by sterilized scissors, and then soaked into pathogen inoculum suspension at $1x10^6$ sporangia/ml for 5 min before transplanting into sterilized soil mixture pot.

The experiment was carried out by using a randomized complete block design (RCBD) with four replications, treatments were performed as follows: T1 = non-treated control, T2 = inoculated with pathogen alone, T3 = treated with antagonist in oil formulation at 10 ml/plant, T4 = treated with antagonist in powder formulation at 10 g/plant, T5 = treated with antagonist in oil formulation at 10 ml/plant and inoculated with pathogen, T6 = treated with antagonist in powder

formulation at 10 g/plant and inoculated with pathogen, and T7 = treated with metalaxyl at recommended rate and inoculated with pathogen. The experimental pots were maintained in outdoor for observation. Moreover, treatments were applied either antagonist or metalaxyl at every 2 weeks for 5 months.

Data were recorded by modified disease severity index (DSI) of Ahmed *et al.* (1999) as described above in root inoculation test. Percentage of disease control was calculated by the formula as follows: disease reduction (%) = [(DSI in treatment where inoculated with pathogen alone – DSI in treatment where treated with either antagonist or metalaxyl)/ DSI in treatment where inoculated with pathogen alone]x100. All data were statistically computed for analysis of variance. Treatment means were compared using the Duncan's Multiple Range Test (DMRT) test at $P \leq 0.05$.

CHAPTER 4

RESULTS

4.1 Collection, Isolation, Identification and Pathogenicity Tests

4.1.1 Collection Samples

Atotal of 104 samples were collected from the disease epidemic planting areas of pineapple var. Pattavia at Petchaburi, Phatthalung, Prachuap Khiri Khan and Rayong provinces from Jan 2006 to May 2008 (Table 4.1). All samples were isolated the pathogen to be pure culture, identification and pathogenicity tests.

Table 4.1 Diseased samples collected from root rot of pineapple.

Collection date	Locations	number of samples	Isolates
	Phatthalung province		
Jan 2006	-Pabon	15	PT101, PT102, PT108, PT301, PT501, PT701, PT901
Apr 2006	-Pabak	12	PT205, PT206, PT207
Apr 2008	-Pabon	12	PT304, PT305, PT307, PT401
	Rayong province		
Dec 2007	-Nicomphatthana	15	RY201, RY301, RY302, RY501, RY502, RY503, RY504, RY505
Dec 2007	-Pluakdeang	12	RY601, RY603, RY701
May 2008	-Nicomphatthana	15	RY801, RY802, RY803, RY804, RY805, RY806, RY807, RY808
	Prachuap Khiri Khan province		
Jan 2008	-Aownoiy	12	PJ104, PJ106, PJ108, PJ201, PJ202
	Petchaburi province		
Feb 2008	-Cha-am	11	PB201, PB108, PB202, PB206, PB301, PB302

Code: PT = Phatthalung, RY = Rayong, PJ = Prachuap Khiri Khan, PB = Petchaburi. The first number was No. of soil sambles followed by No. of isolates.

4.1.2 Isolation and Identification

Forty-four isolates were morphology identified as *Pythium* spp., and 15 isolates were *P. aphanidermatum* which isolated from Phatthalung, Rayong and Petchaburi provinces and 29 isolates were *P. graminicola* which isolated from Phatthalung, Rayong and Prachuap Khiri Khan provinces. The descriptions were presented in Table 4.2, 4.3, Figs. 4.1-4.44 and described as follows:-

Pythium aphanidermatum (Edson) Fitzp., Mycologia 15(4): 168(1923) Figs.4.1-4.15

Colony on PDA, radiate with cottony aerial mycelia; hyphae branched, 1.90-5.03 μm in diameter; sporangia inflacted filamentous that were branched, forming irregular toruloid complexes, Oogonia spherical, terminal and intercalary, measuring 20.24-26.46 μm in diameter ; Antheridia 1-2 per oogonium; oospore aplerotic, spherical, single, smooth-walled, measuring 16.63-22.90 μm in diameter.

Isolate examined: PT305, PT307, RY801, RY802, RY803, RY804, RY805, RY806, RY807, RY808, PJ104, PJ106, PJ108, PJ201 and PJ202.

Collection location: PT=Phatthalung, RY=Rayong, PJ=Prachuap Khiri Khan.

Pythium graminicola Subraman., Bulletin of Agric.Res. Inst.Pus. 177: 1(1928) Figs.4.16-4.44

Colony on PDA, rosette, hyphae branched, 1.83-4.48 μm in diameter; sporangia inflacted filamentous that were branched, forming irregular toruloid complexes; Oogonia spherical, terminal and intercalary, measuring 20.08-25.88 μm in diameter; antheridia 1-6 per oogonium; oospore plerotic, spherical, single, smooth-walled, measuring 15.23-22.48 μm in diameter.

Isolate examined: PT101, PT102, PT108, PT301, PT501, PT701, PT901, PT205, PT206, PT207, PT304, PT401, RY201, RY301, RY302, RY501, RY502, RY503, RY504, RY505, RY601, RY603, RY701, PB108, PB201, PB202, PB206, PB301, PB302.

Collection location: PT=Phatthalung, RY=Rayong, PB=Petchaburi.

Table 4.2 Comparative morphological characters of *Pythium aphanidermatum* causing pineapple root rot.

Isolates	Morphological features[ν]									
	Hyphae wide (µm)	Sporangium shape	Oogonium diam (µm)	Antheridium/ oogonium	Monoclinous or diclinous antheridium	Oospore diam (µm)	Oospore wall thickness (µm)	Aplerotic or plerotic oospore	growth rate on PDA (cm)	colony pattern on PDA (cm)
PT305	2.32-4.21	inflated filamentous	23.50-24.25	1-2	mono or diclinous antheridium	16.78-22.34	1-2	aplerotic	4.12	radiate
PT307	2.21-3.86	inflated filamentous	20.92-25.02	1-2	mono or diclinous antheridium	17.16-21.30	1-2	aplerotic	4.58	radiate
RY801	1.90-4.79	inflated filamentous	22.97-25.32	1-2	mono or diclinous antheridium	18.94-22.12	1-2	aplerotic	4.25	radiate
RY802	2.47-5.03	inflated filamentous	21.21-26.46	1-2	mono or diclinous antheridium	20.10-22.90	1-2	aplerotic	3.98	radiate
RY803	2.29-4.38	inflated filamentous	20.24-25.86	1-2	mono or diclinous antheridium	16.63-20.13	1-2	aplerotic	4.62	radiate
RY804	2.43-3.85	inflated filamentous	22.08-25.94	1-2	mono or diclinous antheridium	19.64-21.35	1-2	aplerotic	4.36	radiate
RY805	1.90-3.52	inflated filamentous	20.75-24.23	1-2	mono or diclinous antheridium	18.47-20.49	1-2	aplerotic	4.28	radiate
RY806	2.07-3.28	inflated filamentous	20.46-26.22	1-2	mono or diclinous antheridium	17.62-20.06	1-2	aplerotic	4.05	radiate
RY807	2.40-4.17	inflated filamentous	23.79-25.94	1-2	mono or diclinous antheridium	19.68-21.83	1-2	aplerotic	4.45	radiate
RY808	2.56-4.73	inflated filamentous	22.93-24.18	1-2	mono or diclinous antheridium	19.34-21.47	1-2	aplerotic	4.28	radiate
PJ104	2.35-3.17	inflated filamentous	23.85-24.75	1-2	mono or diclinous antheridium	17.96-20.04	1-2	aplerotic	4.35	radiate
PJ106	3.27-3.84	inflated filamentous	21.78-23.66	1-2	mono or diclinous antheridium	17.57-20.25	1-2	aplerotic	4.65	radiate
PJ108	2.81-3.28	inflated filamentous	20.75-24.65	1-2	mono or diclinous antheridium	18.26-21.38	1-2	aplerotic	4.55	radiate
PJ201	2.17-4.38	inflated filamentous	23.72-25.60	1-2	mono or diclinous antheridium	20.14-22.50	1-2	aplerotic	4.80	radiate
PJ202	2.75-3.64	inflated filamentous	21.15-24.54	1-2	mono or diclinous antheridium	19.35-21.82	1-2	aplerotic	3.89	radiate

[ν] Isolates of *Pythium aphanidermatum* isolated from the rhizosphere of pineapple were examined with at least 30 organs.

Table 4.3 Comparative morphological characters of *Pythium graminicola* causing pineapple root rot.

Isolates	Morphological features[1/]									
	Hyphae wide (µm)	Sporangium shape	Oogonium diam (µm)	Antheridium/ oogonium	Monoclinous or diclinous antheridium	Oospore diam (µm)	Oospore wall thickness (µm)	Aplerotic or plerotic oospore	growth rate on PDA (cm)	colony on PDA (cm)
PT101	2.40-3.82	inflated filamentous	20.45-24.95	1-6	mono or diclinous antheridium	15.78-22.25	3-8	plerotic	3.79	rosette
PT102	2.24-3.90	inflated filamentous	22.70-25.40	1-6	mono or diclinous antheridium	18.94-20.87-	3-8	plerotic	4.10	rosette
PT108	2.20-3.51	inflated filamentous	23.38-25.75	1-6	mono or diclinous antheridium	17.29-20.68	3-8	plerotic	3.65	rosette
PT301	2.64-3.85	inflated filamentous	21.68-24.15	1-6	mono or diclinous antheridium	17.72-20.66	3-8	plerotic	3.50	rosette
PT501	2.89-3.81	inflated filamentous	22.30-25.58	1-6	mono or diclinous antheridium	20.91-21.40	3-8	plerotic	3.85	rosette
PT701	1.83-3.30	inflated filamentous	21.68-24.28	1-6	mono or diclinous antheridium	16.54-21.30	3-8	plerotic	3.85	rosette
PT901	2.68-4.48	inflated filamentous	20.08-24.70	1-6	mono or diclinous antheridium	15.54-20.39	3-8	plerotic	3.88	rosette
PT205	2.59-3.30	inflated filamentous	20.28-24.75	1-6	mono or diclinous antheridium	19.97-21.64	3-8	plerotic	4.00	rosette
PT206	2.78-3.28	inflated filamentous	21.68-25.58	1-6	mono or diclinous antheridium	17.49-22.21	3-8	plerotic	3.72	rosette
PT207	2.96-3.27	inflated filamentous	20.73-25.88	1-6	mono or diclinous antheridium	16.64-21.58	3-8	plerotic	3.79	rosette
PT304	2.04-3.48	inflated filamentous	22.60-24.15	1-6	mono or diclinous antheridium	15.44-20.81	3-8	plerotic	3.55	rosette
PT401	2.33-4.10	inflated filamentous	21.56-24.14	1-6	mono or diclinous antheridium	17.91-20.39	3-8	plerotic	3.90	rosette
RY201	2.56-3.88	inflated filamentous	20.78-25.37	1-6	mono or diclinous antheridium	15.43-21.62	3-8	plerotic	3.82	rosette
RY301	2.68-4.37	inflated filamentous	20.74-24.29	1-6	mono or diclinous antheridium	16.28-22.48	3-8	plerotic	3.65	rosette
RY302	2.64-3.79	inflated filamentous	20.54-24.32	1-6	mono or diclinous antheridium	17.57-20.23	3-8	plerotic	3.72	rosette

[1/] Isolates of *Pythium graminicola* isolated from the rhizosphere of pineapple were examined with at least 30 organs.

Table 4.3. (Continued) Comparative morphological characters of *Pythium graminicola* causing pineapple root rot.

isolates	Morphological features[1/]									
	Hyphae wide (µm)	Sporangium shape	Oogonium diam (µm)	Antheridium/ oogonium	Monoclinous or diclinous antheridium	Oospore diam (µm)	Oospore wall thickness (µm)	Aplerotic or plerotic oospore	growth rate on PDA (cm)	colony on PDA (cm)
RY501	2.04-4.10	inflated filamentous	21.55-24.71	1-6	mono or diclinous antheridium	16.99-21.18	3-8	plerotic	4.00	rosette
RY502	2.65-3.67	inflated filamentous	20.28-24.10	1-6	mono or diclinous antheridium	17.73-20.29	3-8	plerotic	3.87	rosette
RY503	2.18-3.50	inflated filamentous	22.49-25.84	1-6	mono or diclinous antheridium	17.74-21.32	3-8	plerotic	3.80	rosette
RY504	2.79-3.85	inflated filamentous	21.87-24.12	1-6	mono or diclinous antheridium	15.78-20.26	3-8	plerotic	3.52	rosette
RY505	2.41-3.85	inflated filamentous	22.26-24.15	1-6	mono or diclinous antheridium	18.94-21.41	3-8	plerotic	3.74	rosette
RY601	2.70-3.88	inflated filamentous	20.71-24.43	1-6	mono or diclinous antheridium	15.96-21.80	3-8	plerotic	3.68	rosette
RY603	2.68-4.09	inflated filamentous	22.35-25.74	1-6	mono or diclinous antheridium	16.07-21.61	3-8	plerotic	3.46	rosette
RY701	2.12-3.72	inflated filamentous	21.82-25.06	1-6	mono or diclinous antheridium	17.33-21.40	3-8	plerotic	3.75	rosette
PB108	2.16-3.79	inflated filamentous	21.74-25.63	1-6	mono or diclinous antheridium	17.21-21.42	3-8	plerotic	3.49	rosette
PB201	2.62-3.12	inflated filamentous	20.38-24.67	1-6	mono or diclinous antheridium	15.23-20.27	3-8	plerotic	3.60	rosette
PB202	2.65-3.41	inflated filamentous	21.52-24.62	1-6	mono or diclinous antheridium	16.26-22.04	3-8	plerotic	3.97	rosette
PB206	2.45-3.80	inflated filamentous	20.67-24.25	1-6	mono or diclinous antheridium	15.15-21.36	3-8	plerotic	3.63	rosette
PB301	2.70-3.89	inflated filamentous	22.89-25.48	1-6	mono or diclinous antheridium	16.70-21.39	3-8	plerotic	3.42	rosette
PB302	2.46-3.50	inflated filamentous	20.58-24.31	1-6	mono or diclinous antheridium	16.00-20.83	3-8	plerotic	3.50	rosette

[1/] Isolates of *Pythium graminicola* isolated from the rhizosphere of pineapple were examined with at least 30 organs.

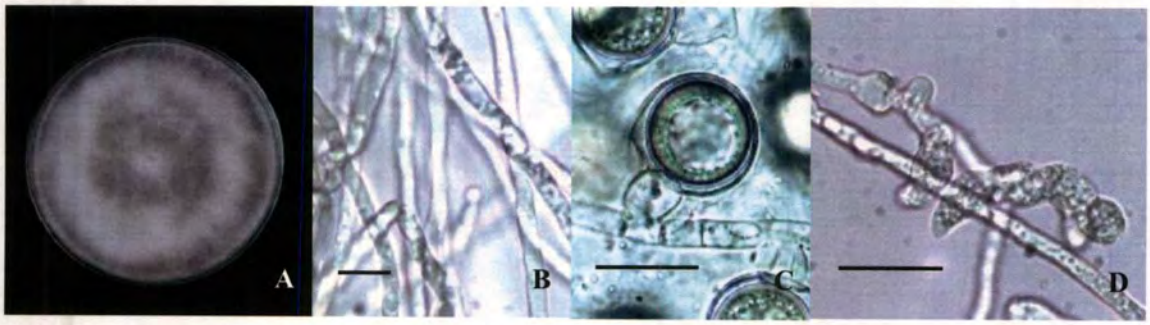

Fig. 4.1 *Pythium aphanidermatum* PT305. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

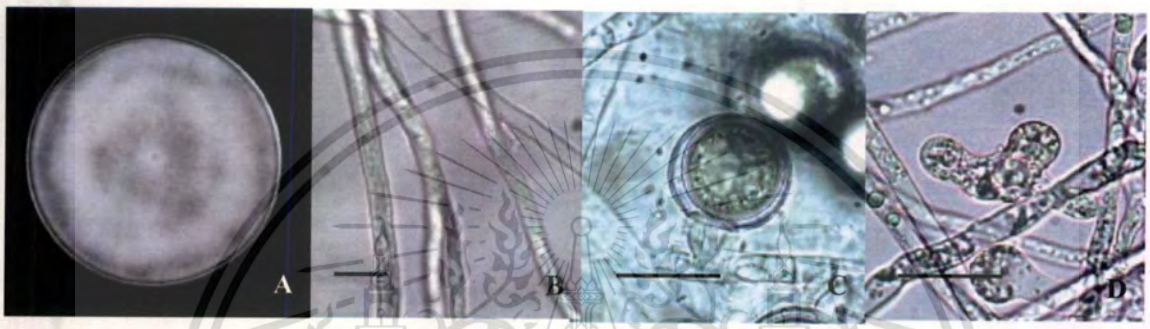

Fig. 4.2 *Pythium aphanidermatum* PT307. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

Fig.4.3 *Pythium aphanidermatum* RY801. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

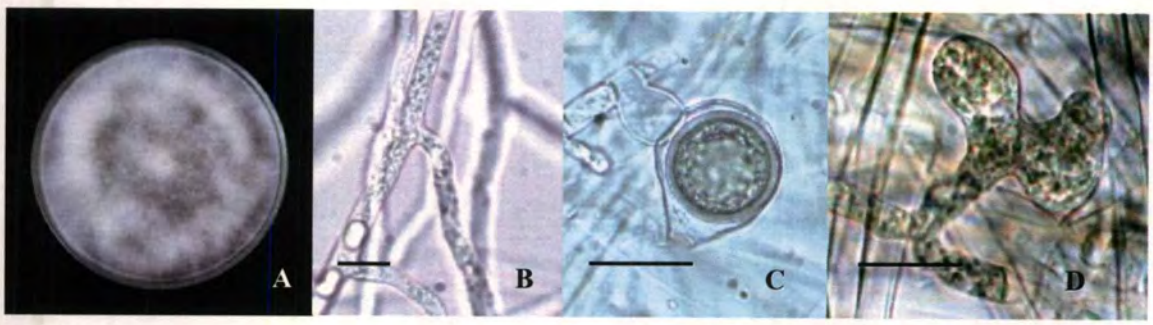

Fig.4.4 *Pythium aphanidermatum* RY802. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

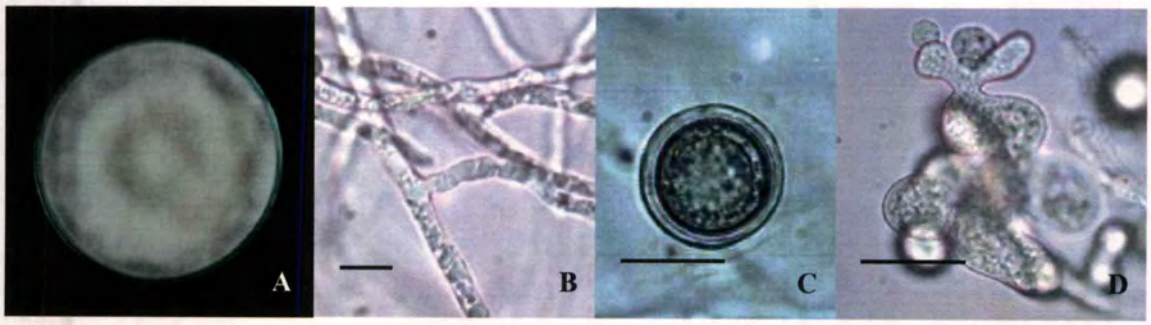

Fig.4.5 *Pythium aphanidermatum* RY803. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

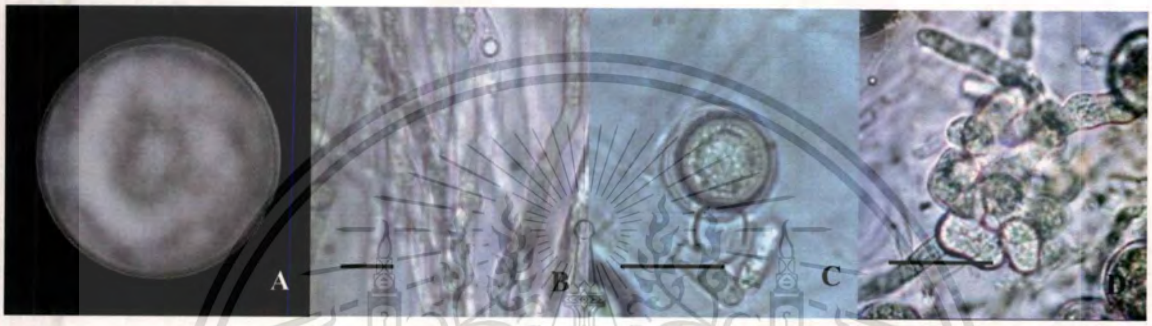

Fig.4.6 *Pythium aphanidermatum* RY804. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

Fig.4.7 *Pythium aphanidermatum* RY805. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

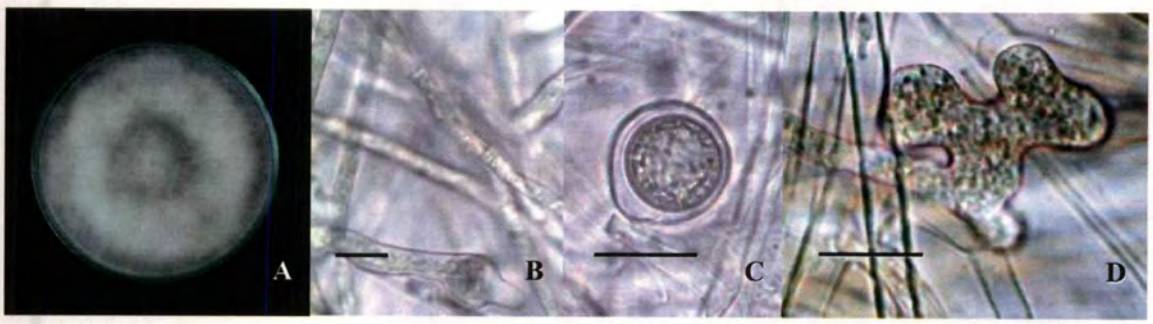

Fig.4.8 *Pythium aphanidermatum* RY806. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

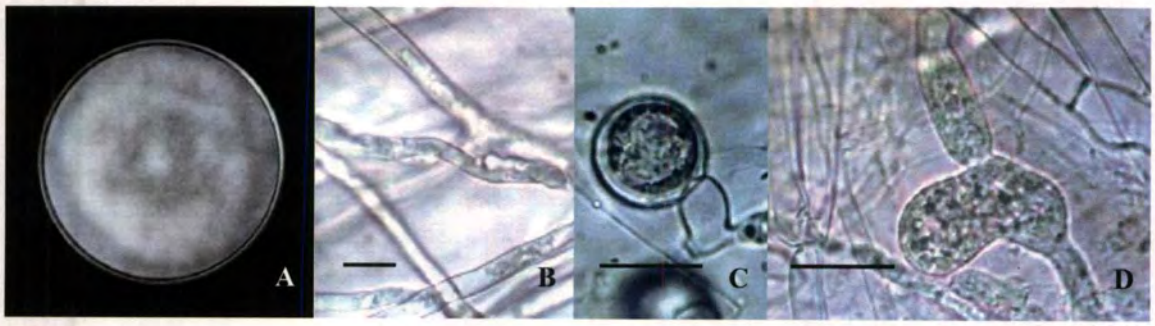

Fig.4.9 *Pythium aphanidermatum* RY807. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 μm, C,D=20 μm.

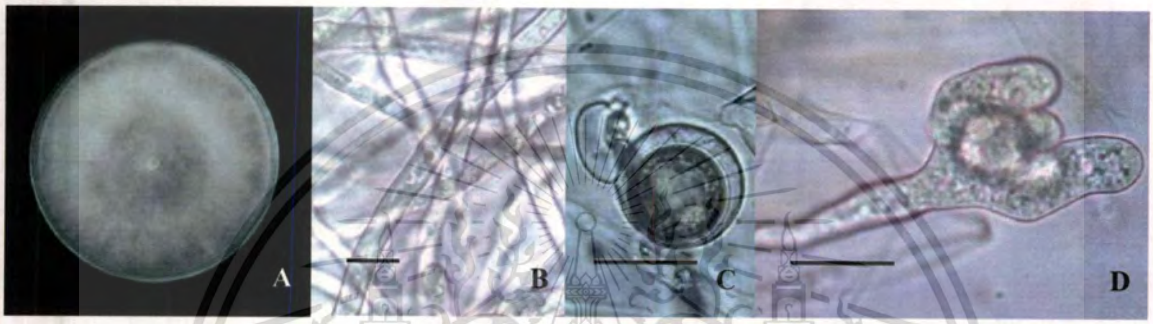

Fig.4.10 *Pythium aphanidermatum* RY808. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 μm, C,D=20 μm.

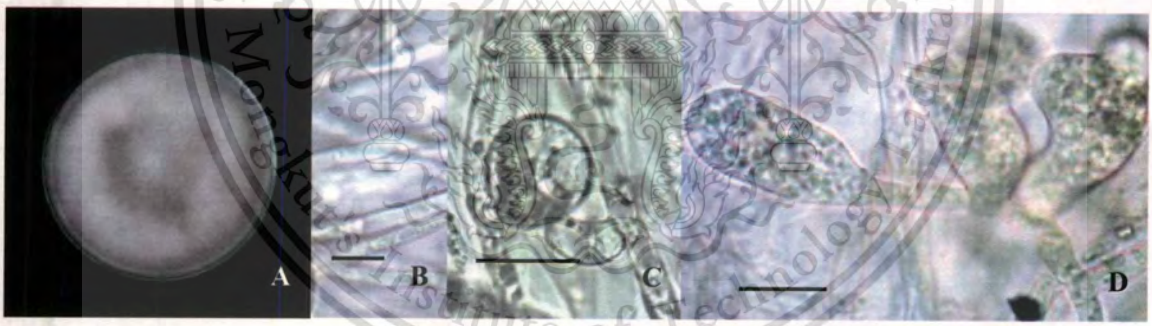

Fig.4.11 *Pythium aphanidermatum* PJ104. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 μm, C,D=20 μm.

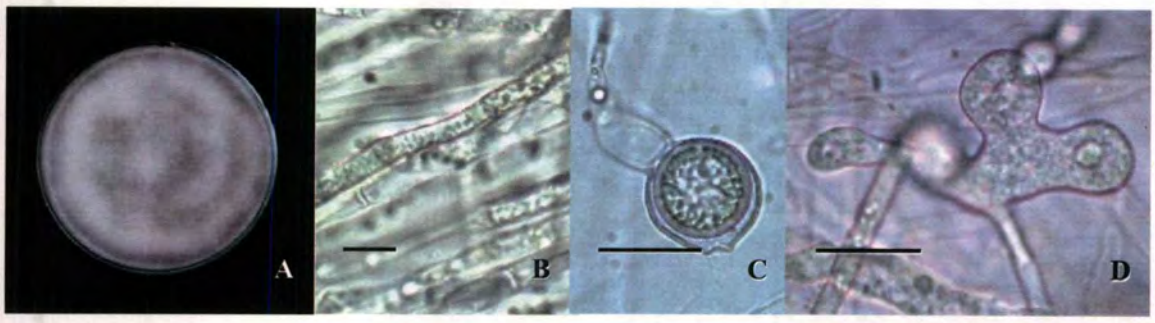

Fig.4.12 *Pythium aphanidermatum* PJ106. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 μm, C,D=20 μm.

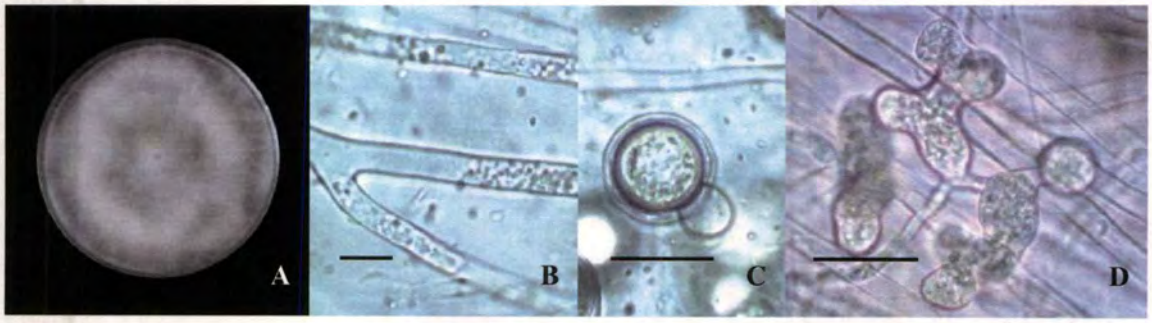

Fig.4.13 *Pythium aphanidermatum* PJ108. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

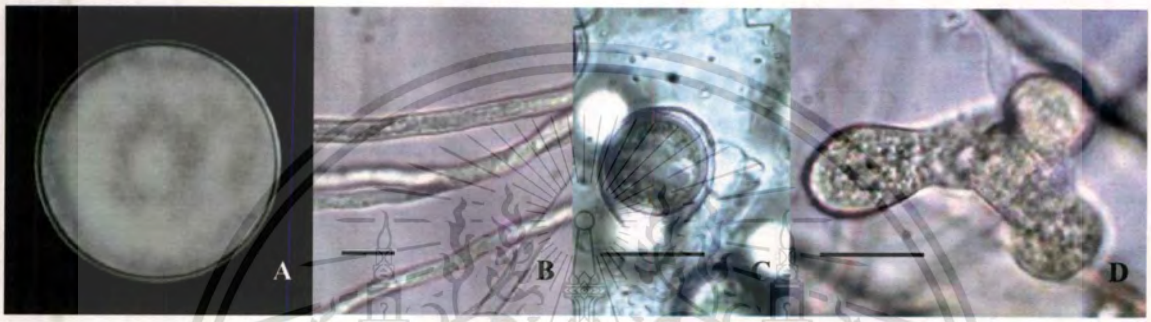

Fig.4.14 *Pythium aphanidermatum* PJ201. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

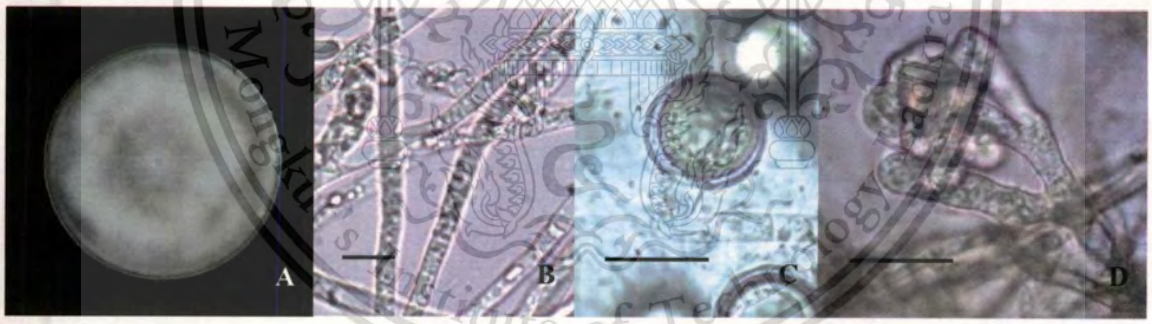

Fig.4.15 *Pythium aphanidermatum* PJ202. A. 3-day-old culture on PDA, B. mycelia, C. oospore, D. sporangium, bars. B=10 µm, C,D=20 µm.

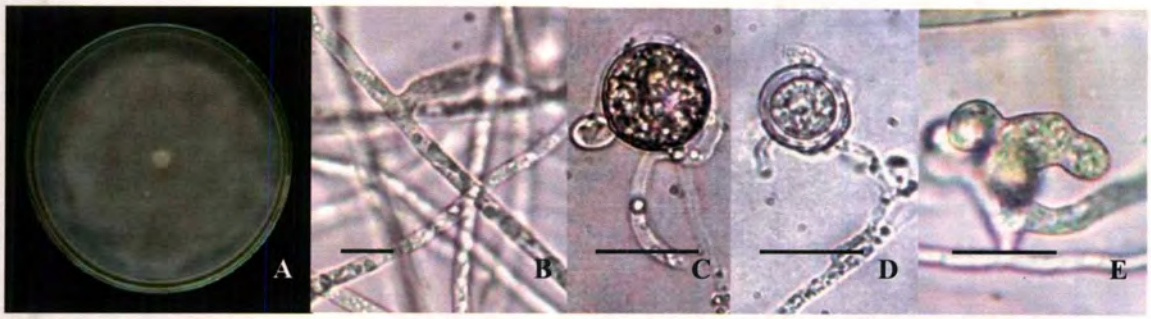

Fig.4.16 *Pythium graminicola* PT101. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

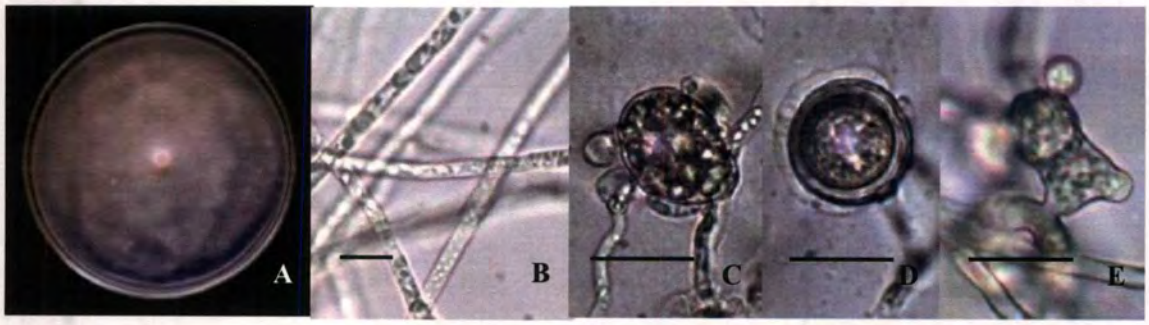

Fig.4.17 *Pythium graminicola* PT102. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

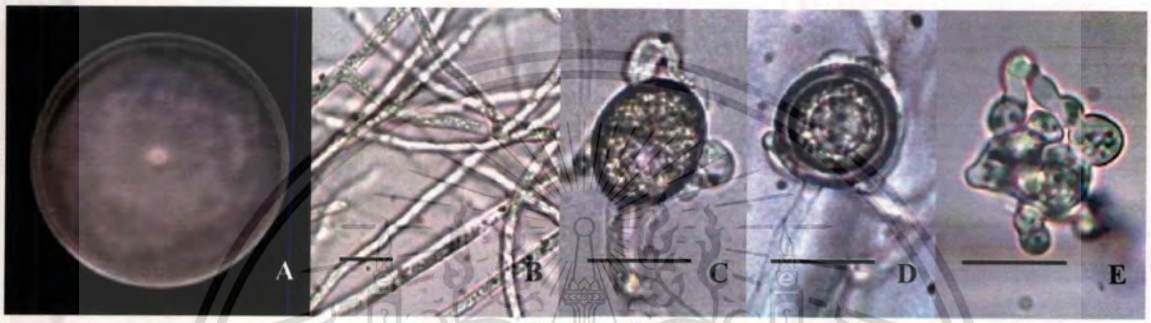

Fig.4.18 *Pythium graminicola* PT108. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

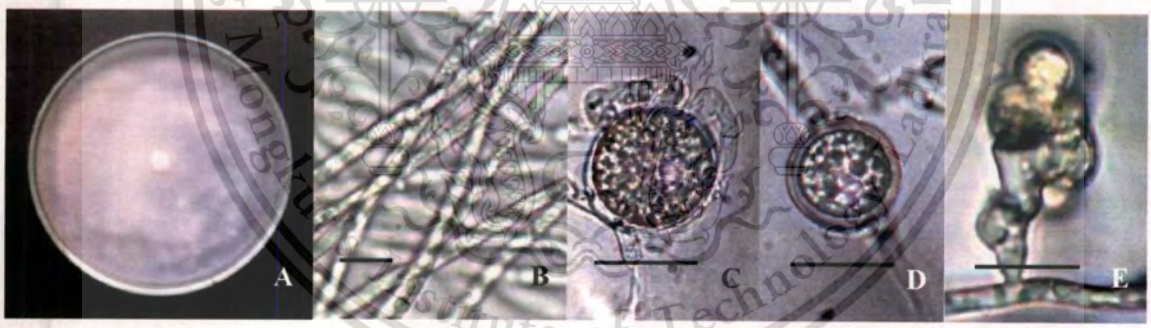

Fig.4.19 *Pythium graminicola* PT301. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

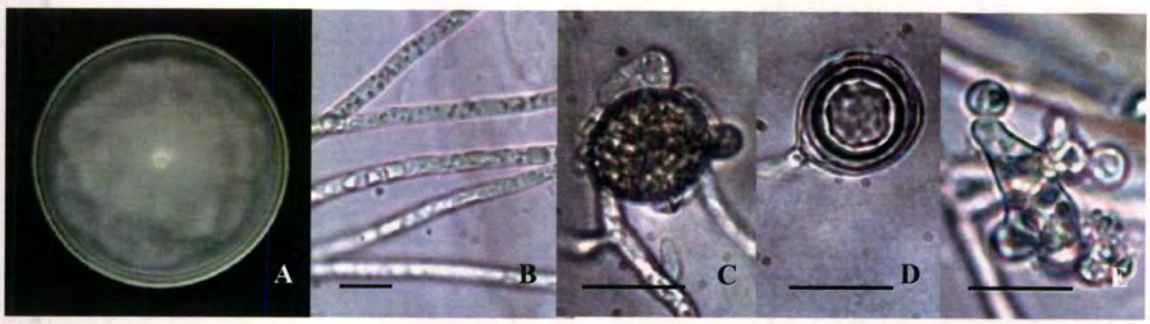

Fig.4.20 *Pythium graminicola* PT501. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

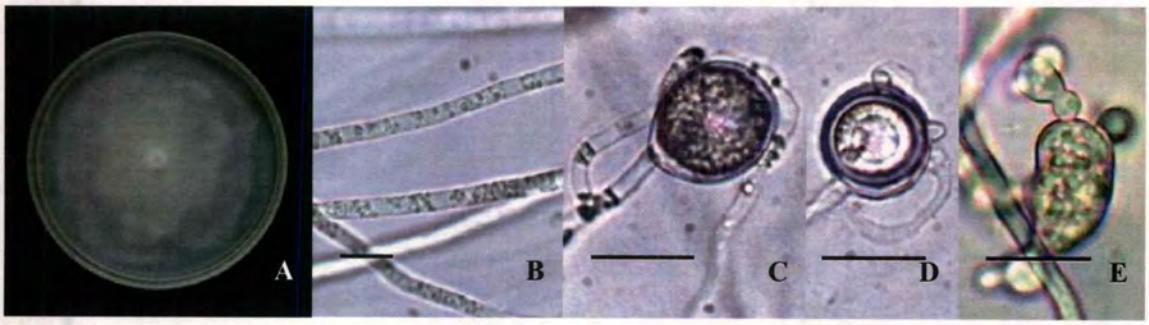

Fig.4.21 *Pythium graminicola* PT701. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

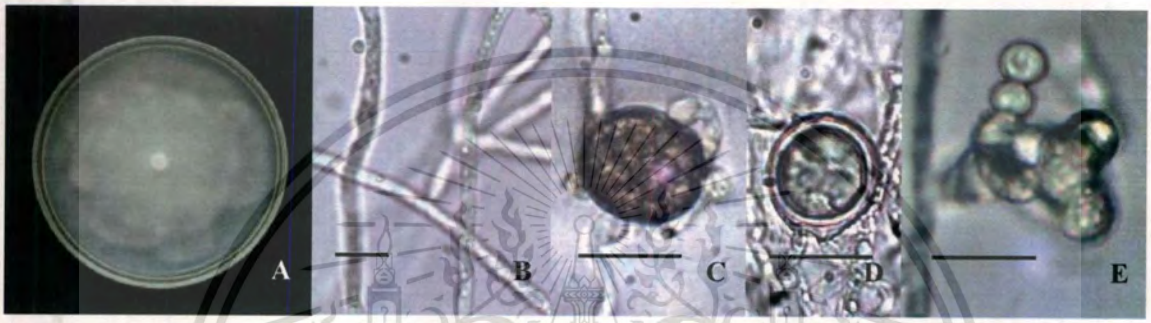

Fig.4.22 *Pythium graminicola* PT901. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

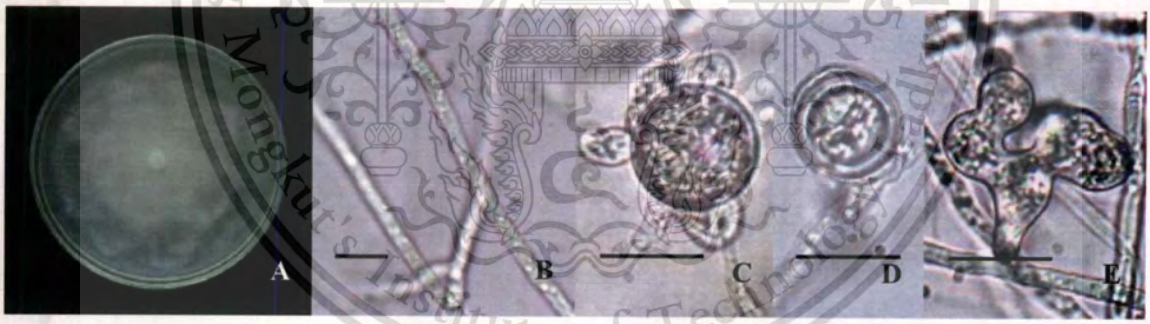

Fig.4.23 *Pythium graminicola* PT205. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

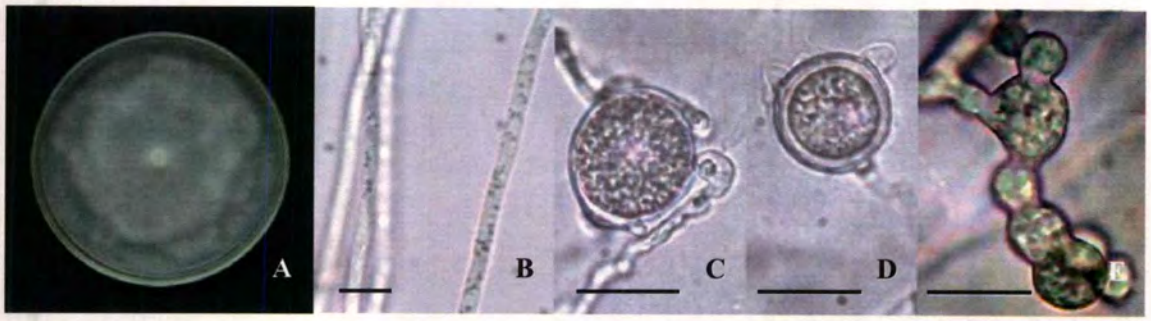

Fig.4.24 *Pythium graminicola* PT206. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

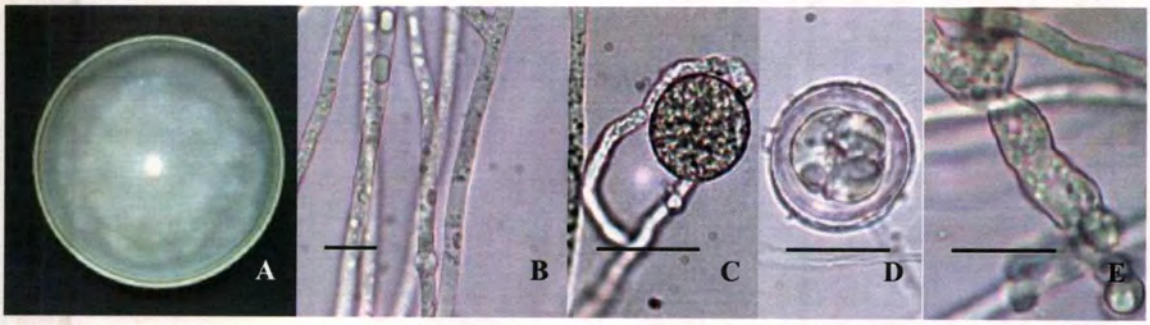

Fig.4.25 *Pythium graminicola* PT207. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

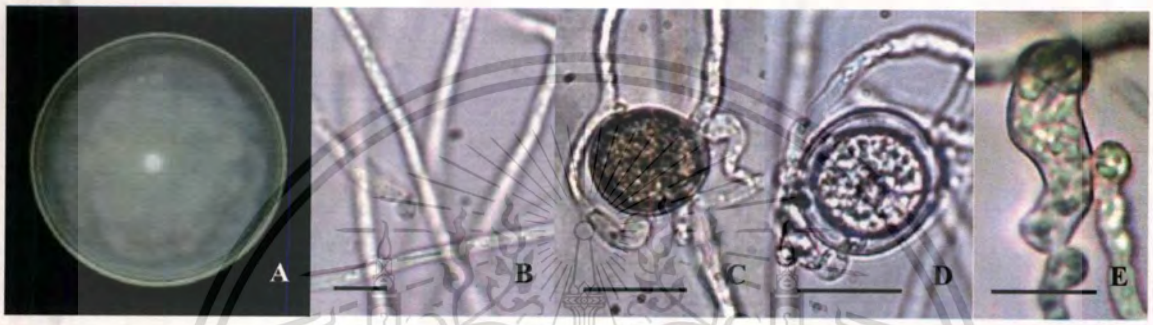

Fig.4.26 *Pythium graminicola* PT304. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

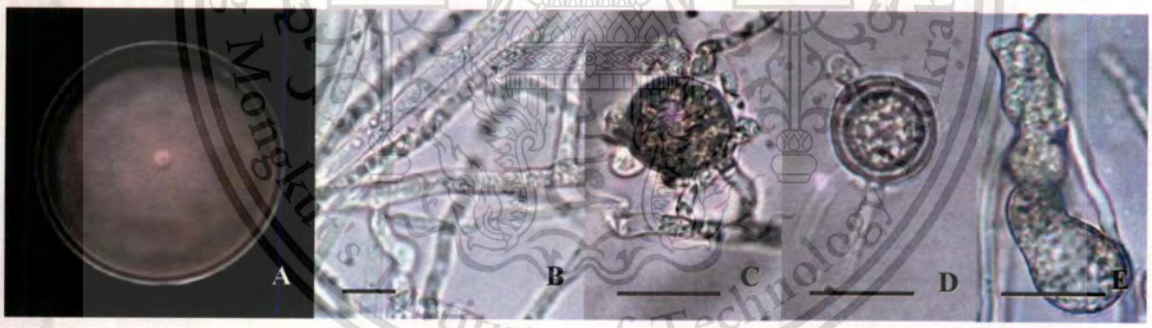

Fig.4.27 *Pythium graminicola* PT401. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

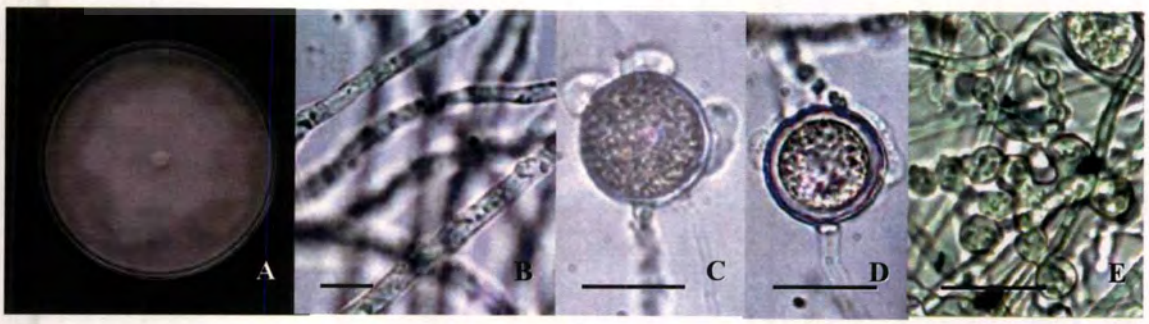

Fig.4.28 *Pythium graminicola* RY201. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

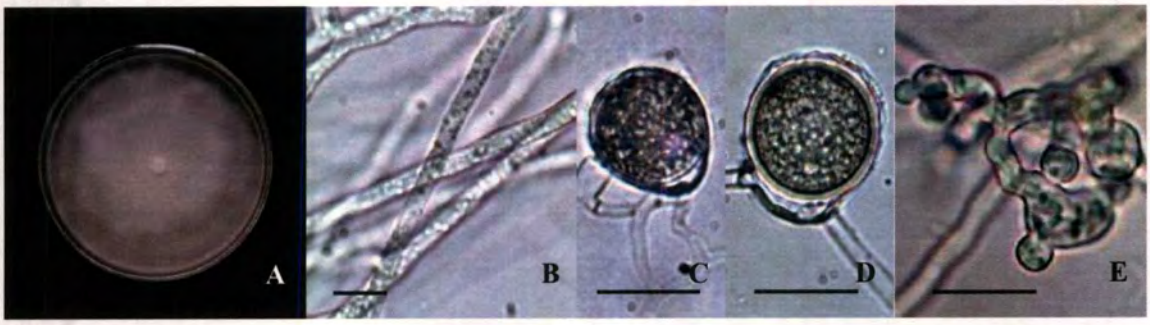

Fig.4.29 *Pythium graminicola* RY301. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

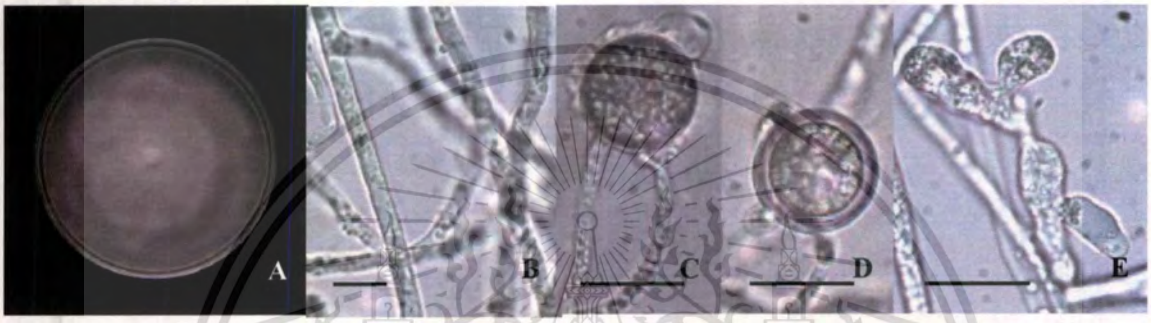

Fig.4.30 *Pythium graminicola* RY302. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

Fig.4.31 *Pythium graminicola* RY501. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

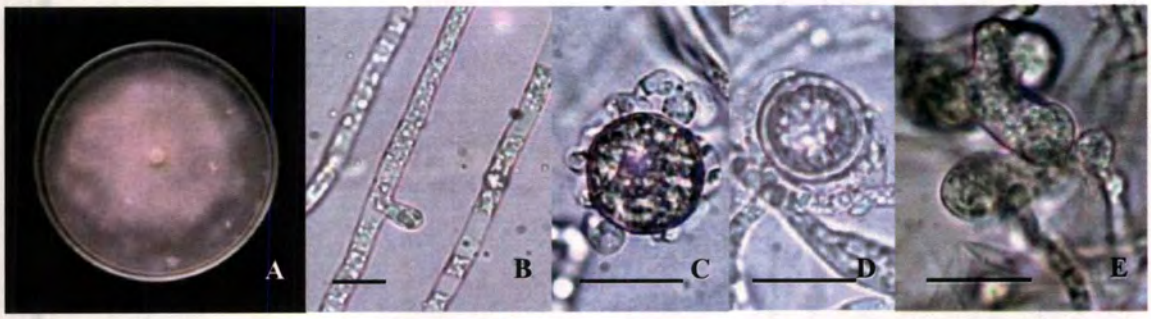

Fig.4.32. *Pythium graminicola* RY502. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

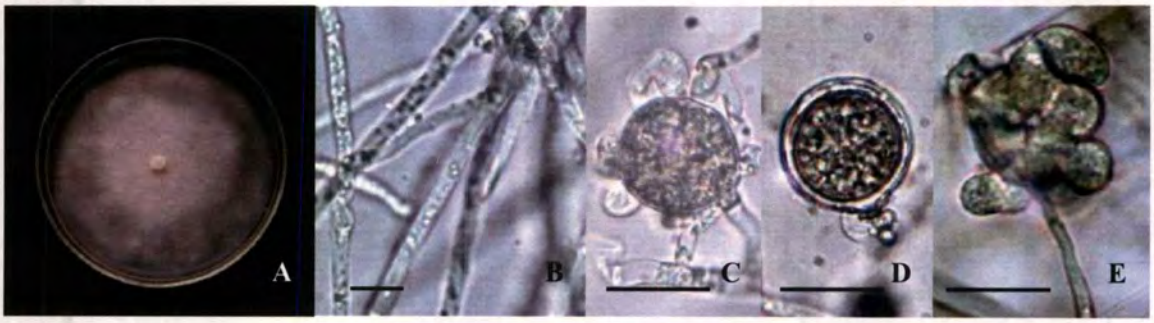

Fig.4.33 *Pythium graminicola* RY503. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

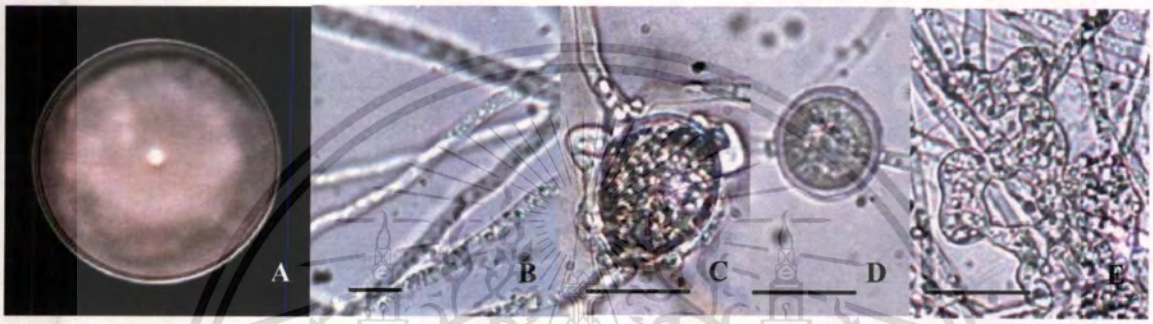

Fig.4.34 *Pythium graminicola* RY504. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

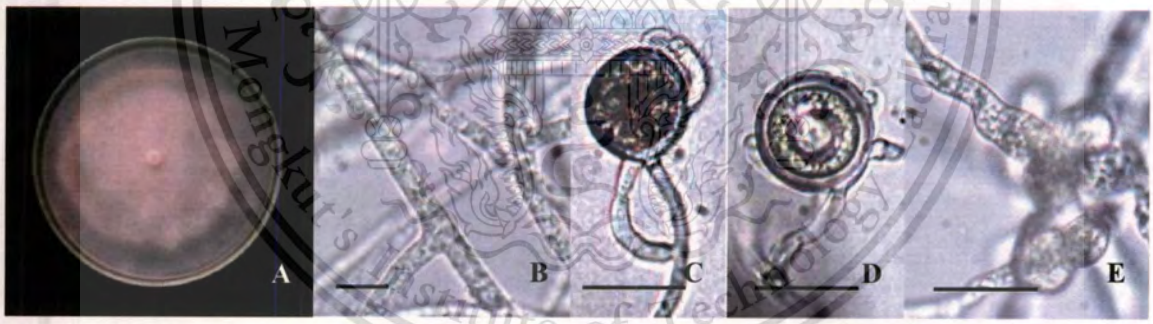

Fig.4.35 *Pythium graminicola* RY505. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

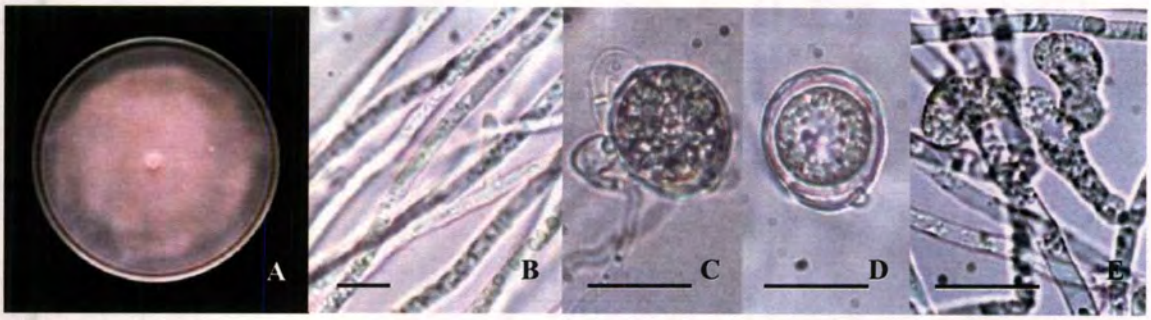

Fig.4.36 *Pythium graminicola* RY601. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

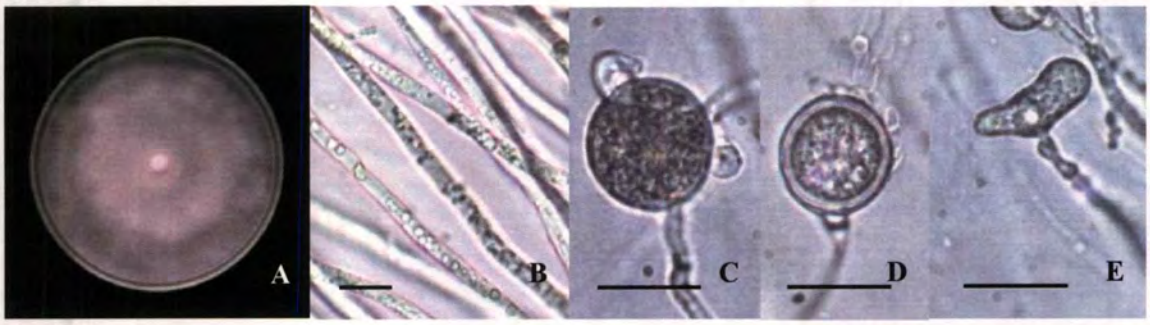

Fig.4.37. *Pythium graminicola* RY603. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

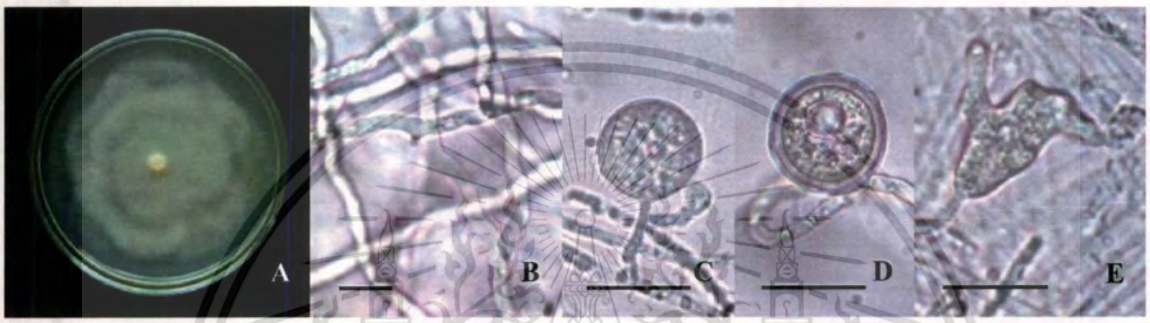

Fig.4.38 *Pythium graminicola* RY701. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

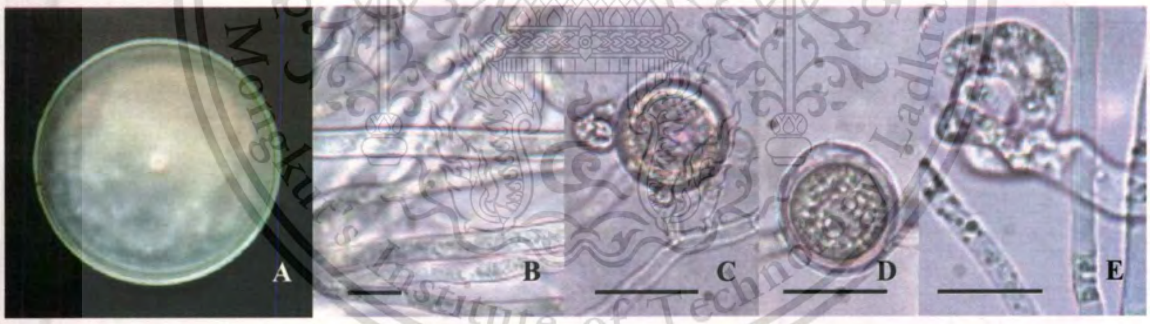

Fig.4.39 *Pythium graminicola* PB108. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

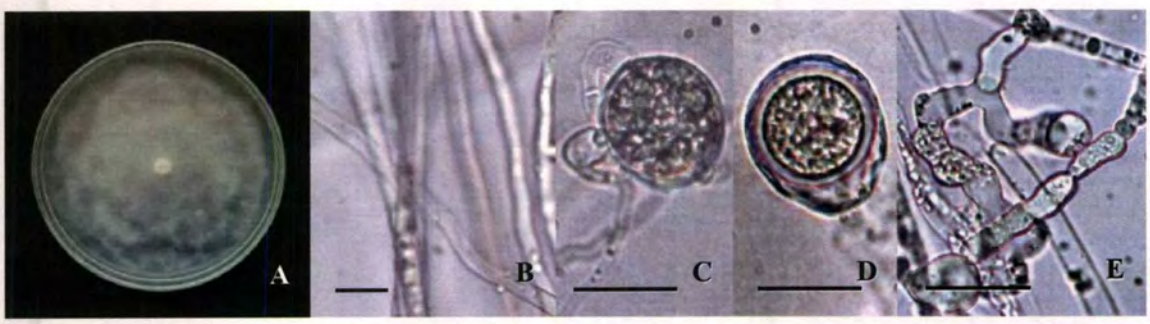

Fig.4.40 *Pythium graminicola* PB201. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

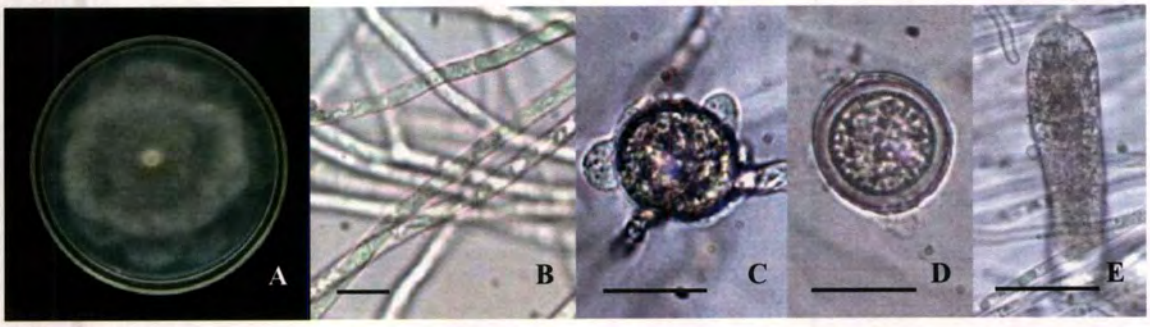

Fig.4.41 *Pythium graminicola* PB202. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

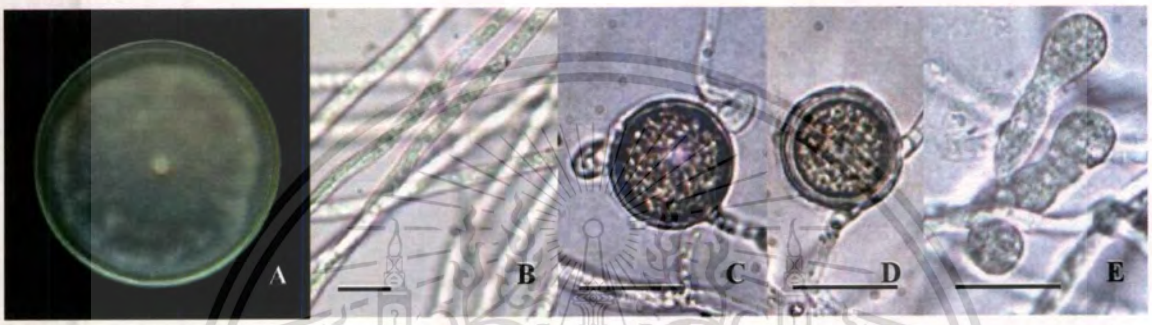

Fig.4.42 *Pythium graminicola* PB206. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

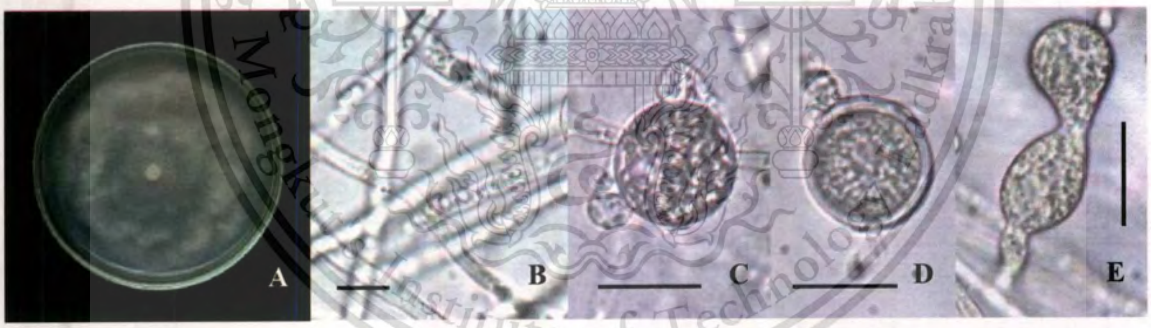

Fig.4.43 *Pythium graminicola* PB301. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

Fig.4.44 *Pythium graminicola* PB302. A. 3-day-old culture on PDA, B. mycelia, E. oogonium, D. oospore, E. sporangium, bars. B=10 µm, C,D,E=25 µm.

Forty-four isolates were identified by morphological characters and yielded 15 isolates belong to *P. aphanidermatum* and 29 isolates belong to *P. graminicola.* Seven isolates of *P. aphanidermatum* (random selection) and 29 isolates of *P. graminicola* were studied to confirm morphological identification by using their DNA sequences of ITS region including 5.8S. Moreover, phylogenetic tree was constructed in order to compare of different diversity of fungi.

Seven isolates of *P. aphanidermatum* and 29 isolates of *P. graminicola* were taken to extract DNA. Their ITS region were sequenced to compare with other species of *Pythium* retrieved from GenBank (Table 4.4). BLAST searches 7 isolates of *P. aphanidermatum* and 29 isolates of *P. graminicola* with other *Pythium* species in GenBank confirmed the *P. aphanidermatum* and *P. graminicola* identification. Phylogenetic tree was presented in Fig.4.45. *Phytophthora cinnamomi* and *P. parasitica* were used as outgroup in the analysis. The parsimony analysis generated 2 major clades representing the *Pythium* species which correlated with morphological characters. The clades were designed as A and B which clade A was described into subclade A1 and A2 as follows:-

Clade A

This clade consisted of *P. periplocum, P. amasculum P. hydnosporum, P. myriotylum, P. tracheiphilum, P. porphyrae, P. deliense, P. aphanidermatum, P. graminicola, P. vanterpoolii, P. volutum, P. arrhenomanes, P. phragmitis, P. catenulatum, P. torulosum P. folliculosum, P. flevoense, P. pachycaule, P. marinum* and *P. diclinum.* The species in this clade were characterized by filamentous sporangia.

Subclade A1

The species that belonged to Subclade A were *P. periplocum, P. hydnosporum* and *P. amasculum.* The species in this subclade were characterized by oogonia ornamented with acute spines.

Subclade A2

The species in this subclade were *P. tracheiphilum, P. porphyrae, P. deliense, P. aphanidermatum, P. graminicola, P. vanterpoolii, P. volutum, P. arrhenomanes, P. phragmitis, P. catenulatum, P. torulosum P. folliculosum, P. flevoense, P. pachycaule, P. marinum* and *P. diclinum.* Species of subclade A2 have spherical oogonia.

Clade B

The species that belonged to clade B were *P. hypogynum, P. erinaceum, P. parvum, P. minus, P. irregulare, P. kunmingense, P. spinosum, P. nagaii, P. violae, P. paddicum,*

P. uncinulatum, *P. orthogonon* and *P. Acanthophoron*. Species of clade B have globose sporangia and spherical oogonia.

The 29 isolates of *P. graminicola* were not shown genetic variation within species whereas 7 isolates of *P. aphanidermatum* were found that isolate PT305 was the most distant species relative to the other isolates.

Table 4.4 List of *Pythium* sequences from GenBank used in this study.

Pythium species*	Host/substrate	Locality	CBS no.	ITS GenBank accession No.
P. acanthophoron	*Ananas sativus*	Hawaii, USA	CBS 337.29	AY598711
P. amasculinum	soil	China	CBS 552.88	AY598671
P. aphanidermatum	-	Japan	-	AB274404
P. aphanidermatum	vegitable soil	Japan	-	AB355599
P. aphanidermatum	-	South Africa	-	FJ415896
P. arrhenomanes	*Zea mays*	USA	-	AY858635
P. catenulatum	Turf grass	USA	CBS 842.68	AY598675
P. deliense	*Nicotiana tabacum*	Indoniasia	CBS 314.33	AY598674
P. diclinum	*Beta vulgaris*	The Netherland	CBS 664.79	AY598690
P. erinaceum	Wheatfield soil	New Zealand	CBS 505.80	AY598694
P. flevoense	soil	The Netherland	CBS 234.72	AY598691
P. folliculosum	soil	Switzerland	CBS 220.94	AY598676
P. graminicola	*Saccharum officinarum*	Jamaica	CBS 327.62	AY598625
P. graminicola	unknown	unknown	-	AY243091
P. hydnosporum	unknown	Germany	CBS 253.60	AY598672
P. hypogynum	soil	France	CBS 234.94	AY598693
P. irregulare	*Phaseolus vulgaris*	The Netherlands	CBS 250.28	AY598702
P. kunmingense	soil	China	CBS 550.88	AY598700
P. marinum	soil	UK	CBS 750.96	AY598689
P. minus	soil	UK	CBS 226.88	AY598698
P. myriotylum	*Arachis hypogaea*	Israel	CBS 254.70	AY598678
P. nagaii	soil	UK	CBS 779.96	AY598705
P. orthogonon	Zea mays	Lebanon	CBS 376.72	AY598710
P. pachycaule	soil	UK	CBS 227.88	AY598687
P. paddicum	*Triticum* sp. and *Hordeum* sp.	Japan	CBS 698.83	AY598707
P. parvum	soil	UK	CBS 225.88	AY598697
P. phragmitis	*Phragmites australis*	Germay	-	AY594259

Table 4.4 (Continued) List of *Pythium* sequences from GenBank used in this study.

Pythium species*	Host/substrate	Locality	CBS no.	ITS GenBank accession No.
P. periplocum	*Citrullus vulgaris*	USA	CBS 289.31	AY598670
P. periilum	soil	USA	169.68	AY598683
P. porphyrae	*Porphyra yezoensis*	Japan	CBS 369.79	AY598673
P. spinosum	compost	The Netherlands	CBS 275.67	AY598701
P. torulosum	unknown	unknown	-	AB095046
P. tracheiphilum	*Lactuca sativa*	Italy	CBS 323.65	AY598677
P. uncinulatum	*Lactuca sativa*	The Netherlands	CBS 518.77	AY598712
P. vanterpoolii	*Triticum sativum*	UK	CBS 295.37	AY598685
P. violae	soil	Australia	CBS 159.64	AY598706
P. volutum	*Triticum* sp. and *Hordeum* sp.	Janpan	CBS 699.83	AY598686
Phytophthora cinnamomi	unknown	unknown	-	FJ801920
Phytophthora parasitica	unknown	unknown	-	AY773100

* ITS sequences of *Pythium* species were retrieved from GenBank

4.1.3 Pathogenicity Tests

Preliminaly tests were done using detached leaves method. All 44 isolates of *Pythium aphanidermatum* and *P. graminicola* were proved to be pathogenic to pineapple variety Pattavia. Comparison between control (non-inoculated) and all isolates indicated that all tested isolates caused lesion on detached leaves. The lesion diameters on detached leaves after 5 days inoculation of all tested isolates were recorded as shown on Fig.4.46 and Table 4.5. The lesion diameters on pineapple leaves caused by *P. aphanidermatum* isolates RY801, RY802, RY803 and RY804 were 7.20, 7.09, 7.26 and 7.08, respectively which were not statistically different and they were significantly higher than other isolates at P=0.05 whereas control leaves did not show any lesions. Therefore, these 4 isolates were selected for pathogenicity test by root inoculation for finding the most aggressive isolate. Disease severity after 2 weeks inoculation of *P. aphanidermatum* isolates RY801, RY802, RY803 and RY804 were 2.75, 3.25, 5.00 and 4.50, respectively (Table 4.6 and Fig.4.47). Root rot caused by *P. aphanidermatum* RY803 was significantly higher disease severity than the others at P=0.01 while control did not show any symptom. Therefore, *P. aphanidermatum* RY803 was re-isolated from infected pineapple root and selected for further study.

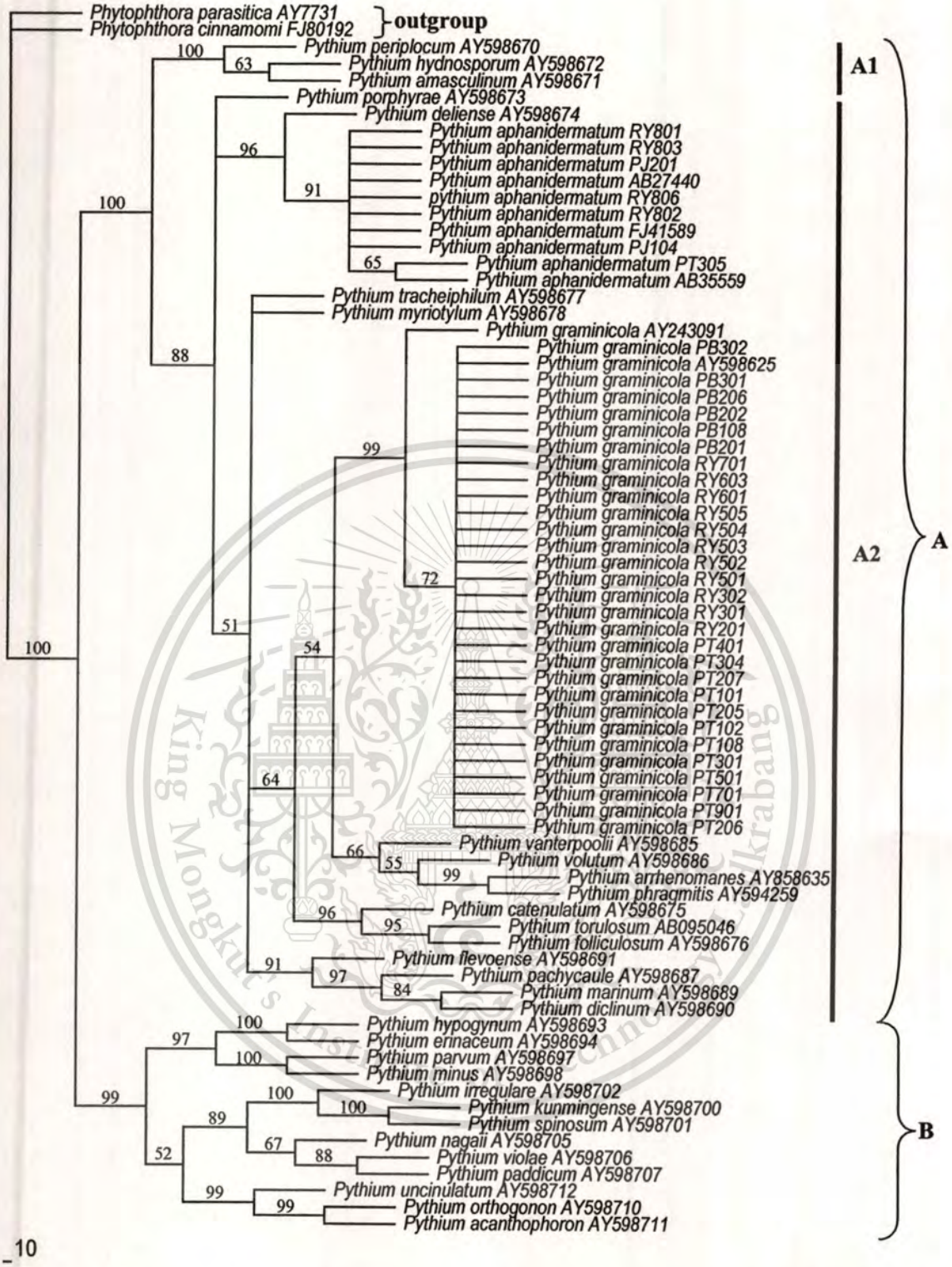

Fig.4.45 Phylogeny of *Pythium* species based on the ITS region including 5.8S of nuclear rDNA. The tree was generated from maximum parsimony analysis using the heuristic search algorithm of PAUP* 4.0b8. Numbers of the branches indicated the bootstrap values resulting from 1000 bootstrap replications. Designated outgroup was *Phytophthora parasitica* and *P. cinnamomi*.

Table 4.5 Pathogenicity test of all isolates using detached leaves method.

Collection sites	Locations	Obtained isolates	Lesion diameter[y] (cm)
control	-	-	0 l
Phatthalung province	Pabon	*Pythium graminicola* PT101	0.91 ghijk
		Pythium graminicola PT102	1.00 ghi
		Pythium graminicola PT108	1.01 ghi
		Pythium graminicola PT301	0.89 ghijk
		Pythium graminicola PT501	0.92 ghijk
		Pythium graminicola PT701	0.94 ghijk
		Pythium graminicola PT901	0.95 ghijk
	Pabak	*Pythium graminicola* PT205	1.00 ghi
		Pythium graminicola PT206	0.80 hijk
		Pythium graminicola PT207	0.97 ghij
	Pabon	*Pythium graminicola* PT304	0.92 ghijk
		Pythium aphanidermatum PT305	3.14 d
		Pythium aphanidermatum PT307	2.24 e
		Pythium graminicola PT401	1.03 ghi
Rayong province	Nicomphatthana	*Pythium graminicola* RY201	0.97 ghij
		Pythium graminicola RY301	0.94 ghijk
		Pythium graminicola RY302	0.93 ghijk
		Pythium graminicola RY501	1.00 ghi
		Pythium graminicola RY502	1.17 fgh
		Pythium graminicola RY503	0.83 ghijk
		Pythium graminicola RY504	0.75 hijk
		Pythium graminicola RY505	0.81 hijk
	Pluakdeang	*Pythium graminicola* RY601	1.03 ghi
		Pythium graminicola RY603	0.89 ghijk
		Pythium graminicola RY701	0.52 jk
	Nicomphatthana	*Pythium aphanidermatum* RY801	7.20 a
		Pythium aphanidermatum RY802	7.09 a
		Pythium aphanidermatum RY803	7.26 a
		Pythium aphanidermatum RY804	7.08 a
		Pythium aphanidermatum RY805	4.69 b
		Pythium aphanidermatum RY806	3.28 d
		Pythium aphanidermatum RY807	3.68 c
		Pythium aphanidermatum RY808	1.27 fg
Prachuap Khiri Khan province	Aownoiy	*Pythium aphanidermatum* PJ104	0.96 ghij
		Pythium aphanidermatum PJ106	0.83 ghijk
		Pythium aphanidermatum PJ108	1.03 ghi
		Pythium aphanidermatum PJ201	1.04 ghi
		Pythium aphanidermatum PJ202	1.49 f
Petchaburi province	Cha-am	*Pythium graminicola* PB108	0.87 ghijk
		Pythium graminicola PB201	1.00 ghi
		Pythium graminicola PB202	0.50 k
		Pythium graminicola PB206	0.51 jk
		Pythium graminicola PB301	0.79 hijk
		Pythium graminicola PB302	0.59 ijk

[y]Average of four replications. Means followed by a common letter in the column were not significantly different (P=0.05) by DMRT.

Fig.4.46 Pathogenicity test of all isolates using detached leaves method.

Table 4.6 Pathogenicity test of four aggressive isolates by root inoculation.

Isolates	disease severity index (DSI)[1/]
P. aphanidermatum RY801	2.75 b
P. aphanidermatum RY802	3.25 b
P. aphanidermatum RY803	5.00 a
P. aphanidermatum RY804	4.50 ab
control	1.00 c

[1/] disease severity index of pineapple root rot, 1 = no root rot, 2= 1-25% root rot, 3=26-50% root rot, 4=51-75% root rot and 5=76-100% root rot (modified from Ahmed *et al.*,1999). Average of four replications. Means followed by a common letter in the column were not significantly different (P=0.01) by Duncan's Multiple Range Test.

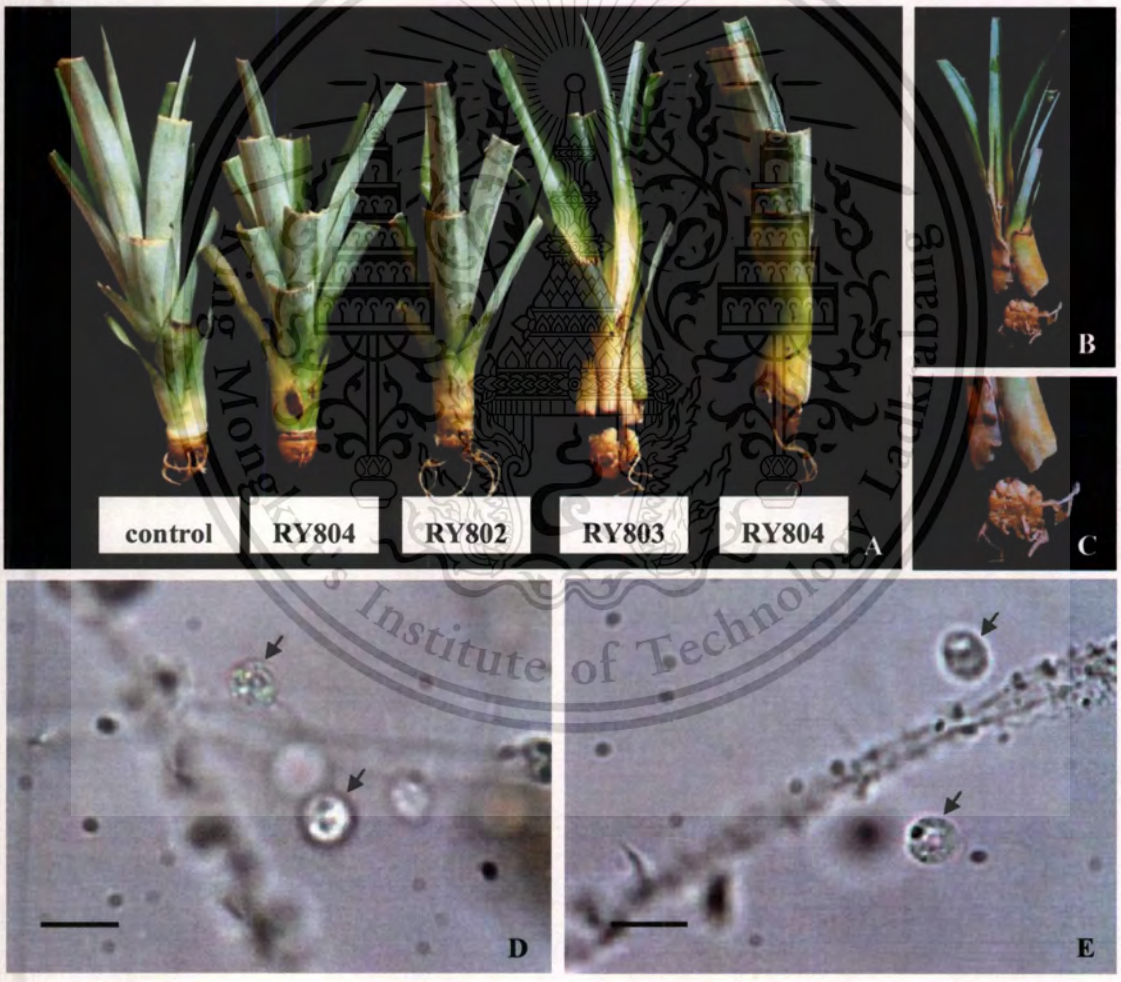

Fig.4.47 Pathogenicity test of four aggressive isolates by root inoculation.

A. = four isolates of pathogen and control, B and C. = RY803, D and E zoospore cysts of RY803 germinating towards pineapple roots. Bars D,E = 10 μm.

4.2 Screening for Antagonistic Fungi and Antagonistic Substances Against the Pathogen Causing Pineapple Root Rot.

4.2.1. Isolation and Identification of Antagonists from Soil

In total of 42 promising antagonist isolates were encountered from soil by baiting and soil plate techniques yielded 29 isolates from baiting technique and 13 isolates from soil plate technique (Table 4.7). Species were encountered by baiting technique included *Chaetomium aureum* strain MB103, MB601, MB603, MB608 and RY102, *C. bostrychodes* strain PR101, PR102, PR103 and NB701, *C. basiliense* strain AM101, *C. carinthiacum* strain NB501, *C. cochliodes* strain RY301, *C. cupreum* strain NB102, RY201, RY202 and RY204, *C. flavigenum* strain MB402, MB604, MB606, MB607 and MB611, *C. fusiforme* strain NB401 and MB605, *C. perlucidum* strain NB202 and MB501 and *C. succineum* strain MB304 and MB305. Species were encountered by soil plate technique included *Aspergillus niger* strain AP101, *C. cupreum* strain SO101, *Emericella nidulans* strain EN101, *E. rugulosa* strain ER101, *Eurotium chevalieri* strain EU101, *Gliocladium* catenulatum strain RY102, RY109 and RY111, *Penicillium janthinellum* strain PN101, *Trichoderma hamatum* strain PT101 and *T. harzianum* strain RY101, RY104 and RY112. The fungal names were corrected by searching from the Index Fungorum database (www.indexfugorum.org/Names/names.asp). Morphological features of all isolates were described and illustrated as follows:-

Aspergillus niger Tiegh., Annls Sci. Nat., Bot., sér. 5 8: 240 (1867) Fig. 4.48

Colonies on PDA at roomtemperature are initially white, quickly becoming black with conidial production. Hyphae are septate and hyaline. Conidiophores are long, smooth and hyaline. Conidial heads are large (up to 300-2000 μm) with long metulae and short phialides covering the entire vesicle. Conidia are brown to black, rough, globose and measure 3.5-5 μm in diameter.

Specimens examined: Thailand, Rayong, isolated from soil under pineapple plantation, 20 August 2007, Chaninun Pornsuriya AP101. The isolate was deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Table 4.7 Promising antagonists isolated from soil in pineapple plantation at different locations in Thailand.

Methods	Species	Isolates	
		Phatthalung	**Rayong**
baiting	*Chaetomium aureum*	MB103, MB601, MB603, MB608,	RY102
	Chaetomium bostrychodes	NB701, PR101, PR102, PR103	-
	Chaetomium brasiliense	-	AM101
	Chaetomium carinthiacum	NB501	-
	Chaetomium cochliodes	-	RY301
	Chaetomium cupreum	NB102	RY201, RY202, RY204
	Chaetomium flavigenum	MB402, MB604, MB606, MB607, MB611	-
	Chaetomium fusiforme	MB605, NB401	-
	Chaetomium perlucidum	MB501, NB202	-
	Chaetomium siamense sp. nov.	MB303, MB502	-
	Chaetomium succineum	MB304, MB305	-
soil plate	*Aspergillus niger*	AP101	-
	Chaetomium cupreum	SO101	-
	Emericella nidulans	-	EN101
	Emericella rugulosa	-	ER101
	Eurotium chevalieri	EU101	-
	Gliocladium catenulatum	-	RY102, RY109, RY111
	Penicillium janthinellum	PN101	-
	Trichoderma hamatum	PT101	-
	Trichoderma harzianum	-	RY101, RY104,RY112

Chaetomium aureum Chivers, Proc. Amer. Acad. Arts & Sci. 48: 86 (1912) Figs. 4.49-4.53

Young colonies usually are white by aerial mycelium. Mature colonies become red by a red pigment exudate. Ascomata are pale green, ovate in shape, 78.5-142.6 x 90.6-180.3 µm. Ascomatal hairs arcuate, septate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are elliptical, 5.0-7.0 x 9.0-12.4 µm, with two apical germ pores.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya MB103, MB601, MB603, MB608; Thailand, Rayong, isolated from soil under pineapple plantation, 20 August 2007, Chaninun Pornsuriya RY102. The isolates were deposited in Biocontrol Research Unit and Mycology Section (KMITL).

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

Figs.4.54-4.57

Colonies are rapidly growing, young colonies usually are white by aerial mycelium, occasionally with a purple pigment exudate. Mature colonies become green to brown with ascomata. Ascomata are olivaceous, maturing within 10-14 days, dark green to brown when old, ovate in shape, 190.2-349.8 x 272-419.8 µm. Ascomatal hairs usually spirally coiled. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are lemoniform, 7.5-9.9 x 8.6-11.3 µm, with an apical germ pore.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya NB701, PR101, PR102, PR103. The isolates were deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Chaetomium brasiliense Batista & Pontual, Bol.Agr.Pernambuco 15:70 (1948) Fig. 4.58

Colonies are moderately growing on PDA, reaching a diameter of 5-6 cm in 10 days, with floccose airial mycelium, becoming dark due to pigmented. Mycelium are grey or nearly white, reverse black when old. Ascomata are superficial or spherical, ostiolate, 80-105.9 x 121.0-200.0 µm, with a dark wall of angular cell. Ascomatal hairs are flexuous, undulate or spirally coiled, septate, dark brown, verrucose or warty. Asci fasciculate, cylindrical, short stalk, with 8 ascospores. Ascospores ovate, bilaterally flattened, dark brown when mature, 5-6 x 6-7 x 7-8.5 µm, with a germ pore at the attenuated end.

Specimens examined: Thailand, Rayong, isolated from soil under pineapple plantation, 20 August 2007, Chaninun Pornsuriya AM101. The isolate was deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Chaetomium carinthiacum Sörgel, Arch. Mikrobiol. 40(4): 393 (1961) Fig. 4.59

Colonies usually are white by aerial mycelium, without a pigment exudate. Mature colonies become green to brown with ascomata. Ascomata are olivaceous, maturing within 10-14 days, dark green when old, ovate in shape, 96.1-146.4x101.8-153.2 µm. Ascomatal hairs irregularly sinuous with roughened hairs. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are elliptical, 4.3-6.2x8.4-11.6 µm, with an apical germ pore.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya NB501. The isolate was deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Chaetomium cochlioides Palliser, North American Flora. 3 (1): 61 (1910) Fig. 4.60

Colonies are rapidly growing, young colonies usually are white by aerial mycelium, occasionally with a purple pigment exudate. Mature colonies become green to brown with ascomata. Ascomata are olivaceous, maturing within 10-14 days, dark green to brown when old, ovate in shape, 107.1-143.1x122.0-209.2 µm. Ascomatal hairs usually irregularly sinuous. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are broadly ovate to lemon-shaped, 4.2-5.9x7.1-10.0 µm, with an apical germ pore.

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

Chaetomium cupreum Ames, Mycologia 41(6): 642 (1949) Figs. 4.61-4.65

Colonies usually are red due to a red pigment exudate. Ascomata are red, maturing within 10-14 days, ovate in shape, 79.7-142.7 x 94.7-151.5 µm. Ascomatal hairs arcuate, apically circinate or coiled, septate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are reniform, 4.7-6.7 x 6.7-10.0 µm, with a single apical germ pore.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya NB102, SO101; Thailand, Rayong, isolated

from soil under pineapple plantation, 20 August 2007, Chaninun Pornsuriya RY201, RY202, RY204. The isolates were deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Chaetomium flavigenum van Warmelo, Mycologia 58: 847 (1966) Figs. 4.66-4.70

Colonies usually are white by aerial mycelium, becoming red or orange due to a red pigment exudate. Mature colonies become green to brown with ascomata. Ascomata are olivaceous to brown, maturing within 10-14 days, dark grey-green when old, ovate in shape, 92.5-134.9 x 113.2-190.3 µm. Ascomatal hairs arcuate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are fusiform, 4.0-6.3 x 6.6-11.2 µm, with two apical germ pores.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya MB402, MB604, MB606, MB607, MB611. The isolates were deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Chaetomium fusiforme Chivers, Proc. Amer. Acad. Arts & Sci. 48: 87 (1912) Figs.4.71-4.72

Colonies usually yellow due to yellow pigment exudates. Ascomata are olivaceous grey, maturing within 10-14 days, ovate in shape, 75.4-161.4x110.2-202.8 µm. Ascomatal hairs arcuate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are fusiform, 5.1-6.9x12.7-17.7 µm, with two apical germ pores.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya MB605, NB401. The isolates were deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Chaetomium perlucidum Sergeeva, Notul. Syst. Sect. Crypt. Inst. Bot. Acad. Sci. U.S.S.R. 11: 108 (1956) Figs. 4.73-4.74

Colonies usually are white or greylish by aerial mycelium, without a pigment exudate. Mature colonies dark grey to black with ascomata. Ascomata are grey, ovate in shape, 91.9-145.5x120.1-190.6 µm. Ascomatal hairs undulate and irregularly sinuous. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are elliptical, 3.4-5.5x6.7-9.2 µm, with an apical germ pore.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya MB501, NB202. The isolates were deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Chaetomium succineum Ames, Mycologia 41(6): 645 (1949) Figs. 4.75-4.76

Colonies usually are dark green or greylish by aerial mycelium, without a pigment exudate. Mature colonies dark grey to black with ascomata. Ascomata are grey, ovate in shape, 107.1-143.1x122.0-209.2 µm. Ascomatal hairs loosely hairs. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are elliptical, 4.2-5.9x7.1-10.0 µm, with an apical germ pore.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya MB304, MB305. The isolates were deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Emericella nidulans (Eidam) Vuil1., Seances Acad. sci. Paris 184: 137 (1927) Fig. 4.77

Anamorph: *Aspergillus nidulans* (Eidam) Winter, Rabenh. Krypt.-Fl. (Leipzig) 1(2): 62 (1884)

Colonies diameters on PDA are 5-6.0 cm in 2 weeks at roomtemperature, green from conidia becoming brownish from ascomata. Ascomata (Cleistothecia) are abundant, dull yellow globose, 122.0-345.0 µm in diameter, surrounded by hülle cells. Hülle cells ellipsoidal to globose, 9.0-20.0 µm in diameter. Asci are globose to subglobose, 8-spored. Ascospores red to purple, smooth, 2.8-4.2 × 3.5-5.7 µm, with 2 narrow longitudinal flanges, with entire margins.

Specimens examined: Thailand, Rayong, isolated from soil under pineapple plantation, 20 August 2007, Chaninun Pornsuriya EN101. The isolate was deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Emericella rugulosa (Thom & Raper) C.R. Benj., Mycologia 47(5): 680 (1955) Fig. 4.78

Anamorph: *Aspergillus rugulovalvus*, formerly *A. rugulosus* Thom & Raper, Mycologia 31: 660 (1939)

Colonies diameters on PDA are 5-6.0 cm in 2 weeks at roomtemperature, green to dark green in colors from conidia. Conidia are globose, rugulose, 3-4 µm in diameter. Conidiophores smooth-walled, pale brownish, 50-80 µm, conidial heads columnar. Colonies become brownish from ascomata. Ascomata (Cleistothecia) are abundant, dull yellow globose, 250.0-400.0 µm in diameter, surrounded by dark brown, globose hülle cells. Hülle cells ellipsoidal to globose, 9.5-

32.0 µm in diameter. Asci are globose to subglobose, 8-spored. Ascospores are purple-red, lenticular, rugulose, with two sinuate equatorial crests, 2.5-4.0 × 3.0-4.5 µm.

Specimens examined: Thailand, Rayong, isolated from soil under pineapple plantation, 20 August 2007, Chaninun Pornsuriya ER101. The isolate was deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Eurotium chevalieri Mangin, Ann. Sci. Nat., Bot.,Sér. 9, 10: 361 (1909) Fig. 4.79

Anamorph: *Aspergillus chevalieri* (Mangin) Thom & Church, The Aspergillus 1-272 (1926)

Colony diameters on PDA 2.8-3.2 cm in 2 weeks roomtemperature; conidial heads radiate, green to blue-green; ascomata (Cleistothecia) white to yellow, subglobose to globose, up to 178 µm wide, asci 8-spored, spherical to subspherical, ascospores lenticular, with wall smooth to finely roughened, with 2 distinct longitudinal trough flanges, 4.2-5.4 × 3.3-4.0 µm.

Specimens examined: Thailand, Rayong, isolated from soil under pineapple plantation, 20 August 2007, Chaninun Pornsuriya EU101. The isolate was deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Gliocladium catenulatum Gilman & Abbott, Journal of Iowa State College, Sci. 1(3): 303 (1927) Figs. 4.80-4.82

Colony are fast growing on PDA, reaching a diameter of 5-6 cm in 3 days, pale to olive-green. Conidiophores penicillate, rather irregularly branched predominating, but primary verticillate. Conidiophores also present. Phialides of penicillate mostly 12-17 µm long and approximately 2.5 µm wide in the basal part. Conidia ellipsoidal, bilaterally symmetrical, with an oblique scar of attachment, mostly 5.0-6.5 X 2.5-3.0 µm.

Specimens examined: Thailand, Rayong, isolated from soil under pineapple plantation, 20 August 2007, Chaninun Pornsuriya RY102, RY109, RY111. The isolates were deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Penicillium janthinellum Biourge, La Cellule 33(1): 258 (1923) Fig. 4.83

Colonies are moderately slow-growing, initially white and become green to blue green. Hyphae are septate, hyaline, 2.5 to 3.0 µm in diameter, conidiophores with few divergent branches, smooth-walled. Conidia are subglobose, mostly 2.5-3 µm in diameter.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya PN101. The isolate was deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Trichoderma hamatum (Bonord.) Bainier, Bull. Soc. mycol. Fr. 22: 131 (1906) Fig.4.84

Colonies are fast growing, initially white and become yellow due to yellow exudate. Conidiophores are mostly curled sterile, branches. Phialides are particularly broad, 3-4 µm wide. Conidia are short-cylindrical, green, smooth-walled, 2.5-3.5 µm in diameter, produced successively from tips of phialides.

Specimens examined: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya PT101. The isolate was deposited in Biocontrol Research Unit and Mycology Section (KMITL).

Trichoderma harzianum Rifai, Mycol. Pap. 116: 38 (1969) Figs. 4.85-4.87

Colonies grow rapidly on PDA, initially glassy-white, becoming green tufts of sporulation. Hyphae hyaline, 1.5-12.0 µm wide. Conidiophores are erect and produce side branches bearing whorls of short phialides. Conidia subglobose to ovoidal, lacking a visible basal abscission scar, smooth, , 2.5-3.0 x 2.0-2.5 µm.

Specimens examined: Thailand, Rayong, isolated from soil under pineapple plantation, 20 August 2007, Chaninun Pornsuriya RY101, RY104, RY112. The isolates were deposited in Biocontrol Research Unit and Mycology Section (KMITL).

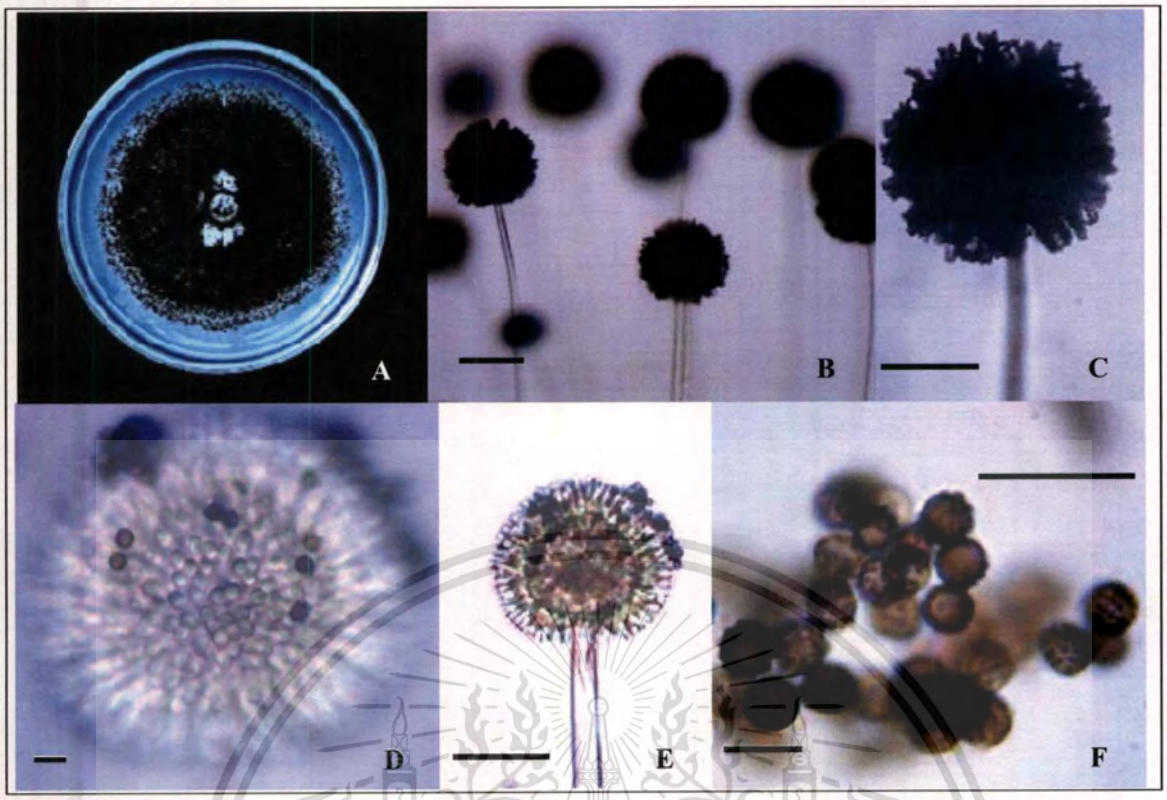

Fig. 4.48 *Aspergillus niger* AP101. A. 10-day-old culture on PDA, B,C,D. Conidial head, E. 1-layer of phialide, F.Conidiospores. Bars B = 100 µm. C, E = 50 µm. D, F = 10 µm.

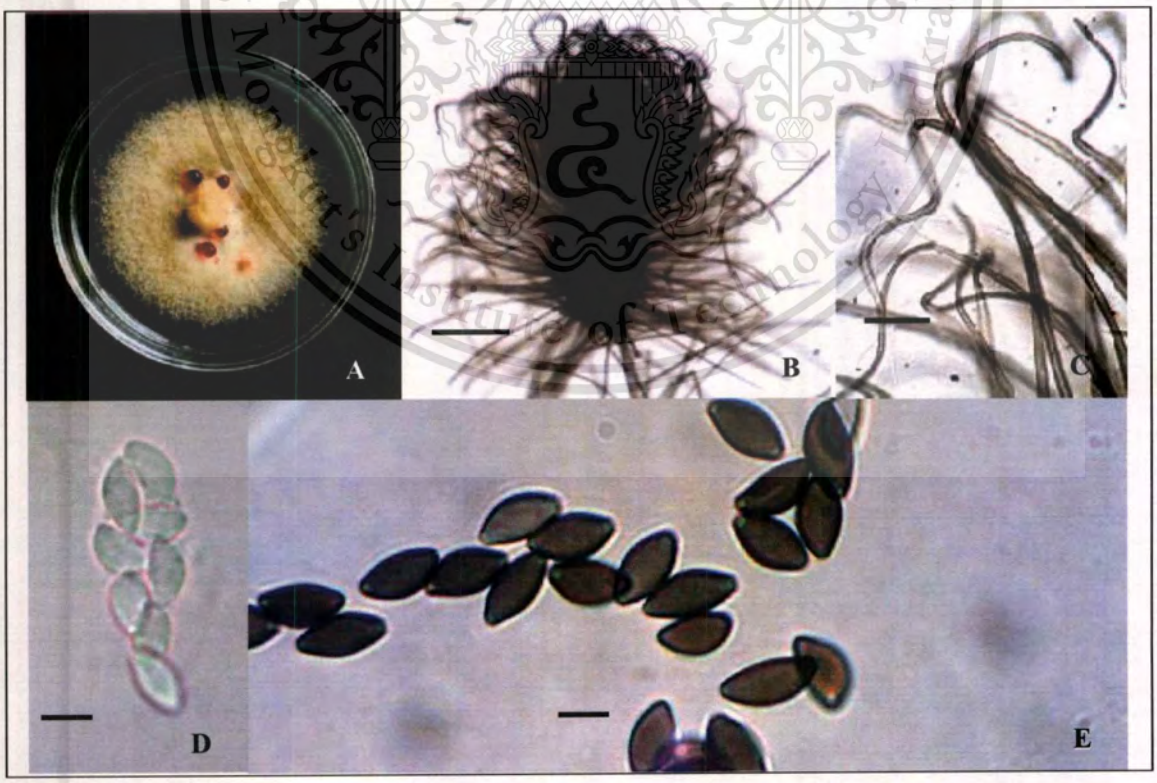

Fig. 4.49 *Chaetomium aureum* MB103. A. 10-day-old culture on PDA, B. ascomata, C. ascomatal hairs, D. 8 ascospores in an ascus, E. ascospores. Bar. B=100 µm, C,D=10 µm.

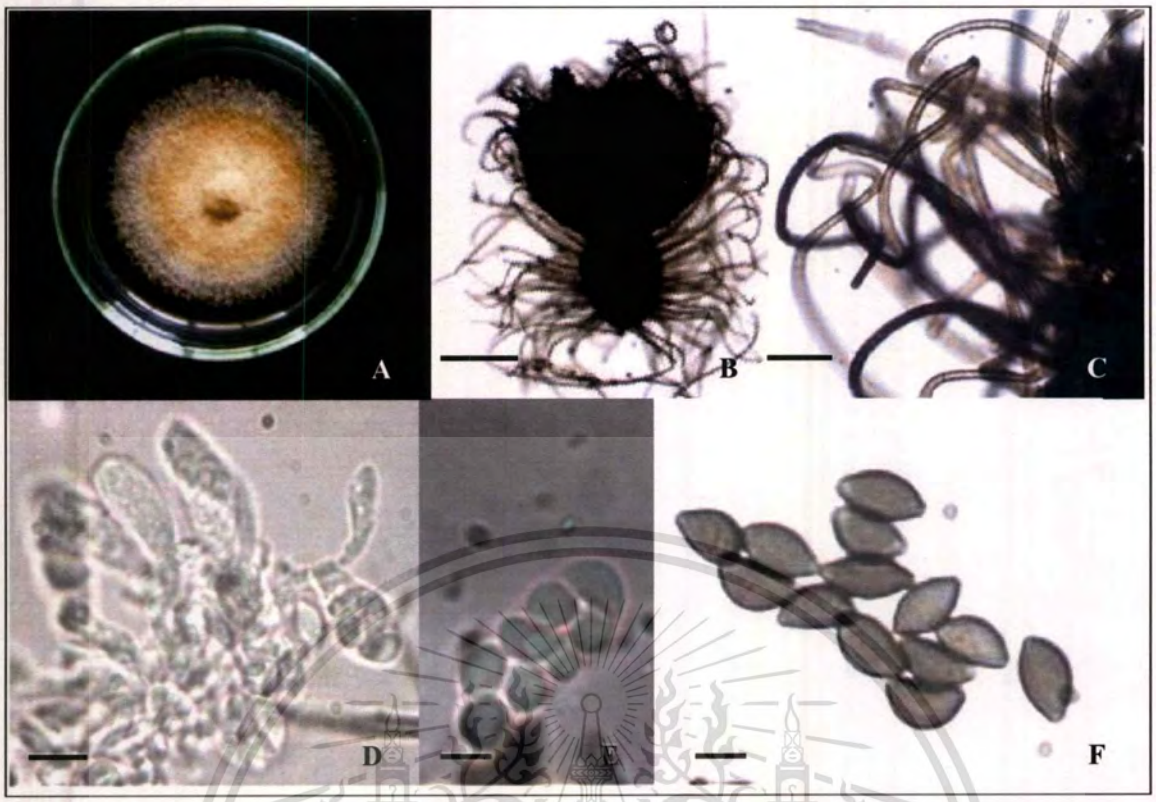

Fig. 4.50 *Chaetomium aureum* MB601. A. 10-day-old culture on PDA, B. ascomata, C. ascomatal hairs, D.young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10µm.

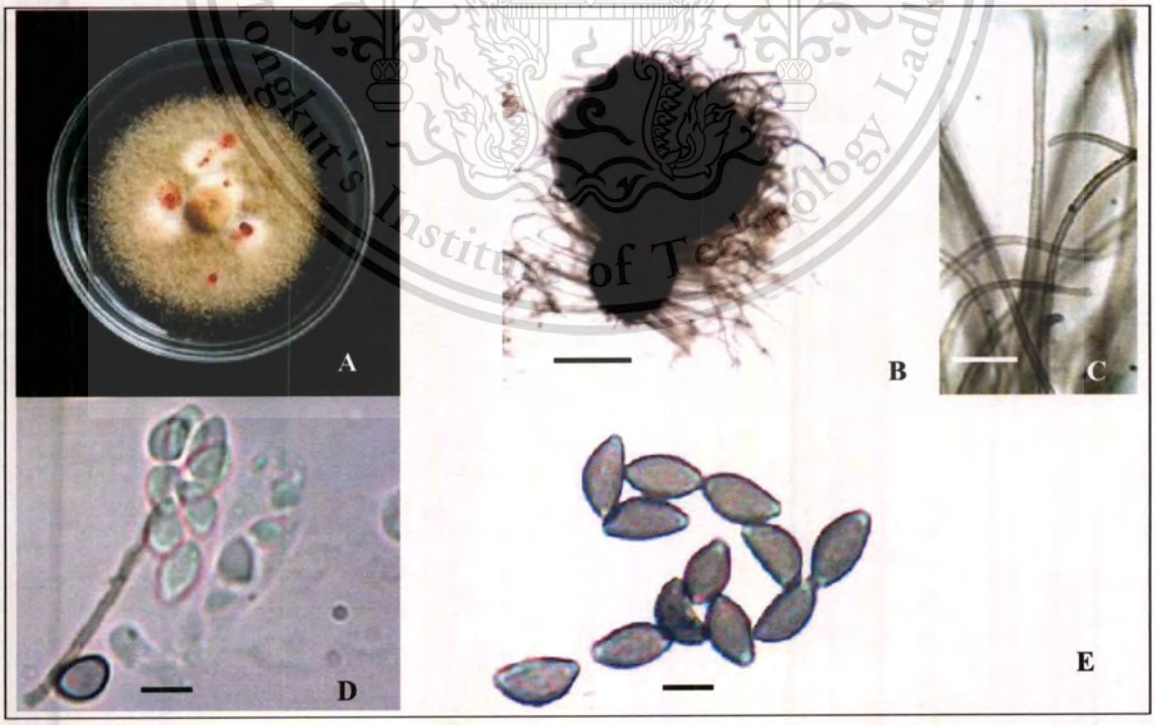

Fig. 4.51 *Chaetomium aureum* MB603. A. 10-day-old culture on PDA, B. ascomata, C. ascomatal hairs, D. 8 ascospores in an ascus, E. ascospores. Bar. B=100 µm, C,D,E=10µm.

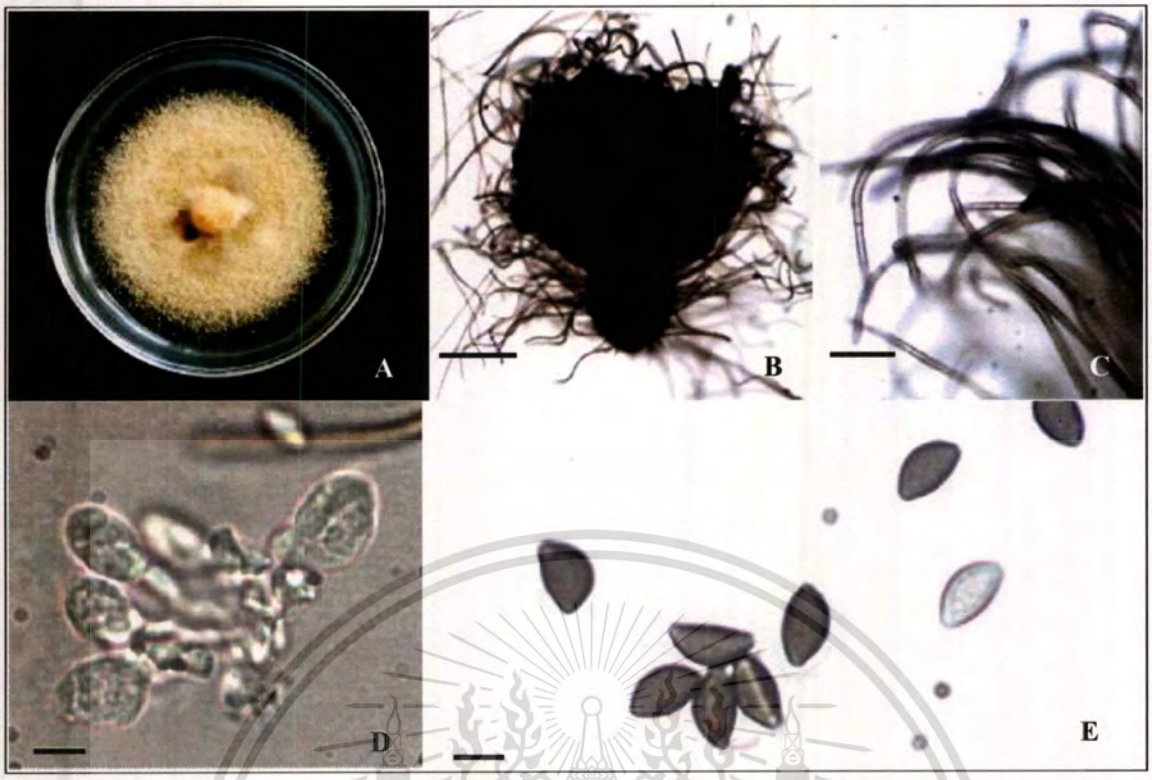

Fig. 4.52 *Chaetomium aureum* MB608. A. 10-day-old culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 μm, C,D,E,F=10μm.

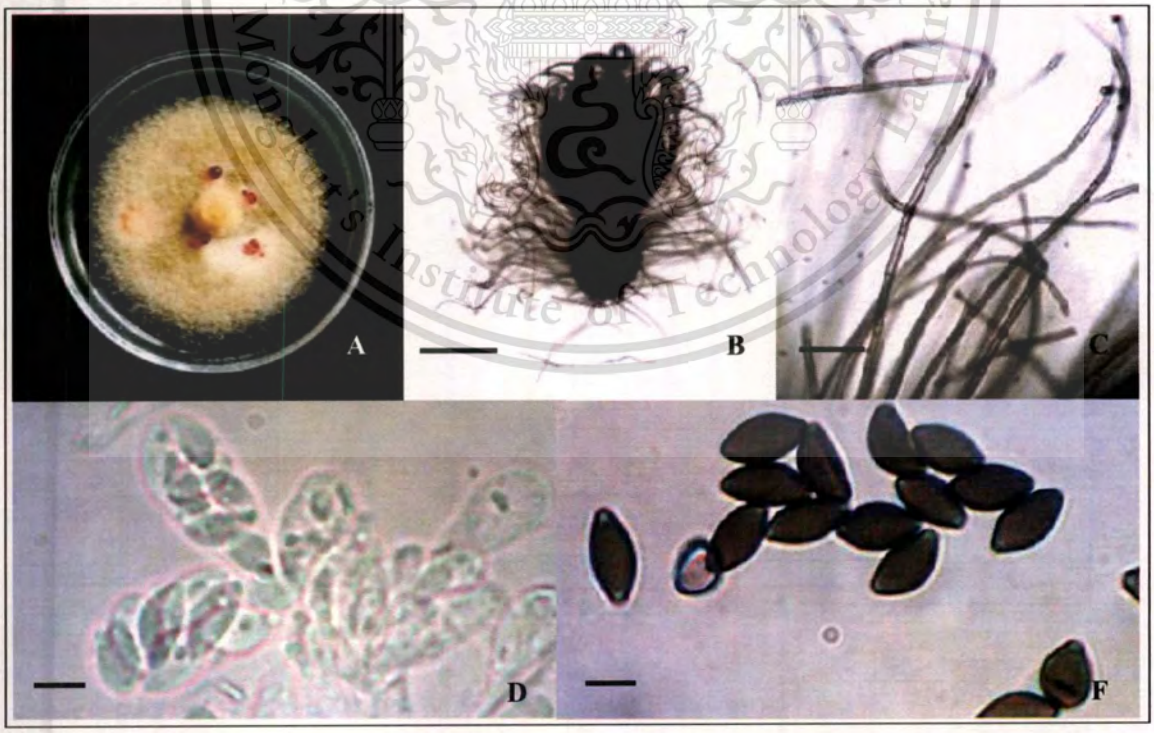

Fig. 4.53 *Chaetomium aureum* RY102. A. 10-day-old culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 μm, C,D,E,F=10μm.

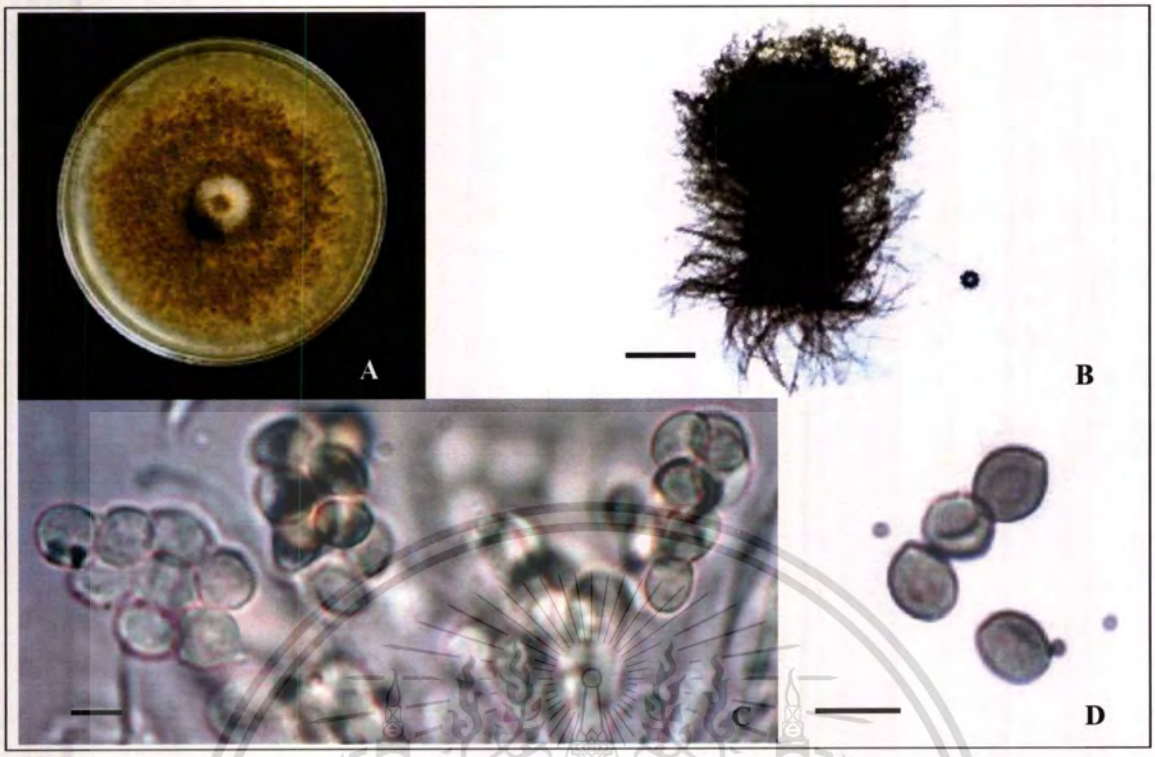

Fig. 4.54 *Chaetomium bostrychodes* NB701. A. 10-day-old-culture on PDA, B. ascomata, C. 8 ascospores in an ascus, D. ascospores. Bar. B=200 µm, C,D=10 µm.

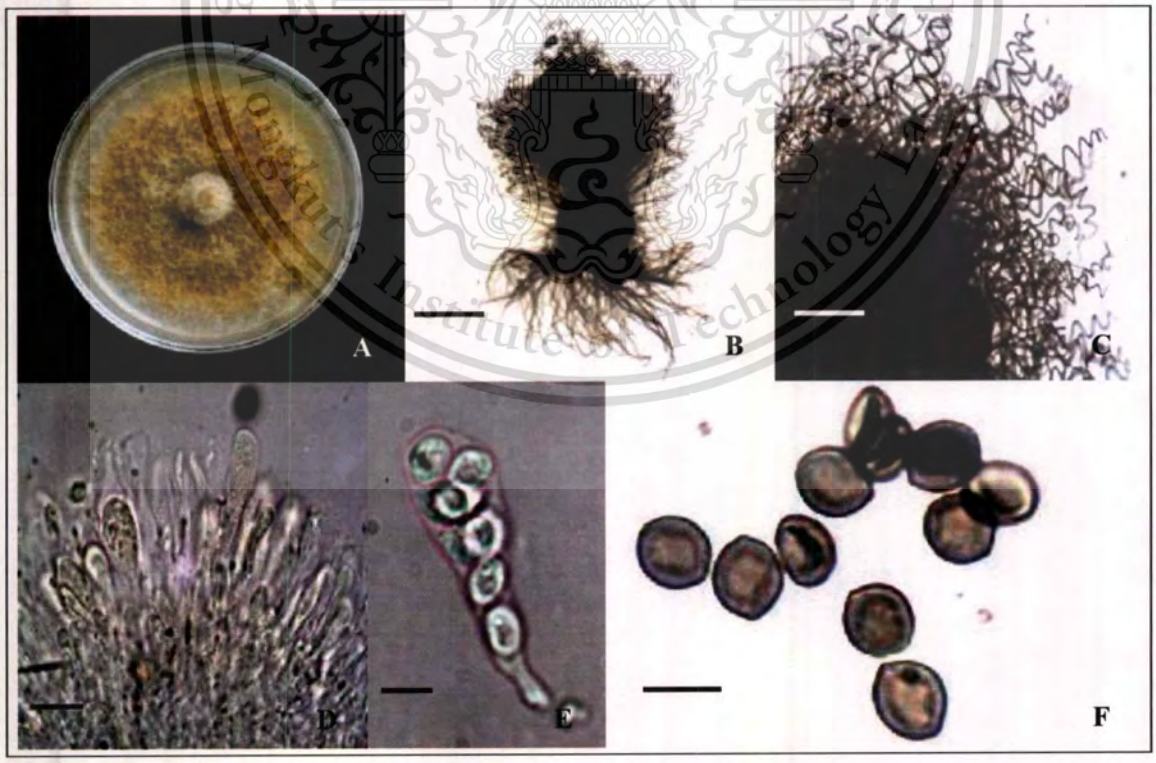

Fig. 4.55 *Chaetomium bostrychodes* PR101. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=200 µm, C,D,E,F=10 µm.

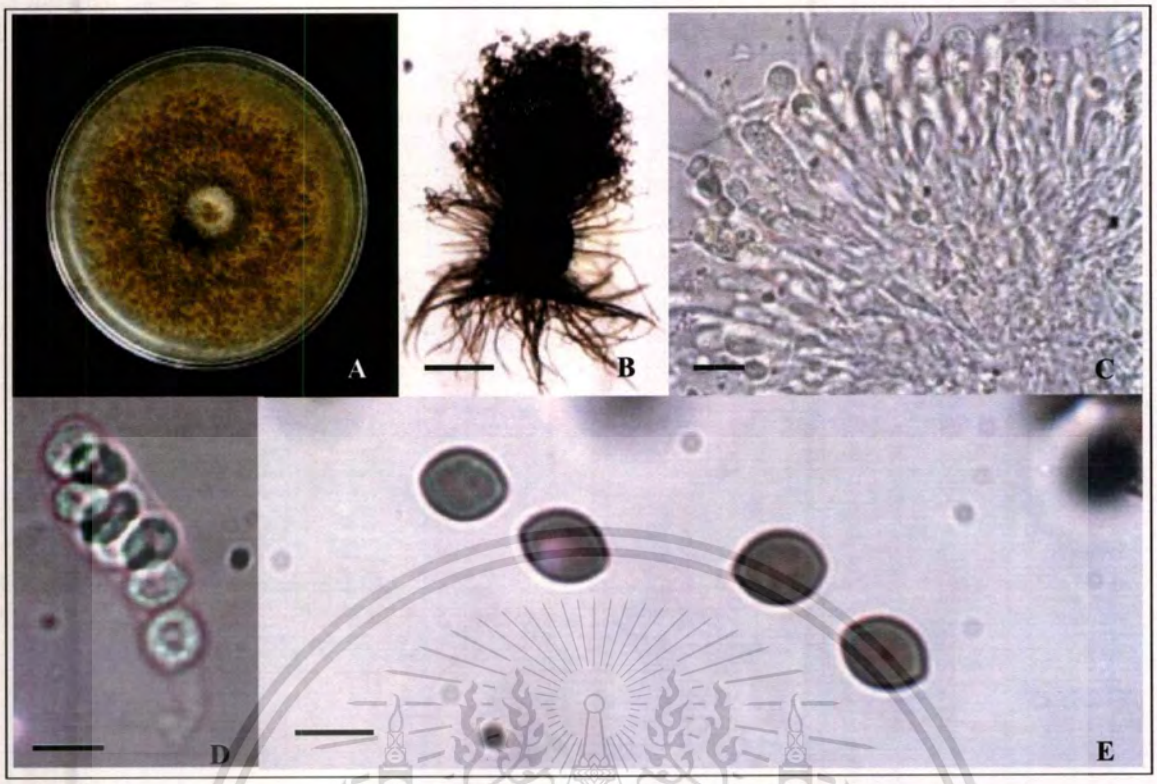

Fig. 4.56 *Chaetomium bostrychodes* PR102. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=200 µm, C,D,E=10 µm.

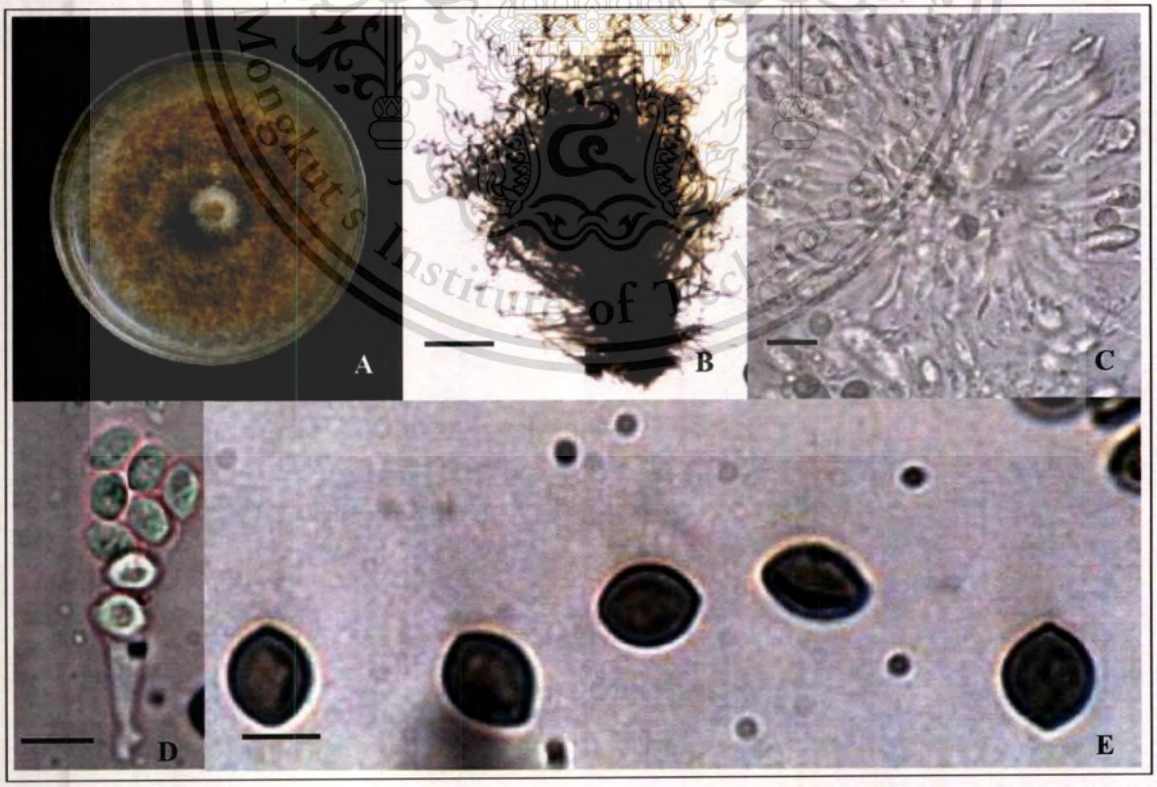

Fig. 4.57 *Chaetomium bostrychodes* PR103. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=200 µm, C,D,E=10 µm.

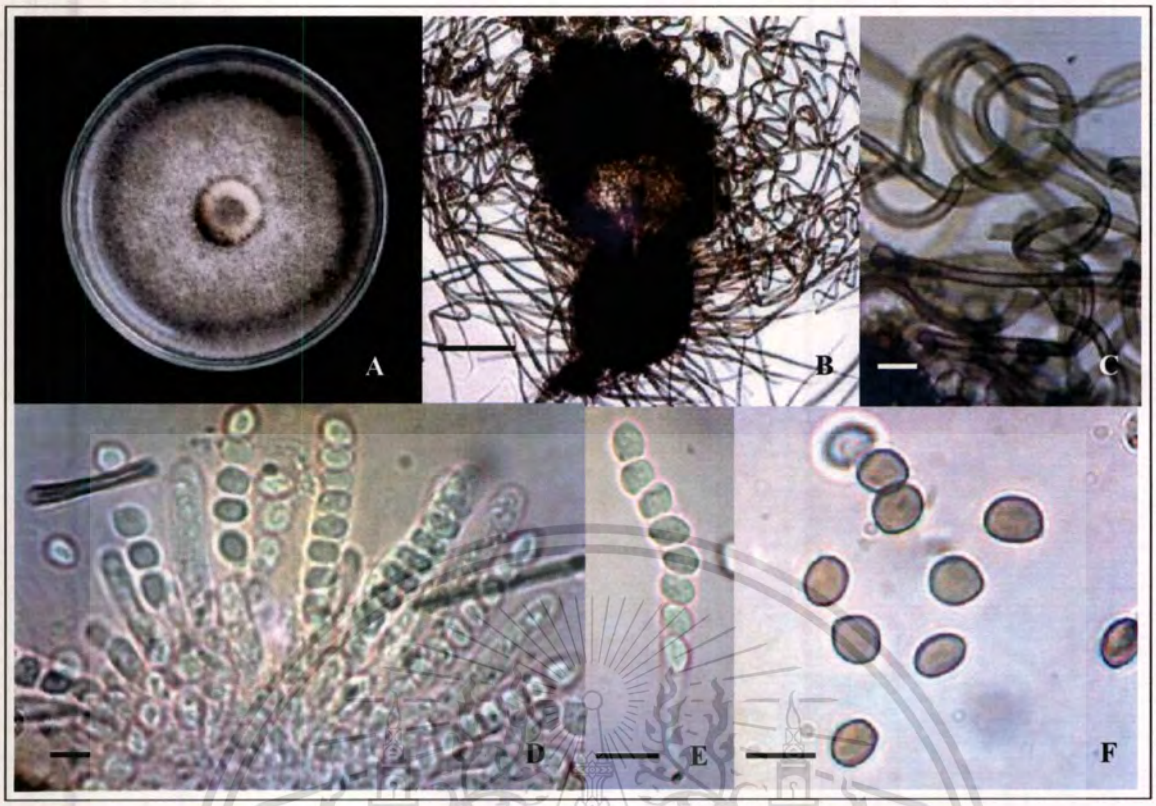

Fig. 4.58 *Chaetomium brasiliense* AM101. A. 10-day-old culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10µm.

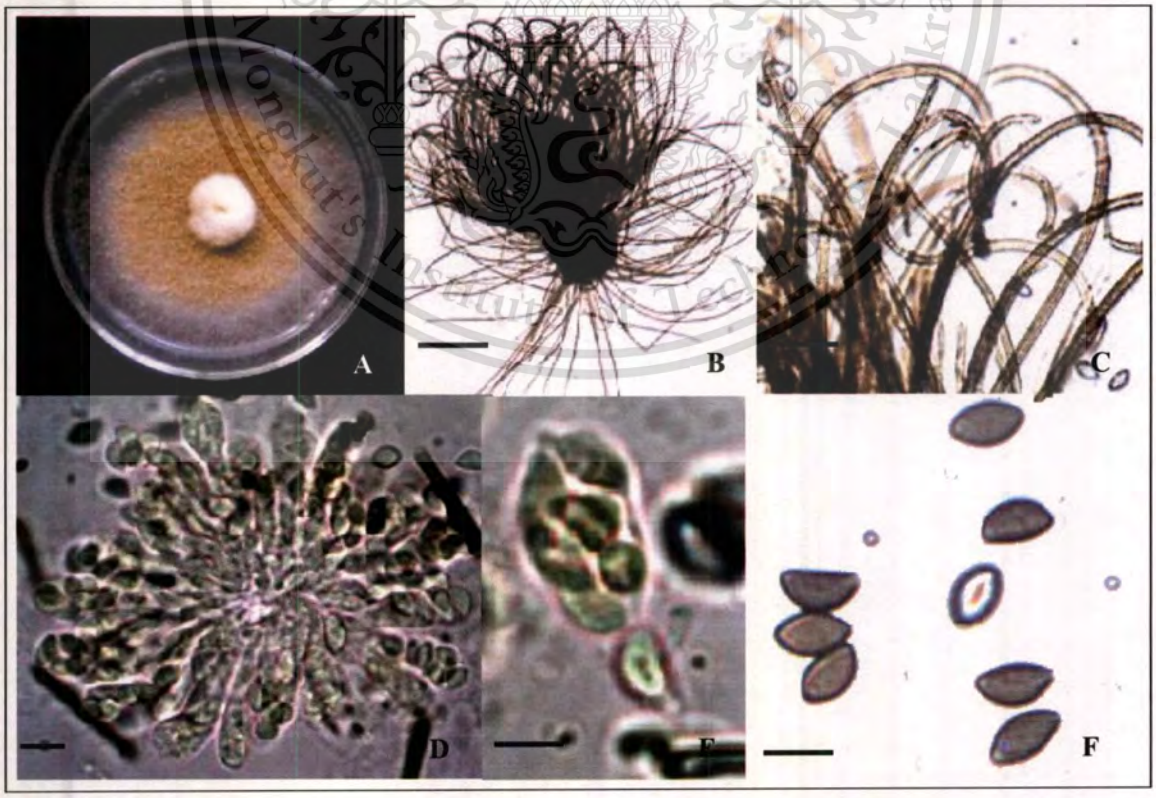

Fig. 4.59 *Chaetomium carinthiacum* NB501 A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10 µm.

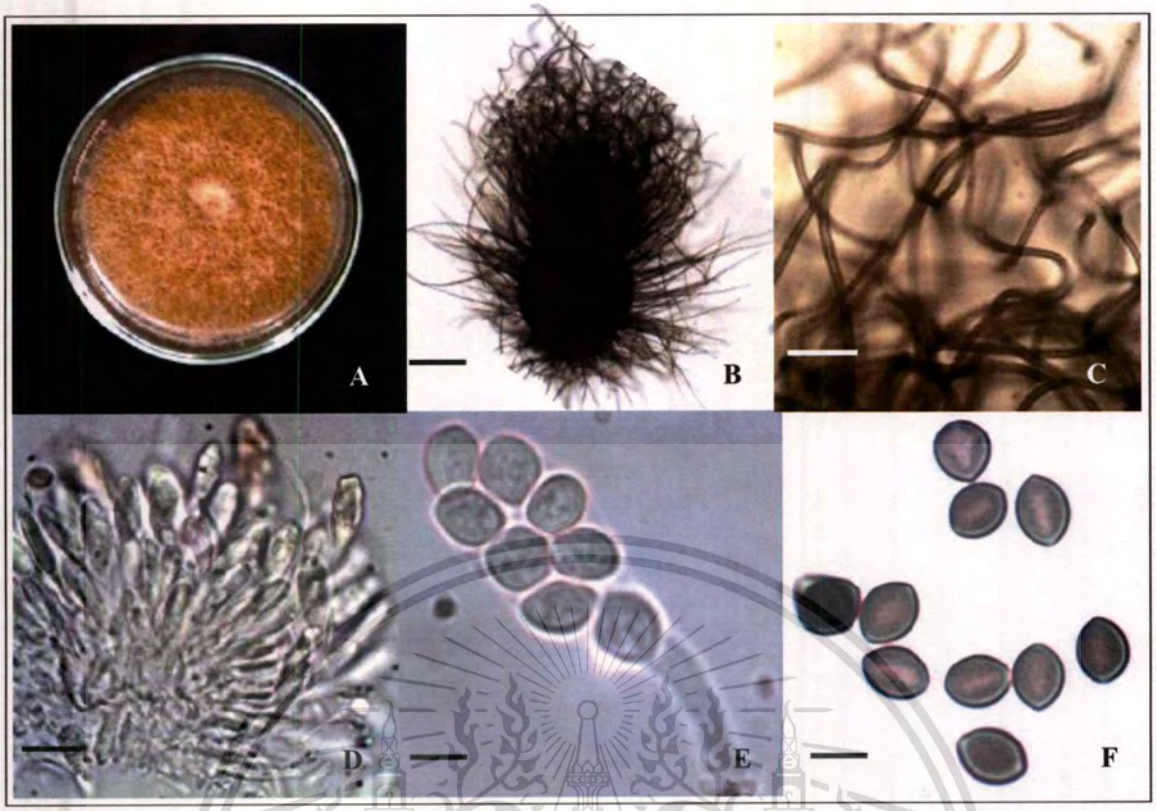

Fig. 4.60 *Chaetomium cochliodes* RY301. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 μm, C,D,E,F=10 μm.

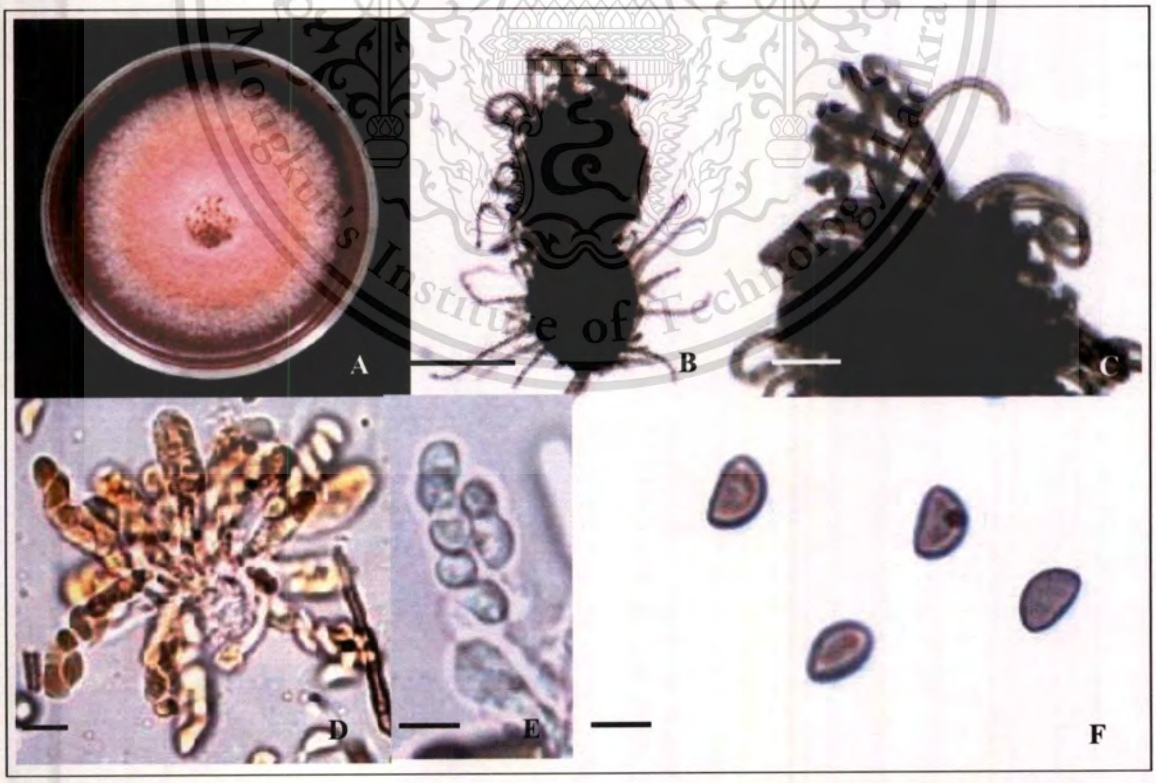

Fig. 4.61 *Chaetomium cupreum* NB102. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 μm, C,D,E,F=10 μm.

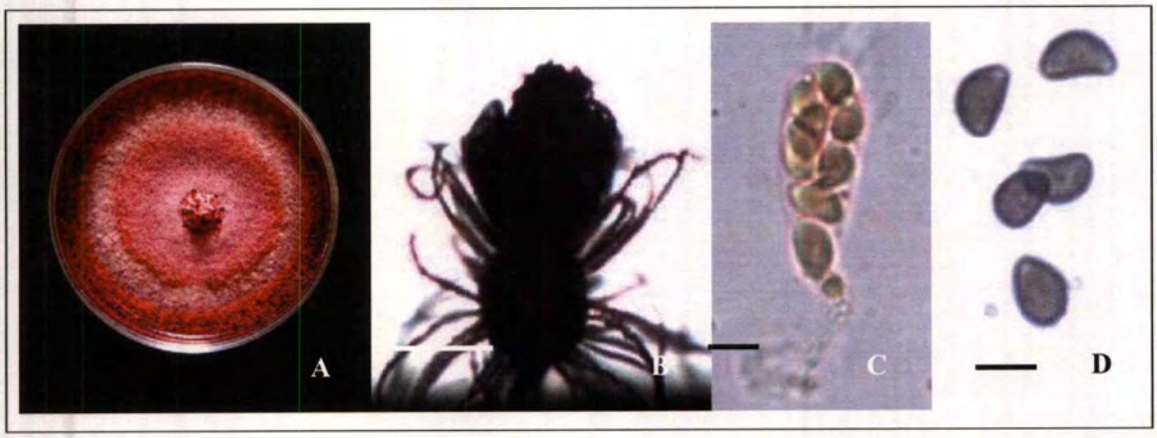

Fig. 4.62 *Chaetomium cupreum* RY201. A. 10-day-old-culture on PDA, B. ascomata, C. 8 ascospores in an ascus, D. ascospores. Bar. B=100 µm, C,D=10 µm

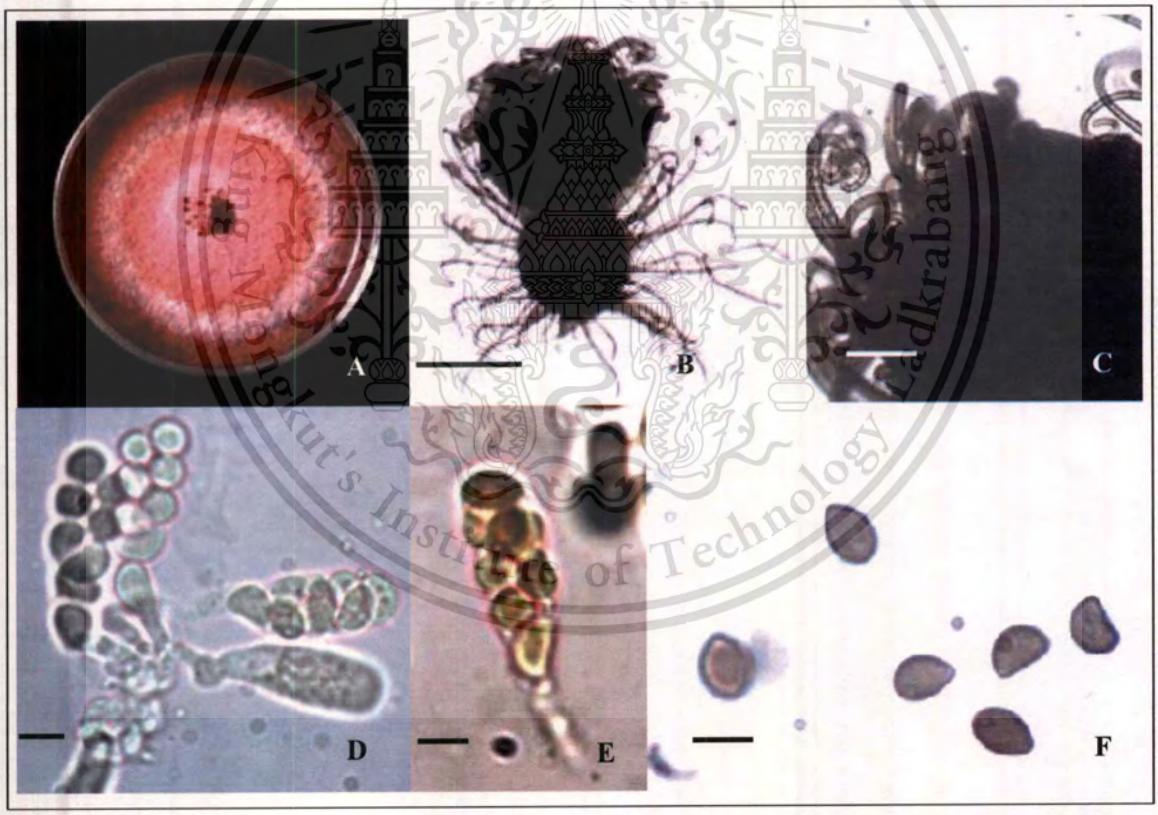

Fig. 4.63 *Chaetomium cupreum* RY202. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10 µm.

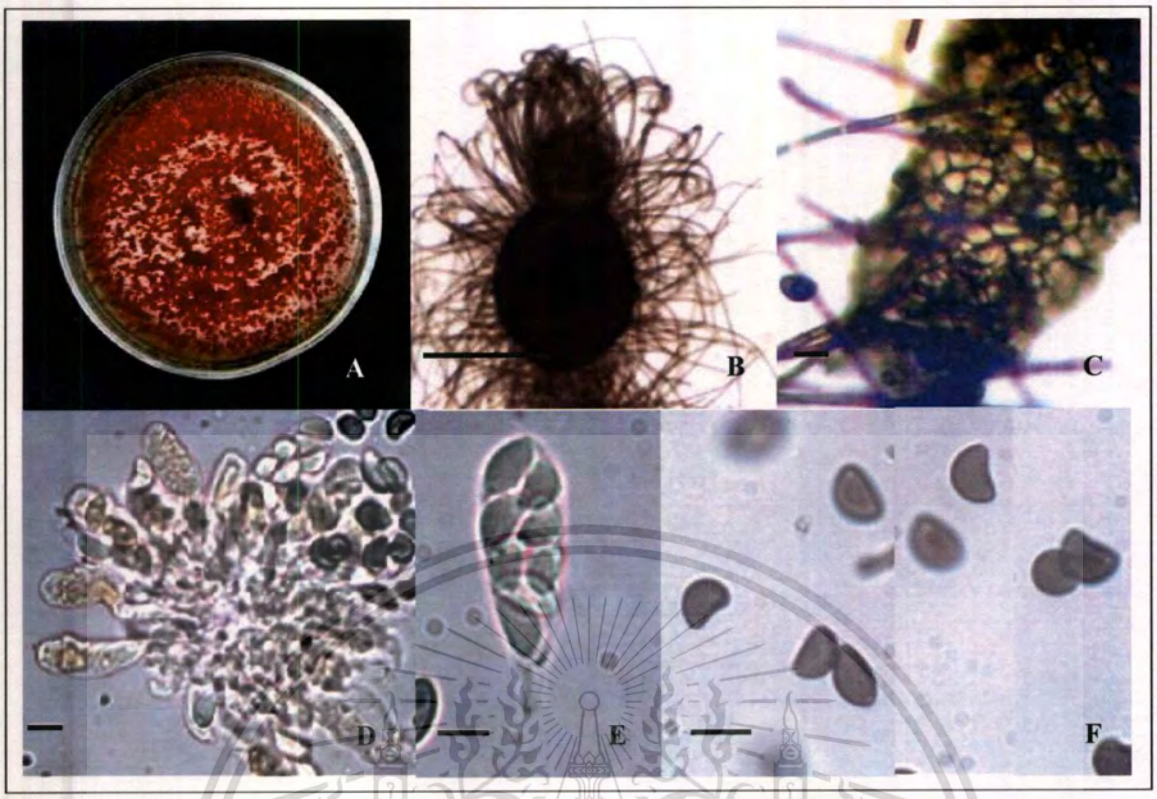

Fig. 4.64 *Chaetomium cupreum* RY204. A. 10-day-old-culture on PDA, B. ascomata, C. peridium, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10 µm.

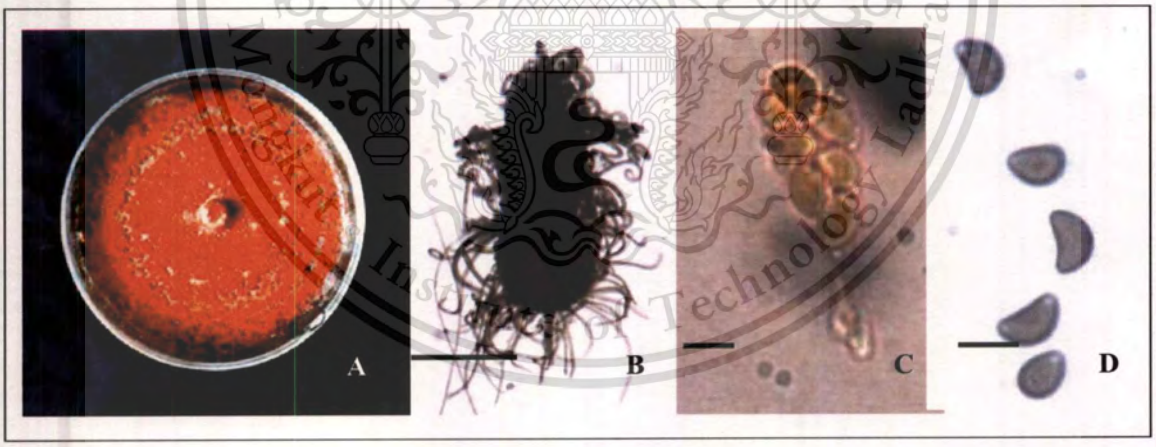

Fig. 4.65 *Chaetomium cupreum* SO101. A. 10-day-old-culture on PDA, B. ascomata, C. 8 ascospores in an ascus, D. ascospores. Bar. B=100 µm, C,D=10 µm.

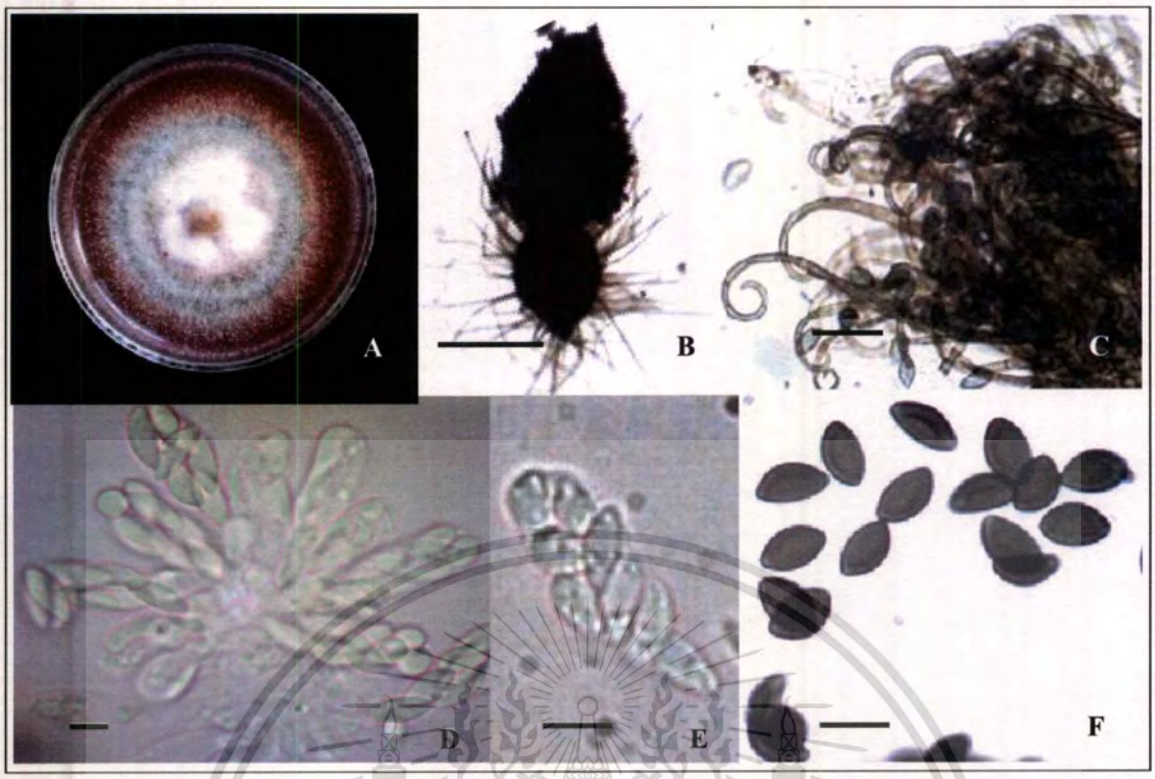

Fig. 4.66 *Chaetomium flavigenum* MB402. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10 µm.

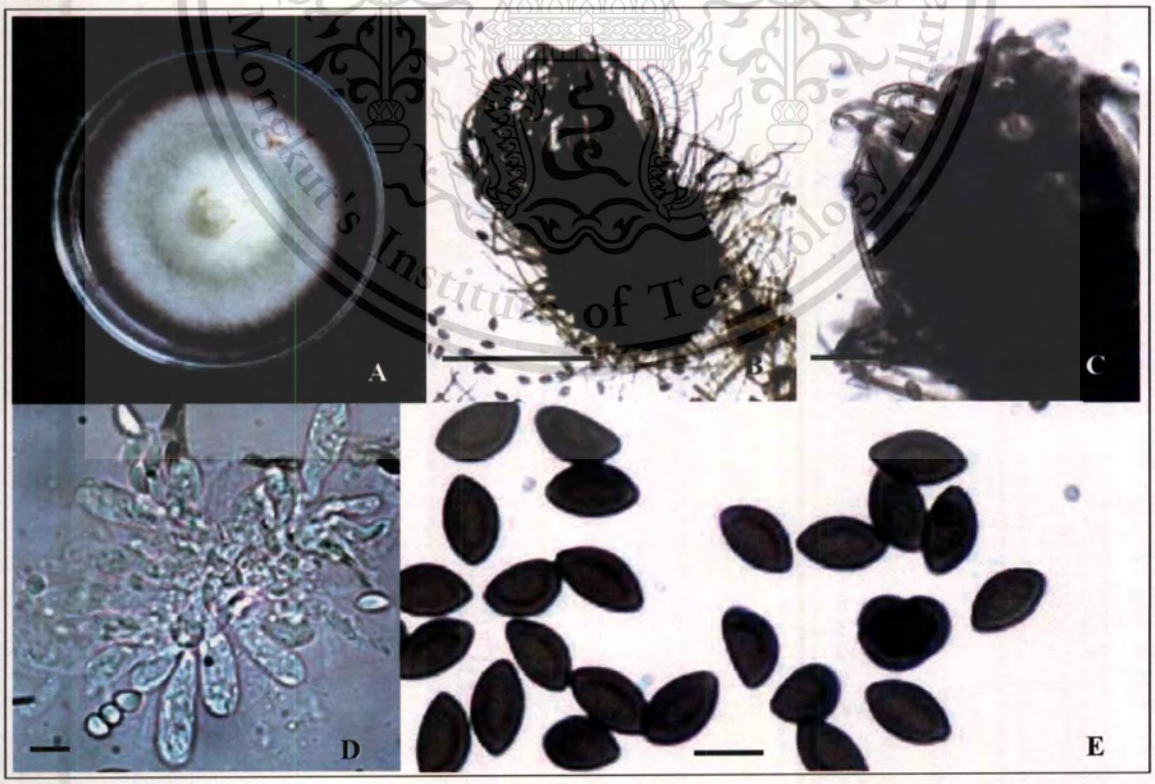

Fig. 4.67 *Chaetomium flavigenum* MB604. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. ascospores. Bar. B=100 µm, C,D,E=10 µm.

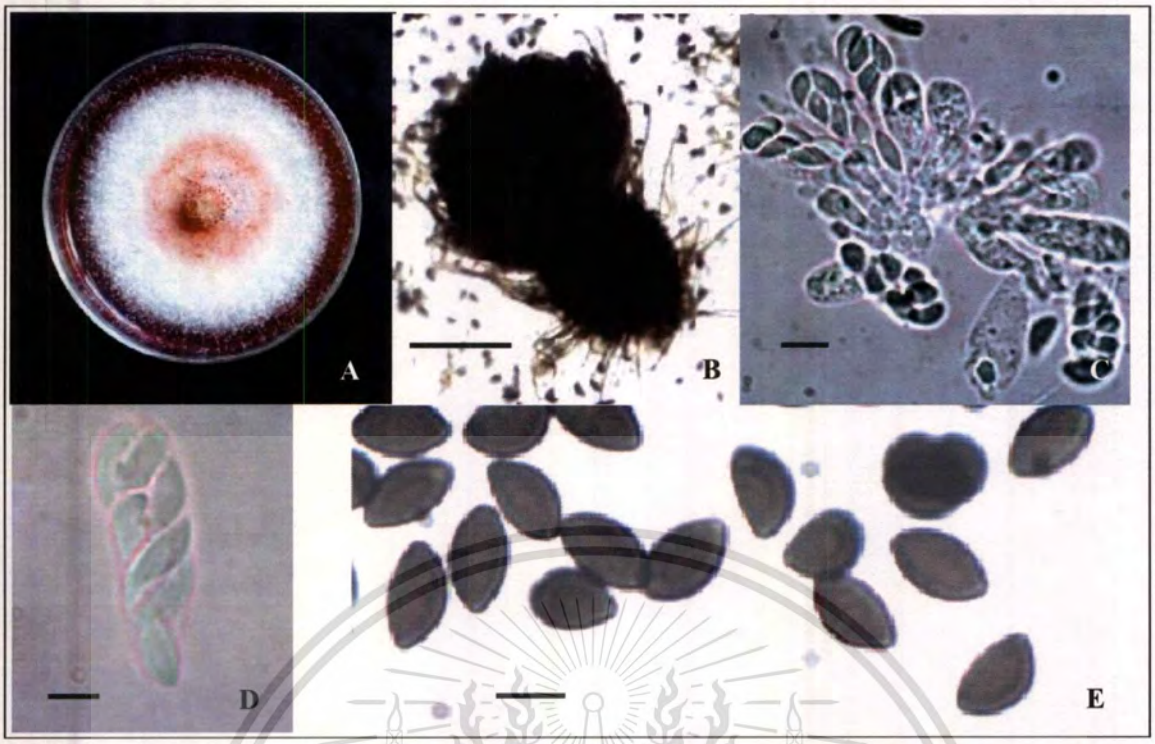

Fig. 4.68 *Chaetomium flavigenum* MB606. A. 10-day-old-culture on PDA, B. ascomata, C. young asci, D. 8 ascospores in an ascus, E. ascospores. Bar. B=100 μm, C, D,E=10 μm.

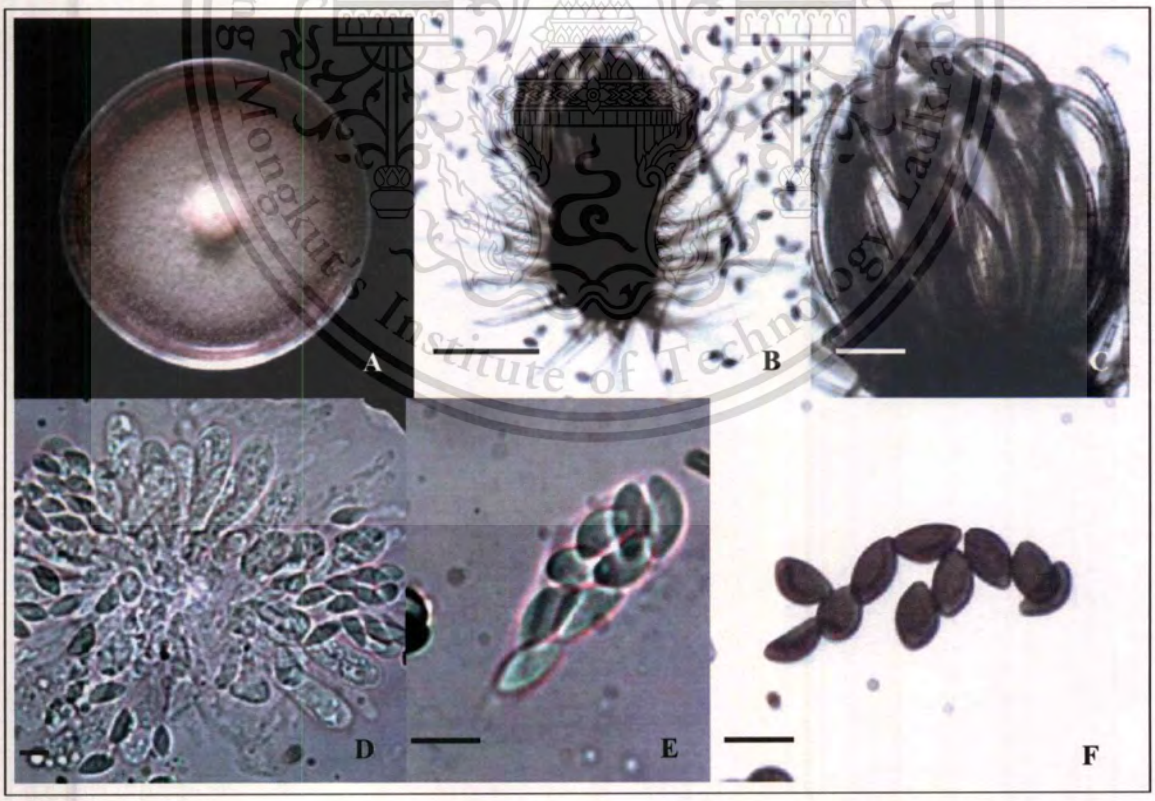

Fig. 4.69 *Chaetomium flavigenum* MB607. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 μm, C,D,E,F=10 μm.

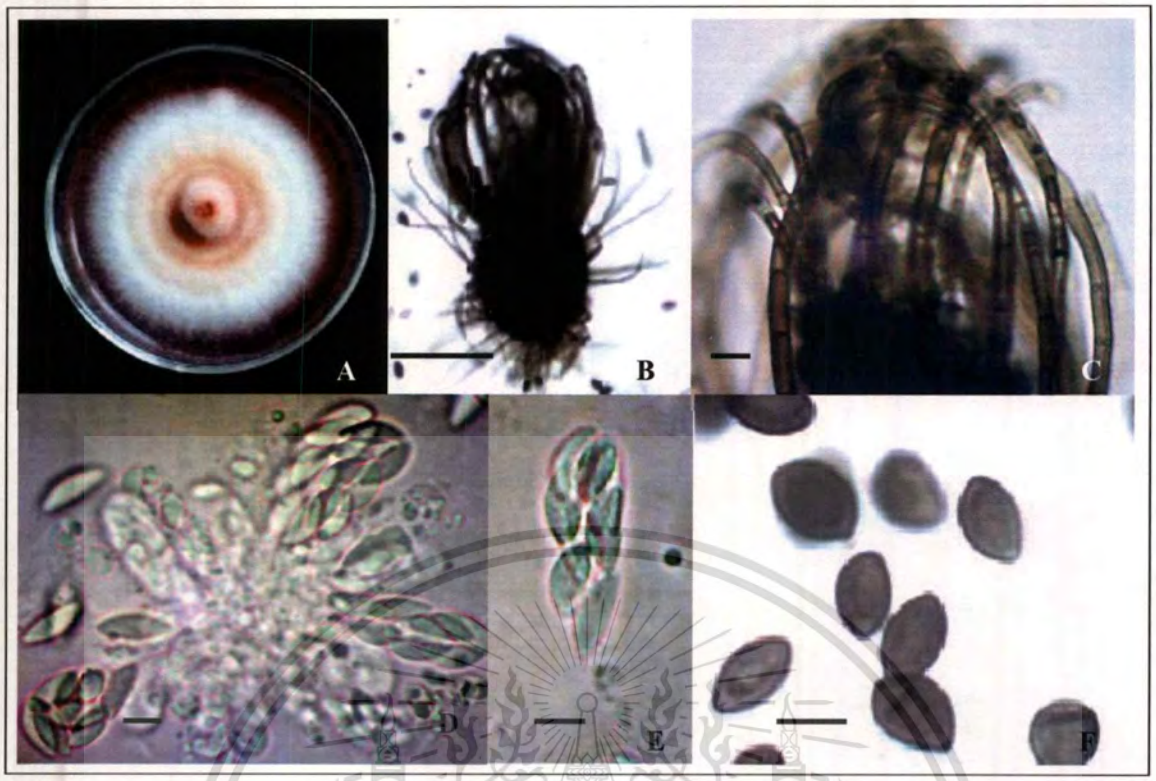

Fig. 4.70 *Chaetomium flavigenum* MB611. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 μm, C,D,E,F=10 μm.

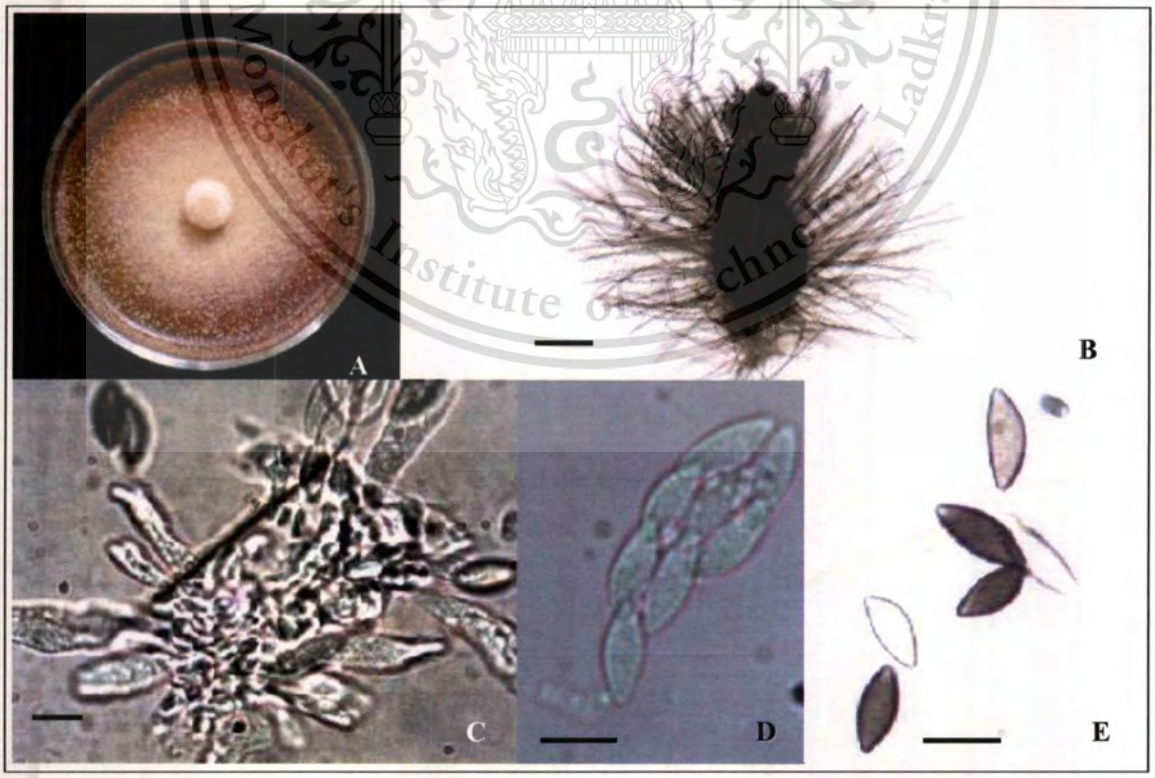

Fig. 4.71 *Chaetomium fusiforme* MB605. A. 10-day-old-culture on PDA, B. ascomata, C. young asci, D. 8 ascospores in an ascus, E. ascospores. Bar. B=100 μm, C,D,E =10 μm.

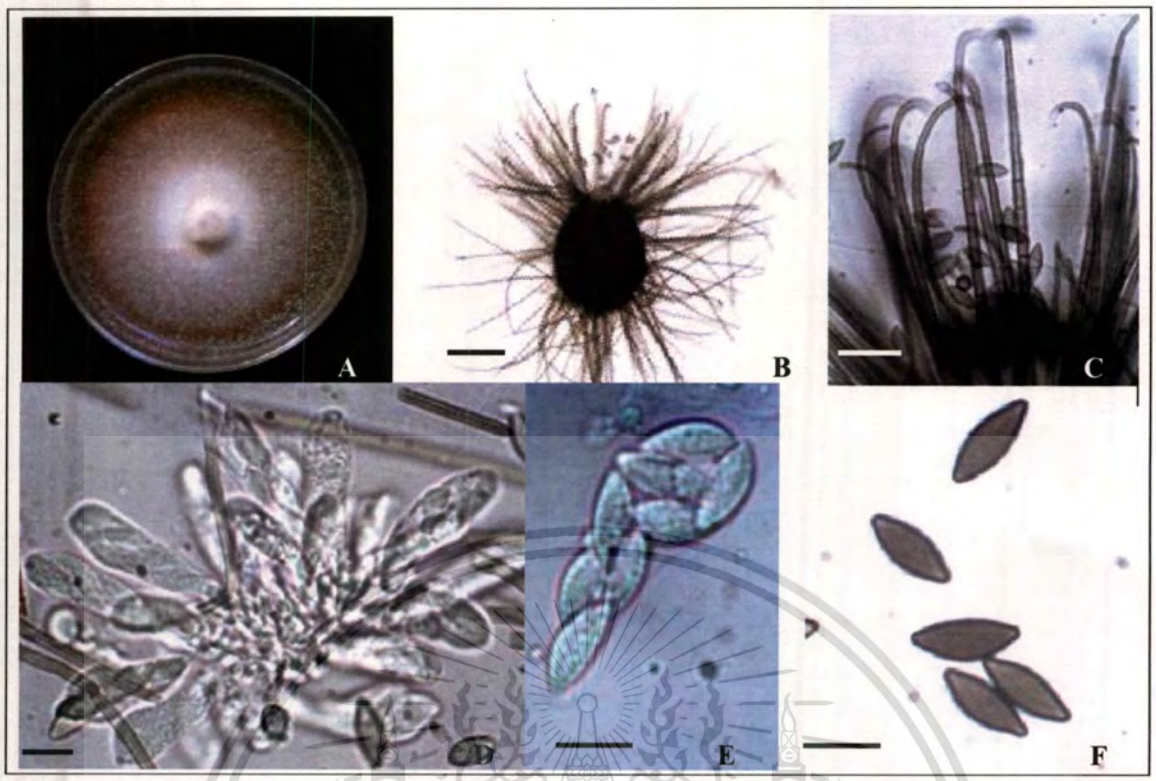

Fig. 4.72 *Chaetomium fusiforme* NB401. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10 µm.

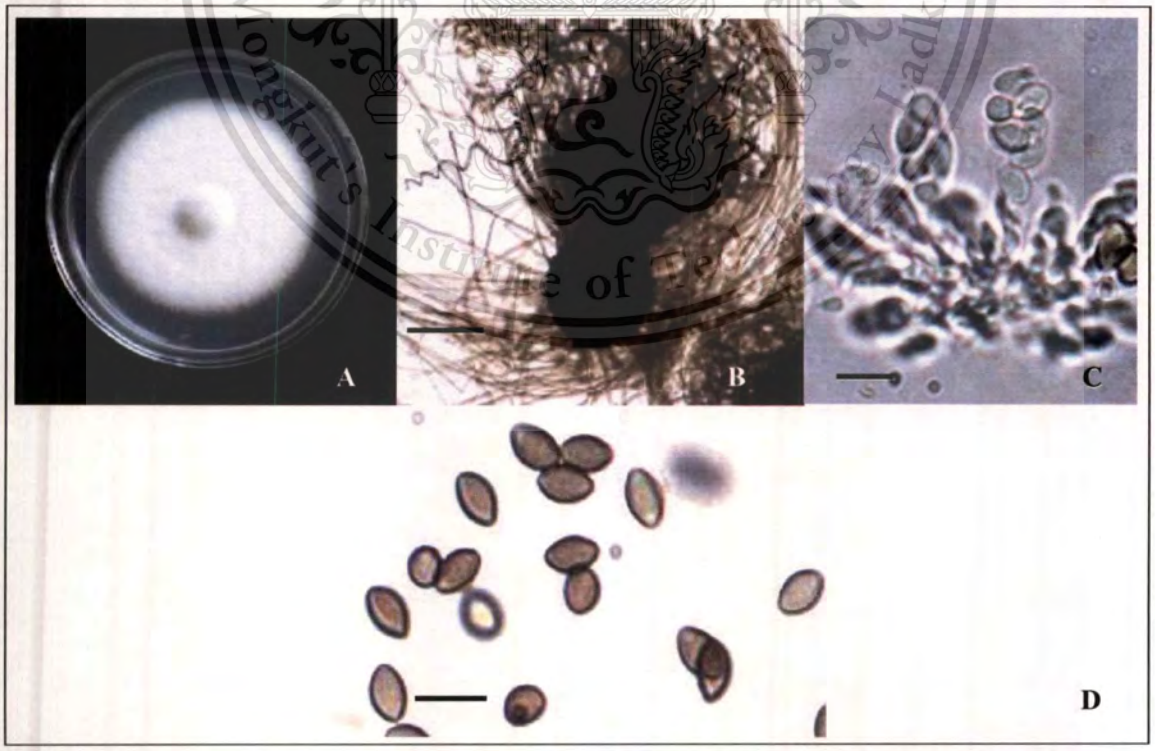

Fig. 4.73 *Chaetomium perlucidum* NB501. A. 10-day-old-culture on PDA, B. ascomata, C. young asci, D. ascospores. Bar. B=100 µm, C,D =10 µm.

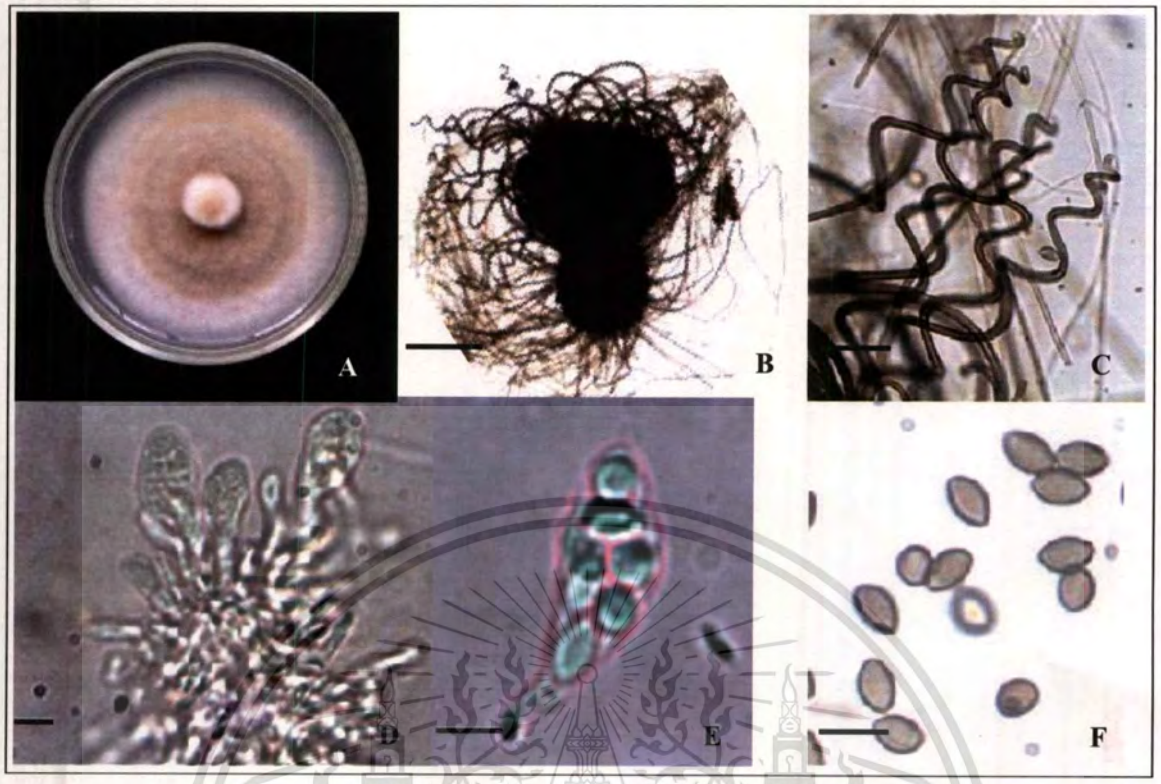

Fig. 4.74 *Chaetomium perlucidum* NB202. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10 µm.

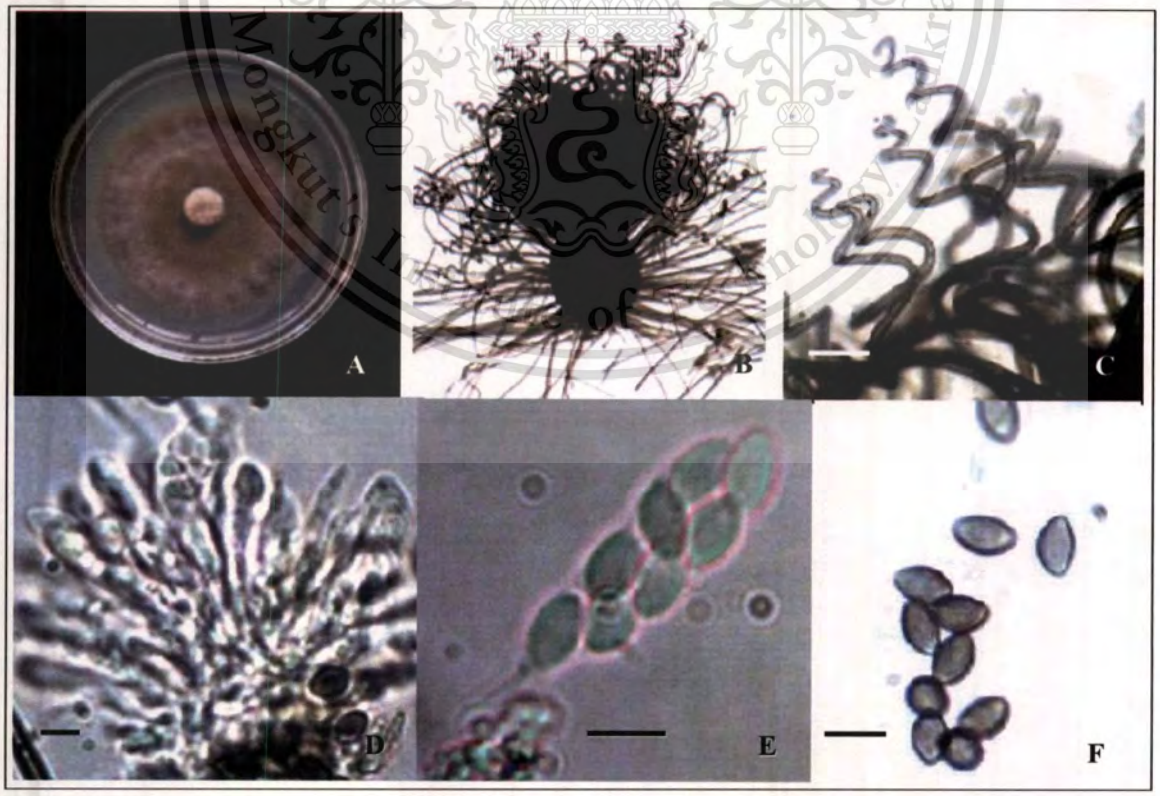

Fig. 4.75 *Chaetomium succineum* MB304. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10 µm.

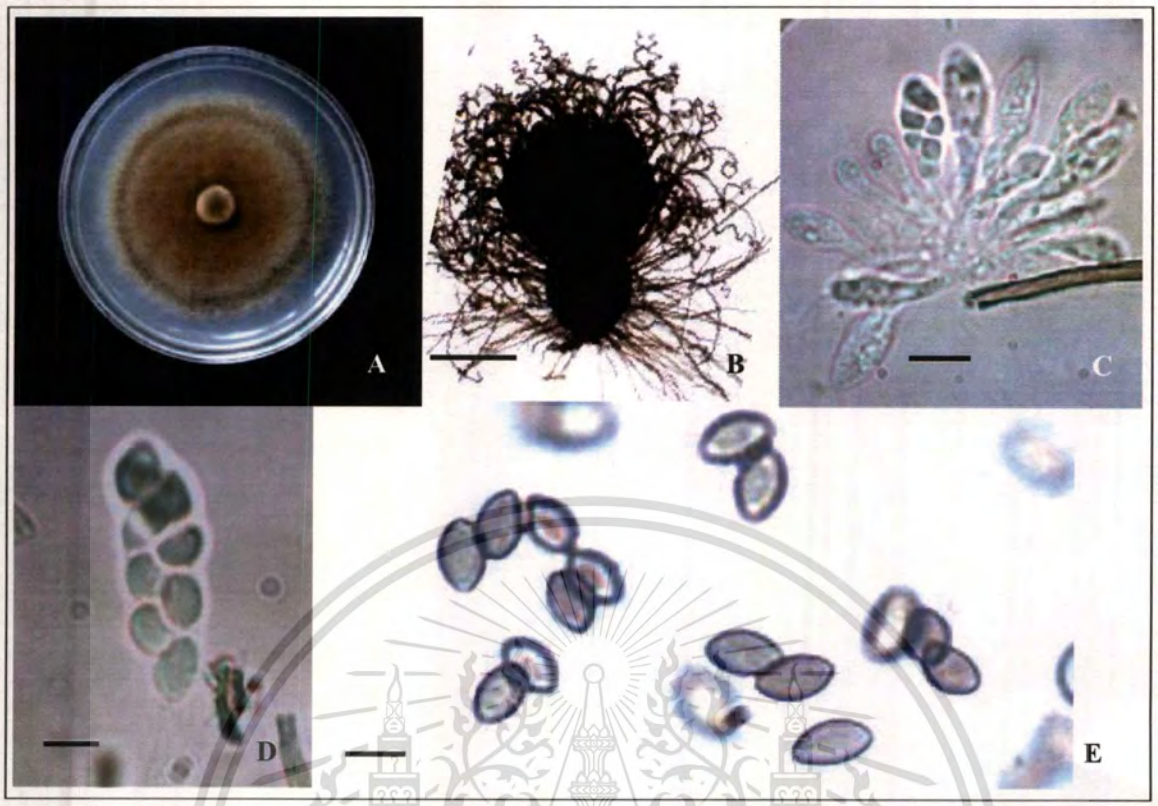

Fig. 4.76 *Chaetomium succineum* MB305. A. 10-day-old-culture on PDA, B. ascomata, C. young asci, D. 8 ascospores in an ascus, E. ascospores. Bar. B=100 µm, C,D,E=10 µm.

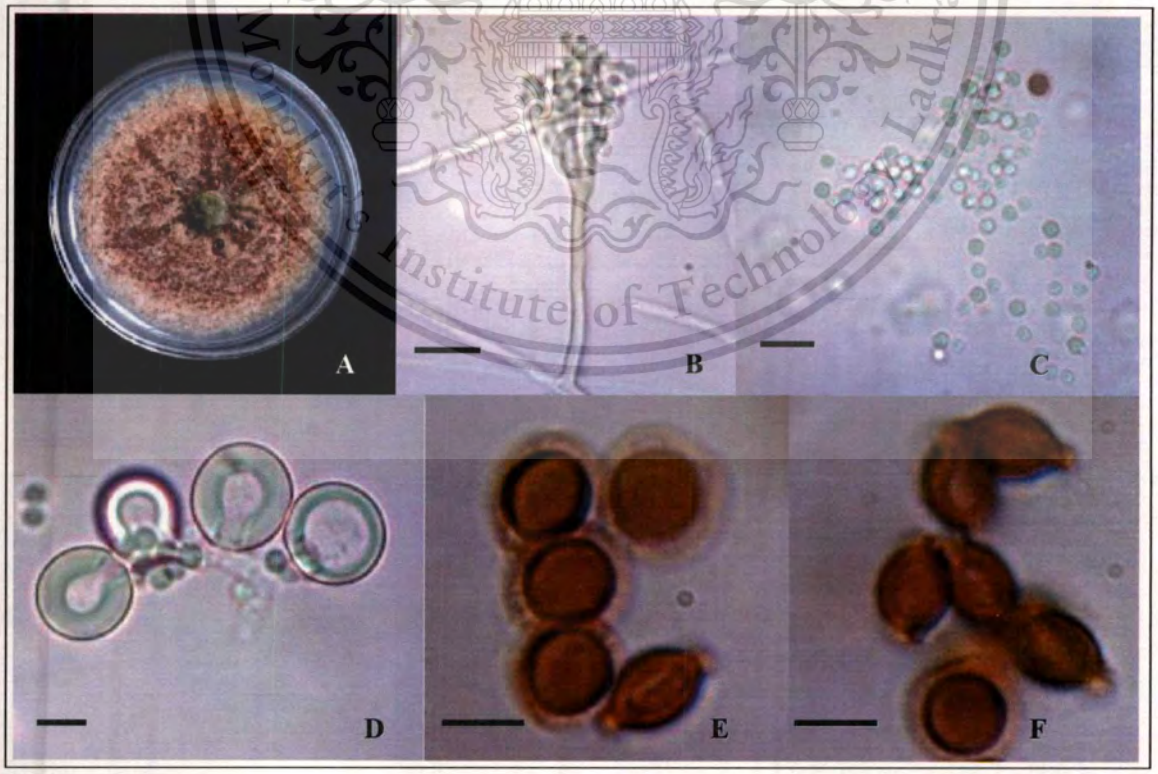

Fig. 4.77 *Emericella nidulans* EN101. A. 10-day-old-culture on PDA, B.conidia of *Aspergillus nidulans*, C. conidiospores, D. hulle cells, E.F ascospores. Bars. B = 50 µm, C, D, E, F =10 µm.

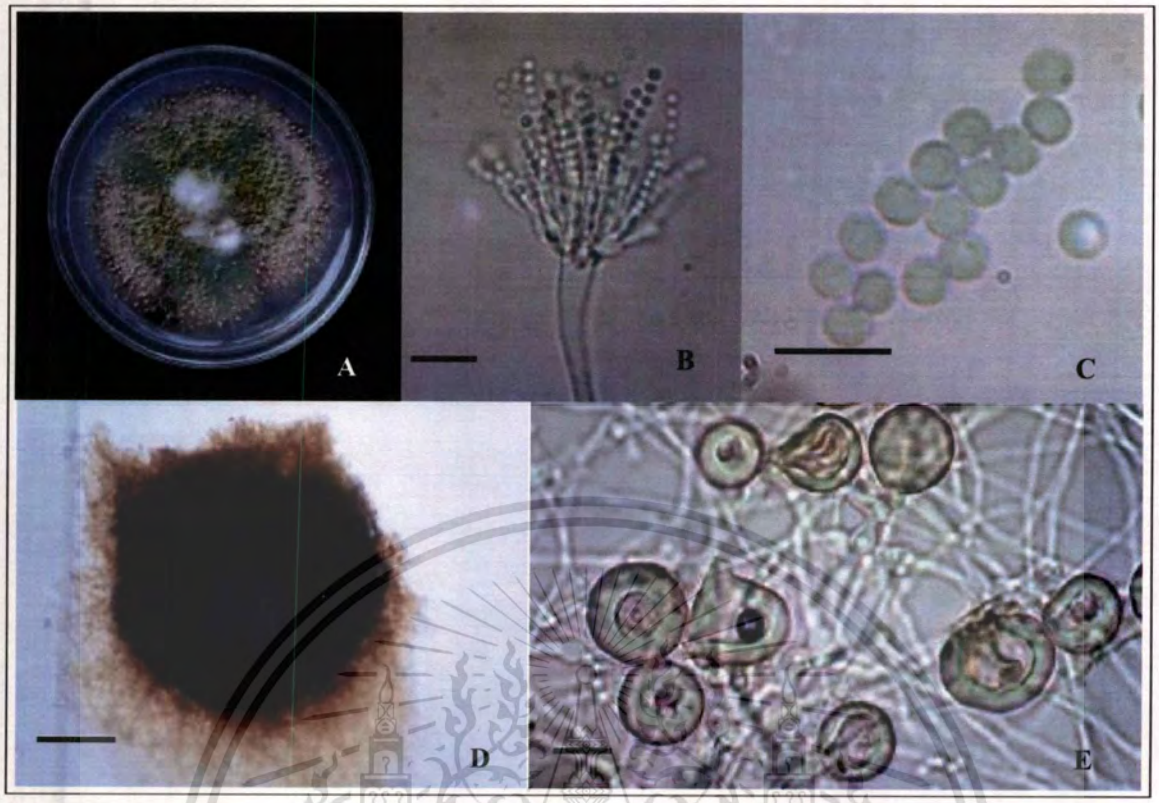

Fig. 4.78 *Emericella rugulosa* ER101. . A. 10-day-old-culture on PDA, B.conidia of *Aspergillus rugulosa*, C. conidiospores, D.ascomata, E. hulle. Bars. B, D = 50 µm, C, E = 10 µm.

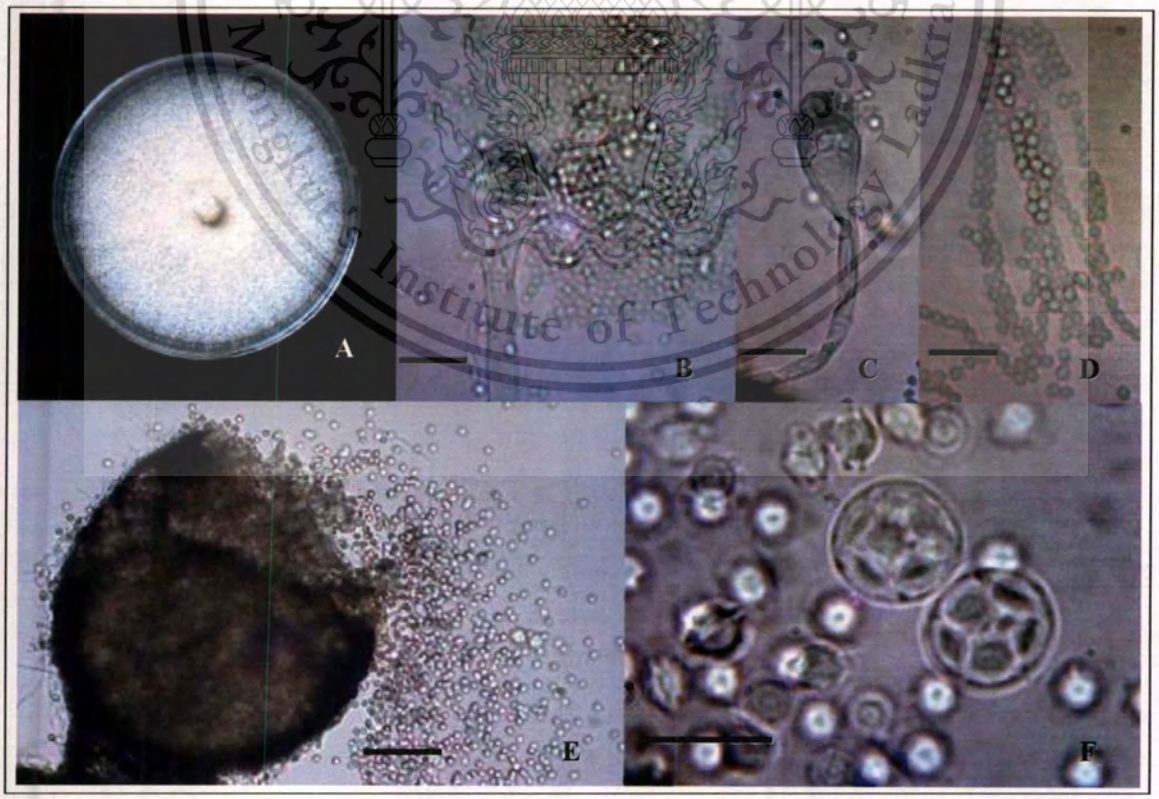

Fig. 4.79 *Eurotium chevalieri* RY101. A. 10-day-old-culture on PDA, B,.conidia, C 1 layer phialide, D.conidiospores, E.ascomata, F. ascus and ascospores. Bars B,C, D = 20 µm, E = 50 µm, F = 10 µm.

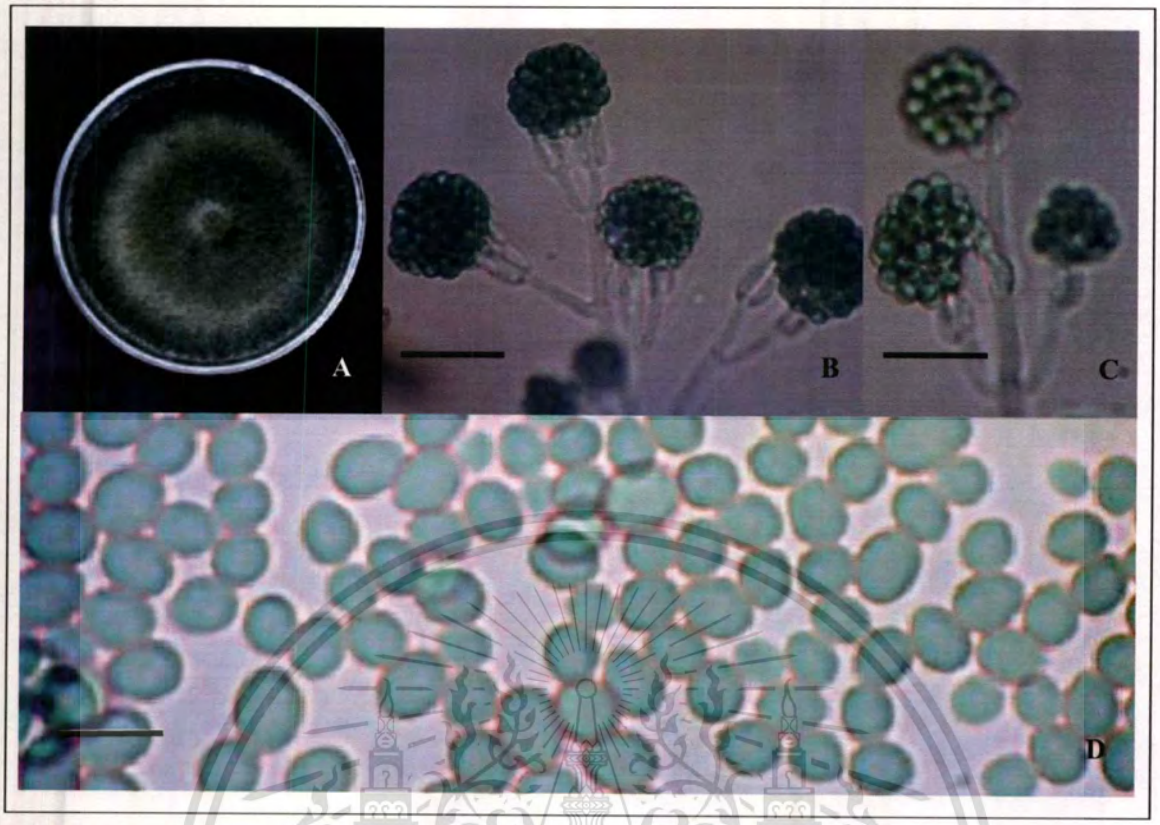

Fig. 4.80 *Gliocladium catenulatum* RY102. A. 10-day-old-culture on PDA, B,C. conidia, D. conidiospores. Bars B,C = 10 μm, D = 5 μm.

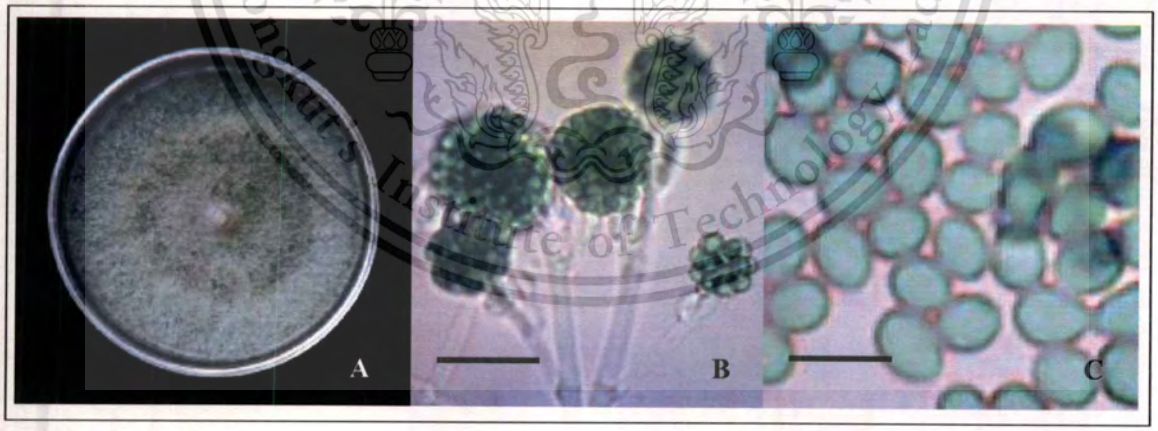

Fig. 4.81 *Gliocladium catenulatum* RY109. A. 10-day-old-culture on PDA, B. conidia, C. conidiospores. Bars B = 10 μm, C = 5 μm.

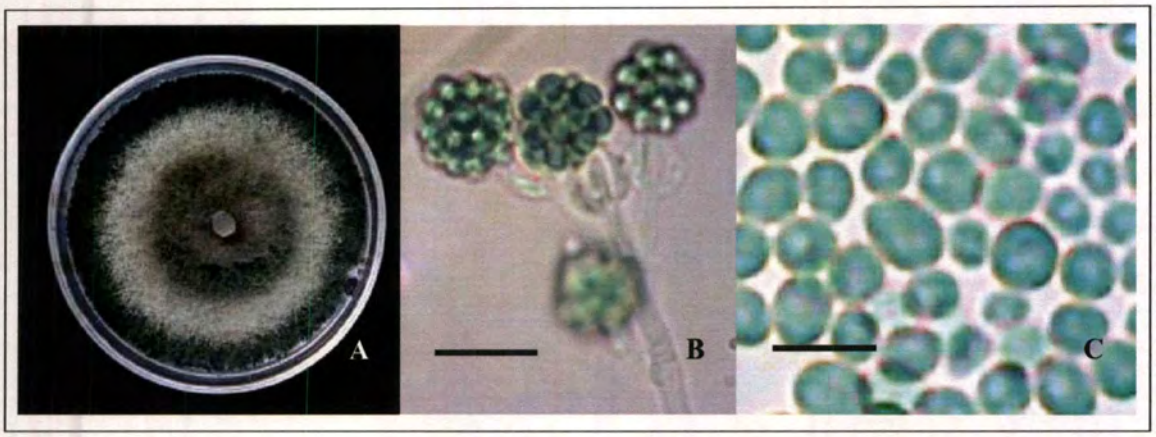

Fig. 4.82 *Gliocladium catenulatum* RY111. A. 10-day-old-culture on PDA, B. conidia, C. conidiospores. Bars B = 10 μm, C = 5 μm.

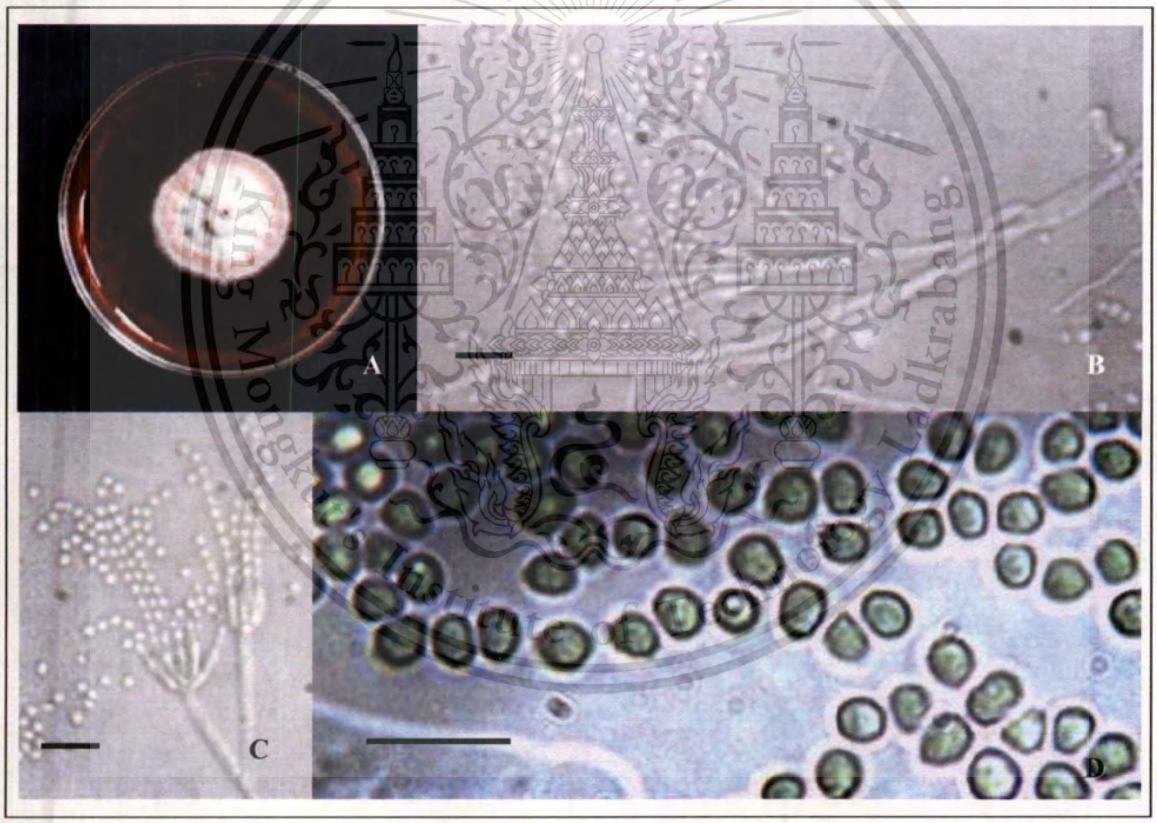

Fig. 4.83 *Penicillium janthinellum* PN101. A. 10-day-old-culture on PDA, B,C. conidia, D. conidiospores. Bars B,C = 10 μm, D = 5 μm.

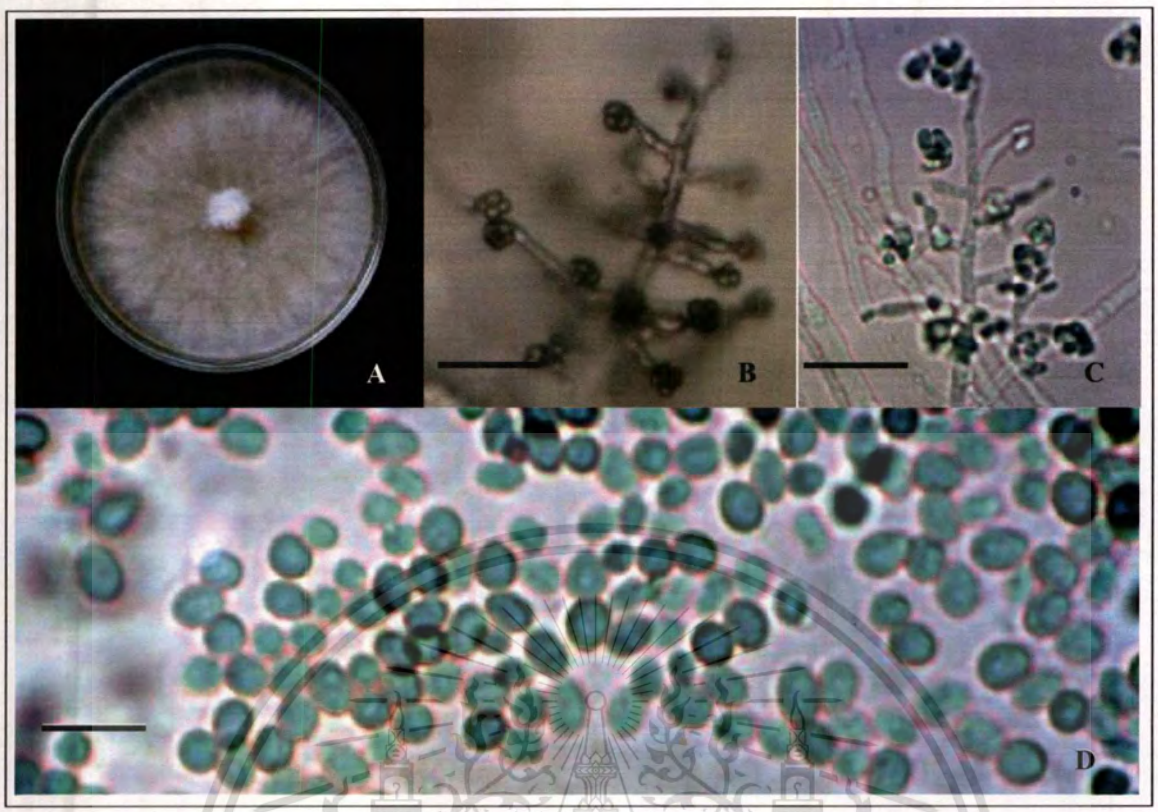

Fig. 4.84 *Trichoderma hamatum* PT101. A. 10-day-old-culture on PDA, B,C. conidia, D. conidiospores. Bars B,C = 10 µm, D = 5 µm.

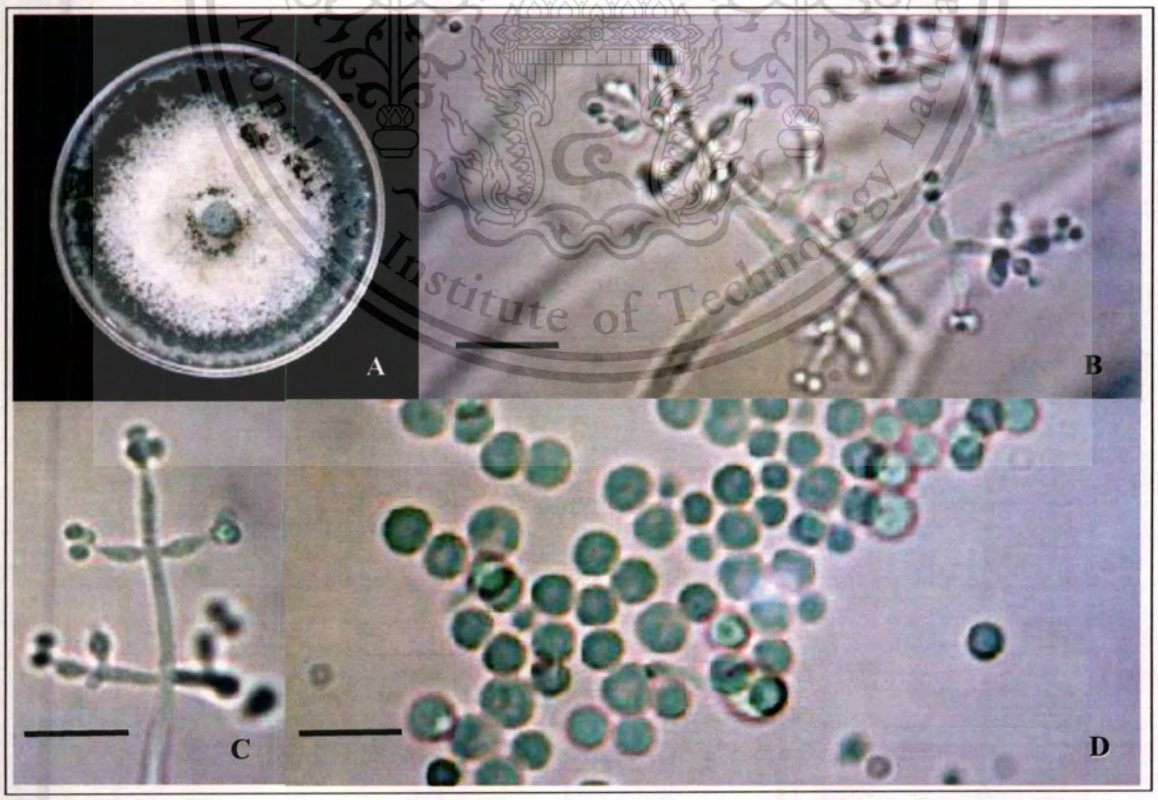

Fig. 4.85 *Trichoderma harzianum* RY101. A. 10-day-old-culture on PDA, B,C. conidia, D. conidiospores. Bars B,C = 10 µm, D = 5 µm.

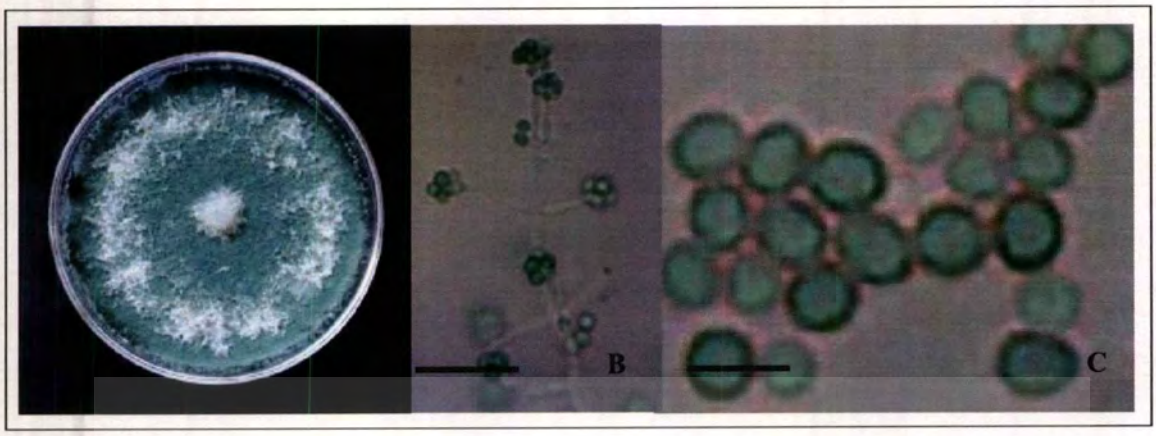

Fig. 4.86 *Trichoderma harzianum* RY104. A. 10-day-old-culture on PDA, B. conidia, C. conidiospores. Bars B = 10 μm, C = 5 μm.

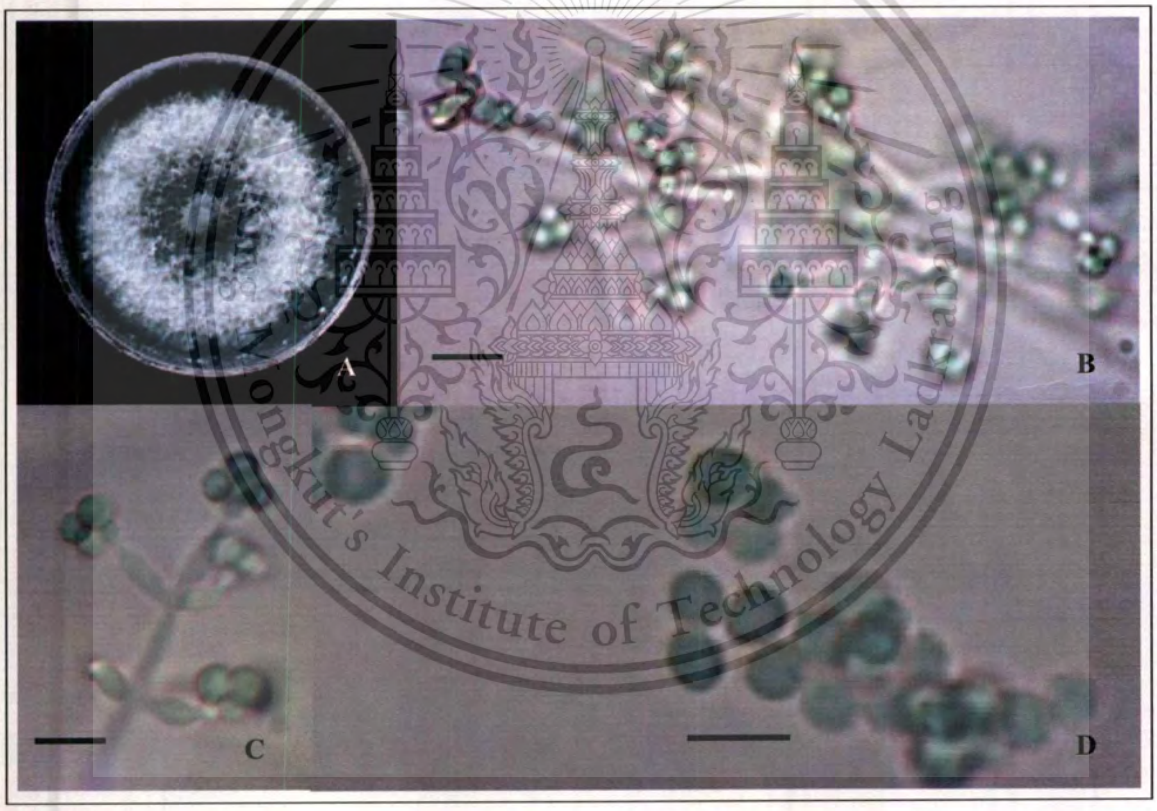

Fig. 4.87 *Trichoderma harzianum* RY112. A. 10-day-old-culture on PDA, B,C. conidia, D. conidiospores. Bars B,C = 10 μm, D = 5 μm.

During the experiment of screening antagonistic fungi against pineapple root rot, 2 isolates of *Chatomium* (MB303 and MB502) encountered by baiting technique could not be identified as any species of *Chaetomium* and were therefore described as new. These species were named *Chaetomium siamense* sp. nov. (Figs. 4.88-4.90). The new species was described details

on its morphology and provided molecular evidence to support its status as a new texon as follows:-.

Chaetomium siamense Pornsuriya & Soytong **sp. nov.** Fig. 4.88-4.90

MYCOBANK MB 506801

Coloniae modica bardus crescents in agaris 'PDA' et 'CMA', cum mycelio aerio; reversum rubus. Mycelium ex hyphis hyalinis vel subhyalinis, septatis, laevibus, 1.8-2.3 µm diam compositum. Ascomata superficialia, globosae, ovata, ostiolata, fuscobrunneum in refulgens, paramariae ad 87-148 x 107-177 µm. Peridium fuscobrunneum et ex textura angularis; epidermoidea ex fuscus, paramariae ad 2.8-5.7x5.1-10.4 µm. Ascomatal pili abundantibus, simplici, aurantiacus in refulgens, spira in ultima parte et ex septati, 1.3-4 µm diam, 170 µm longi. Pili laterals recti, leviter verrucosi, paramariae ad 56-160 µm longi. Asci numerosis, fasciculati, clavali, pedicellati, 8-spori, 9-14x28-40 µm. Ascosporae fusiformia, fascobrunnneum, laevibus, 4.3-6.0 x 11.1-13.5 µm, cum poro germinalibus apicali visibile.

ETYMOLOGY: *siamense* refers to appear in Thailand

Colonies in PDA and CMA growing moderately slow, covering the Petri dish surface after 14 days at room temperature, with aerial mycelium; become red due to a red pigment exudates when young, yellow when old and formation of ascomata. Mycelium composed of hyaline to subhyaline, septate, smooth hyphae, 1.8-2.3 µm diam. Ascomata discrete, maturing within 10-14 days, superficial globose or ovate, ostiolate, dark brown in reflected light, 87-148 x 107-177 µm. Peridium dark brown, with textural angular; outer layer consisted of brown and thick-walled cells measuring 87-148 x 107-177 µm. Ascomatal hairs abundant, unbranched, red or orange-red in reflected light, apically circinate or coiled, regularly septate and forming a dense tuft around the ostiole, finely verrucose toward the tip, up to 170 µm long, 1.3-4 µm diam near the base. Lateral hairs less abundant, straight, finely verrucose, 56-160 µm long. Asci numerous, fasciculate, clavate, stalked, 8-spored, 9-14x28-40 µm. Ascospores fusiform, dark brown at maturity smooth- and rather thick-walled cells, 4.3-6.0 x 11.1-13.5 µm, with two apical germ pores.

Holotype: Thailand, Phatthalung, isolated from soil under pineapple plantation, 27 September 2007, Chaninun Pornsuriya TMACC001. The holotype was deposited in the Thai Mycological Association Culture Collection (TMACC) at KMITL, Bangkok, Thailand.

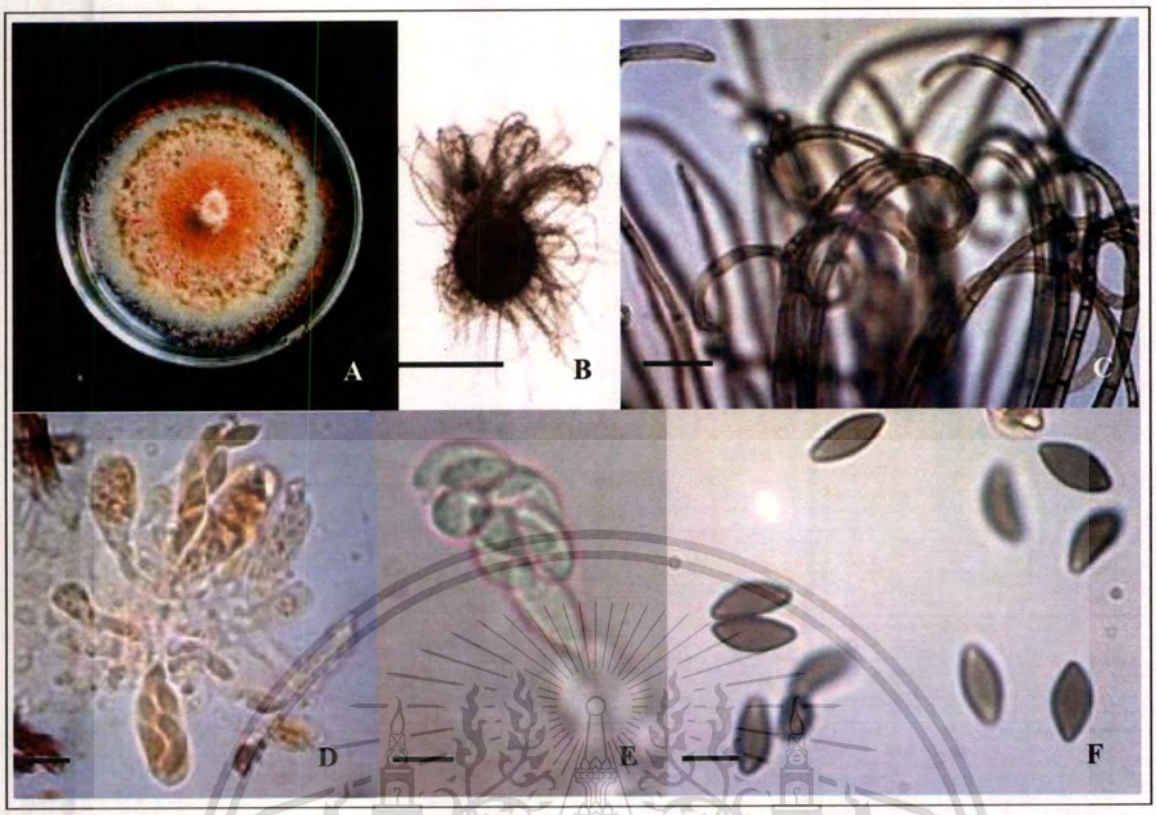

Fig. 4.88 *Chaetomium siamense* MB303. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10 µm.

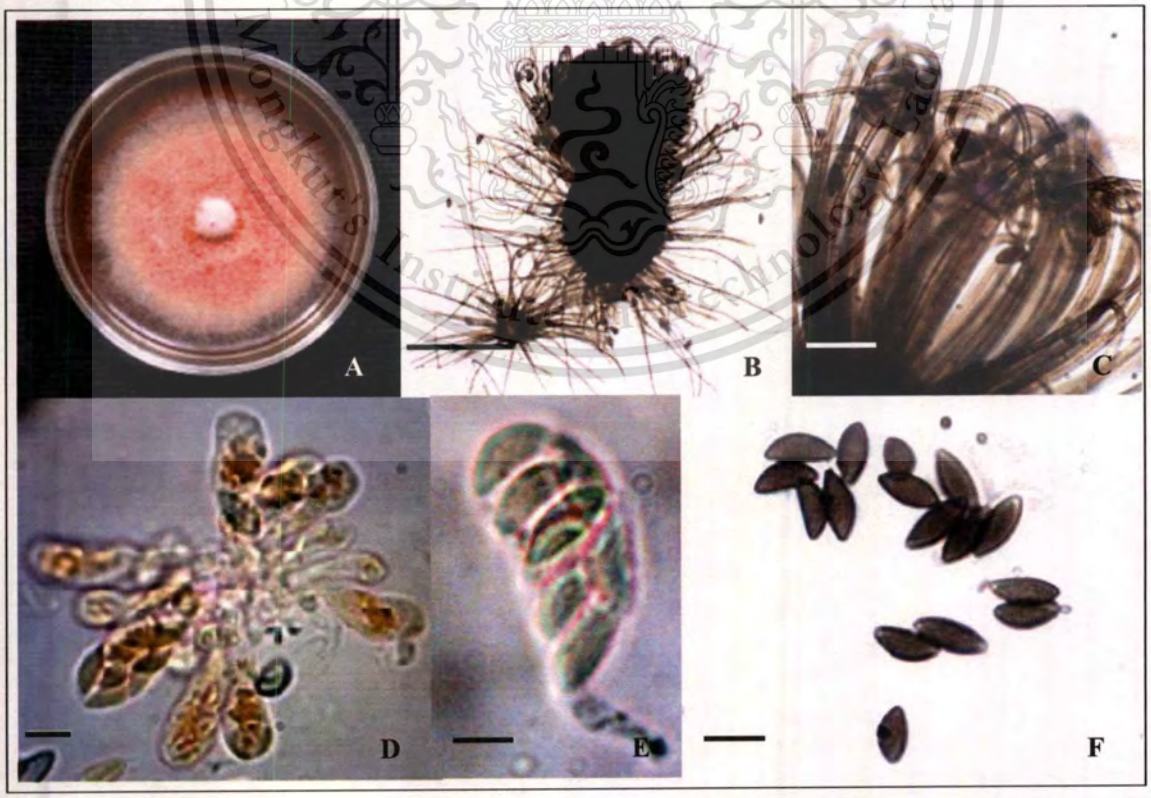

Fig. 4.89 *Chaetomium siamense* MB502. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores. Bar. B=100 µm, C,D,E,F=10 µm.

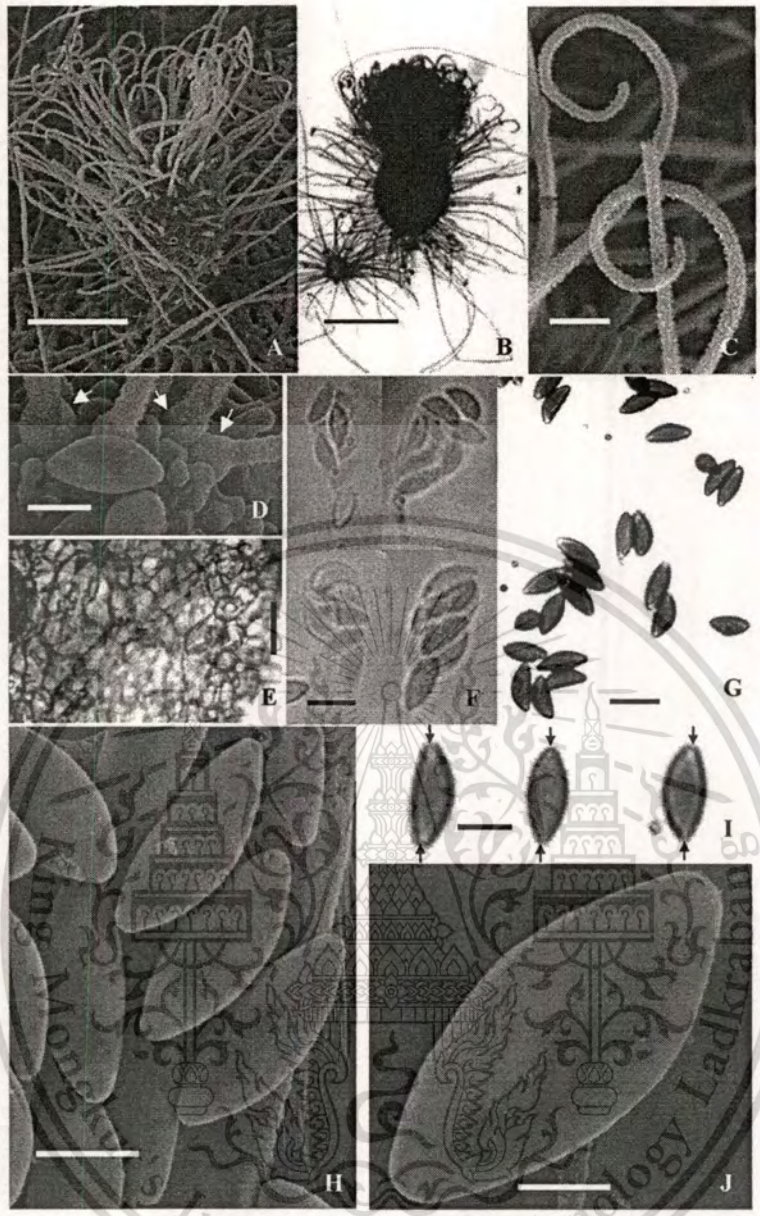

Fig 4.90 *Chaetomium siamense* MB502. A. Ascomata viewed under a scanning electron microscope.B. Ascomata viewed under a light microscope. C-D. Arcuate ascomatal hairs with swollen basal cells viewed under a scanning electron microscope. E. Peridium viewed under a light microscope. F. Asci viewed under a light microscope. G. Ascospores viewed under a light microscope. H. Ascospores viewed under a scanning electron microscope. I. Fusiform ascospores with two apical germ pores. J. Ascospore viewed under a scanning electron microscope. Bars A, B=50 µm. C, D, E, F=10 µm. G, H=5 µm. I=2 µm.

The new species was similar to *C. cupreum* but differed by ascospores shape. In *C. siamense* ascospores were fusiform with two apical germ pores while in *C. cupreum* ascospores were reniform with a singal germ pore (Fig. 4.91).

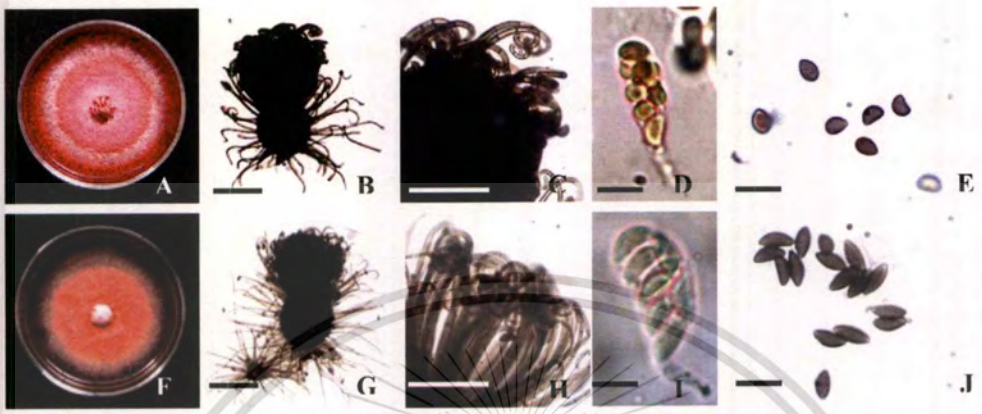

Fig.4.91 Comparison of the characteristics between *Chaetomium cupreum* and *Chaetomium siamense* A-E. *Chaetomium cupreum*, F-J. *Chaetomium siamense*. A,F.10-day-old-culture on PDA, B,G. ascomata, C, H. ascomatal hairs, D,I. 8 ascospores in an ascus, E,J. ascospores. Scale bars: Fig. B, C, G, H 50 µm; Fig. D, E, I, J 10 µm.

In addition, the phylogenetic analysis (Fig. 4.92) clearly indicated that *C. siamense* related to *C. cupreum* by this new species shared a common ancestor with *C. cupreum* but the sequence differences were clearly consistent with a separated branch linking of the two taxa and with *C. siamense* being a distinct species.

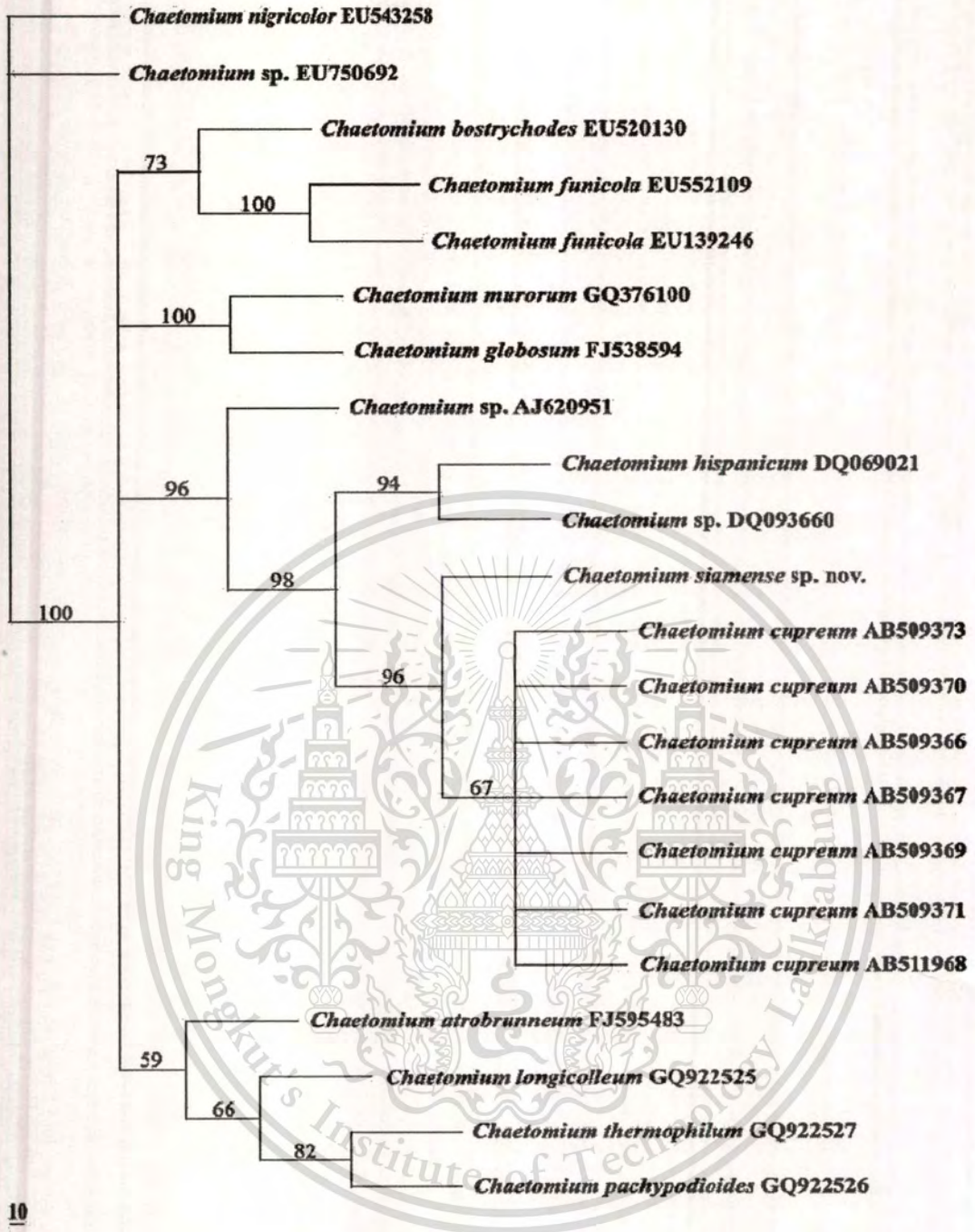

Fig. 4. 92 Phylogenetic tree of *Chaetomium* spp. from GenBank including *Chaetomium siamense* and *Chaetomium cupreum* constructed after distance-based analysis of ITS1, 5.8S and ITS2 regions of rDNA. GenBank accession numbers are shown if available. Numbers at the brances indicate the percentage of bootstrap values after 1000 replications. The designated outgroup was *Chaetomium funicola*.

4.2.2 Screening of Antagonistic Fungi

Screening of antagonistic fungi was determined by testing bi-culture plates and followed by testing antagonistic substances. In a preliminary test, the 42 isolates of promising antagonistic fungi isolated from soil under pineapple plantations in Phatthalung and Rayong provinces as shown in Table 4.7 and Figs. 4.48-4.89 were tested for screening antagonist against *P. aphanidermatum* RY803 in bi-culture plates. Colony diameters of the promising antagonists and *P. aphanidermatum* RY803 were transformed into percentage of mycelial growth inhibition and number of oospores of *P. aphanidermatum* RY803 were transformed into percentage of oospore inhibition. Among the promising antagonists tested, *Gliocladium catenulatum* RY102, *Trichoderma harzianum* RY104 and *T. harzianum* RY112 showed significantly higher (P=0.01) inhibition on mycelial growth than the other isolates by 100, 100 and 100% inhibition over control, respectively (Figs.4.127, 4.133, 4.134 and Table 4.8) which were followed by *Chaetomium bostrychodes* PR101, *T. harzianum* RY101, *C. cupreum* NB102, *C. cochliodes* RY301, *G. catenulatum* RY111, *C. flavigenum* MB607, *C. cupreum* RY201, *C. aureum* MB601 and *C cupreum* RY202 by 98.06, 90.28, 89.72, 89.00, 88.89, , 87.50, 86.95, 82.25 and 81.67% inhibition over control, respectively (Figs.4.100, 4.132, 4.106, 4.105, 4.129, 4.114, 4.107, 4.95, 4.108 and Table 4.8). Moreover, *T. harzianum* RY104 and *T. harzianum* RY112 showed significantly higher (P=0.01) inhibition on oospore formation than the others by 100 and 100% inhibition over control, respectively (Figs.4.133-4.134 and Table 4.8) followed by *C. cochliodes* RY301, *Gliocladium catenulatum* RY102, *C. bostrychodes* PR101, *C. cupreum* NB102, *C. aureum* MB601, *G. catenulatum* RY111, *C cupreum* RY202, *T. harzianum* RY101, *C. cupreum* RY201 and *C. flavigenum* MB607 by 92.17, 91.44, 86.93, 85.16, 84.40, 83.74, 81.67, 80.27, 75.24 and 65.88% inhibition over control, respectively (Figs.4.105, 4.127, 4.100, 4.106, 4.95, 4.129, 4.108, 4.132, 4.107, 4.114 and Table 4.8). However, 10 isolates of promising antagonistic fungi that gave both percent inhibitions of mycelia growth and oospore formation over 80% as follows: *C. aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *G. catenulatum* RY102, *Gliocladium catenulatum* RY111, *Trichoderma harzianum* RY 101, *Trichoderma harzianum* RY 104 and *Trichoderma harzianum* RY 112 were re-isolated from bi-culture plates to keep in PDA slant for further culturing to extract their metabolites and tested for mycelial growth and oospore inhibitions to screen only one isolate that was the most effective antagonist against *P. aphanidermatum* RY803 causing root rot of pineapple.

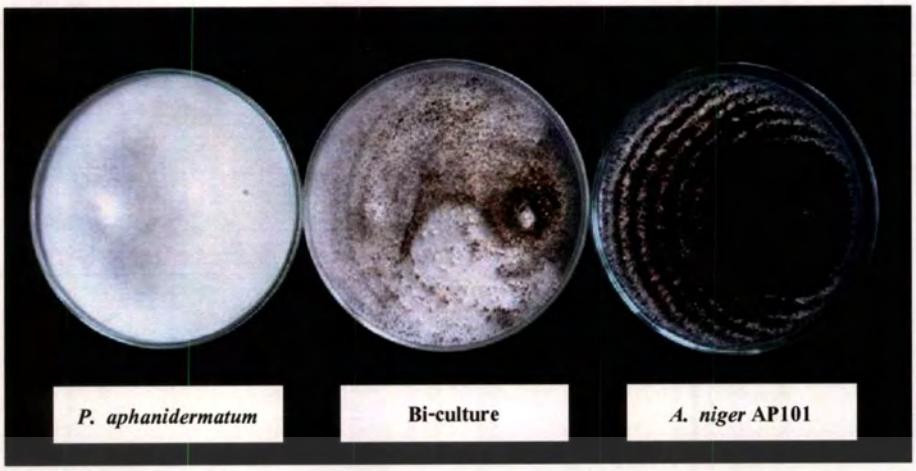

Fig.4.93 Bi-culture test of *Aspergillus niger* AP101 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Fig.4.94 Bi-culture test of *Chaetomium aureum* MB103 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

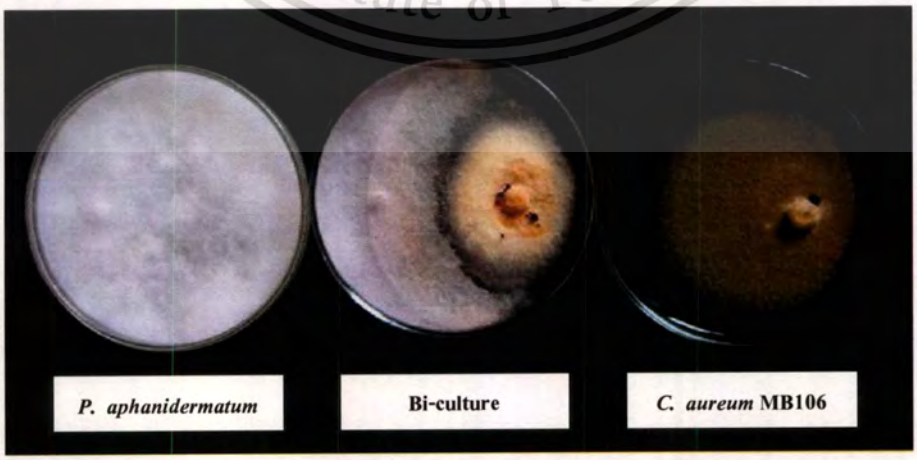

Fig.4.95 Bi-culture test of *Chaetomium aureum* MB601 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

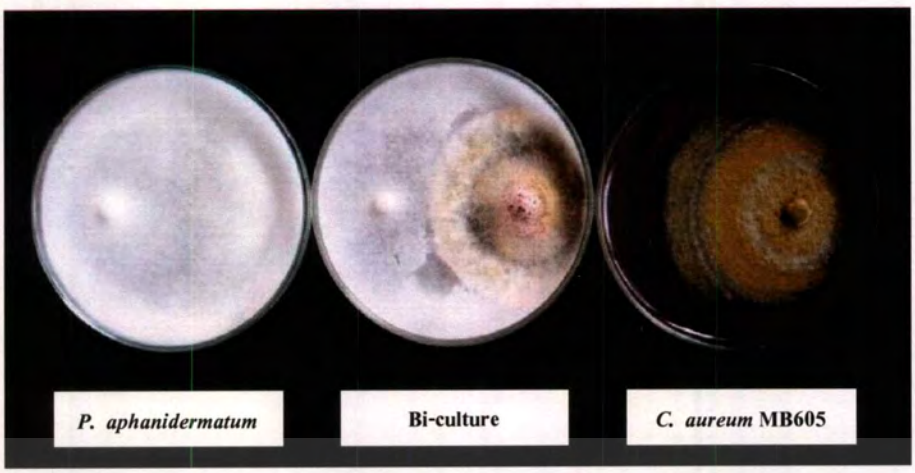

Fig.4.96 Bi-culture test of *Chaetomium aureum* MB605 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

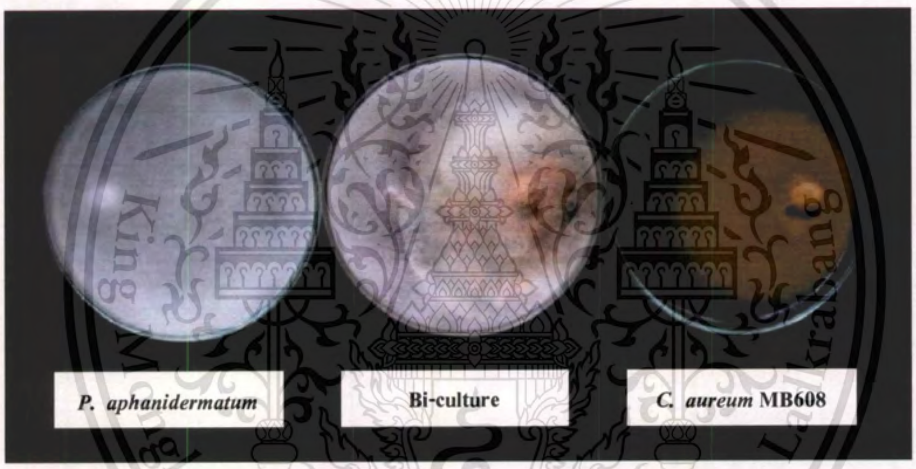

Fig.4.97 Bi-culture test of *Chaetomium aureum* MB608 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

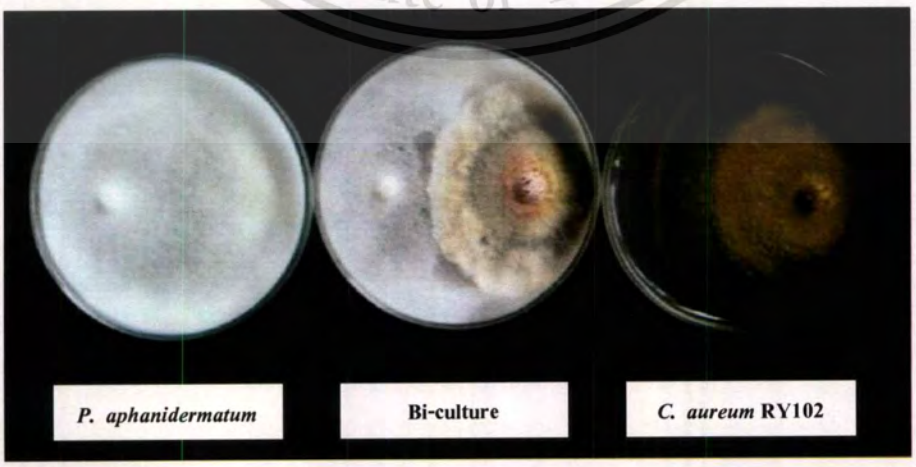

Fig.4.98 Bi-culture test of *Chaetomium aureum* RY102 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

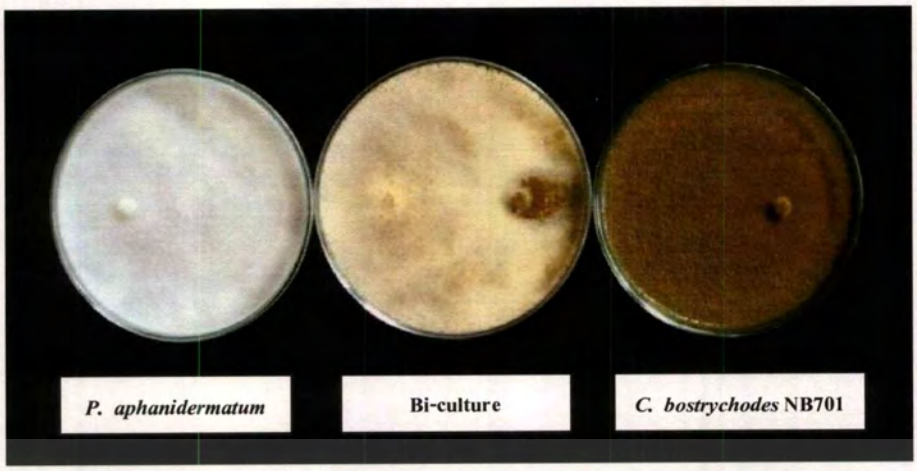

Fig.4.99 Bi-culture test of *Chaetomium bostrychodes* NB701 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Fig.4.100 Bi-culture test of *Chaetomium bostrychodes* PR101 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

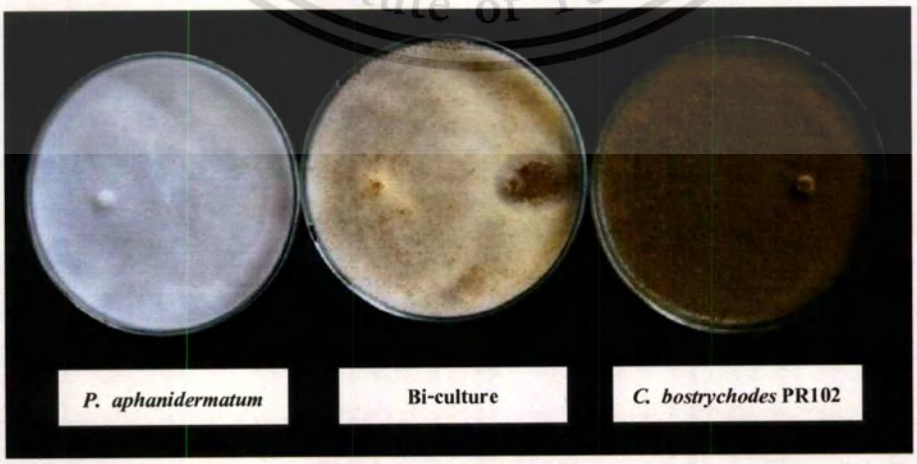

Fig.4.101 Bi-culture test of *Chaetomium bostrychodes* PR102 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Fig.4.102 Bi-culture test of *Chaetomium bostrychodes* PR103 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Fig.4.103 Bi-culture test of *Chaetomium brasiliense* AM101against *Pythium aphanidermatum* RY803 on PDA at 20 days.

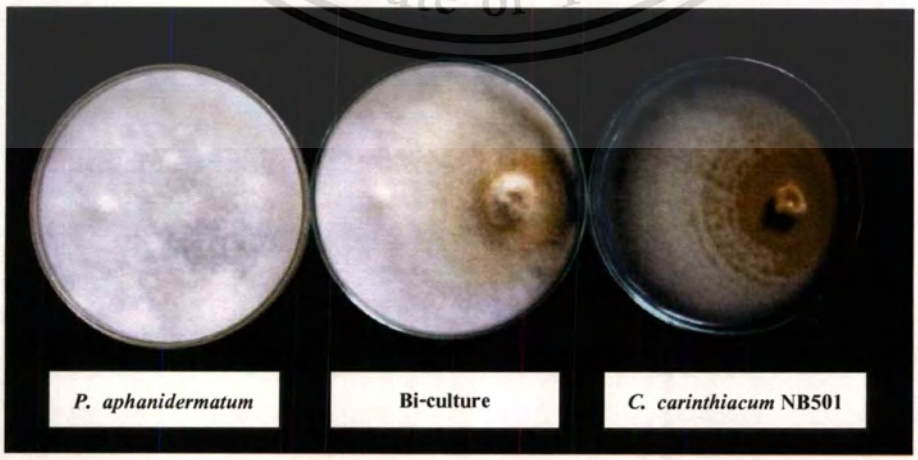

Fig.4.104 Bi-culture test of *Chaetomium carinthiacum* NB501 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Fig.4.105 Bi-culture test of *Chaetomium cochliodes* RY301 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

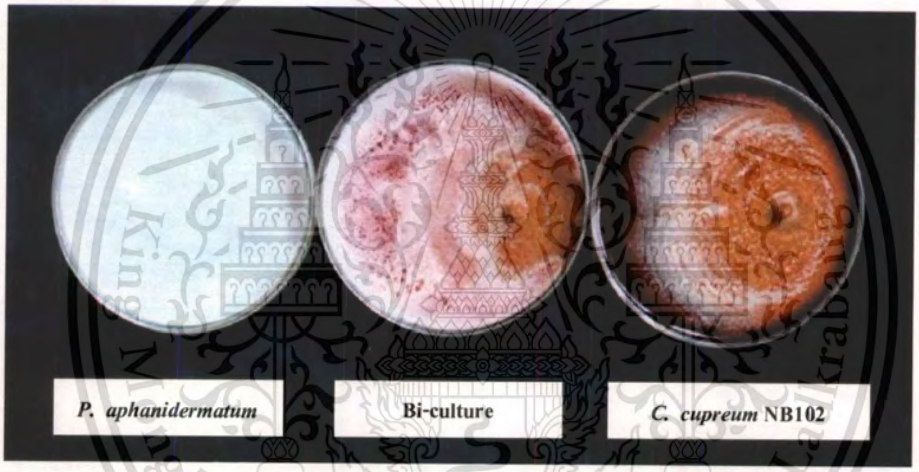

Fig.4.106 Bi-culture test of *Chaetomium cupreum* NB102 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

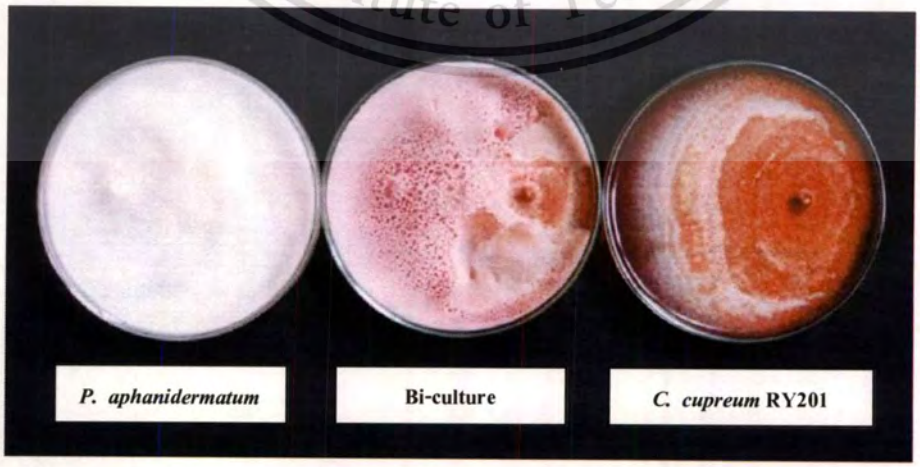

Fig.4.107 Bi-culture test of *Chaetomium cupreum* RY201 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

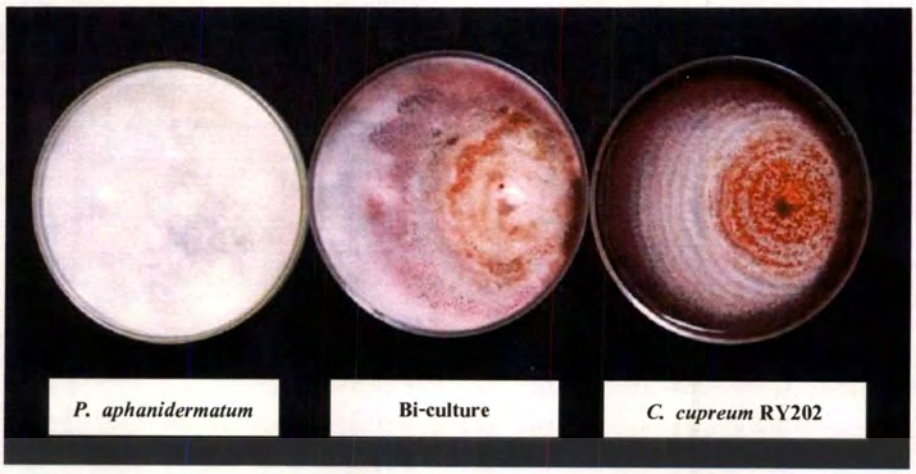

Fig.4.108 Bi-culture test of *Chaetomium cupreum* RY202 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

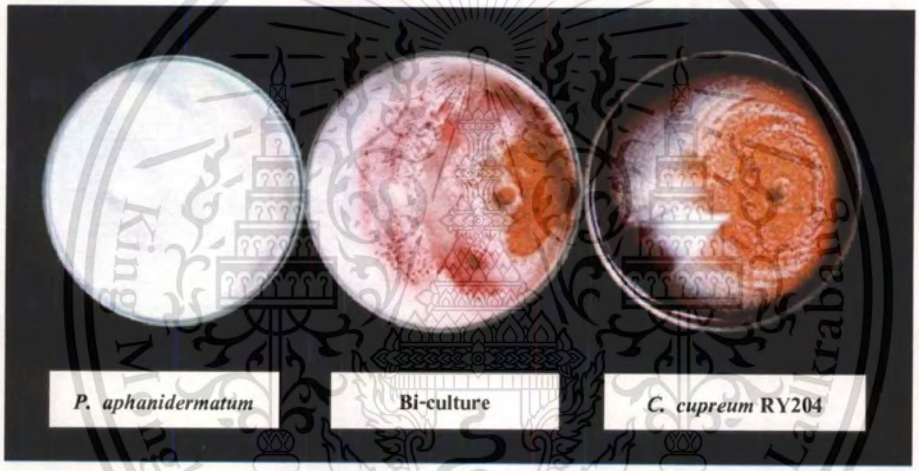

Fig.4.109 Bi-culture test of *Chaetomium cupreum* RY204 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

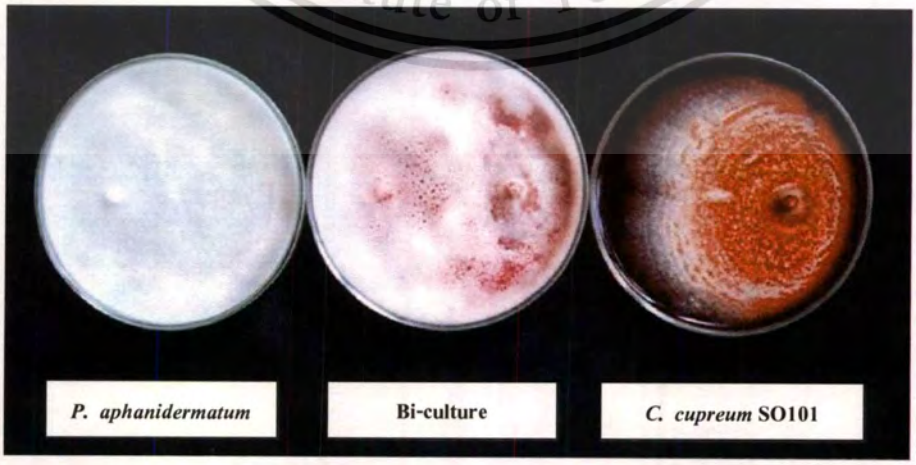

Fig.4.110 Bi-culture test of *Chaetomium cupreum* SO101 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

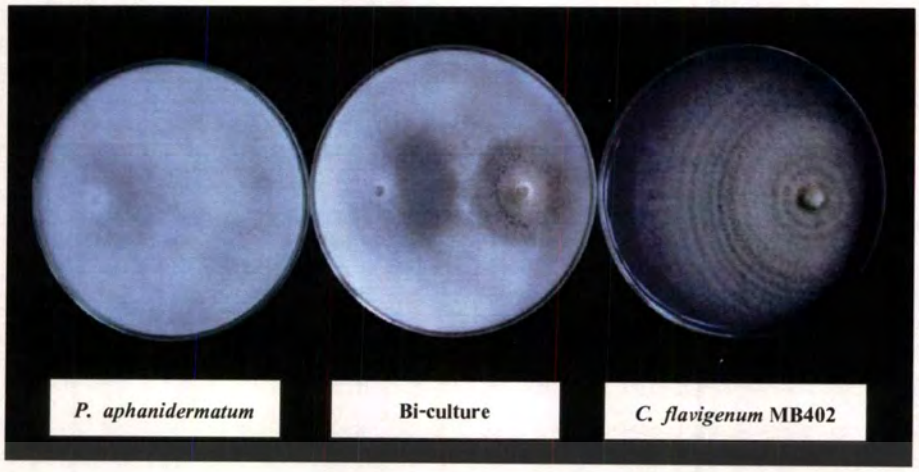

Fig.4.111 Bi-culture test of *Chaetomium flavigenum* MB402 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

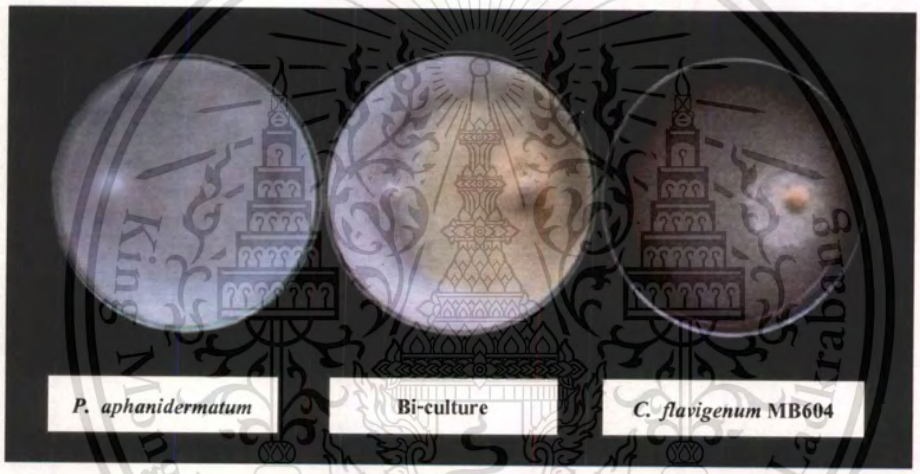

Fig.4.112 Bi-culture test of *Chaetomium flavigenum* MB604 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

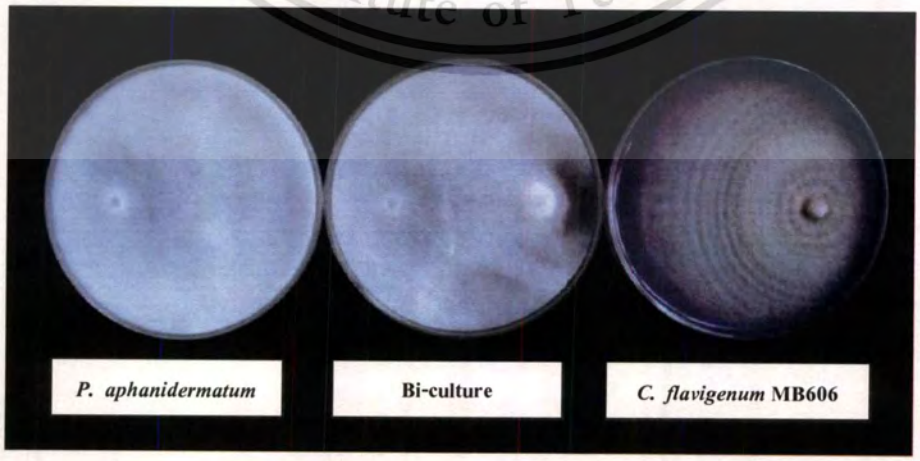

Fig.4.113 Bi-culture test of *Chaetomium flavigenum* MB606 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

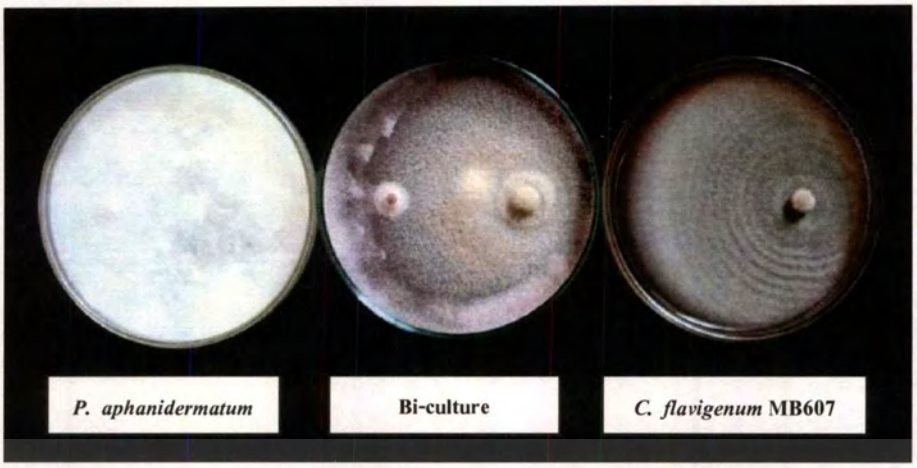

Fig.4.114 Bi-culture test of *Chaetomium flavigenum* MB607 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

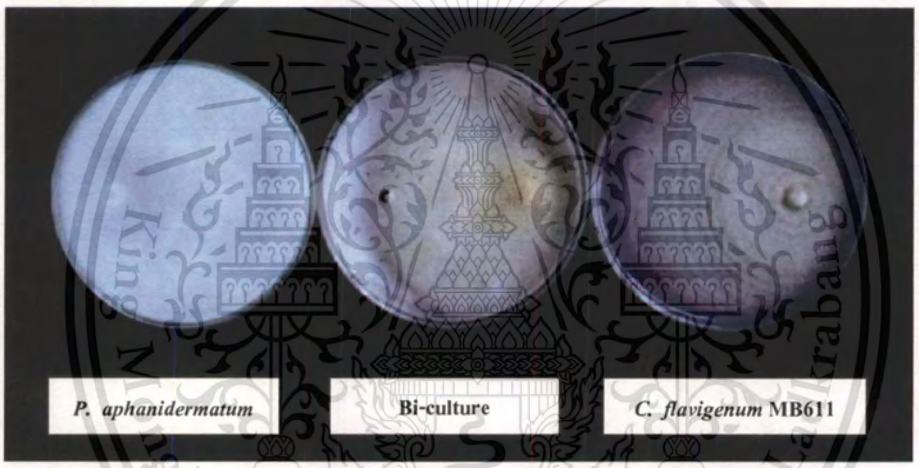

Fig.4.115 Bi-culture test of *Chaetomium flavigenum* MB611 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

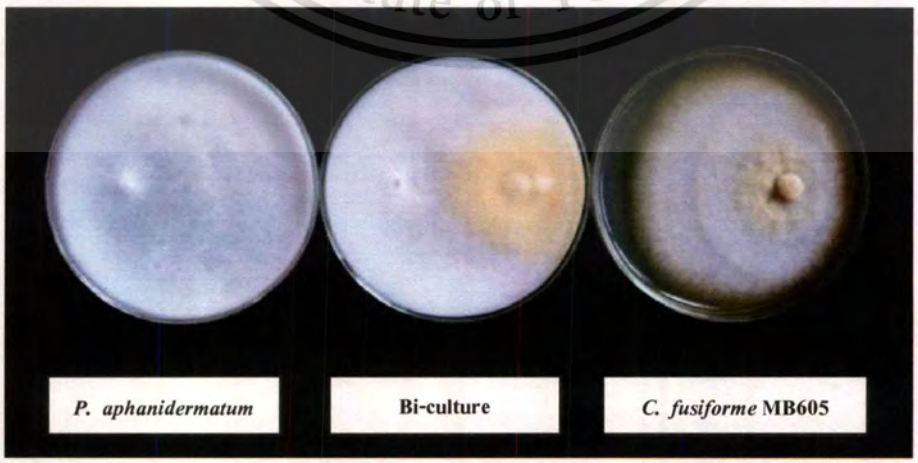

Fig.4.116 Bi-culture test of *Chaetomium fusiforme* MB605 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

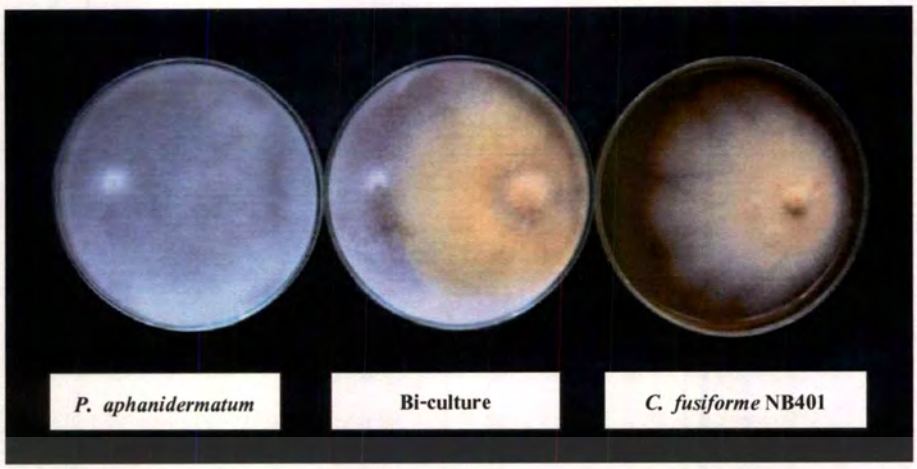

Fig.4.117 Bi-culture test of *Chaetomium fusiforme* NB401 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

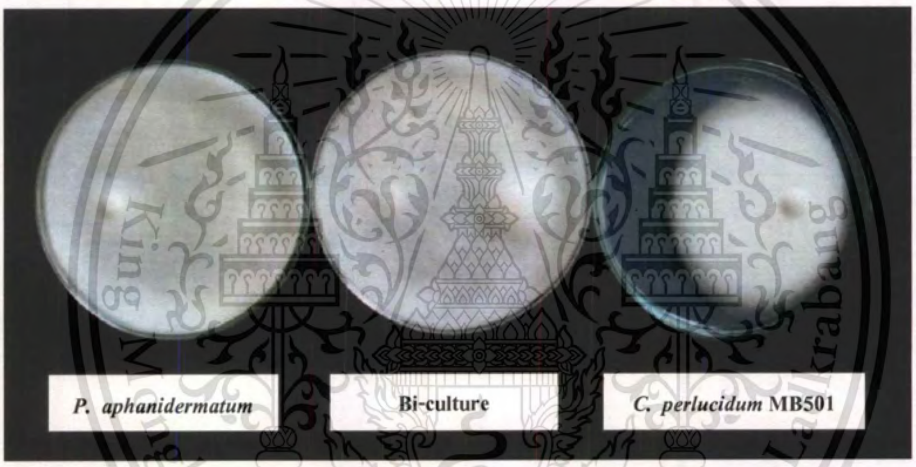

Fig.4.118 Bi-culture test of *Chaetomium perlucidum*MB501 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

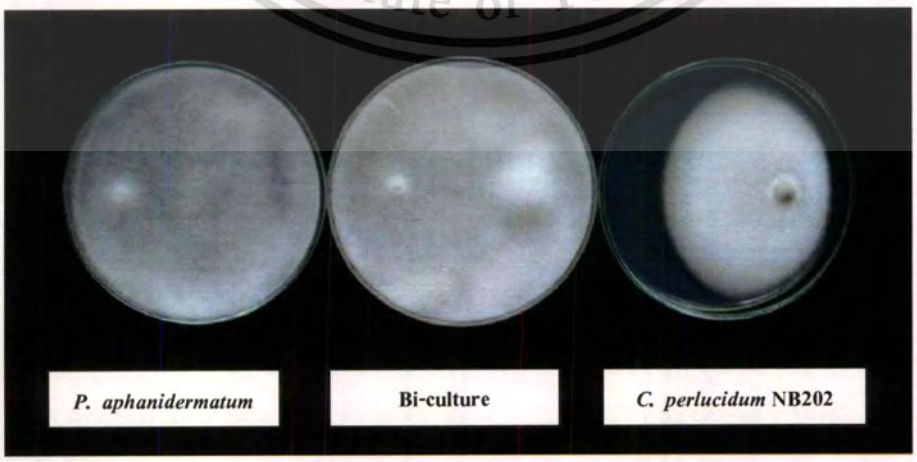

Fig.4.119 Bi-culture test of *Chaetomium perlucidum* NB202 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

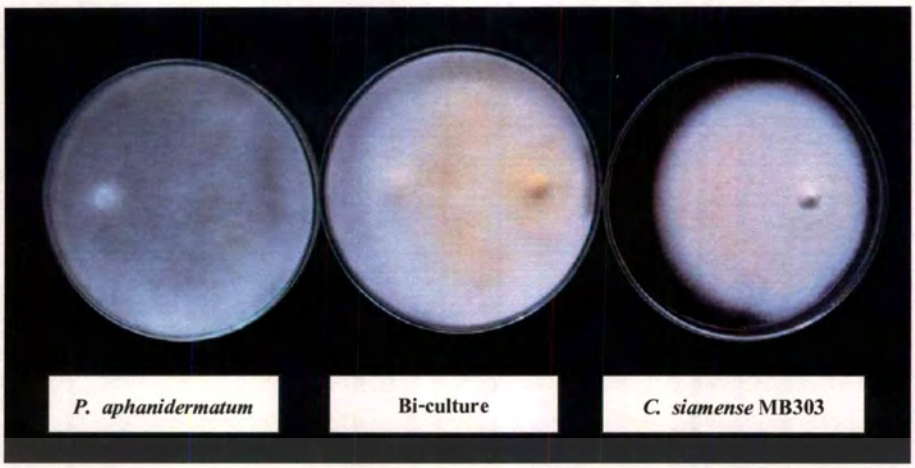

Fig.4.120 Bi-culture test of *Chaetomium siamense* MB303 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

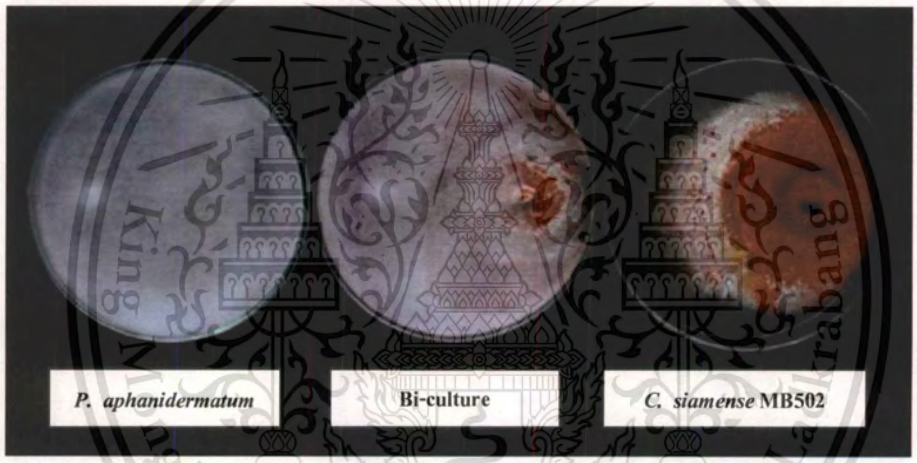

Fig.4.121 Bi-culture test of *Chaetomium siamense* MB502 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

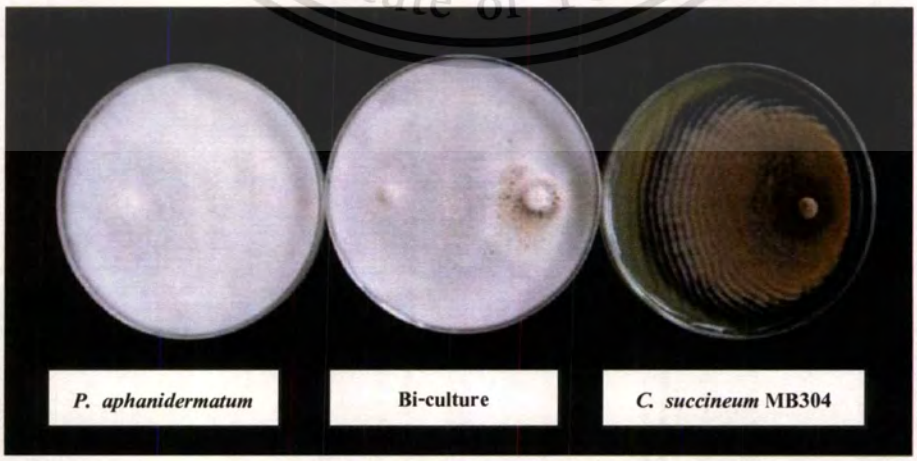

Fig.4.122 Bi-culture test of *Chaetomium succineum* MB304 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

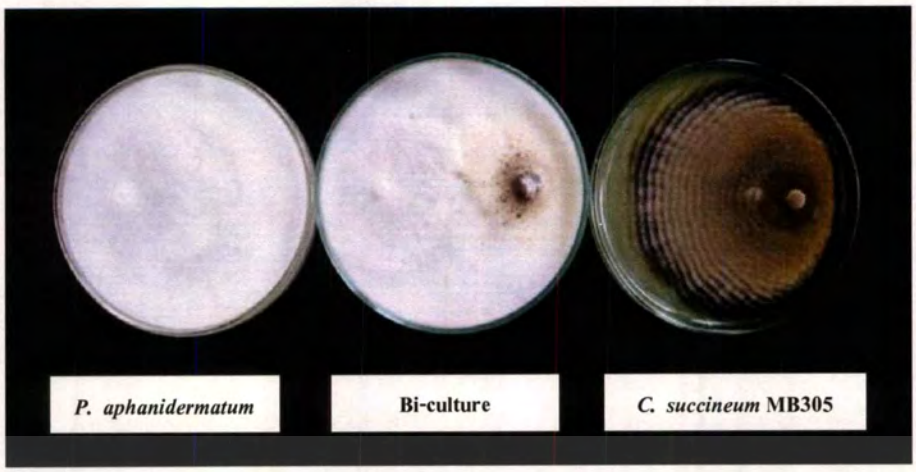

Fig.4.123 Bi-culture test of *Chaetomium succineum* MB305 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Fig.4.124 Bi-culture test of *Emericella nidulans* EN101 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

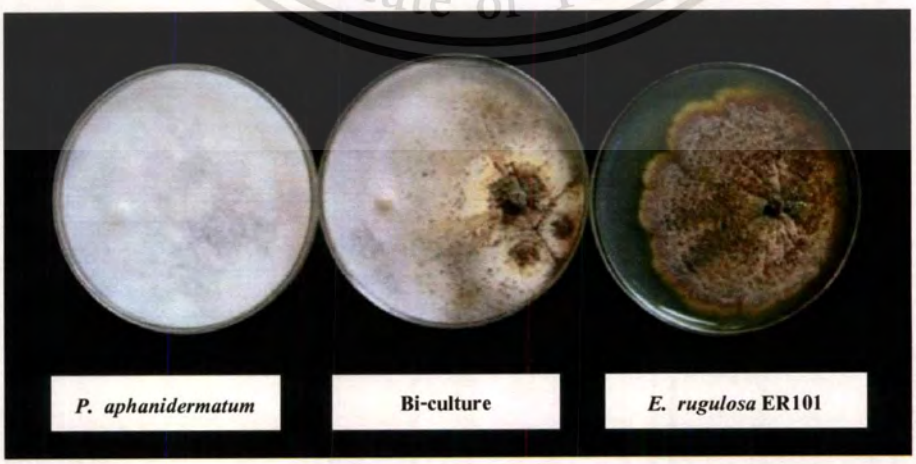

Fig.4.125 Bi-culture test of *Emericella rugulosa* ER101 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Fig.4.126 Bi-culture test of *Eurotium chevalieri* EU101 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Fig.4.127 Bi-culture test of *Gliocladium catenulatum* RY102 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

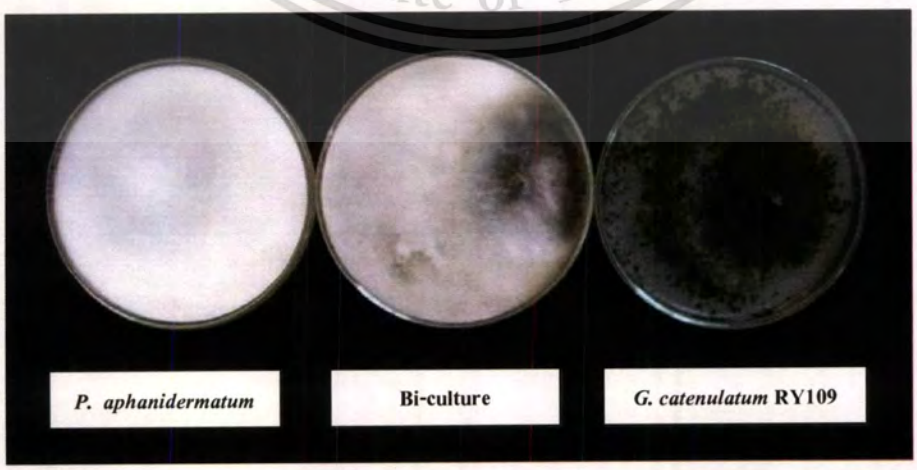

Fig.4.128 Bi-culture test of *Gliocladium catenulatum* RY109 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

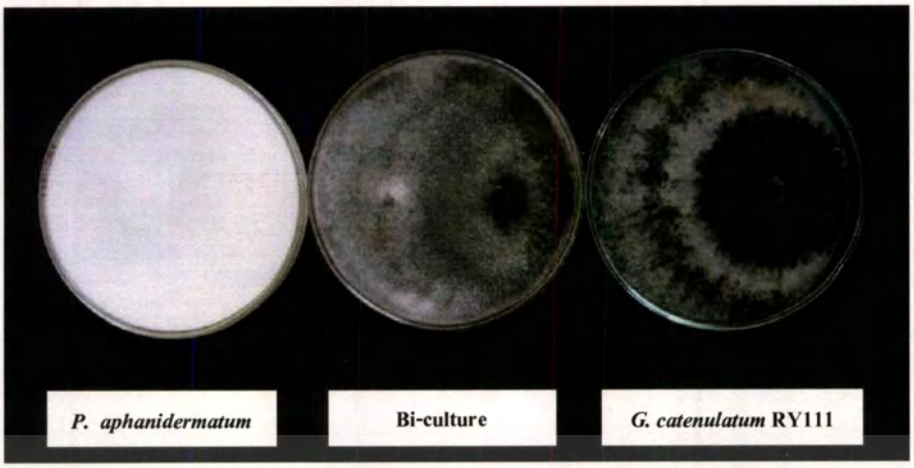

Fig.4.129 Bi-culture test of *Gliocladium catenulatum* RY111 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

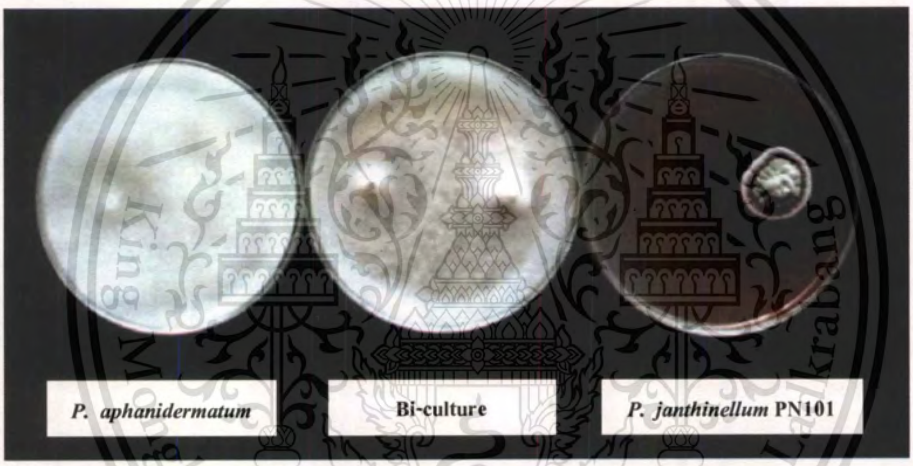

Fig.4.130 Bi-culture test of *Penicillium janthinellum* PN101 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Fig.4.131 Bi-culture test of *Trichoderma hamatum* PT101 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

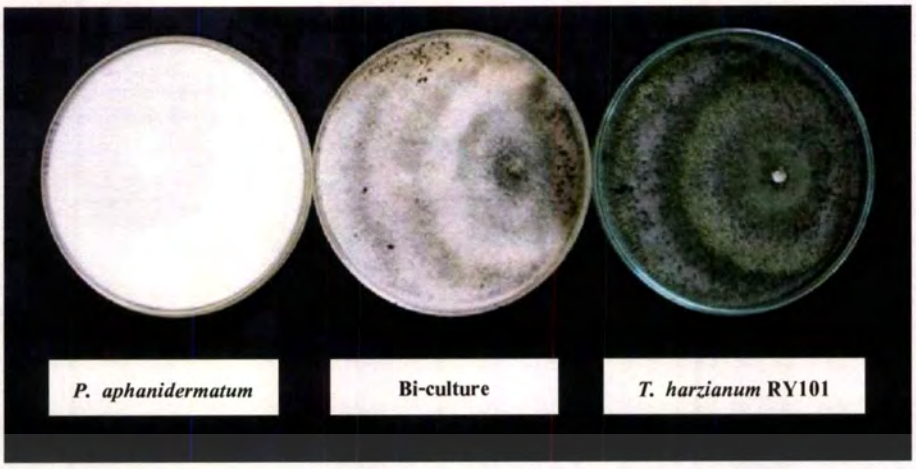

Fig.4.132 Bi-culture test of *Trichoderma harzianum* RY101 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

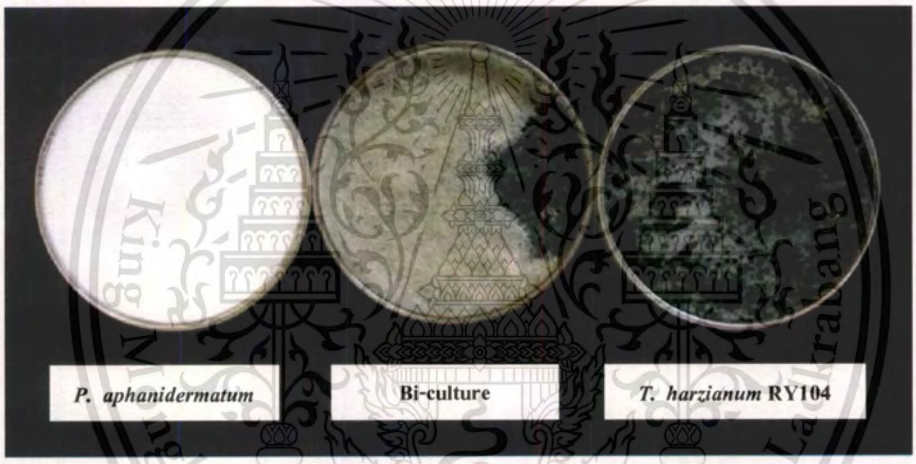

Fig.4.133 Bi-culture test of *Trichoderma harzianum* RY104 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

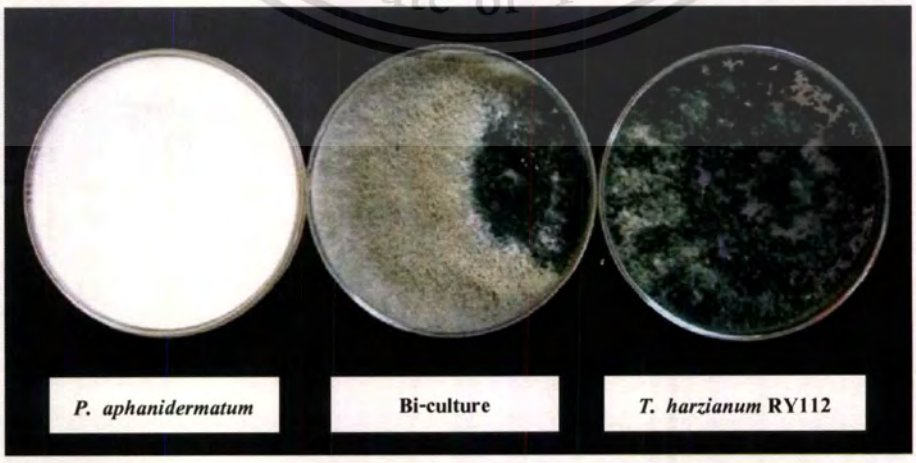

Fig.4.134 Bi-culture test of *Trichoderma harzianum* RY112 against *Pythium aphanidermatum* RY803 on PDA at 20 days.

Table 4.8 Percent inhibition on growth and oospores of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

promising antagonistic fungi	colony diameter (cm)			Growth inhibition (%)[1/]	numberof oospores x 10^7		Oospore inhibition (%)[2/]
	P. aphanidermatum RY803 (control))	*P. aphanidermatum* RY803 in bi-culture plate	promising antagonist (control)		*P. aphanidermatum* RY803 (control)	*P. aphanidermatum* RY803 bi-culture plate	
Aspergillus niger AP101	9.00	9.00	9.00	0.06 m	10.21	10.18	0.25 t
Chaetomium aureum MB103	9.00	2.63	7.80	70.84 ef	10.71	3.29	70.84 fg
Chaetomium aureum MB601	9.00	2.48	7.53	82.25 cd	10.44	1.63	84.40 de
Chaetomium aureum MB603	9.00	5.20	7.75	42.22 i	10.59	7.37	42.22 mn
Chaetomium aureum MB608	9.00	3.67	7.80	59.20 gh	10.91	5.04	49.24 kl
Chaetomium aureum RY102	9.00	4.80	7.75	46.67 i	10.31	5.58	48.80 kl
Chaetomium bostrychodes NB701	9.00	8.35	9.00	7.22 lm	10.43	7.44	28.70 o
Chaetomium bostrychodes PR101	9.00	0.18	9.00	98.06 ab	10.55	1.38	86.93 cd
Chaetomium bostrychodes PR102	9.00	7.34	9.00	71.33 lm	10.80	5.50	48.80 kl
Chaetomium bostrychodes PR103	9.00	2.34	9.00	8.42 klm	10.54	6.54	37.87 n
Chaetomium brasiliense AM101	9.00	2.44	9.00	72.92 def	10.57	3.19	69.80 g
Chaetomium carinthiacum NB501	9.00	7.75	8.50	13.89 jkl	10.30	9.55	7.34 qr
Chaetomium cochliodes RY301	9.00	1.35	9.00	89.00 abc	10.30	1.10	92.17 b
Chaetomium cupreum NB102	9.00	0.93	8.00	89.72 abc	10.53	1.56	85.16 de
Chaetomium cupreum RY201	9.00	1.18	8.75	86.95 bc	10.18	2.52	75.24 f
Chaetomium cupreum RY202	9.00	1.65	8.00	81.67 cde	10.49	1.92	81.67 de
Chaetomium cupreum RY204	9.00	3.83	8.75	57.50 h	10.41	4.24	59.29 h
Chaetomium cupreum SO101	9.00	4.83	7.54	46.39 i	10.37	4.92	52.52 ijk
Chaetomium flavigenum MB402	9.00	8.78	8.72	2.50 lm	10.46	9.47	9.52 q

Table 4.8 (continued) Percent inhibition on growth and oospores of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

promising antagonistic fungi	colony diameter (cm)			Growth inhibition (%)[1/]	number of oospores x 10^7		Oospore inhibition (%)[2/]
	P. aphanidermatum RY803 (control)	*P. aphanidermatum* RY803 in bi-culture plate	promising antagonist (control)		*P. aphanidermatum* RY803 (control)	*P. aphanidermatum* RY803 bi-culture plate	
Chaetomium flavigenum MB604	9.00	8.83	8.82	68.61 fg	10.46	4.74	54.65 hij
Chaetomium flavigenum MB606	9.00	9.00	8.90	0.00 m	10.43	9.76	6.38 qrs
Chaetomium flavigenum MB607	9.00	1.13	8.50	87.50 bc	10.39	3.35	65.88 g
Chaetomium flavigenum MB611	9.00	2.68	8.86	70.28 f	10.25	4.62	54.89 hij
Chaetomium fusiforme MB605	9.00	7.25	7.90	22.22 j	10.31	8.49	17.57 p
Chaetomium fusiforme NB401	9.00	2.58	7.58	71.39 def	10.40	5.18	50.11 jkl
Chaetomium perlucidum MB501	9.00	9.00	6.83	0.00 m	10.51	10.34	2.65 rst
Chaetomium perlucidum NB202	9.00	9.00	6.76	0.00 m	10.34	10.20	1.37 st
Chaetomium siamense MB303	9.00	8.55	8.52	5.00 lm	10.65	9.13	15.20 p
Chaetomium siamense MB502	9.00	3.88	7.59	56.95 h	10.32	3.52	56.95 hi
Chaetomium succineum MB304	9.00	8.03	9.00	10.83 klm	10.88	10.16	6.62 qrs
Chaetomium succineum MB305	9.00	8.50	9.00	5.56 lm	10.33	10.09	2.50 rst
Emericella nidulans EN101	9.00	7.75	9.00	13.89 jkl	10.36	5.32	48.63 kl
Emericella rugulosa ER101	9.00	2.75	7.86	69.45 fg	10.56	5.61	46.81 lm
Eurotium chevalieri EU101	9.00	6.00	9.00	36.11 i	10.54	6.45	38.15 n
Gliocladium catenulatum RY102	9.00	0.00	9.00	100 a	10.44	1.89	91.44 bc
Gliocladium catenulatum RY109	9.00	7.75	9.00	13.89 jkl	10.55	8.50	19.42 p
Gliocladium catenulatum. RY111	9.00	1.00	9.00	88.89 abc	10.72	1.74	83.74 de
Penicillium janthinellum PN101	9.00	9.00	1.20	0.00 m	10.53	10.43	3.68 rst

Table 4.8 (continued) Percent inhibition on growth and oospores of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

promising antagonistic fungi	colony diameter (cm)			Growth inhibition (%)[y]	number of oospores x 10^7		Oospore inhibition (%)[z]
	P. aphanidermatum RY803 (control)	*P. aphanidermatum* RY803 in bi-culture plate	promising antagonist (control)		*P. aphanidermatum* RY803 (control)	*P. aphanidermatum* RY803 bi-culture plate	
Trichoderma hamatum PT101	9.00	7.00	9.00	19.44 jk	10.27	4.35	56.95 hi
Trichoderma harzianum RY101	9.00	8.13	9.00	90.28 abc	10.25	2.12	80.27 e
Trichoderma harzianum RY104	9.00	0.00	9.00	100 a	10.26	0.00	100 a
Trichoderma harzianum RY112	9.00	0.00	9.00	100 a	10.64	0.00	100 a

[y] Average of four replications. Means of growth inhibition followed by a common letter were not significantly different (P=0.01) by DMRT.

[z] Average of four replications. Means of oospores inhibition followed by a common letter were not significantly different (P=0.01) by DMRT.

4.2.3 Testing Antagonistic Substances in Laboratory

The selected antagonistic isolates from previous experiment (*C. aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocladium catenulatum* RY102, *G. catenulatum* RY111, *Trichoderma harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112) were cultured in PDB for 4 weeks. Dried mycelial mats of each fungus were ground and extracted successively with solvents as follows: Hexane, Ethyl acetate (EtOAc) and Methanol (MeOH). The filtrates were evaporated to yield crude hexane, EtOAc and MeOH extracts, respectively. The obtained crude extracts were presented in Fig. 4.135 and yields of crude extracts were recorded as shown in Table 4.9. Colors of crude extracts were pale yellow or red to dark brown depended on different species of antagonistic fungi and extracting solvents (Fig. 4.135). Crude textures were oil, wax or solid (Fig. 4.135). Crude MeOH extract from *Trichoderma harzianum* RY104 gave the highest yield (4.113 g) followed by crude MeOH extract from *Chaetomium bostrychodes* PR101, crude EtOAc extract from *C. aureum* MB601, crude EtOAc extract from *C. cupreum* NB201 and crude hexane extract from *T. harzianum* RY104 that gave yield of crude extract 3.492, 3.004, 2.032 and 2.331 g, respectively. The yields of crude extracts varied according to fungal isolates, dry weight of mycelial mats and kind of solvents. Each crude extract was redissolved with 2% dimethylsulfoxide (DMSO), and then prepared in 6 concentrations (0, 10, 50, 100, 500 and 1,000 µg/ml) to test antifungal activities of each crude extract against mycelial growth and oospore formation of *P. aphanidermatum* RY803 on PDA (Figs.4.136-4.145 and Tables 4.10-4.13).

Table 4.9 Yields of mycelial mats and crude extracts from 10 antagonistic isolates.

Fungi	Dried weight of mycelial mats (g)	Yields of crude extracts (g)		
		hexane	EtOAc	MeOH
Chaetomium aureum MB601	49.93	1.335 (2.7%)	3.004 (6.0%)	1.892 (3.8%)
Chaetomium bostrychodes PR101	36.49	0.625 (1.7%)	1.098 (3.0%)	3.492 (9.6%)
Chaetomium cochliodes RY301	37.09	0.430 (1.2%)	1.352 (3.6%)	1.503 (4.1%)
Chaetomium cupreum NB201	49.92	0.927 (1.9%)	2.032 (4.1%)	1.327 (2.7%)
Chaetomium cupreum RY202	48.54	1.012 (2.1%)	1.083 (2.2%)	2.316 (4.8%)
Gliocladium catenulatum RY102	45.38	0.343 (0.8%)	0.257 (0.6%)	1.322 (2.9%)
Gliocladium catenulatum RY111	38.65	0.415 (1.1%)	0.916 (2.4%)	1.234 (3.2%)
Trichoderma harzianum RY 101	40.20	0.996 (2.5%)	0.450 (1.1%)	1.207 (3.0%)
Trichoderma harzianum RY 104	70.27	2.331 (3.3%)	1.333 (1.9%)	4.113 (5.9%)
Trichoderma harzianum RY 112	42.37	1.172 (2.8%)	0.453 (1.1%)	1.365 (3.2%)

Fig. 4.135 Crude extracts from 10 antagonistic fungi. A. *Chaetomium aureum* MB601, B. *Chaetomium bostrychodes* PR101, C. *Chaetomium cochliodes* RY301, D. *Chaetomium cupreum* NB201, E. *Chaetomium cupreum* RY202, F. *Gliocladium catenulatum* RY102, G. *Gliocladium catenulatum* RY111, H. *Trichoderma harzianum* RY101, I. *Trichoderma harzianum* RY104, J. *Trichoderma harzianum* RY112.

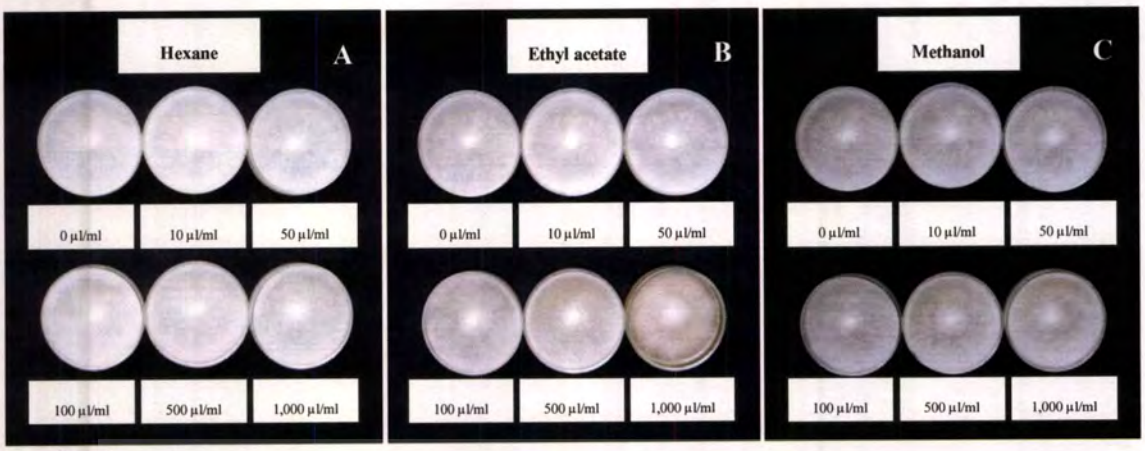

Fig. 4. 136 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Chaetomium aureum* MB601 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

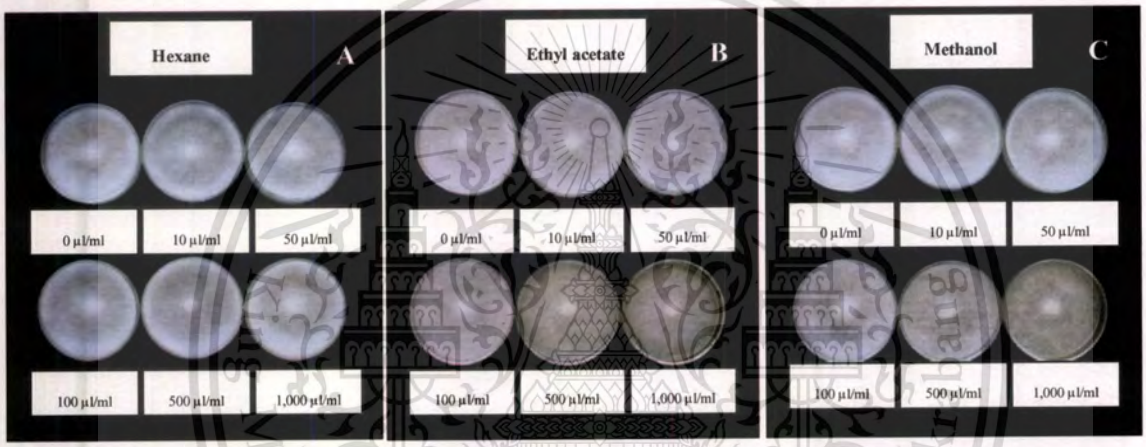

Fig. 4.137 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Chaetomium bostrychodes* PR101 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

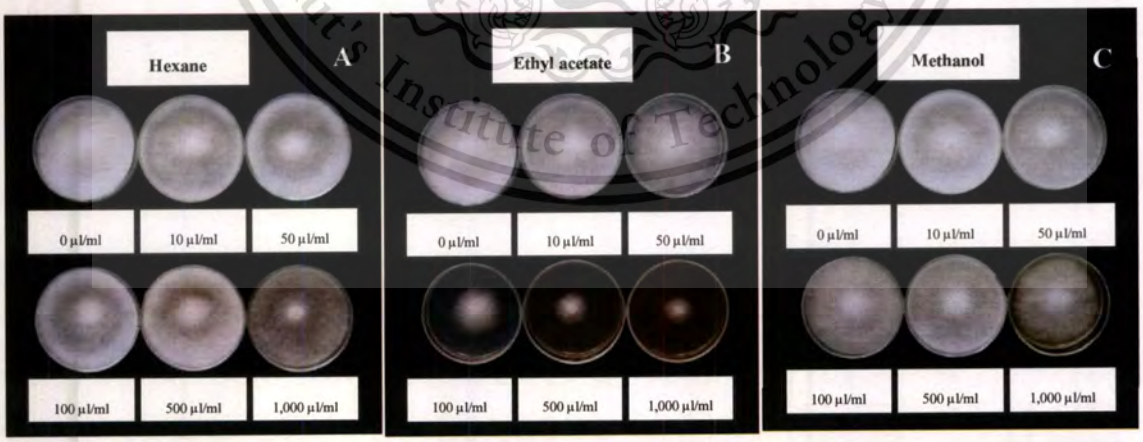

Fig. 4.138 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Chaetomium cochliodes* RY301 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

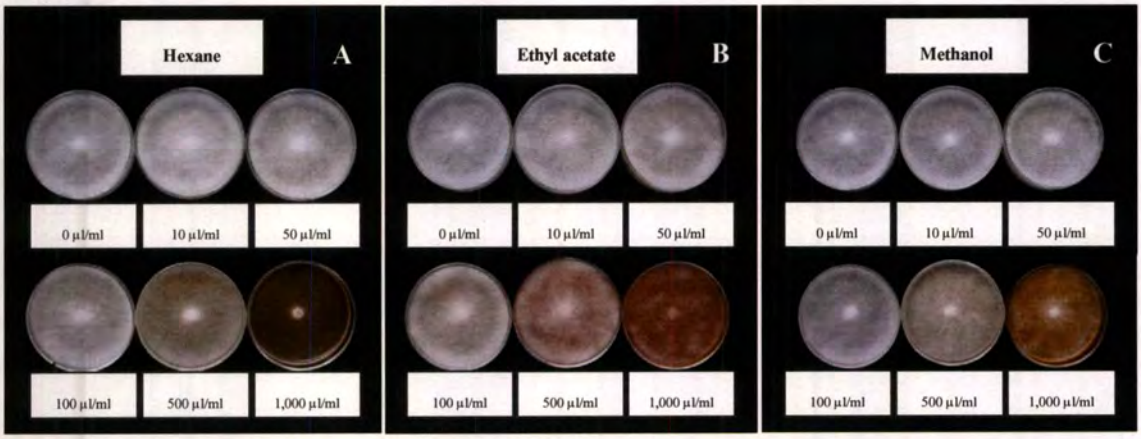

Fig. 4.139 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Chaetomium cupreum* NB201 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

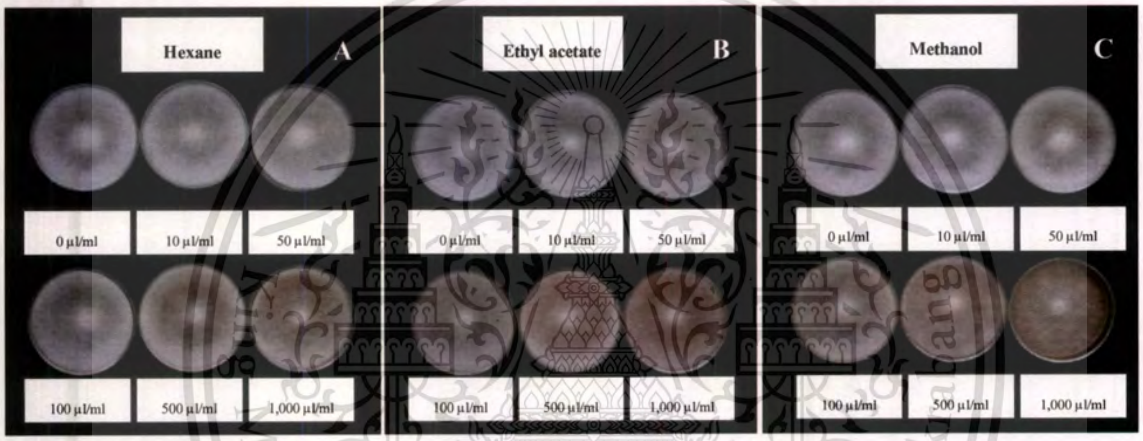

Fig. 4.140 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Chaetomium cupreum* RY202 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

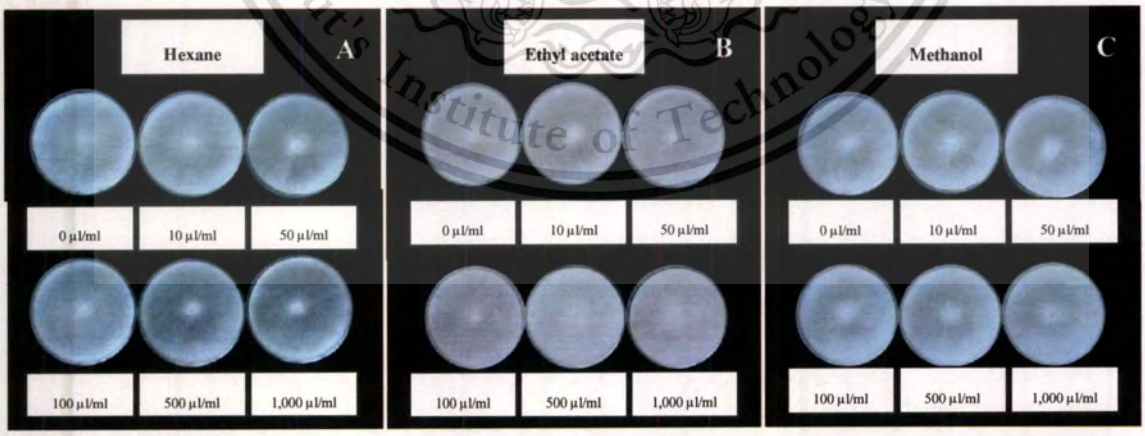

Fig. 4.141 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Gliocladium catenulatum* RY102 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

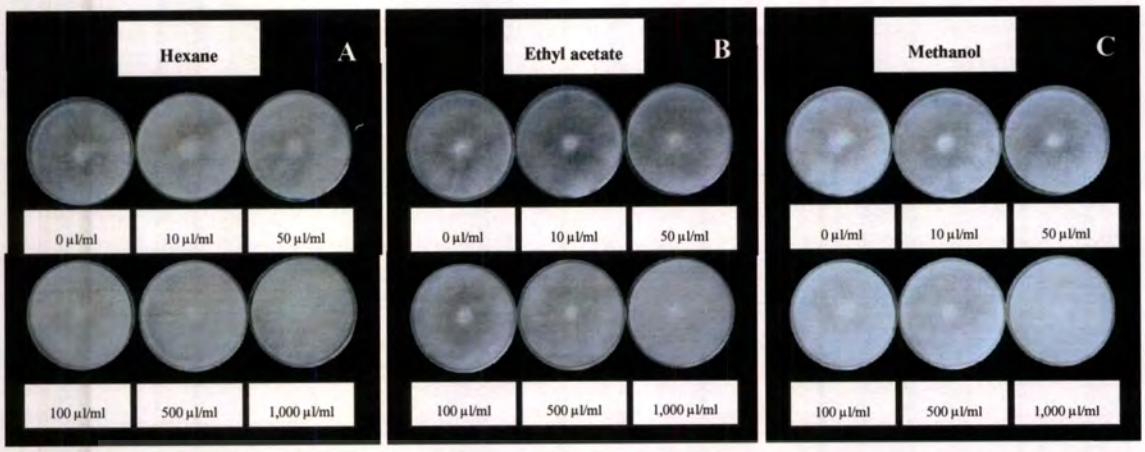

Fig. 4.142 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Gliocladium catenulatum* RY111 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

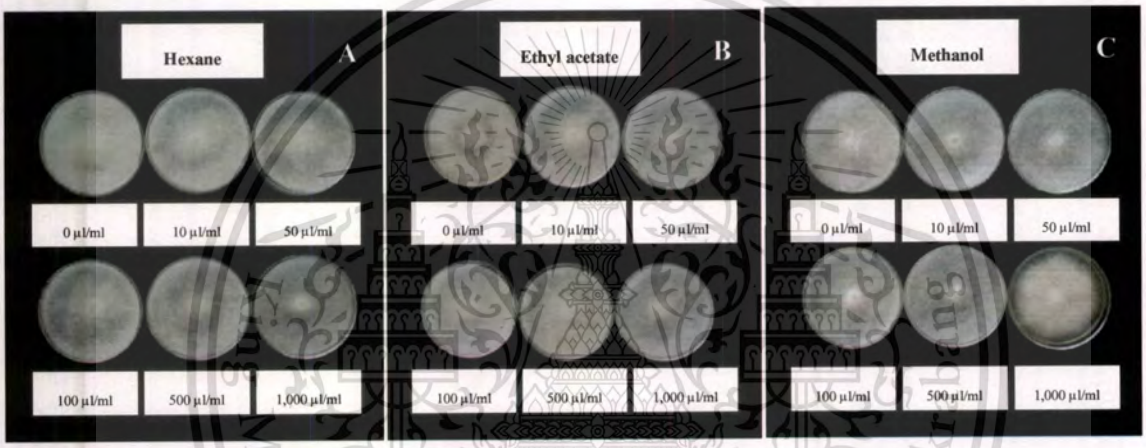

Fig. 4.143 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Trichoderma harzianum* RY101 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentration.

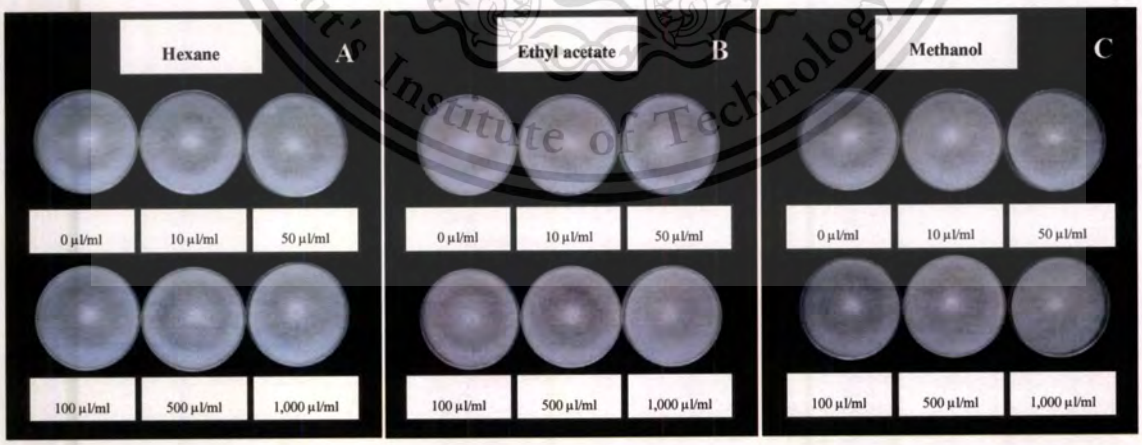

Fig. 4.144 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Trichoderma harzianum* RY104 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

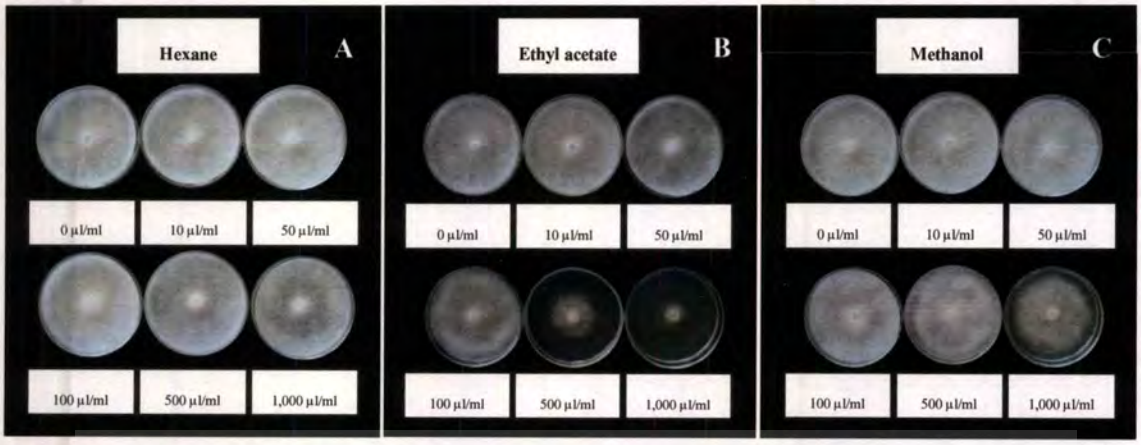

Fig. 4.145 Two-day-old colony of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from *Trichoderma harzianum* RY112 at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

Effect of crude extracts on mycelial growth inhibition of *Pythium aphanidermatum* RY803 was recorded at 2 days (Figs.4.136-4.145 and Tables 4.10-4.11). Crude EtOAc extract from *Chaetomium cochliodes* RY301 at concentration of 100, 500 and 1,000 µg/ml and *Trichoderma harzianum* RY112 at concentration of 500 and 1,000 µg/ml gave colony diameter of *P. aphanidermatum* RY803 by 3.23, 1.83, 1.45, 2.75 and 1.48 cm, respectively whereas colony diameters of other crude extracts were 5 cm (Table 4.10). The data of colony diameter were transformed into percent inhibition of mycelial growth (Table 4.11). The greatest inhibition on mycelial growth of *P. aphanidermatum* RY803 was obtained from crude EtOAc extract of *C. cochliodes* RY301 that gave percent inhibition by 63.40 and 71% at concentration of 500 and 1,000 µg/ml, respectively (Fig. 4.138 and Table 4.11) while crude EtOAc extract from *T. harzianum* RY112 could inhibit the mycelial growth of *P. aphanidermatum* RY803 by 70.04% at concentration of 1,000 µg/ml (Fig. 4.145 and Table 4.11), and the other crude extracts inhibited mycelial growth of *P. aphanidermatum* RY803 less than 50% at all tested concentrations.

Effect of crude extracts on oospore inhibition of *P. aphanidermatum* RY803 were recorded at 10 days (Tables 4.12). Number of oospores (Table 4.12) were transformed into percent inhibition of oospore formation (Table 4.13). All tested crude extracts significantly inhibited oospore formation of *P. aphanidermatum* RY803. Particularly, crude EtOAc extract of *C. cochliodes* RY301 at 1,000 µg/ml gave the highest inhibition on oospore formation of *P. aphanidermatum* RY803 averaged 88.95% (Tables 4.13).

Table. 4.10 Effect of crude extracts from promising antagonistic fungi on mycelial growth of *Pythium aphanidermatum* RY803.

Crude extracts of promising antagonistic fungi	Colony diameter[ν] of *Pythium aphanidermatum* RY803 at each concentration (cm)					
	0	10	50	100	500	1,000
Chaetomium aureum MB601						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium bostrychodes PR101						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium cochliodes RY301						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	3.23 b	1.83 c	1.45 b
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium cupreum NB201						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium cupreum RY202						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Gliocladium catenulatum RY102						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Gliocladium catenulatum RY111						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Trichoderma harzianum RY101						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Trichoderma harzianum RY104						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Trichoderma harzianum RY112						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	2.75 b	1.48 b
MeOH	5 a	5 a	5 a	5 a	5 a	5 a

[ν]Average of four replications. Means followed by a common letter in each column were not significantly different at P=0.01 by Duncan's Multiple Range Test.

Table 4.11 Percent inhibition of mycelial growth of *Pythium aphanidermatum* RY803 on PDA containing crude hexane, EtOAc and MeOH extracts from promising antagonistic fungi at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations.

Crude extracts of promising antagonistic fungi	Inhibition of mycelial growth (%) of *Pythium aphanidermatum* RY803 at each concentration (µg/ml)					
	0	10	50	100	500	1,000
Chaetomium aureum MB601						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	0	0	0
MeOH	0	0	0	0	0	0
Chaetomium bostrychodes PR101						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	0	0	0
MeOH	0	0	0	0	0	0
Chaetomium cochliodes RY301						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	35.40	63.40	71.00
MeOH	0	0	0	0	0	0
Chaetomium cupreum NB201						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	0	0	0
MeOH	0	0	0	0	0	0
Chaetomium cupreum RY202						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	0	0	0
MeOH	0	0	0	0	0	0
Gliocladium catenulatum RY102						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	0	0	0
MeOH	0	0	0	0	0	0
Gliocladium catenulatum RY111						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	0	0	0
MeOH	0	0	0	0	0	0
Trichoderma harzianum RY101						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	0	0	0
MeOH	0	0	0	0	0	0
Trichoderma harzianum RY104						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	0	0	0
MeOH	0	0	0	0	0	0
Trichoderma harzianum RY112						
Hexane	0	0	0	0	0	0
EtOAc	0	0	0	0	45.00	70.40
MeOH	0	0	0	0	0	0

Table. 4.12 Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803.

Crude extracts of promising antagonistic fungi	Number of ospores[y] (x 10^4 oospores/ml)					
	0	10	50	100	500	1,000
Chaetomium aureum MB601						
Hexane	48.79 a	44.71 cdef	44.05 bc	42.66 a	42.52 a	42.52 a
EtOAc	48.67 a	39.98 k	32.16 kl	28.43 i	24.66 i	20.32 k
MeOH	48.29 a	44.36 efg	44.17 bc	33.96 f	33.52 cd	33.07 e
Chaetomium bostrychodes PR101						
Hexane	48.55 a	48.20 a	46.06 a	35.80 d	34.25 c	33.28 e
EtOAc	48.58 a	43.84 fgh	31.59 kl	31.42 g	30.68 fg	21.82 i
MeOH	48.65 a	41.13 j	30.99 lm	30.50 h	30.49 g	20.41 k
Chaetomium cochliodes RY301						
Hexane	48.70 a	35.86 m	29.79 m	21.01 m	20.87 k	20.59 jk
EtOAc	48.42 a	36.26 m	32.46 k	18.43 n	8.37 m	5.35 n
MeOH	48.75 a	38.81 l	20.65 n	20.60 m	18.56 l	14.57 m
Chaetomium cupreum NB201						
Hexane	48.63 a	47.70 a	39.38 h	31.61 g	30.60 g	18.51 l
EtOAc	48.45 ab	48.35 a	35.72 j	23.94 l	20.27 k	20.25 k
MeOH	48.39 ab	44.37 efg	37.22 i	26.32 k	23.13 j	20.40 k
Chaetomium cupreum RY202						
Hexane	48.75 a	48.42 a	44.85 b	35.17 de	32.55 de	25.27 h
EtOAc	48.88 a	43.59 gh	39.51 h	35.08 e	32.33 e	21.17 j
MeOH	49.07 a	42.25 i	41.72 fg	31.22 g	30.68 fg	20.36 k
Gliocladium catenulatum RY102						
Hexane	48.71 a	45.20 cde	43.52 cd	41.93 a	38.45 b	36.46 c
EtOAc	48.88 a	43.82 fgh	42.25 def	39.46 c	31.51 efg	30.42 f
MeOH	49.07 a	45.54 c	41.30 fg	33.90 f	31.80 ef	29.86 f
Gliocladium catenulatum RY111						
Hexane	48.86 a	45.08 cge	43.41 cd	42.12 a	38.70 b	35.63 d
EtOAc	49.13 a	42.89 hi	41.98 ef	39.36 c	32.61 de	30.17 f
MeOH	48.32 a	46.47 b	41.20 fg	40.51 b	38.55 b	32.86 e
Trichoderma harzianum RY101						
Hexane	48.22 a	45.57 c	43.55 cd	42.66 a	41.52 a	40.52 b
EtOAc	48.60 a	39.48 kl	32.16 kl	27.43 j	24.41 i	22.32 i
MeOH	48.64 a	44.44 defg	40.42 gh	33.83 f	32.52 de	30.32 f
Trichoderma harzianum RY104						
Hexane	48.91 a	45.00 cde	43.28 cde	42.04 a	38.59 b	36.94 c
EtOAc	49.13 a	43.55 gh	42.24 def	39.46 c	31.52 efg	30.36 f
MeOH	48.29 a	45.27 cde	41.30 fg	33.85 f	31.84 e	29.71 f
Trichoderma harzianum RY112						
Hexane	48.45 a	45.03 cde	43.13 cde	41.92 a	38.51 b	36.62 c
EtOAc	48.39 a	43.54 gh	42.02 ef	39.23 c	26.53 h	18.45 l
MeOH	48.17 a	45.39 cd	41.12 fg	33.78 f	31.98 e	28.81 g

[y]Average of four replications. Means followed by a common letter in each column were not significantly different at P=0.01 by Duncan's Multiple Range Test.

Table 4.13 Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi and effective dose (ED_{50}) values at each crude extract.

Crude extracts of promising antagonistic fungi	Percent inhibition of oospore formation[v] of *Pythium aphanidermatum* RY803 at each concentration (µg/ml)					ED_{50} values (µg/ml)
	10	50	100	500	1,000	
Chaetomium aureum MB601						
Hexane	8.35 ghij	9.71 jklm	12.54 mn	12.83 j	12.83 n	NF
EtOAc	17.85 bc	33.92 bc	41.59 e	49.33 de	58.27 d	368
MeOH	8.13 hijk	8.53 klm	29.66 gh	30.59 gh	31.51 j	NF
Chaetomium bostrychodes PR101						
Hexane	0.72 o	5.12 m	26.27 i	29.46 h	31.40 j	NF
EtOAc	9.75 fghi	34.98 bc	35.26 f	36.84 f	55.09 ef	753
MeOH	15.47 cd	36.30 bc	37.31 f	37.33 f	58.05 d	662
Chaetomium cochliodes RY301						
Hexane	26.33 a	38.77 b	56.83 b	57.12 c	57.69 d	178
EtOAc	25.11 a	32.96 c	61.93 a	82.72 a	88.95 a	64
MeOH	20.38 b	57.63 a	57.72 b	61.91 b	70.10 b	84
Chaetomium cupreum NB201						
Hexane	1.90 no	19.00 efgh	35.00 f	37.08 f	61.93 c	575
EtOAc	1.34 no	26.28 d	50.60 c	58.17 bc	58.20 d	297
MeOH	8.32 ghij	23.08 ge	45.61 d	52.21 d	57.86 d	543
Chaetomium cupreum RY202						
Hexane	0.66 0	8.01 lm	27.88 hi	33.24 fgh	48.17 g	NF
EtOAc	10.82 fgh	19.17efgh	28.23 ghi	33.85 fgh	56.68 de	994
MeOH	13.90 de	20.34 ef	36.39 f	37.49 f	58.51 d	762
Gliocladium catenulatum RY102						
Hexane	7.78 hijkl	10.96 jklm	13.92 lmn	21.04 i	25.14 kl	NF
EtOAc	8.67 ghij	12.38 ijkl	18.18 jk	34.66 fg	36.94 i	NF
MeOH	5.03 lm	15.05 fghij	29.70 gh	34.08 fgh	38.08 hi	NF
Gliocladium catenulatum RY111						
Hexane	8.28 ghij	11.08 ijkl	13.76 mn	20.73 i	27.07 k	NF
EtOAc	12.60 ef	14.48 ghij	19.81 j	33.56 fgh	38.53 hi	NF
MeOH	3.66 mn	14.74 fghij	16.16 kl	20.20 i	32.00 j	NF
Trichoderma harzianum RY101						
Hexane	5.29 klm	9.69 jklm	11.52 n	13.89 j	15.97 m	NF
EtOAc	18.44 b	33.82 bc	43.56 de	49.77 d	54.07 f	473
MeOH	8.61 ghij	16.72 h	30.46 g	33.15 fgh	37.66 i	NF
Trichoderma harzianum RY104						
Hexane	8.26 ghij	11.40 ijkl	13.98 lm	21.02 i	24.45 l	NF
EtOAc	11.27 efg	13.96 ghijk	19.63 j	35.79 f	38.15 hi	NF
MeOH	6.24 jklm	14.47 ghij	29.89 gh	34.06 fgh	38.47 hi	NF
Trichoderma harzianum RY112						
Hexane	7.05 ijkl	10.97 ijkl	13.47 mn	20.50 i	24.41 l	NF
EtOAc	10.03 fghi	13.18 hijkl	18.94 j	45.18 e	61.87 c	617
MeOH	5.77 jklm	14.64 fghij	29.88 gh	37.99 f	40.19 h	NF

[v]Average of three replications. Means followed by a common letter in each column were not significantly different at P=0.01 by Duncan's Multiple Range Test. NF= no effect.

The data of oospore formation were computed into the effective dose (ED_{50}) values at each crude extract. Crude EtOAc, MeOH and hexane extracts from *C. cochliodes* RY301 gave the greatest oospore inhibition of *P. aphanidermatum* RY803 which the ED_{50} values was 64, 84 and 178 µg/ml, respectively followed by crude EtOAc extract from *C. aureum* MB601 and *T. harzianum* RY101 which gave ED_{50} values at 368 and 473 µg/ml, respectively as well as mycelial growth and oospore formation of *P. aphanidermatum* RY803 on PDA added with crude EtOAc extract from *C. cochliodes* RY301 shown abnormal features of hyphae (Fig.4.146C, F and J), oogonia (Fig.4.146D, E, G and H) and oospores (A, B, F, I, K and L). *P. aphanidermatum* RY803 formed abnormal protoplasm in cells and demonstrated uncommon shapes. Moreover, after inducing the sporangia by distilled water, sporangia were not produced in crude EtOAc extract of *C. cochliodes* RY301 at 100, 500 and 1,000 µg/ml.

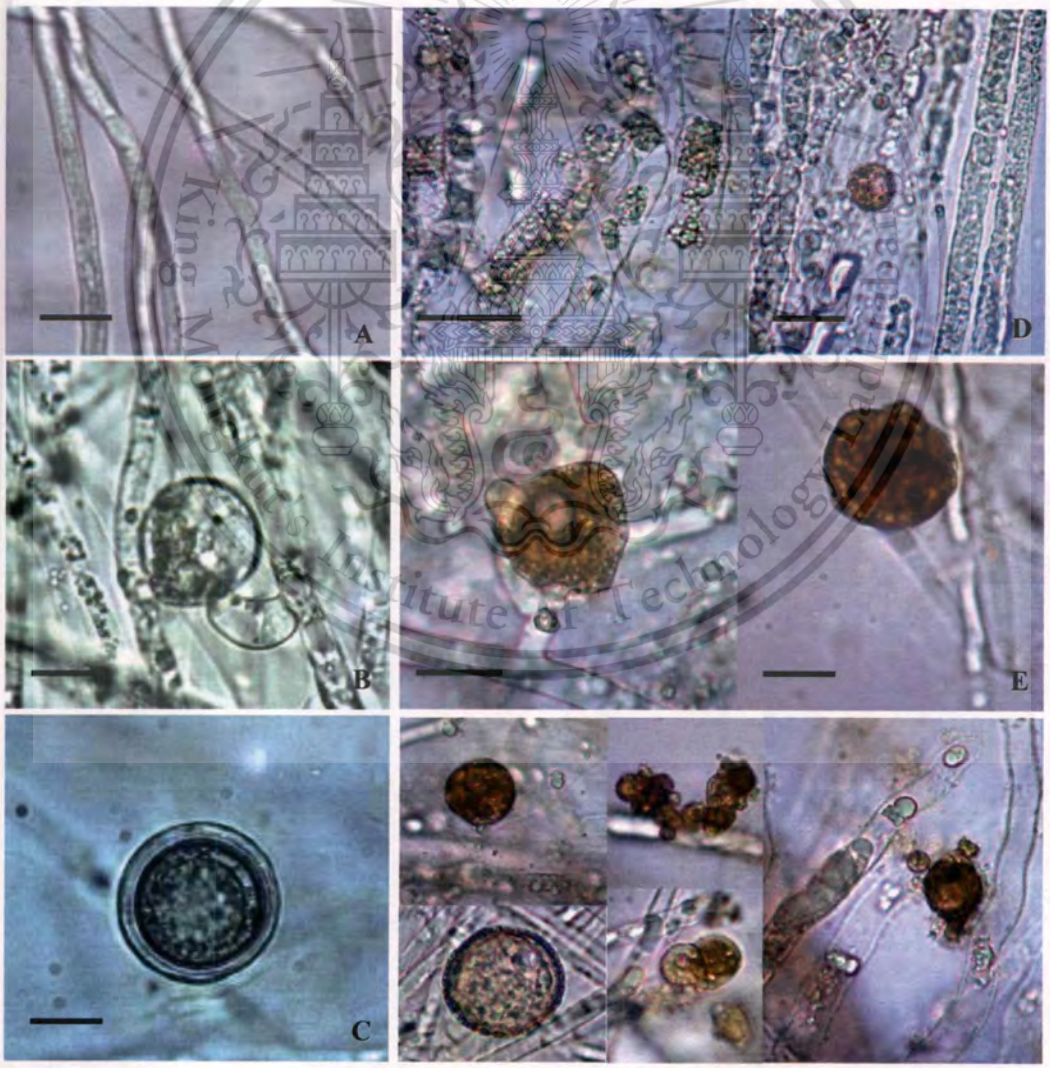

Fig. 4.146 Comparison of normal and abnormal mycelia, oogonia and oospore of *Pythium aphanidermatum* RY803 on PDA amended with crude extract from *Chaetomium cochliodes* RY301. A, B and C showed normal mycelia, oogonium and oospore, respectively. D, E and F showed abnormal mycelia, oogonia and oospores, respectively. Bars.=10 µm.

In this study, it was pointed out that *Chaetomium cochliodes* RY301 could significantly inhibit mycelial growth oospore and sporangium formation of *P. aphanidermatum* RY803. Therefore, it was selected to develop as biofungicide to control pineapple root rot caused by *P. aphanidermatum* RY803 for further experiment.

4.3 Biological Control of Pineapple Root Rot Using Biofungicides in Pot Experiment

Chaetomium cochliodes RY301 used as the effective antagonist for controlling pineapple root rot was cultured on PDB for 4 weeks. Biofungicide from *Chaetomium cochliodes* RY301 as oil and powder formulation was formulated. In oil formulation ascospores were mixed into sterilized bio-oil and adjusted to $1x10^6$ spores/ml (Fig.4.147A, B) while in powder formulation was made by dried mycelial mats with ascospores ground and mixed in sterilized alginate, glucose, sodium humate and talcum as powder formulation which contains $1x10^6$ spores/g according to the method of Dr. Kasem Soytong (Fig.4.147C, D). Pineapple plants were inoculated with sporangial suspension of *Pythium aphanidermadum* RY803 at concentration of $1x10^6$ spores/ml by dipped root technique and then grown in sterilized soil.

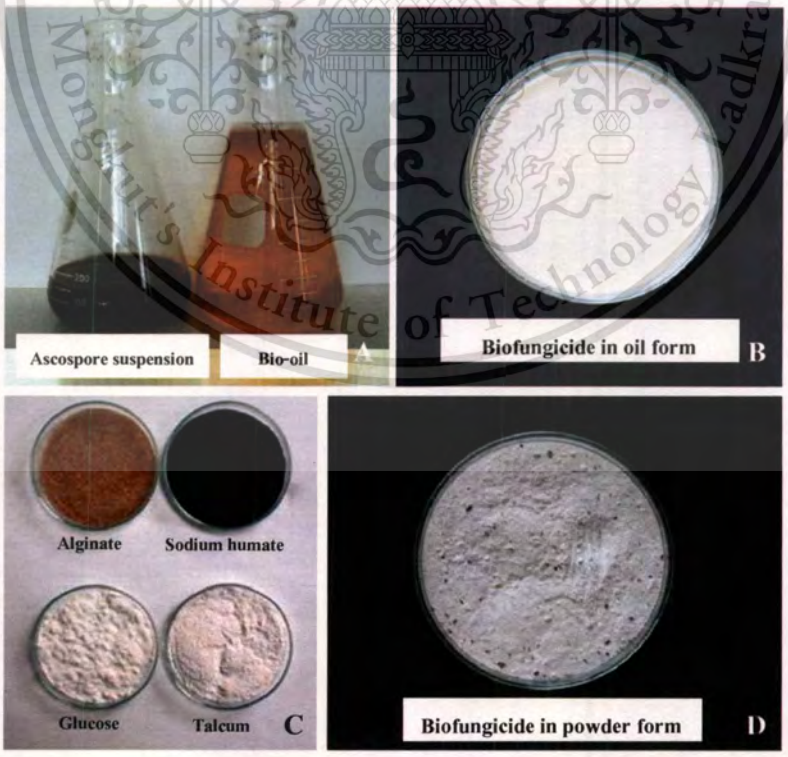

Fig.4.147 Ingradients for preparing biofungicides. A. Ingradients for preparing biofungicides in oil form, B. Biofungicides in oil form, C. Ingradients for preparing biofungicides in powder form, D. Biofungicides in powder form.

Biological control of pineapple root rot using biofungicide formulated from *C. cochliodes* RY301 was determined in pot experiments for 5 months. The results were recoded disease severity indexs of pineapple plants which were treated with biofungicide in oil and powder formulations at 10 ml/plant and 10 g/plant, respectively every 2 weeks and compared with the nontreated control and treated with fungicide metalaxyl.

Based on the result, biofungicide in oil and powder formulations of *C. cochliodes* RY301 gave nonsignificant differences from treated with metalaxyl to control pineapple root rot caused by *P. aphanidermadum* RY803 in every month which the disease index at 5 month were 2.40 and 2.60 where the disease reduction was 52 and 48%, respectively compared to the treatment of non-treated control (disease index was 1.00) and treated with *P. aphanidermadum* RY803 alone (disease index was 5.00) (Tables 4.14 and 4.15).

Plant fresh weight of leaves and stems, and root were shown in Table 4.16. The applying biofungicide in oil and powder formulations of *C. cochliodes* RY301 and treated with metalaxyl fresh were nonsignificantly different in fresh weight of leaves and stems (369, 332 and 357 g, respectively), and root (21, 18 and 19 g, respectively). Moreover, biofungicide in oil formulation gave significantly highest weight of leaves and stems (505 g), and root (28 g) followed by applying biofungicide in powder formulation (373 and 23 g, respectively) which nonsignificantly different when compared to the non-treated control. This implied that *C. cochliodes* RY301 could act as plant growth stimulant.

Table 4.14 Disease severity index of pineapple root rot after applying biofungicide in pot for 5 months.

Treatments	disease severity index[1] (DSI) in 5 months				
	1	2	3	4	5
Non-treated control (T1)	1.00 b	1.00 b	1.00 c	1.00 c	1.00 c
Pythium aphanidermadum RY803 alone (T2)	2.40 a	3.00 a	3.60 a	4.20 a	5.00 a
oil formulation at 10 ml/plant every 2 weeks (T3)	1.00 b	1.00 b	1.00 c	1.00 c	1.00 c
powder formulation at 10 g/plant every 2 weeks (T4)	1.00 b	1.00 b	1.00 c	1.00 c	1.00 c
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	1.40 b	1.60 b	1.60 bc	2.20 b	2.40 b
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	1.60 b	1.60 b	1.80 b	2.40 b	2.60 b
Treated with metalaxyl at recommended rate + pathogen (T7)	1.20 b	1.40 b	1.50 bc	2.10 b	2.30 b

[1] disease severity index of pineapple root rot, 1 = no root rot, 2= 1-25% root rot, 3=26-50% root rot, 4=51-75% root rot and 5= 76-100% root rot (modified from Ahmed *et al.*,1999) average of five replications. Means followed by a common letter in each column were not significantly different (P=0.01) by Duncan's Multiple Range Test.

Table 4.15 Disease reduction of pineapple root rot after applying biofungicide in pot for 5 months.

Treatments	Disease reduction (%)[1] in 5 months				
	1	2	3	4	5
Non-treated control (T1)	-	-	-	-	-
Pythium aphanidermadum RY803 alone (T2)	-	-	-	-	-
oil formulation at 10 ml/plant every 2 weeks (T3)	58.33	66.67	72.22	76.19	80
powder formulation at 10 g/plant every 2 weeks (T4)	58.33	66.67	72.22	76.19	80
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	41.67	46.67	55.56	47.62	52
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	33.33	46.67	50	42.86	48
Treated with metalaxyl at recommended rate + pathogen (T7)	50	53.33	58.33	50	54

[1]Percentage of Disease reduction = (DSI of inoculated with pathogen alone– DSI of treated with either biofungicide or metalaxyl)/DSI of inoculated with pathogen alone X 100.

Table 4.16 Effect of biofungicides on fresh weight of pineapple after growing in pot for 5 months.

Treatments	Total fresh weigh (g)[1]	
	Leaves and stems	Roots
Non-treated control (T1)	375b	26 ab
Pythium aphanidermadum RY803 alone (T2)	174 c	6 c
oil formulation at 10 ml/plant every 2 weeks (T3)	505 a	28 a
powder formulation at 10 g/plant every 2 weeks (T4)	373 b	23 ab
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	369 b	21 ab
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	332 b	18 b
Treated with metalaxyl at recommended rate + pathogen (T7)	357 b	19 ab

[1]The average of five replications. Means followed by a common letter in each column were not significantly different (P=0.01) by Duncan's Multiple Range Test.

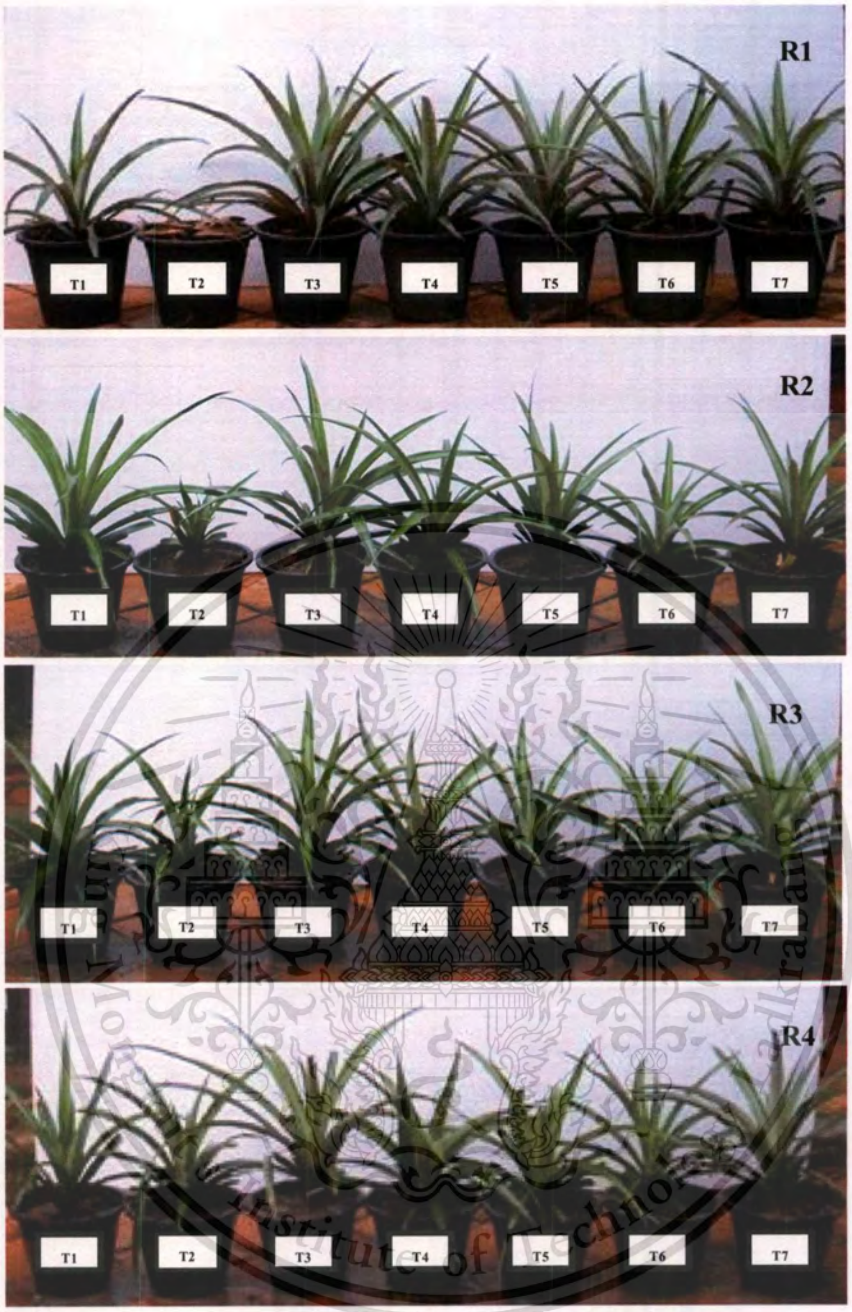

Fig.4.148 Testing of biofungicide formulated from *Chaetomium cochliodes* RY301 to control pineapple root rot. T1 = Non-treated control, T2 = *Pythium aphanidermadum* RY803 alone, T3 = oil formulation at 10 ml/plant every 2 weeks, T4 = powder formulation at 10 g/plant every 2 weeks, T5 = oil formulation at 10 ml/plant every 2 weeks + pathogen, T6 = powder formulation at 10 g/plant every 2 weeks + pathogen, T7 = Treated with metalaxyl at recommended rate + pathogen.

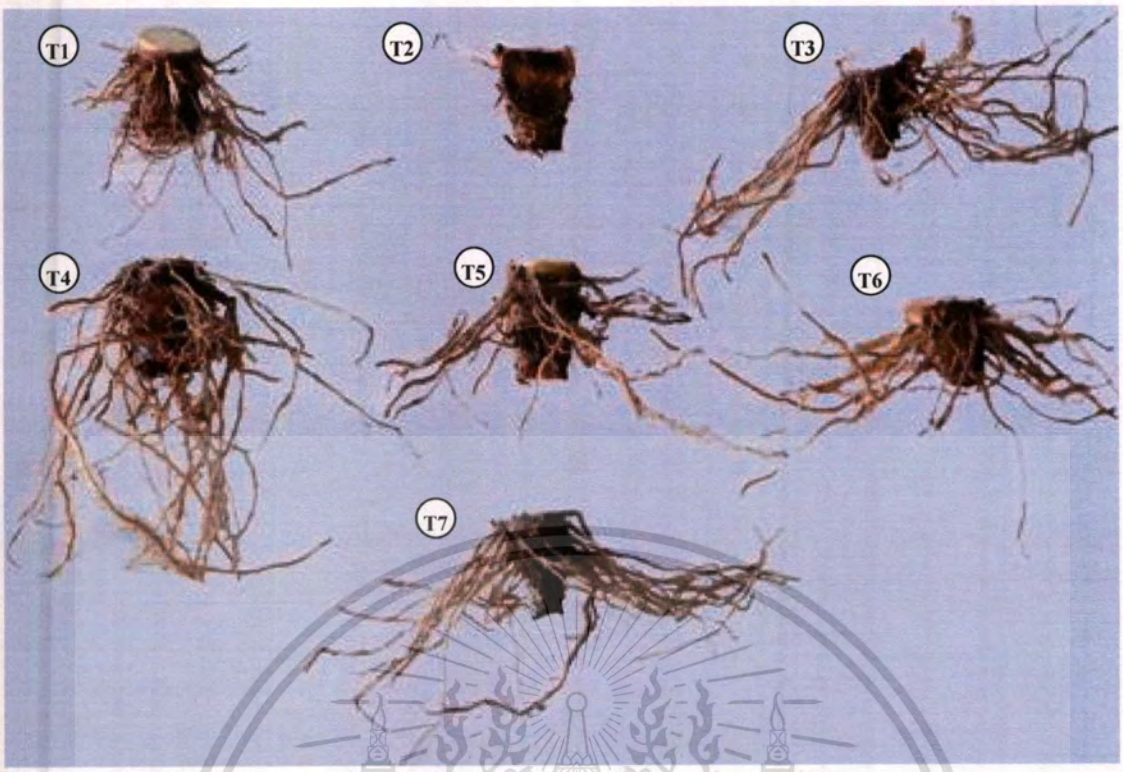

Fig.4.149 Effect of biofungicide formulated from *Chaetomium cochliodes* RY301 on pineapple root in different treatments as follows: T1 = Non-treated control, T2 = *Pythium aphanidermadum* RY803 alone, T3 = oil formulation at 10 ml/plant every 2 weeks, T4 = powder formulation at 10 g/plant every 2 weeks, T5 = oil formulation at 10 ml/plant every 2 weeks + pathogen, T6 = powder formulation at 10 g/plant every 2 weeks + pathogen, T7 = Treated with metalaxyl at recommended rate + pathogen.

CHAPTER 5

DISCUSSION

In this study, 2 species of *Pythium* were isolated from soil and roots of pineapple causing root rot by baiting technique. They were identified as *P. aphanidermatum* and *P. graminicola.* Both *P. aphanidermatum* and *P. graminicola* belong to Oomycetes in order Peronosporales. The hypha is hyaline, no septate except in old. The asexual spores are filamentous-inflated sporangia, branched forming irregular toruloid complexes. To differentiate *P. aphanidermatum* from *P. graminicola* required examination under a compound microscope of oogonia and antheridia. Oospores are the sexual spores and in case of *P. aphanidermatum*, they are apluerotic oogonium (oospore does not fill the oogonium) with 1-2 antheridia while *P. graminicola* forms pluerotic oogonium (oospore fill the oogonium) with 1-6 antheridia. The size and shape of sporangia, oogonia, antheridia and oospores of *P. aphanidermatum* and *P. graminicola* isolated from pineapple root rot were in agreement with the descriptions in the literature (van der Plaats-Niterink. 1981). Morphological examinations in *P. aphanidermatum* demonstrated that the size of oogonia and oospores, and the sporangia feature are similar to *P. graminicola.* However, they can be distinguished by the number of antheridium/oogonium. In a study of the ITS rDNA sequence, both *P. aphanidermatum* and *P. graminicola* belonged to clade A which were characterized by filamentous sporangia. These results suggested that *P. aphanidermatum* and *P. graminicola* were closely related. The phylogeny based on ITS sequences in this study agrees well with the phylogeny constructed from 116 species of *Pythium* based on the ITS region of the nuclear ribosomal DNA (André Lévesque and De Cock. 2004). The clusters A-D and E-J in the study by André Lévesque and De Cock (2004) correspond to the cluters A and B in this study, respectively.

Although 15 isolates of *P. aphanidermatum* and 29 isolates of *P. graminicola* were proved to be pathogenic to pineapple on detached leaves, *P. aphanidermatum* RY803 showed significantly highest of disease serevity index on root. The results suggested that *P. aphanidermatum* RY803 was the most virulent on pineapple root. Pineapple root rot caused by *P. aphanidermatum* has been previously reported by Gonsalves (1994), Department of Agriculture, Ministry of Agriculture and cooperatives, Thailand (2008). Furthermore, *P. aphanidermatum* has

been reported as a pathogen of several plants eg. beets, chrysanthemum, cotton, cucurbits, grasses and pepper (Gonsalves. 1994).

Bi-culture test between 42 isolates of promissing antagonistic fungi and *P. aphanidermatum* RY803 were studied. *C. aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocladium catenulatum* RY102, *G.. catenulatum* RY111, *Trichoderma harzianum* RY 101, *T. harzianum* RY 104 and *Trichoderma harzianum* RY 112 could inhibit the mycelial growth and oospore formation of *P. aphanidermatum* RY803 over 80%. With this, *C. cupreum*, *G. catenulatum* and *T. harzianum* have well known to be antagonist for plant disease control (Soytong. 1992a; 1992b; Biren *et al.* 1999; Ezziyyani *et al.* 2007). *C. cupreum* had been reported to reduce leaf spot disease of corn caused by *Curvularia lunata*, rice blast caused by *Pyricularia oryzae*, sheath blight of rice caused by *Rhizoctonia oryzae* and tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* (Soytong. 1992a; 1992b). *G. catenulatum* was a mycoparasite of several fungal genera including *Aspergillus flavus* and *Sclerotium cepivorum* (Biren *et al.* 1999; Tsigbey *et al.* 1999). *T. harzianum* had also been reported as biocontrol agent for controlling *Phytophthora capsici* (Ezziyyani *et al.* 2007) and *Pythium ultimum* (Naseby *et al.* 2000). The results indicated that one of them could be an effective antagonist against *P. aphanidermatum* RY803 causing pineapple root rot. Therefore, crude extracts from selected isolates were tested to screen the most efficient antagonist for controlling pineapple root rot caused by *P. aphanidermatum* RY803. Based on the result crude EtOAc extract of *C. cochliodes* RY301 was found to be the greatest antagonist against *P. aphanidermatum* RY803 causing root rot of pineapple due to it could inhibit oospore formation of *P. aphanidermatum* RY803 at ED_{50} value of 64 μm/ml and formed abnormal of hyphae, oogonia and oospores. This research finding may involve in antibiosis for control mechanism. However, *C. cochliodes* RY301 may produce some antibiotic substance as previously reported by Phonkerd *et al.* (2008) who indicated that *C. cochliodes* VTh01 and *C. cochliodes* CTh05 could produce four new dimeric spiro-azaplilones, cochliodones A-D, two new azaphiliones, chaetoviridines E and F, a new epi-chaetoviridin A, and known compounds, chaetoviridin A, ergosterol, chaetochalasin A. Chaetoviridines E and chaetochalasin A exhibited antimalarial activity against *Plasmodium falcipulum* while cochliodones C, chaetoviridines E and F, chaetochalasin A expressed antimycobacterial activity against *Mycobacterium tuberculosis*. Furthermore, *C. cochliodes* VTh01 and *C. cochliodes* CTh05 were reported to be antagonistic to *Fusarium oxysporum* f. Sp. *lycopersici* causing tomato wilt (Phonkerd *et al.* 2008). Therefore, it

is implied that *C. cochliodes* RY301 might have the similar active compounds that could be antagonist against *P. aphanidermatum* RY803. In this study, it was pointed out that *C. cochliodes* RY301 could exhibit inhibitory activity against *P. aphanidermatum* RY803.

During a study on antagonistic fungi for controlling root rot disease of pineapple, numerous strains of *Chaetomium* were isolated from soil by baiting technique. Four species were reported to be new records in Thailand as follows: *C. carinthiacum*, *C. flavigenum*, *C. perlucidum* and *C. succineum*. One of the isolates could not be identified as any species of *Chaetomium* and is therefore described as new. In this study describes this species as *Chaetomium siamense* sp. nov. *Chaetomium* is a large ascomycetous fungus with species inhabiting various substrates containing cellulose including paper and plant compost. Since the genus was reviewed by von Arx.*et al.* (1986), some new species have been described as follows:- *C. biporatum* (Cano and Guarro. 1987), *C. histoplasmoides* (Carris and Glawe. 1987), *C. sinaiense* (Mustafa and Ezz El din. 1989), *C. subcurvisporum* (Abdullah and Al-Bader. 1989), *C. myricicola* (Horie and Udagawa. 1990), *C. mesopotamicum* (Abdullah and Zora. 1993), *C. novae-caledonicum* (Udagawa *et al.* 1994), *C. floriforme* (Gené and Guarro. 1996), *C. cuyabenoensis* (Decock and Hennebert. 1997), *C. umbratile* (Udagawa *et al.* 1997), *C. macrostiolatum*, *C. olivicolor* and *C. tarraconensis* (Rodríguez *et al.* 2002), *C. acropullum* (Wang and Zheng. 2005a) and *C. ampulliellum* (Wang and Zheng. 2005b). *C. siamense* sp. nov. can be distinguished from the previously published species by superficial, spherical or ovate ascomata with angular brown-walled; ascomatal hairs red or orange-red in reflected light, arcuate, apically circinate or coiled, septate, fusiform ascospores with two apical germ pores. At first sight, the new species was suspected to be *C. cupreum* Ames. because its morphological characters were similar to *C. cupreum* which have been described by Seth (1970), von Arx *et al.* (1986), Soytong and Quimio (1989), Petcharat and Soytong (1991). Both of the species share features of diffusing red pigment in to agar medium and their ascomata superficial, spherical or ovate with angular brown-walled; ascomatal hairs red, arcuate, apically circinate or coiled, septate. However, the new species can be easily distinguished from *C. cupreum* by the ascospore morphology according to scanning electron microscopic features. *C. siamense* sp. nov. have longer and fusiform ascospores (4.3-6.0 x 11.1-13.5 μm) with two apical germ pores whereas ascospores of *C. cupreum* are reniform (4.5-6 x 7-10 μm) with a single apical germ pore.

In pot experiment *C. cochliodes* RY301 applied as formulations of oil and powder were not significantly different from the metalaxyl treatment in reducing root rot of pineapple. The

experiment revealed that the biofungicide from *C. cochliodes* RY301 in formulation of oil and powder have been successfully used to control pineapple root rot. Moreover, *C. cochliodes* RY301 in formulation of oil gave the highest fresh weigh of plant and root. Biofungicide from *C. cochliodes* have not been reported but there are reported that *C. cochliodes* VTh 01 and *C. cochliodes* CTh 05 could act as antagonist for controlling tomato wilt caused by *Fusarium oxysporum* f sp *lycopersici* (Phonkerd *et al.* 2008). The previously research has successfully been applied biofungicide in the form of pellet and powder formulations developed from *Chaetomium cupreum* and *C. globosum* for the long term protection of Durian and Black Pepper caused by *Phytophthora palmivora*, Tangerine caused by *Ph. parasitica* and Strawberry caused by *Ph. cactorum* (Soytong *et al.* 2001).

CHAPTER 6

CONCLUSION

Biological control is an alternative disease management strategy when growers are concerned about phytotoxicity from fungicides. For study on biological control of pineapple root rot, forty-four isolates of P*ythium* spp. were isolated from the disease epidemic planting areas of pineapple variety. Pattavia at Petchaburi, Phatthalung, Prachuap Khiri Khan and Rayong provinces from Jan 2006 to May 2008. Forty-four isolates of pathogen were encountered by baiting technique. All isolates were identified using the monograph provided by Plaats-Niterink and confirmed by their ITS region of the nuclear rDNA. Fifteen isolates from Phatthalung, Rayong and Petchaburi provinces were identified by their morphological characters as *P. aphanidermatum* whereas 29 isolates from Phatthalung, Rayong and Prachuap Khiri Khan provinces were identified as *P. graminicola*. Morphological examinations in *P. aphanidermatum* were found that the size of oogonia and oospores, and the sporangia feature were similar to *P. graminicola*. However, they can be distinguished by the number of antheridium/oogonium. Both *P. aphanidermatum* and *P. graminicola* were characterized by hyaline hyphae, mycelium no septate except in old, filamentous-inflated sporangia. To differentiate *P. aphanidermatum* from *P. graminicola* required examination under a microscope of oogonia and antheridia. Oospores of *P. aphanidermatum* were apluerotic oogonium with 1-2 antheridia while *P. graminicola* forms pluerotic oogonium with 1-6 antheridia.

All isolates of *P. aphanidermatum*, *P. graminicola* from pineapple root rot and other species retrieved from GenBank were compared from molecular and morphological viewpoints. They were divided into four groups (A, B, C and D) based on the phylogenetic analysis of ITS sequences. Clade A, B, C and D comprised 3, 53, 4 and 13 isolates, respectively. All isolates were proved to be pathogenic to pineapple root by detached leaves and root test. *Pythium aphanidermatum* RY803 from Rayong provinces gave more virulent than any other isolates while *Pythium graminicola* was the first reported causing root rot of pineapple in Thailand. Therefore, *P. aphanidermatum* RY803 was used for screening antatonistic fungi by bi-culture plate and crude extract test.

Forty-two isolates of soil fungi isolated by baiting technique and soil plate technique for screening antagonist. Species discovered by baiting technique included: *Chaetomium aureum* (MB103, MB601, MB603, MB608 and RY102), *C. bostrychodes* (PR101, PR102, PR103 and NB701), *C. basiliense* (AM101), *C. carinthiacum* (NB501), *C. cochliodes* (RY301), *C. cupreum* (NB102, RY201, RY202 and RY204), *C. flavigenum* (MB402, MB604, MB606, MB607 and MB611), *C. fusiforme* (NB401 and MB605), *C. perlucidum* (NB202 and MB501) and *C. succineum* (MB304 and MB305), and species discovered by soil plate technique included: *Aspergillus niger*(AP101), *C. cupreum* (SO101), *Emericella nidulans* (EN101), *E. rugulosa* (ER101), *Eurotium chevalieri* (EU101), *Gliocladium catenulatum* (RY102, RY109 and RY111), *Penicillium janthinellum* (PN101), *Trichoderma hamatum* (PT101) and *T. harzianum* (RY101, RY104 and RY112). During a study on antagonistic fungi for controlling root rot disease of pineapple, 2 isolates of *Chatomium* (MB303 and MB502) could not be identified as any species of *Chaetomium* and were therefore described as new. These species were describes as *Chaetomium siamense* sp. nov. Morphological features of this new species, together with the sequences of the ITS region of its rDNA and its comparison with related species are discussed. The fungus is distinguished by its ovate ascomata with red circinate ascomatal hairs, and fusiform ascospores with two apical germ pores. In addition, Phylogenetic analysis of ITS rDNA sequence data supports that *C. siamense* as a distinct species.

All of the promising antagonists were tested for their ability to control *P. aphanidermatum* RY803 in bi-culture plate. Bi-culture test for antagonism showed that *C. aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocadium catenulatum* RY102, *G. catenulatum* RY111, *Trichoderma harzianum* RY 101, *Trichoderma harzianum* RY 104 and *Trichoderma harzianum* RY 112 gave both mycelial growth and oospores inhibition activity to *P. aphanidermatum* RY803 over 80%. These 10 promising antagonists were extracted with Hexane, Ethy acetate and Methanol. Each crude extract was prepared to different concentrations (0, 10, 50, 100, 500 and 1,000 µg/ml) and tested against *P. aphanidermatum* RY803. The crude Ethyl acetate extracts of *C. cochliodes* RY301 at the rate of 1,000 µg/ml gave significantly the highest inhibition of mycelial growth and oospores formation by 71.00 and 88.95%, respectively and ED_{50} at 64 µg/ml. Moreover, the hyphae, oogonia and oospores of the pathogen formed abnormal protoplasm in cell and demonstrated uncommon shape. Therefore, *C. cochliodes* RY301 was used as antagonist to control root rot of pineapple in pot experiment.

Biological control of pineapple root rot using *Chaetomium cochliodes* RY301 was determined in pot experiments at 5 months. *C. cochliodes* RY301 was prepared in formulations of oil and powder before applying to infested pots at 10 ml/plant and 10 g/plant, respectively. The result showed that *C. cochliodes* RY301 in oil form gave highest fresh weight of pineapple plant while non-treated plant, treated with *C. cochliodes* RY301 in powder form and treated with metalaxyl were nonsignificant. Moreover, *C. cochliodes* RY301 in oil form gave greater plant growth than non-treated plants. This implies that *C. cochliodes* RY301 could act as biofungicide and plant growth stimulant.

It is concluded from the study that *P aphanidermatum* RY803 is a pathogen causing root rot of pineapple and it can be effectively managed by using biocontrol agents. *C. cochliodes* RY301 in oil and powder formulations were effectively inhibited *P aphanidermatum* RY803. Moreover, the oil formulation of *C. cochliodes* RY301 can be used as plant growth promotion and the materials used in the preparation of the oil formulation are readily available and inexpensive.

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APPENDIX A

STATISTICAL ANALYSIS

Table A.1 Pathogenicity test of all isolates using detached leaves method.

Obtained isolates	Lesion diameter(cm)				
	R1	R2	R3	R4	Means
control	0	0	0	0	0
Pythium graminicola PT101	0.9	0.9	0.9	0.93	0.91
Pythium graminicola PT102	0.98	1.03	1.01	0.98	1.00
Pythium graminicola PT108	1	1	1.01	1.03	1.01
Pythium graminicola PT301	0.9	0.88	0.88	0.9	0.89
Pythium graminicola PT501	0.93	0.9	0.9	0.93	0.92
Pythium graminicola PT701	0.98	0.9	0.96	0.9	0.94
Pythium graminicola PT901	0.96	0.94	0.95	0.96	0.95
Pythium graminicola PT205	1.05	0.95	1	1	1.00
Pythium graminicola PT206	0.8	0.8	0.8	0.8	0.80
Pythium graminicola PT207	0.94	0.96	0.98	0.98	0.97
Pythium graminicola PT304	0.9	0.93	0.9	0.93	0.92
Pythium aphanidermatum PT305	2.87	3.42	3.13	3.15	3.14
Pythium aphanidermatum PT307	2.5	2.09	2.38	2	2.24
Pythium graminicola PT401	1.05	1	1.05	1	1.03
Pythium graminicola RY201	0.93	1.05	0.98	0.9	0.97
Pythium graminicola RY301	0.94	0.94	0.93	0.94	0.94
Pythium graminicola RY302	0.94	0.93	0.92	0.94	0.93
Pythium graminicola RY501	1.05	0.95	1	1	1.00
Pythium graminicola RY502	1.12	1.2	1.2	1.15	1.17
Pythium graminicola RY503	0.8	0.85	0.83	0.83	0.83
Pythium graminicola RY504	0.75	0.73	0.8	0.7	0.75
Pythium graminicola RY505	0.85	0.75	0.83	0.8	0.81
Pythium graminicola RY601	1	0.95	1.08	1.1	1.03
Pythium graminicola RY603	0.85	0.9	0.9	0.9	0.89

Table A.1 (continued) Pathogenicity test of all isolates using detached leaves method.

Obtained isolates	Lesion diameter(cm)				
	R1	R2	R3	R4	Means
Pythium graminicola RY701	0.5	0.58	0.5	0.5	0.52
Pythium aphanidermatum RY801	7.25	7.25	7.5	6.8	7.20
Pythium aphanidermatum RY802	7.5	6.8	7.5	6.55	7.09
Pythium aphanidermatum RY803	6.55	7.5	7.5	7.5	7.26
Pythium aphanidermatum RY804	7.45	5.85	7.5	7.5	7.08
Pythium aphanidermatum RY805	4.38	4.85	4.72	4.8	4.69
Pythium aphanidermatum RY806	2.8	3.15	3.66	3.5	3.28
Pythium aphanidermatum RY807	3.68	3.6	3.72	3.7	3.68
Pythium aphanidermatum RY808	1.2	1	1.2	1.68	1.27
Pythium aphanidermatum PJ104	0.95	0.96	0.98	0.95	0.96
Pythium aphanidermatum PJ106	0.8	0.85	0.85	0.83	0.83
Pythium aphanidermatum PJ108	0.9	0.9	1.1	1.2	1.03
Pythium aphanidermatum PJ201	1	1.32	0.95	0.9	1.04
Pythium aphanidermatum PJ202	0.95	1.9	1.5	1.6	1.49
Pythium graminicola PB108	0.8	0.98	0.85	0.85	0.87
Pythium graminicola PB201	1	1	1.05	0.95	1.00
Pythium graminicola PB202	0.5	0.5	0.5	0.5	0.50
Pythium graminicola PB206	0.55	0.5	0.5	0.5	0.51
Pythium graminicola PB301	0.7	0.85	0.8	0.8	0.79
Pythium graminicola PB302	0.6	0.6	0.5	0.65	0.59

Table A.1 Analysis of variance of pathogenicity test of all isolates using detached leaves method.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	44	650.1952	14.7772	358.00**	1.50	1.76
Ex. Error	35	5.5724	0.0413			
Total	79	655.7676	3.6635			

** significant difference at P=0.01, GRAND MEAN = 1.7258 , CV = 11.7721%

LSD 0.05 = 0.2844, LSD 0.01 = 0.3759

Table A.2 Pathogenicity test of four aggressive isolates by root inoculation.

Isolates	disease severity index (DSI)				
	R1	R2	R3	R4	Means
P. aphanidermatum RY801	2	3	3	3	2.75
P. aphanidermatum RY802	3	3	4	3	3.25
P. aphanidermatum RY803	5	5	5	5	5.00
P. aphanidermatum RY804	5	4	5	4	4.50
control	1	1	1	1	1.00

Table A.2 Analysis of variance of pathogenicity test of four aggressive isolates by root inoculation.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	4	39.7000	9.9250	59.55**	3.06	4.89
Ex. Error	15	2.5000	0.1667			
Total	19	42.2000	2.2211			

** significant difference at P=0.01, GRAND MEAN = 3.3

CV = 12.3712%

LSD 0.05 = 0.6151

LSD 0.01 = 0.8507

Table A.3 Percent inhibition on growth of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

promising antagonistic fungi	Growth inhibition (%)				
	R1	R2	R3	R4	Means
Aspergillus niger AP101	0.00	0.25	0.00	0.00	0.06
Chaetomium aureum MB103	68.89	65.56	77.78	71.11	70.84
Chaetomium aureum MB601	84.56	83.33	78.89	82.22	82.25
Chaetomium aureum MB603	44.22	38.11	41.00	45.56	42.22
Chaetomium aureum MB608	57.78	54.11	64.22	60.67	59.20
Chaetomium aureum RY102	50.00	46.67	44.44	45.56	46.67

Table A.3 (Continued) Percent inhibition on growth of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

promising antagonistic fungi	Growth inhibition (%)				
	R1	R2	R3	R4	Means
Chaetomium bostrychodes NB701	10.00	4.44	5.56	8.89	7.22
Chaetomium bostrychodes PR101	100.00	94.44	97.78	100.00	98.06
Chaetomium bostrychodes PR102	70.44	74.67	70.00	70.22	71.33
Chaetomium bostrychodes PR103	8.11	8.67	6.78	10.11	8.42
Chaetomium brasiliense AM101	77.44	69.78	72.44	72.00	72.92
Chaetomium carinthiacum NB501	14.44	14.44	13.33	13.33	13.89
Chaetomium cochliodes RY301	89.33	88.89	87.78	90.00	89.00
Chaetomium cupreum NB102	90.00	88.89	91.11	88.89	89.72
Chaetomium cupreum RY201	88.89	86.67	83.33	88.89	86.95
Chaetomium cupreum RY202	83.33	80.00	80.00	83.33	81.67
Chaetomium cupreum RY204	57.78	55.56	61.11	55.56	57.50
Chaetomium cupreum SO101	50.00	44.44	46.67	44.44	46.39
Chaetomium flavigenum MB402	0.00	5.56	4.44	0.00	2.50
Chaetomium flavigenum MB604	72.22	66.67	68.89	66.67	68.61
Chaetomium flavigenum MB606	0.00	0.00	0.00	0.00	0.00
Chaetomium flavigenum MB607	88.89	83.33	88.89	88.89	87.50
Chaetomium flavigenum MB611	77.78	75.56	61.11	66.67	70.28
Chaetomium fusiforme MB605	38.89	0.00	50.00	0.00	22.22
Chaetomium fusiforme NB401	66.67	72.22	77.78	68.89	71.39
Chaetomium perlucidum MB501	0.00	0.00	0.00	0.00	0.00
Chaetomium perlucidum NB202	0.00	0.00	0.00	0.00	0.00
Chaetomium siamense MB303	0.00	8.89	11.11	0.00	5.00
Chaetomium siamense MB502	61.11	61.11	55.56	50.00	56.95
Chaetomium succineum MB304	8.89	12.22	11.11	11.11	10.83
Chaetomium succineum MB305	0.00	11.11	0.00	11.11	5.56
Emericella nidulans EN101	14.57	13.46	13.62	13.89	13.89
Emericella rugulosa ER101	66.67	72.22	66.67	72.22	69.45
Eurotium chevalieri EU101	38.89	33.33	33.33	38.88	36.11
Gliocladium catenulatum RY102	100.00	100.00	100.00	100.00	100

Table A.3 (Continued) Percent inhibition on growth of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

promising antagonistic fungi	Growth inhibition (%)				
	R1	R2	R3	R4	Means
Gliocladium catenulatum RY109	16.67	11.11	11.11	16.67	13.89
Gliocladium catenulatum. RY111	77.78	77.78	100.00	100.00	88.89
Penicillium janthinellum PN101	0.00	0.00	0.00	0.00	0.00
Trichoderma hamatum PT101	22.22	11.11	22.22	22.22	19.44
Trichoderma harzianum RY101	88.89	94.44	88.89	88.89	90.28
Trichoderma harzianum RY104	100.00	100.00	100.00	100.00	100
Trichoderma harzianum RY112	100.00	100.00	100.00	100.00	100

Table A.3 Analysis of variance of percent inhibition on growth of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	41	210304	5129.3871	168.34**	1.50	1.67
Ex. Error	26	3839.2270	30.4701			
Total	67	214144.0971	1282.3000			

** significant difference at P=0.01

GRAND MEAN = 49.6058

CV = 11.1276 %

LSD 0.05 = 7.7283

LSD 0.01 = 10.2146

Table A.4 (Continued) Percent inhibition on oospores of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

promising antagonistic fungi	Oospore inhibition (%)				
	R1	R2	R3	R4	Means
Aspergillus niger AP101	0.30	0.20	0.29	0.19	0.25
Chaetomium aureum MB103	68.89	65.56	77.78	71.11	70.84
Chaetomium aureum MB601	85.01	86.51	83.92	82.17	84.40

Table A.4 (Continued) Percent inhibition on oospores of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

promising antagonistic fungi	Oospore inhibition (%)				
	R1	R2	R3	R4	Means
Chaetomium aureum MB603	44.22	38.11	41.00	45.56	42.22
Chaetomium aureum MB608	51.98	45.54	53.17	46.26	49.24
Chaetomium aureum RY102	50.00	47.32	50.47	47.41	48.80
Chaetomium bostrychodes NB701	28.72	27.98	29.30	28.81	28.70
Chaetomium bostrychodes PR101	88.63	83.50	86.61	88.96	86.93
Chaetomium bostrychodes PR102	50.00	47.32	50.47	47.41	48.80
Chaetomium bostrychodes PR103	32.41	41.36	36.39	41.32	37.87
Chaetomium brasiliense AM101	70.06	65.27	69.20	74.65	69.80
Chaetomium carinthiacum NB501	6.62	8.70	7.60	6.42	7.34
Chaetomium cochliodes RY301	90.16	92.23	93.87	92.43	92.17
Chaetomium cupreum NB102	80.82	89.95	90.07	79.80	85.16
Chaetomium cupreum RY201	78.48	71.93	76.09	74.46	75.24
Chaetomium cupreum RY202	79.02	85.38	80.84	81.42	81.67
Chaetomium cupreum RY204	61.26	59.78	58.25	57.85	59.29
Chaetomium cupreum SO101	54.90	52.00	53.64	49.55	52.52
Chaetomium flavigenum MB402	9.74	9.64	9.98	8.73	9.52
Chaetomium flavigenum MB604	50.24	54.23	55.83	58.28	54.65
Chaetomium flavigenum MB606	5.95	8.94	4.59	6.02	6.38
Chaetomium flavigenum MB607	66.47	65.78	66.47	64.81	65.88
Chaetomium flavigenum MB611	57.25	55.03	55.24	52.05	54.89
Chaetomium fusiforme MB605	22.02	16.67	12.18	19.40	17.57
Chaetomium fusiforme NB401	54.06	47.97	50.09	48.32	50.11
Chaetomium perlucidum MB501	1.99	1.42	2.87	4.31	2.65
Chaetomium perlucidum NB202	2.95	0.19	0.10	2.22	1.37
Chaetomium siamense MB303	12.17	17.97	17.77	12.87	15.20
Chaetomium siamense MB502	61.11	61.11	55.56	50.00	56.95
Chaetomium succineum MB304	6.59	7.49	6.27	6.12	6.62
Chaetomium succineum MB305	3.57	2.38	1.39	2.65	2.50

Table A.4 (Continued) Percent inhibition on oospores of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

promising antagonistic fungi	Oospore inhibition (%)				
	R1	R2	R3	R4	Means
Emericella nidulans EN101	47.94	47.24	47.27	52.06	48.63
Emericella rugulosa ER101	50.51	46.94	50.09	39.70	46.81
Eurotium chevalieri EU101	41.68	37.21	38.05	35.66	38.15
Gliocladium catenulatum RY102	92.61	92.44	87.99	92.71	91.44
Gliocladium catenulatum RY109	18.00	20.22	19.85	19.60	19.42
Gliocladium catenulatum. RY111	85.52	82.37	84.00	83.08	83.74
Penicillium janthinellum PN101	1.76	5.33	3.53	4.09	3.68
Trichoderma hamatum PT101	58.07	57.31	57.52	57.77	56.95
Trichoderma harzianum RY101	80.04	80.52	79.24	81.27	80.27
Trichoderma harzianum RY104	100.00	100.00	100.00	100.00	100
Trichoderma harzianum RY112	100.00	100.00	100.00	100.00	100

Table A.4 Analysis of variance of percent inhibition on oospores of *Pythium aphanidermatum* RY803 by promising antagonistic fungi on PDA at room temperature for 20 days.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	41	159767.3232	3896.7640	552.92**	1.50	1.76
Ex. Error	26	887.9959	7.0476			
Total	67	160655.3192	962.0079			

** significant difference at P=0.01

GRAND MEAN = 48.4589

CV = 5.4783 %

LSD 0.05 = 3.7168

LSD 0.01 = 4.9125

Table. A.5 Effect of crude extracts from promising antagonistic fungi on mycelial growth of *Pythium aphanidermatum* RY803 at 100 µg/ml.

promising antagonistic fungi	Crude extracts	Colony diameter of *Pythium aphanidermatum* RY803 (cm)				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Chaetomium bostrychodes PR101	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Chaetomium cochliodes RY301	Hexane	5	5	5	5	5
	EtOAc	2.80	3.40	3.40	3.30	3.23
	MeOH	5	5	5	5	5
Chaetomium cupreum NB201	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Chaetomium cupreum RY202	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Gliocladium catenulatum RY102	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Gliocladium catenulatum RY111	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Trichoderma harzianum RY101	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Trichoderma harzianum RY104	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Trichoderma harzianum RY112	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5

Table A.5 Analysis of variance of effect of crude extracts from promising antagonistic fungi on mycelial growth of *Pythium aphanidermatum* RY803 at 100 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	12.1824	0.4201	152.76**	1.65	2.03
Ex. Error	90	0.2475	0.0028			
Total	119	12.4299	0.1045			

** significant difference at P=0.01

GRAND MEAN = 4.9408

CV = 1.0614 %

LSD 0.05 = 0.0734

LSD 0.01 = 9.7040

Table. A.6 (Continued) Effect of crude extracts from promising antagonistic fungi on mycelial growth of *Pythium aphanidermatum* RY803 at 500 µg/ml.

Promising antagonistic fungi	Crude extracts	Colony diameter of *Pythium aphanidermatum* RY803 (cm)				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Chaetomium bostrychodes PR101	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Chaetomium cochliodes RY301	Hexane	5	5	5	5	5
	EtOAc	1.90	1.80	1.70	1.90	1.83
	MeOH	5	5	5	5	5
Chaetomium cupreum NB201	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Chaetomium cupreum RY202	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5

Table. A.6 (Continued) Effect of crude extracts from promising antagonistic fungi on mycelial growth of *Pythium aphanidermatum* RY803 at 500 µg/ml.

Promising antagonistic fungi	Crude extracts	Colony diameter of *Pythium aphanidermatum* RY803 (cm)				
		R1	R2	R3	R4	Means
Gliocladium catenulatum RY102	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Gliocladium catenulatum RY111	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Trichoderma harzianum RY101	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Trichoderma harzianum RY104	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Trichoderma harzianum RY112	Hexane	5	5	5	5	5
	EtOAc	2.80	3.00	2.70	2.50	2.75
	MeOH	5	5	5	5	5

Table A.6 Analysis of variance of effect of crude extracts from promising antagonistic fungi on mycelial growth of *Pythium aphanidermatum* RY803 at 500 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	56.6484	1.9534	1116.23**	1.65	2.03
Ex. Error	90	0.1575	0.0017			
Total	119	56.8059	0.4774			

** significant difference at P=0.01

GRAND MEAN = 4.8191

CV = 0.8681 %

LSD 0.05 = 5.8569

LSD 0.01 = 7.7411

Table. A.7 (Continued) Effect of crude extracts from promising antagonistic fungi on mycelial growth of *Pythium aphanidermatum* RY803 at 1,000 µg/ml.

Promising antagonistic fungi	Crude extracts	Colony diameter[y] of *Pythium aphanidermatum* RY803				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Chaetomium bostrychodes PR101	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Chaetomium cochliodes RY301	Hexane	5	5	5	5	5
	EtOAc	1.40	1.50	1.50	1.40	1.45
	MeOH	5	5	5	5	5
Chaetomium cupreum NB201	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Chaetomium cupreum RY202	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Gliocladium catenulatum RY102	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Gliocladium catenulatum RY111	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Trichoderma harzianum RY101	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Trichoderma harzianum RY104	Hexane	5	5	5	5	5
	EtOAc	5	5	5	5	5
	MeOH	5	5	5	5	5
Trichoderma harzianum RY112	Hexane	5	5	5	5	5
	EtOAc	1.60	1.40	1.30	1.60	1.48
	MeOH	5	5	5	5	5

Table A.7 Analysis of variance of effect of crude extracts from promising antagonistic fungi on mycelial growth of *Pythium aphanidermatum* RY803 at 1,000 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	93.4383	3.2220	3741.69**	1.65	2.03
Ex. Error	90	0.0775	0.0009			
Total	119	93.5159	0.7858			

** significant difference at P=0.01

GRAND MEAN = 4.7641

CV = 0.6159 %

LSD 0.05 = 4.1084

LSD 0.01 = 5.4302

Table. A.8 Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 0 µg/ml.

Promising antagonistic fungi	Crude extracts	Number of ospores (x 10^4 oospores/ml)				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	47.88	49.03	49.12	49.11	48.79
	EtOAc	48.69	48.47	48.64	48.89	48.67
	MeOH	47.64	48.52	48.63	48.36	48.29
Chaetomium bostrychodes PR101	Hexane	48.86	48.1	48.96	48.27	48.55
	EtOAc	48.75	48.51	48.69	48.38	48.58
	MeOH	48.79	48.61	48.53	48.67	48.65
Chaetomium cochliodes RY301	Hexane	48.75	46.97	50.84	48.23	48.70
	EtOAc	48.44	48.27	48.42	48.55	48.42
	MeOH	48.36	47.64	50.36	48.64	48.75
Chaetomium cupreum NB201	Hexane	49.72	48.56	48.38	47.87	48.63
	EtOAc	48.56	48.49	48.54	48.19	48.45
	MeOH	48.38	48.93	48.45	47.81	48.39
Chaetomium cupreum RY202	Hexane	49.75	48.24	48.29	48.72	48.75
	EtOAc	49.61	48.56	48.57	48.78	48.88
	MeOH	49.77	48.72	48.82	48.98	49.07

Table. A.8 (Continued) Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 0 µg/ml.

Promising antagonistic fungi	Crude extracts	Number of ospores (x 10^4 oospores/ml)				
		R1	R2	R3	R4	Means
Gliocladium catenulatum RY102	Hexane	48.28	47.77	50.12	48.68	48.71
	EtOAc	48.89	48.98	48.67	48.96	48.88
	MeOH	48.86	48.95	49.18	49.28	49.07
Gliocladium catenulatum RY111	Hexane	48.74	47.62	50.47	48.6	48.86
	EtOAc	48.74	47.61	51.7	48.47	49.13
	MeOH	48.69	47.57	48.67	48.33	48.32
Trichoderma harzianum RY101	Hexane	48.46	48.14	48.12	48.15	48.22
	EtOAc	48.58	48.57	48.95	48.29	48.60
	MeOH	48.64	48.58	48.83	48.52	48.64
Trichoderma harzianum RY104	Hexane	48.36	47.75	51.17	48.35	48.91
	EtOAc	48.67	47.76	51.56	48.53	49.13
	MeOH	48.27	47.64	48.72	48.52	48.29
Trichoderma harzianum RY112	Hexane	48.56	48.49	48.54	48.19	48.45
	EtOAc	48.38	47.93	48.45	48.81	48.39
	MeOH	48.44	47.42	48.27	48.55	48.17

Table A.8 Analysis of variance of effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 0 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	8.6018	0.2966	0.48	1.65	2.03
Ex. Error	90	55.2008	0.6133			
Total	119	63.8026	0.5362			

Non significant difference

GRAND MEAN = 48.6437

CV = 1.6100 %

LSD 0.05 = 1.0964

LSD 0.01 = 1.4492

Table. A.9 Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 10 µg/ml.

Promising antagonistic fungi	Crude extracts	Number of ospores (x 10^4oospores/ml)				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	44.49	44.69	44.85	44.8	44.71
	EtOAc	39.01	40.83	40.41	39.68	39.98
	MeOH	44.28	44.42	44.31	44.42	44.36
Chaetomium bostrychodes PR101	Hexane	48.22	47.93	48.42	48.21	48.20
	EtOAc	43.63	44.07	43.68	43.97	43.84
	MeOH	40.85	41.2	41.34	41.11	41.13
Chaetomium cochliodes RY301	Hexane	35.91	35.1	36.28	36.14	35.86
	EtOAc	35.75	36.62	36.68	36	36.26
	MeOH	39.3	38.27	39.44	38.21	38.81
Chaetomium cupreum NB201	Hexane	47.42	47.51	48.07	47.79	47.70
	EtOAc	48.46	48.33	48.38	48.23	48.35
	MeOH	44.3	43.77	46.29	43.1	44.37
Chaetomium cupreum RY202	Hexane	48.77	48.1	48.16	48.66	48.42
	EtOAc	43.16	44.02	43.86	43.3	43.59
	MeOH	42.38	42.19	42.13	42.3	42.25
Gliocladium catenulatum RY102	Hexane	45.69	44.64	44.68	45.79	45.20
	EtOAc	43.55	43.6	43.72	44.42	43.82
	MeOH	45.33	45.25	45.62	45.94	45.54
Gliocladium catenulatum RY111	Hexane	45.85	44.25	44.82	45.4	45.08
	EtOAc	42.59	42.69	42.56	43.72	42.89
	MeOH	46.6	46.72	46.56	45.98	46.47
Trichoderma harzianum RY101	Hexane	45.52	45.35	45.59	45.83	45.57
	EtOAc	39.01	39.83	39.41	39.68	39.48
	MeOH	44.38	44.42	44.53	44.42	44.44
Trichoderma harzianum RY104	Hexane	45.27	45.18	45.08	44.48	45.00
	EtOAc	43	43.74	43.43	44.04	43.55
	MeOH	45.39	45.36	45.22	45.11	45.27
Trichoderma harzianum RY112	Hexane	45.3	44.97	44.31	45.53	45.03
	EtOAc	43.79	43.16	43.15	44.06	43.54
	MeOH	45.45	45.12	45.21	45.77	45.39

Table A.9 nalysis of variance of effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 10 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	1166.1896	40.2134	181.14**	1.65	2.03
Ex. Error	90	19.9803	0.2220			
Total	119	1186.1699	9.9678			

** significant difference at P=0.01

GRAND MEAN = 43.8021

CV = 1.0757 %

LSD 0.05 = 0.6596

LSD 0.01 = 0.8719

Table. A.10 Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 50 µg/ml.

Promising antagonistic fungi	Crude extracts	Number of ospores (x 10^4 oospores/ml)				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	44.12	43.98	43.84	44.24	44.05
	EtOAc	32.02	32.22	32.47	31.94	32.16
	MeOH	44.14	44.25	44.31	43.97	44.17
Chaetomium bostrychodes PR101	Hexane	46.1	45.94	45.92	46.28	46.06
	EtOAc	31.56	31.57	31.61	31.62	31.59
	MeOH	30.89	31	30.96	31.12	30.99
Chaetomium cochliodes RY301	Hexane	29.85	30.11	29.54	29.66	29.79
	EtOAc	32.24	32.47	32.48	32.65	32.46
	MeOH	20.65	20.62	20.67	20.65	20.65
Chaetomium cupreum NB201	Hexane	39.03	39.76	39.09	39.65	39.38
	EtOAc	35.28	36.18	35.81	35.59	35.72
	MeOH	37.01	37.49	37.18	37.21	37.22
Chaetomium cupreum RY202	Hexane	46.39	42.51	46.86	43.63	44.85
	EtOAc	39.77	38.54	39.48	40.25	39.51
	MeOH	41.8	41.52	41.8	41.77	41.72

Table. A.10 (Continued) Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 50 µg/ml.

Promising antagonistic fungi	Crude extracts	Number of ospores (x 10^4 oospores/ml)				
		R1	R2	R3	R4	Means
Gliocladium catenulatum RY102	Hexane	43.65	44.55	42.67	43.19	43.52
	EtOAc	41.6	42.56	42.45	42.4	42.25
	MeOH	41.88	40.2	42.15	40.96	41.30
Gliocladium catenulatum RY111	Hexane	43.7	44.56	42.24	43.14	43.41
	EtOAc	41.25	42.18	42.12	42.37	41.98
	MeOH	41.24	40.41	42.28	40.85	41.20
Trichoderma harzianum RY101	Hexane	43.12	43.98	43.84	43.24	43.55
	EtOAc	32.02	32.22	32.47	31.94	32.16
	MeOH	40.14	40.25	40.31	40.97	40.42
Trichoderma harzianum RY104	Hexane	42.39	42.84	44.34	43.54	43.28
	EtOAc	41.8	42.75	42.38	42.02	42.24
	MeOH	41.95	40.83	42.23	40.18	41.30
Trichoderma harzianum RY112	Hexane	43.36	43.64	42.36	43.17	43.13
	EtOAc	41.15	42.27	42.14	42.5	42.02
	MeOH	41.29	40.79	42.34	40.04	41.12

Table A.10 Analysis of variance of effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 50 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	3965.2853	136.7340	320.32**	1.65	2.03
Ex. Error	90	38.4181	0.4269			
Total	119	4003.7034	33.6446			

** significant difference at P=0.01

GRAND MEAN = 39.1055

CV = 1.6707%

LSD 0.05 = 0.9147

LSD 0.01 = 1.2090

Table. A.11 Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 100 µg/ml.

Promising antagonistic fungi	Crude extracts	Number of ospores (x 10^4 oospores/ml)				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	42.75	42.69	42.67	42.54	42.66
	EtOAc	28.28	28.59	28.39	28.46	28.43
	MeOH	33.92	33.97	33.94	34.02	33.96
Chaetomium bostrychodes PR101	Hexane	35.78	35.75	35.91	35.74	35.80
	EtOAc	31.38	31.4	31.55	31.34	31.42
	MeOH	30.42	30.44	30.6	30.54	30.50
Chaetomium cochliodes RY301	Hexane	21.04	21.01	20.9	21.07	21.01
	EtOAc	18.45	18.42	18.45	18.41	18.43
	MeOH	20.56	20.67	20.62	20.56	20.60
Chaetomium cupreum NB201	Hexane	31.57	31.6	31.71	31.54	31.61
	EtOAc	23.82	23.98	24.07	23.87	23.94
	MeOH	26.2	26.23	26.4	26.45	26.32
Chaetomium cupreum RY202	Hexane	36.78	33.85	35.46	34.59	35.17
	EtOAc	35.39	35.11	35.22	34.6	35.08
	MeOH	31.21	31.04	31.32	31.29	31.22
Gliocladium catenulatum RY102	Hexane	41.77	41.34	42.13	42.46	41.93
	EtOAc	38.63	39.47	39.98	39.74	39.46
	MeOH	34.25	33.87	33.65	33.83	33.90
Gliocladium catenulatum RY111	Hexane	41.74	41.61	42.67	42.47	42.12
	EtOAc	38.78	39.62	39.45	39.59	39.36
	MeOH	40.74	40.23	40.76	40.3	40.51
Trichoderma harzianum RY101	Hexane	42.75	42.69	42.67	42.54	42.66
	EtOAc	27.28	27.59	27.39	27.46	27.43
	MeOH	33.92	33.78	33.74	33.87	33.83
Trichoderma harzianum RY104	Hexane	41.8	41.88	42.36	42.13	42.04
	EtOAc	38.98	39.2	39.9	39.76	39.46
	MeOH	34.18	33.7	33.62	33.9	33.85
Trichoderma harzianum RY112	Hexane	41.29	41.68	42.26	42.45	41.92
	EtOAc	38.64	39.1	39.5	39.66	39.23
	MeOH	34.3	33.45	33.4	33.96	33.78

Table A.11 Analysis of variance of effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 100 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	5709.9568	196.8951	1481.38**	1.65	2.03
Ex. Error	90	11.9622	0.1329			
Total	119	5721.9190	48.0834			

** significant difference at P=0.01

GRAND MEAN = 33.9199

CV = 1.0748%

LSD 0.05 = 0.5104

LSD 0.01 = 0.6746

Table. A.12 Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 500 µg/ml.

Promising antagonistic fungi	Crude extracts	Number of ospores (x 10^4 oospores/ml)				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	42.53	42.33	42.55	42.67	42.52
	EtOAc	24.38	25.17	24.66	24.44	24.66
	MeOH	33.47	33.24	33.41	33.94	33.52
Chaetomium bostrychodes PR101	Hexane	34.23	34	34.54	34.21	34.25
	EtOAc	30.31	31.48	29.56	31.38	30.68
	MeOH	30.44	30.42	30.58	30.52	30.49
Chaetomium cochliodes RY301	Hexane	20.95	20.79	20.9	20.83	20.87
	EtOAc	9.12	8.11	8.14	8.11	8.37
	MeOH	18.56	18.51	18.56	18.62	18.56
Chaetomium cupreum NB201	Hexane	30.53	30.56	30.67	30.62	30.60
	EtOAc	20.22	20.28	20.34	20.22	20.27
	MeOH	22.92	22.86	23.59	23.14	23.13
Chaetomium cupreum RY202	Hexane	33.16	32.51	31.56	32.95	32.55
	EtOAc	32.38	32.3	32.41	32.24	32.33
	MeOH	30.59	30.65	30.76	30.7	30.68

Table. A.12 (Continued) Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 500 µg/ml.

Promising antagonistic fungi	Crude extracts	Number of ospores (x 10^4 oospores/ml)				
		R1	R2	R3	R4	Means
Gliocladium catenulatum RY102	Hexane	38.48	38.22	37.87	39.23	38.45
	EtOAc	31.17	31.89	31.76	31.22	31.51
	MeOH	31.96	31.8	32.31	31.14	31.80
Gliocladium catenulatum RY111	Hexane	38.6	38.96	37.58	39.67	38.70
	EtOAc	32.79	32.56	32.24	32.83	32.61
	MeOH	38.96	38.8	38.31	38.14	38.55
Trichoderma harzianum RY101	Hexane	41.53	41.33	41.55	41.67	41.52
	EtOAc	24.38	24.17	24.66	24.44	24.41
	MeOH	32.47	32.24	32.41	32.94	32.52
Trichoderma harzianum RY104	Hexane	38.98	38.22	37.97	39.2	38.59
	EtOAc	31.21	31.91	31.6	31.35	31.52
	MeOH	31.8	31.94	32.02	31.6	31.84
Trichoderma harzianum RY112	Hexane	38.25	38.95	37.29	39.55	38.51
	EtOAc	24.58	27.29	25.31	28.94	26.53
	MeOH	31.78	31.94	32.84	31.35	31.98

Table A.12 Analysis of variance of effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 500 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	6428.0177	221.6558	694.74**	1.65	2.03
Ex. Error	90	28.7144	0.3190			
Total	119	6456.7320	54.2583			

** significant difference at P=0.01

GRAND MEAN = 30.7497

CV = 1.8369%

LSD 0.05 = 0.7908

LSD 0.01 = 1.0452

Table. A.13 Effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 1,000 µg/ml.

Promising antagonistic fungi	Crude extracts	Number of ospores (x 10^4 oospores/ml)				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	42.53	42.41	42.5	42.64	42.52
	EtOAc	20.22	20.34	20.39	20.34	20.32
	MeOH	33.02	33.24	32.82	33.21	33.07
Chaetomium bostrychodes PR101	Hexane	33.18	33.24	33.27	33.42	33.28
	EtOAc	21.81	22.24	22.15	21.07	21.82
	MeOH	20.36	20.33	20.41	20.53	20.41
Chaetomium cochliodes RY301	Hexane	20.67	20.56	20.51	20.62	20.59
	EtOAc	5.32	5.35	5.39	5.34	5.35
	MeOH	14.22	14.34	14.39	15.34	14.57
Chaetomium cupreum NB201	Hexane	18.55	18.35	18.74	18.41	18.51
	EtOAc	20.22	20.22	20.28	20.28	20.25
	MeOH	20.51	20.45	20.34	20.28	20.40
Chaetomium cupreum RY202	Hexane	24.79	25.24	24.1	26.93	25.27
	EtOAc	21.21	21.18	21.23	21.07	21.17
	MeOH	20.22	20.45	20.39	20.37	20.36
Gliocladium catenulatum RY102	Hexane	36.25	36.12	37.11	36.37	36.46
	EtOAc	30.89	30.22	30.36	30.19	30.42
	MeOH	29.98	29.9	29.83	29.71	29.86
Gliocladium catenulatum RY111	Hexane	35.92	35.25	36.2	35.13	35.63
	EtOAc	30.25	30.18	30.09	30.17	30.17
	MeOH	32.98	32.9	32.83	32.71	32.86
Trichoderma harzianum RY101	Hexane	40.53	40.41	40.5	40.64	40.52
	EtOAc	22.22	22.34	22.39	22.34	22.32
	MeOH	30.02	30.24	30.82	30.21	30.32
Trichoderma harzianum RY104	Hexane	36.73	36.37	37.69	36.96	36.94
	EtOAc	30.22	30.45	30.36	30.42	30.36
	MeOH	29.59	29.79	29.67	29.79	29.71
Trichoderma harzianum RY112	Hexane	36.19	36.81	37.32	36.16	36.62
	EtOAc	18.76	18.3	18.64	18.11	18.45
	MeOH	28.89	28.93	28.57	28.84	28.81

Table A.13 Analysis of variance of effect of crude extracts from promising antagonistic fungi on oospore formation of *Pythium aphanidermatum* RY803 at 1,000 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	8273.8678	285.3058	2362.26**	1.65	2.03
Ex. Error	90	10.8699	0.1208			
Total	119	8284.7376	69.6196			

** significant difference at P=0.01

GRAND MEAN = 26.9107

CV = 1.2914%

LSD 0.05 = 0.4865

LSD 0.01 = 0.6431

Table A.14 Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 10 µg/ml.

Promising antagonistic fungi	Crude extracts	Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	7.08	8.85	8.69	8.78	8.35
	EtOAc	19.88	15.76	16.92	18.84	17.85
	MeOH	7.05	8.45	8.88	8.15	8.13
Chaetomium bostrychodes PR101	Hexane	1.31	0.35	1.1	0.12	0.72
	EtOAc	10.5	9.15	10.23	9.12	9.75
	MeOH	16.27	15.24	14.82	15.53	15.47
Chaetomium cochliodes RY301	Hexane	26.34	25.27	28.64	25.07	26.33
	EtOAc	26.2	24.14	24.25	25.85	25.11
	MeOH	18.73	19.67	21.68	21.44	20.38
Chaetomium cupreum NB201	Hexane	4.63	2.16	0.64	0.17	1.90
	EtOAc	0.54	2.39	0.43	1.99	1.34
	MeOH	8.43	10.55	4.46	9.85	8.32
Chaetomium cupreum RY202	Hexane	1.97	0.29	0.27	0.12	0.66
	EtOAc	13	9.35	9.7	11.23	10.82
	MeOH	14.85	13.4	13.7	13.64	13.90
Gliocladium catenulatum RY102	Hexane	8.17	7.85	7.34	7.76	7.78

Table A.14 (Continued) Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 10 µg/ml.

Promising antagonistic fungi	Crude extracts	Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803				
		R1	R2	R3	R4	Means
	EtOAc	7.36	8.54	9.85	8.94	8.67
	MeOH	5.31	6.11	4.41	4.3	5.03
Gliocladium catenulatum RY111	Hexane	6.17	6.17	14.01	6.78	8.28
	EtOAc	12.62	10.33	17.68	9.8	12.60
	MeOH	4.06	3.71	3.26	3.62	3.66
Trichoderma harzianum RY101	Hexane	5.17	5.49	6.32	4.16	5.29
	EtOAc	17.97	19.46	17.83	18.51	18.44
	MeOH	8.52	8.81	8.43	8.69	8.61
Trichoderma harzianum RY104	Hexane	8.41	8.34	8.43	7.87	8.26
	EtOAc	11.32	11.23	11.43	11.1	11.27
	MeOH	6.76	6.26	5.82	6.13	6.24
Trichoderma harzianum RY112	Hexane	7.06	8.17	6.82	6.13	7.05
	EtOAc	9.49	9.95	10.94	9.73	10.03
	MeOH	6.19	6.37	5.86	4.65	5.77

Table A.14 Analysis of variance of percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 10 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	5018.8036	173.0622	84.86**	1.65	2.03
Ex. Error	90	183.5387	2.0393			
Total	119	5202.3423	43.7172			

** significant difference at P=0.01

GRAND MEAN = 9.8673

CV = 14.4725%

LSD 0.05 = 1.9993

LSD 0.01 = 2.6425

Table A.15 Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 50 µg/ml.

Promising antagonistic fungi	Crude extracts	Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	7.85	10.3	10.75	9.92	9.71
	EtOAc	34.24	33.53	33.24	34.67	33.92
	MeOH	7.35	8.8	8.88	9.08	8.53
Chaetomium bostrychodes PR101	Hexane	5.65	4.49	6.21	4.12	5.12
	EtOAc	35.26	34.92	35.08	34.64	34.98
	MeOH	36.69	36.23	36.2	36.06	36.30
Chaetomium cochliodes RY301	Hexane	38.77	35.9	41.9	38.5	38.77
	EtOAc	33.44	32.73	32.92	32.75	32.96
	MeOH	57.3	56.72	58.96	57.55	57.63
Chaetomium cupreum NB201	Hexane	21.5	18.12	19.2	17.17	19.00
	EtOAc	27.35	25.39	26.23	26.15	26.28
	MeOH	23.5	23.38	23.26	22.17	23.08
Chaetomium cupreum RY202	Hexane	6.75	11.88	2.96	10.44	8.01
	EtOAc	19.83	20.63	18.72	17.49	19.17
	MeOH	16.01	14.78	35.85	14.72	20.34
Gliocladium catenulatum RY102	Hexane	9.59	6.74	14.86	11.28	10.62
	EtOAc	14.53	10.17	12.38	12.43	12.38
	MeOH	14.25	15.05	13.02	17.87	15.05
Gliocladium catenulatum RY111	Hexane	10.34	6.43	16.31	11.23	11.08
	EtOAc	15.37	11.41	18.53	12.59	14.48
	MeOH	15.3	15.05	13.13	15.48	14.74
Trichoderma harzianum RY101	Hexane	11.02	8.64	8.89	10.2	9.69
	EtOAc	34.09	33.66	33.67	33.86	33.82
	MeOH	17.48	17.15	17.45	15.56	16.91
Trichoderma harzianum RY104	Hexane	10.38	7.41	17.92	9.89	11.4
	EtOAc	14.12	10.49	17.8	13.41	13.96
	MeOH	13.09	14.29	13.32	17.19	14.47
Trichoderma harzianum RY112	Hexane	10.71	10	12.73	10.42	10.97
	EtOAc	14.94	11.81	13.02	12.93	13.18
	MeOH	14.76	13.98	12.29	17.53	14.64

Table A.15 Analysis of variance of percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 50 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	16737.1782	577.1441	75.15**	1.65	2.03
Ex. Error	90	691.1629	7.6796			
Total	119	17428.3411	146.4566			

** significant difference at P=0.01

GRAND MEAN = 19.7043

CV = 14.0639%

LSD 0.05 = 3.8798

LSD 0.01 = 5.1281

Table A.16 Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 100 µg/ml.

Promising antagonistic fungi	Crude extracts	Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	10.71	12.93	13.13	13.38	10.71
	EtOAc	41.92	41.02	41.63	41.79	41.92
	MeOH	28.8	29.99	30.21	29.65	28.8
Chaetomium bostrychodes PR101	Hexane	26.77	25.68	26.65	25.96	26.77
	EtOAc	35.63	34.98	35.2	35.22	35.63
	MeOH	37.65	37.38	36.95	37.25	37.65
Chaetomium cochliodes RY301	Hexane	56.84	55.27	58.89	56.31	56.84
	EtOAc	61.91	61.84	61.9	62.08	61.91
	MeOH	57.49	56.61	59.05	57.73	57.49
Chaetomium cupreum NB201	Hexane	36.5	34.93	34.46	34.11	36.5
	EtOAc	50.95	50.55	50.41	50.47	50.95
	MeOH	45.85	46.39	45.51	44.68	45.85
Chaetomium cupreum RY202	Hexane	26.07	29.83	26.57	29.06	26.07
	EtOAc	28.66	27.7	27.49	29.07	28.66
	MeOH	37.29	36.29	35.85	36.12	37.29

Table A.16 (Continued) Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 100 µg/ml.

Promising antagonistic fungi	Crude extracts	Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803				
		R1	R2	R3	R4	Means
Gliocladium catenulatum RY102	Hexane	13.48	13.46	15.94	12.78	13.48
	EtOAc	20.63	16.69	17.48	17.93	20.63
	MeOH	29.87	28.42	30.56	29.93	29.87
Gliocladium catenulatum RY111	Hexane	14.36	12.62	15.45	12.61	14.36
	EtOAc	20.43	16.78	23.69	18.32	20.43
	MeOH	16.33	15.43	16.25	16.61	16.33
Trichoderma harzianum RY101	Hexane	11.78	11.32	11.33	11.65	11.78
	EtOAc	43.85	43.2	44.04	43.14	43.85
	MeOH	30.26	30.47	30.9	30.19	30.26
Trichoderma harzianum RY104	Hexane	13.56	12.29	17.22	12.86	13.56
	EtOAc	19.91	17.92	22.61	18.07	19.91
	MeOH	29.19	29.26	30.99	30.13	29.19
Trichoderma harzianum RY112	Hexane	14.97	14.04	12.94	11.91	14.97
	EtOAc	20.13	18.42	18.47	18.75	20.13
	MeOH	29.19	29.46	30.81	30.05	29.19

Table A.16 Analysis of variance of percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 100 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	24101.9317	831.1011	587.73**	1.65	2.03
Ex. Error	90	127.2674	1.4141			
Total	119	24229.1991	203.6067			

** significant difference at P=0.01

GRAND MEAN = 30.1878

CV = 3.9392%

LSD 0.05 = 1.6648

LSD 0.01 = 2.2005

Table A.17 Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 500 µg/ml.

Promising antagonistic fungi	Crude extracts	Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	11.17	13.67	13.38	13.11	12.83
	EtOAc	49.93	48.07	49.3	50.01	49.33
	MeOH	29.74	31.49	31.3	29.82	30.59
Chaetomium bostrychodes PR101	Hexane	29.94	29.31	29.45	29.13	29.46
	EtOAc	37.83	35.11	39.29	35.14	36.84
	MeOH	37.61	37.42	36.99	37.29	37.33
Chaetomium cochliodes RY301	Hexane	57.03	55.74	58.89	56.81	57.12
	EtOAc	81.17	83.2	83.19	83.3	82.72
	MeOH	61.62	61.15	63.15	61.72	61.91
Chaetomium cupreum NB201	Hexane	38.6	37.07	36.61	36.04	37.08
	EtOAc	58.36	58.18	58.1	58.04	58.17
	MeOH	52.63	53.28	51.31	51.6	52.21
Chaetomium cupreum RY202	Hexane	33.35	32.61	34.64	32.37	33.24
	EtOAc	34.73	33.48	33.27	33.91	33.85
	MeOH	38.54	37.09	36.99	37.32	37.49
Gliocladium catenulatum RY102	Hexane	20.3	19.99	24.44	19.41	21.04
	EtOAc	35.96	32.69	34.45	35.52	34.66
	MeOH	34.68	32.8	33.33	35.5	34.08
Gliocladium catenulatum RY111	Hexane	20.8	18.19	25.54	18.37	20.73
	EtOAc	32.72	31.61	37.64	32.27	33.56
	MeOH	19.98	18.44	21.29	21.08	20.20
Trichoderma harzianum RY101	Hexane	14.3	14.15	13.65	13.46	13.89
	EtOAc	49.81	50.24	49.62	49.39	49.77
	MeOH	33.24	33.63	33.63	32.11	33.15
Trichoderma harzianum RY104	Hexane	19.4	19.96	25.8	18.92	21.02
	EtOAc	35.87	33.19	38.71	35.4	35.79
	MeOH	34.12	32.96	34.28	34.87	34.06
Trichoderma harzianum RY112	Hexane	21.23	19.67	23.18	17.93	20.50
	EtOAc	49.19	43.06	47.76	40.71	45.18
	MeOH	34.39	50.16	31.97	35.43	37.99

Table A.17 Analysis of variance of percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 500 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	27190.4921	937.6032	186.80**	1.65	2.03
Ex. Error	90	451.7321	5.0192			
Total	119	27642.2242	232.2876			

** significant difference at P=0.01

GRAND MEAN = 36.8581

CV = 6.0783%

LSD 0.05 = 3.1366

LSD 0.01 = 4.1457

Table A.18 Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 1,000 µg/ml.

Promising antagonistic fungi	Crude extracts	Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803				
		R1	R2	R3	R4	Means
Chaetomium aureum MB601	Hexane	11.17	13.5	13.48	13.17	12.83
	EtOAc	58.47	58.04	58.08	58.5	58.27
	MeOH	30.69	31.49	32.51	31.33	31.51
Chaetomium bostrychodes PR101	Hexane	32.09	30.89	32.05	30.57	31.40
	EtOAc	55.26	54.15	54.51	56.45	55.09
	MeOH	58.27	58.18	57.94	57.82	58.05
Chaetomium cochliodes RY301	Hexane	57.6	56.23	59.66	57.27	57.69
	EtOAc	89.02	88.92	88.87	89	88.95
	MeOH	70.6	69.9	71.43	68.46	70.10
Chaetomium cupreum NB201	Hexane	62.69	62.21	61.26	61.54	61.93
	EtOAc	58.36	58.3	58.22	57.92	58.20
	MeOH	57.61	58.21	58.02	57.58	57.86
Chaetomium cupreum RY202	Hexane	50.17	47.68	50.09	44.72	48.17
	EtOAc	57.25	56.38	56.29	56.81	56.68
	MeOH	59.37	58.03	58.23	58.41	58.51

Table A.18 (Continued) Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 1,000 µg/ml.

Promising antagonistic fungi	Crude extracts	Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803				
		R1	R2	R3	R4	Means
Gliocladium catenulatum RY102	Hexane	24.92	24.39	25.96	25.29	25.14
	EtOAc	36.53	36.23	37.34	37.65	36.94
	MeOH	38.62	36.81	38.44	38.46	38.08
Gliocladium catenulatum RY111	Hexane	26.3	25.98	28.27	27.72	27.07
	EtOAc	37.94	36.61	41.8	37.76	38.53
	MeOH	32.27	30.84	32.55	32.32	32.00
Trichoderma harzianum RY101	Hexane	16.36	16.06	15.84	15.6	15.97
	EtOAc	54.26	54	54.26	53.74	54.07
	MeOH	38.28	37.75	36.88	37.74	37.66
Trichoderma harzianum RY104	Hexane	24.05	23.83	26.34	23.56	24.45
	EtOAc	37.91	36.24	41.12	37.32	38.15
	MeOH	38.7	37.47	39.1	38.6	38.47
Trichoderma harzianum RY112	Hexane	25.47	24.09	23.11	24.96	24.41
	EtOAc	61.22	61.82	61.53	62.9	61.87
	MeOH	40.36	38.99	40.81	40.6	40.19

Table A.18 Analysis of variance of percent inhibition of oospore formation of *Pythium aphanidermatum* RY803 on PDA containing crude extracts from promising antagonistic fungi at 1,000 µg/ml.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	29	35144.7575	1211.8882	1109.06**	1.65	2.03
Ex. Error	90	98.3441	1.0927			
Total	119	35243.1016	296.1605			

** significant difference at P=0.01

GRAND MEAN = 44.6065

CV = 2.3434%

LSD 0.05 = 1.4635

LSD 0.01 = 1.9343

Table A.19 Disease severity index of pineapple root rot after applying biofungicide in pot at 1st month.

Treatments	disease severity index^{y} (DSI) in 5 months					
	R1	R2	R3	R4	R5	Means
Non-treated control (T1)	1	1	1	1	1	1
Pythium aphanidermadum RY803 alone (T2)	2	2	3	2	3	2.4
oil formulation at 10 ml/plant every 2 weeks (T3)	1	1	1	1	1	1
powder formulation at 10 g/plant every 2 weeks (T4)	1	1	1	1	1	1
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	1	1	2	2	1	1.4
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	1	2	2	2	1	1.6
Treated with metalaxyl at recommended rate + pathogen (T7)	2	1	1	1	1	1.2

Table A.19 Analysis of variance of disease severity index of pineapple root rot after applying biofungicide in pot at 1st month.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	6	7.7714	1.2952	8.24**	2.45	3.53
Ex. Error	28	4.4000	0.1571			
Total	34	12.1714	0.3580			

** significant difference at P=0.01

GRAND MEAN = 1.3714

CV = 28.9051%

LSD 0.05 = 0.5134

LSD 0.01 = 0.6927

Table A.20 Disease severity index of pineapple root rot after applying biofungicide in pot at 2nd month.

Treatments	disease severity index^{y} (DSI) in 5 months					
	R1	R2	R3	R4	R5	Means
Non-treated control (T1)	1	1	1	1	1	1
Pythium aphanidermadum RY803 alone (T2)	3	2	3	3	4	3
oil formulation at 10 ml/plant every 2 weeks (T3)	1	1	1	1	1	1
powder formulation at 10 g/plant every 2 weeks (T4)	1	1	1	1	1	1
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	2	2	1	1	2	1.6
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	2	2	1	1	2	1.6
Treated with metalaxyl at recommended rate + pathogen (T7)	1	2	1	1	2	1.4

Table A.20 Analysis of variance of disease severity index of pineapple root rot after applying biofungicide in pot at 2nd month.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	6	15.1429	2.5238	12.62**	2.45	3.53
Ex. Error	28	5.6000	0.2000			
Total	34	20.7429	0.6101			

** significant difference at P=0.01

GRAND MEAN = 1.5142

CV = 29.5330%

LSD 0.05 = 0.5792

LSD 0.01 = 0.7814

Table A.21 Disease severity index of pineapple root rot after applying biofungicide in pot at 3rd month.

Treatments	disease severity index[y] (DSI) in 5 months					
	R1	R2	R3	R4	R5	Means
Non-treated control (T1)	1	1	1	1	1	1
Pythium aphanidermadum RY803 alone (T2)	3	4	4	3	4	3.6
oil formulation at 10 ml/plant every 2 weeks (T3)	1	1	1	1	1	1
powder formulation at 10 g/plant every 2 weeks (T4)	1	1	1	1	1	1
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	1	2	2	2	1	1.6
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	2	2	1	2	2	1.8
Treated with metalaxyl at recommended rate + pathogen (T7)	2	1.5	1	1	2	1.5

Table A.21 Analysis of variance of disease severity index of pineapple root rot after applying biofungicide in pot at 3rd month.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	6	25.5857	4.2643	28.43**	2.45	3.53
Ex. Error	28	4.2000	0.1500			
Total	34	29.7857	0.8761			

** significant difference at P=0.01, GRAND MEAN = 1.6428, CV = 23.5747%

LSD 0.05 = 0.5016

LSD 0.01 = 0.6767

Table A.22 Disease severity index of pineapple root rot after applying biofungicide in pot at 4th month.

Treatments	disease severity index[y] (DSI) in 5 months					
	R1	R2	R3	R4	R5	Means
Non-treated control (T1)	1	1	1	1	1	1
Pythium aphanidermadum RY803 alone (T2)	4	5	3	5	4	4.20
oil formulation at 10 ml/plant every 2 weeks (T3)	1	1	1	1	1	1
powder formulation at 10 g/plant every 2 weeks (T4)	1	1	1	1	1	1
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	2	2	2	2	3	2.20
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	2	2	2	3	3	2.40
Treated with metalaxyl at recommended rate + pathogen (T7)	2	2	2	2.5	2	2.10

Table A.22 Analysis of variance of disease severity index of pineapple root rot after applying biofungicide in pot at 4th month.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	6	40.2429	6.7071	37.56**	2.45	3.53
Ex. Error	28	5.0000	0.1786			
Total	34	45.2429	1.3307			

** significant difference at P=0.01

GRAND MEAN = 1.9857

CV = 21.2809%

LSD 0.05 = 0.5473

LSD 0.01 = 0.7384

Table A.23 Disease severity index of pineapple root rot after applying biofungicide in pot at 5th month.

Treatments	disease severity index[y] (DSI) in 5 months					
	R1	R2	R3	R4	R5	Means
Non-treated control (T1)	1	1	1	1	1	1
Pythium aphanidermadum RY803 alone (T2)	5	5	5	5	5	5
oil formulation at 10 ml/plant every 2 weeks (T3)	1	1	1	1	1	1
powder formulation at 10 g/plant every 2 weeks (T4)	1	1	1	1	1	1
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	2	2	2	3	3	2.4
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	2	3	2	3	3	2.6
Treated with metalaxyl at recommended rate + pathogen (T7)	2	2	2.5	2	3	2.3

Table A.23 Analysis of variance of disease severity index of pineapple root rot after applying biofungicide in pot at 5th month.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	6	61.8429	10.3071	90.19**	2.45	3.53
Ex. Error	28	3.2000	0.1143			
Total	34	65.0429	1.9130			

** significant difference at P=0.01, GRAND MEAN = 2.1857, CV = 15.4669%

LSD 0.05 = 0.4378

LSD 0.01 = 0.5907

Table A.24 Effect of biofungicides on fresh weight of pineapple leaves and stems after growing in pot for 5 months.

Treatments	Total fresh weigh of leaves and stems (g)					
	R1	R2	R3	R4	R5	Means
Non-treated control (T1)	370	370	485	315	335	375
Pythium aphanidermadum RY803 alone (T2)	275	10	190	95	300	174
oil formulation at 10 ml/plant every 2 weeks (T3)	445	520	505	525	530	505
powder formulation at 10 g/plant every 2 weeks (T4)	300	325	325	470	445	373
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	325	435	285	330	470	369
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	370	375	325	250	340	332
Treated with metalaxyl at recommended rate + pathogen (T7)	380	270	415	335	385	357

Table A.24 Analysis of variance of effect of biofungicides on fresh weight of pineapple leaves and stems after growing in pot for 5 months.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	6	283570.0000	47261.6667	8.45**	2.45	3.53
Ex. Error	28	154130.0000	5504.6429			
Total	34	437700.0000	12873.5294			

** significant difference at P=0.01, GRAND MEAN = 355

CV = 20.8995%

LSD 0.05 = 96.1002

LSD 0.01 = 129.6508

Table A.25 Effect of biofungicides on fresh weight of pineapple root after growing in pot for 5 months.

Treatments	Total fresh weigh of leaves and stems (g)					
	R1	R2	R3	R4	R5	Means
Non-treated control (T1)	30	30	20	20	30	26
Pythium aphanidermadum RY803 alone (T2)	15	0	0	0	15	6
oil formulation at 10 ml/plant every 2 weeks (T3)	30	25	30	25	30	28
powder formulation at 10 g/plant every 2 weeks (T4)	25	30	20	15	25	23
oil formulation at 10 ml/plant every 2 weeks + pathogen (T5)	20	20	20	25	20	21
powder formulation at 10 g/plant every 2 weeks + pathogen (T6)	20	15	20	20	15	18
Treated with metalaxyl at recommended rate + pathogen (T7)	20	20	15	20	20	19

Table A.25 Analysis of variance of effect of biofungicides on fresh weight of pineapple root after growing in pot for 5 months.

Source of variation	df	SS	MS	F	F.05	F.01
Treatment	6	1554.2857	259.0476	11.70**	2.45	3.53
Ex. Error	28	620.0000	22.1429			
Total	34	2174.2857	63.9496			

** significant difference at P=0.01

GRAND MEAN = 20.1428

CV = 23.3612%

LSD 0.05 = 6.0950

LSD 0.01 = 8.2229

APPENDIX B

PUBLICATIONS

Pornsuriya, C., Wang, H. K., Lin, F. C. and Soytong, K. 2007. "Characterisation of *Pythium* sp. isolated from the rhizosphere of pineapple and its ITS region of rDNA." 615-618. in **International Conference on Engineering, Applied Science, and Technology.** Bangkok : Research Center for Communications and Information Technology and King Mongkut's Institute of Technology Ladkrabang.

Pornsuriya, C., Wang, H. K., Lin, F. C. and Soytong, K. 2007. "Morphology and molecular phylogeny of *Pythium sylvaticum* isolated from pineapple roots." 129-133. in **International Conference on Integration of Science & Technology for Sustainable Development.** Bangkok : King Mongkut's Institute of Technology Ladkrabang.

Pornsuriya, C., Lin, F.C., Wang, H.K. and Soytong, K. 2007. "*Pythium* spp. Isolated from Rhizosphere of Pineapple Rot: Morphology and ITS Region of rDNA". 8. in **The 2nd Annual Meeting of Thai Mycological Association and Mycology Conference in Thailand.** Bangkok : Thamasart.

Pornsuriya, C., Wang, H.K., Lin, F.C. and Soytong, K. 2008. "First report of pineapple root rot caused by *Pythium graminicola* in Thailand." Journal of Agricultural Technology 4(1) : 139-150.

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Conference Guide....

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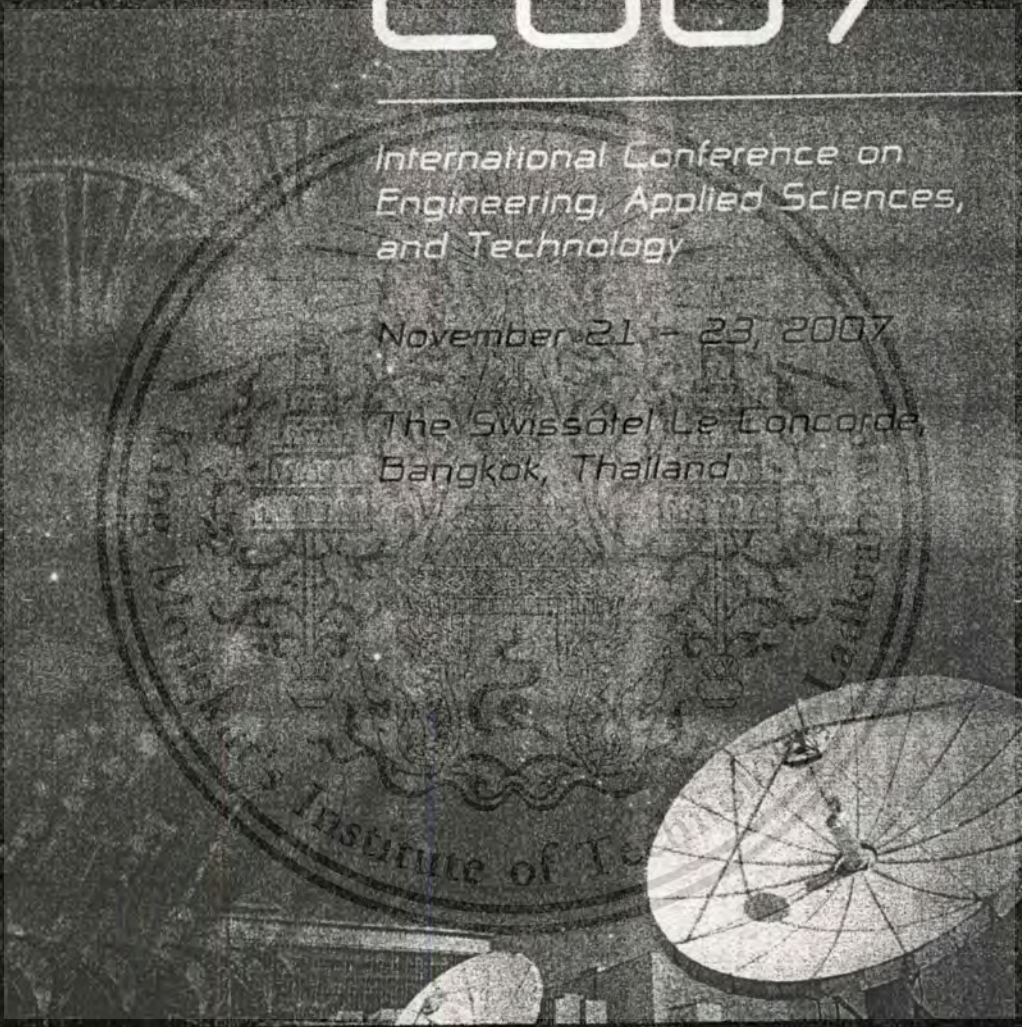

Organized by:

Research Center for Communications and Information Technology, King Mongkut's Institute of Technology Ladkrabang, Thailand

Characterisation of *Pythium* sp. isolated from the rhizosphere of pineapple and its ITS region of rDNA

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Abstract—***Pythium*** **sp. was isolated from soil samples taken from a pineapple plantation in Phattalung province, Thailand during December 2005-March 2006. This fungus is characterized by its smooth-walled, spherical sporangia and usually formed sexual structures. The antheridial 1-5 per oogonium, stalks usually monoclinous, often also diclinous are presented. The ITS region of the ribosomal nuclear DNA was amplified and sequenced for further identify to confirm the species.**

Keywords—***Pythium*****, Antheridia, Oogonia, ITS region, Ribosomal DNA**

I. INTRODUCTION

The genus *Pythium* belongs to Oomycetes which was discovered by Pringsheim in 1858. These fungi are phenotypically true fungi having coenocytic branched mycelia. This genus is often known as phytopathogen causing root rot and damping-off disease. Most of these fungi can live as saprophytes, while others live as facultative saprophytes, becoming plant pathogen from time to time and causing extensive damage to economically important crops worldwide [1]. Plant diseases caused by oomycetes are often encountered under water-logged or wet soil conditions as found in littoral ecosystems. The taxonomy of the genus *Pythium* is mainly based on the morphological descriptions and the keys provided by Middleton [2], Waterhouse [3] and Plaats-Niterink [4]. However, morphological observations are now being suplemented with molecular characteristics. The internal transcribed region (ITS) of the ribosomal nuclear DNA and the nucleotide sequence of this region has become a useful tool in fungal taxonomy and is currently used to identify different species of *Pythium* [5,6].

The morphological and reproductive details of *Pythium* sp., together with the sequence of the PCR amplified ITS region of ribosomal nuclear DNA are presented in this paper for further identify the species.

II. METHODOLOGY

A. Source of Isolates

All of the isolates were obtained from soil collected from the rhizosphere of pineapple showing symptom of root rot and brought to the laboratory at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand for isolation to pure culture.

B. Fungal isolation

The soil samples were mixed with sterile water (1/4 w/v) and baited with leaflets of pineapples (1x1 cm) floated on the surface of the water. After 2-3 days incubation at room temperature, discolured baits were blotted on sterile paper towel to remove excess water and plated onto selective medium which contains Potato dextrose agar (PDA) amended with 25 mg l^{-1}benomyl, 10 mgl^{-1} rifampicin, 200 mg l^{-1} ampicillin, 0.05 ml l^{-1} nystatin. Colonies growing from the baits were transferred to PDA, corn meal agar (CMA) and Potato-Carrot agar (PCA) for studying their morphology. Cultures were stored on PDA slant with sterile parafin oil.

C. Morphological characterization

The morphological characters were examined dimension as follows: oogonia and antheridia. Isolates were pre-grown on PDA plates at room temperature, then agar disks were taken from actively growing colony margins and inoculated onto PCA and CMA plates. Cultures were incubated for 3 to 4 d at room temperature for morphological observation. At least 30 structures were examined for each isolate for all the characters studied.

978-974-8308-56-2

D. DNA extraction and PCR

The isolates were grown in 50 ml test tubes containing 20 ml PDB (Potato dextrose broth) at 28°C on an arbital shaker (180 rpm) for 3-10 d. The mycelium was harvested by filtration. Excess water was removed from mycelium by pressing in a paper towel. A mycelial mat was placed in a prechilled mortar, frozen with liquid nitrogen, and ground to fine powder. Mycelial powder was suspended in 600 µl CTAB buffer (cetyltri-methyl-ammonium bromide), vortex and incubated at 65°C for 30 min, and 600 µl CIA (chloroform: isoamyl alcohol, 24:1 (v/v)) was added. The solution was incubated for 25 min on a shaking platform and centrifuged at 7,000 rpm for 5 min at 4°C. The aqueous phase (top) was transferred to a new microcentrifuge tube and repeat CIA extraction. After the second CIA was washed 300 µl isopropanal was added and mixed by inverting the tube several times. The tube was stored at room temperature, and then was centrifuged at 10,000 rpm for 10 min, the supernatant was decanted and drained on a paper towel for 30 min, the pellet was resuspended with 50 ml sterile double distill water.

The polymerase chain reaction (PCR) with universal primers ITS1(TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) were carried out in 50 µl volumes containing 1 µl genomic DNA, 3 µl $MgCl_2$, 1 µl dNTPs, o.4 µl of each primer and 1 µl *Taq* DNA polymerase in 5 µl PCR buffer. Amplifications were done with the following temperature cycling parameters: denaturation at 94°C for 3 min for first cycle and 1 min each for subsequent cycles, annealing for 30 sec at 55°C, and elongation for 1 min at 72°C. To assess the efficiency of the amplification, 5 µl aliquats of PCR products were electrophoresed in a 1% agarose gel in 1xTAE buffer. The remaining volumes of the PCR amplicons were purified for DNA sequencing. The sequence editing and aligning was carried out using BioEdit, version 7.

III. RESULTS AND DISCUSSION

A total of 70 soil samples from the rhizosphere of pineapple showing symptom of root rot (Fig 1) were collected by random. Thirty-two isolates of *Pythium* presence within the rhizospere of pineapple roots indicates its parasitic and perhaps saprophytic activity towards this plant. Colonies on PCA and CMA are submerged, on PDA showed a rosette pattern (Fig 1). Main hyphae 3-5 µm wide. Hyphal swellings frequent. Oogonia 25-29 µm diam abundantly produced in single culture, globose, smooth-walled, and borne terminally or intercalary. Antheridia usually monoclinous, often also diclinous, 1-5 per oogonium, always wrapping around the oogonium (Fig.2). Morphologically these isolates come close to species like *P. sylvaticum, P. scleroteichum, P. periilum and P. graminicola* [4].

All isolates of *Pythium* sp. had identical ITS sequences. The ITS region of rDNA of this fungus is comprised of 814 bases (Fig.3)

BLAST searches indicated the species' close relatedness to *P. graminicola* and *Pythium periilum*. In order to identify the species of *Pythium* sp., the relationship will be performed in the next stage of this work for comparisons among the *Pythium* sp. and the related species [5,6].

Fig. 1. Symptom of root rot of pineapple (A) *Pythium* sp. colony on PDA. at 3 day-old (B)

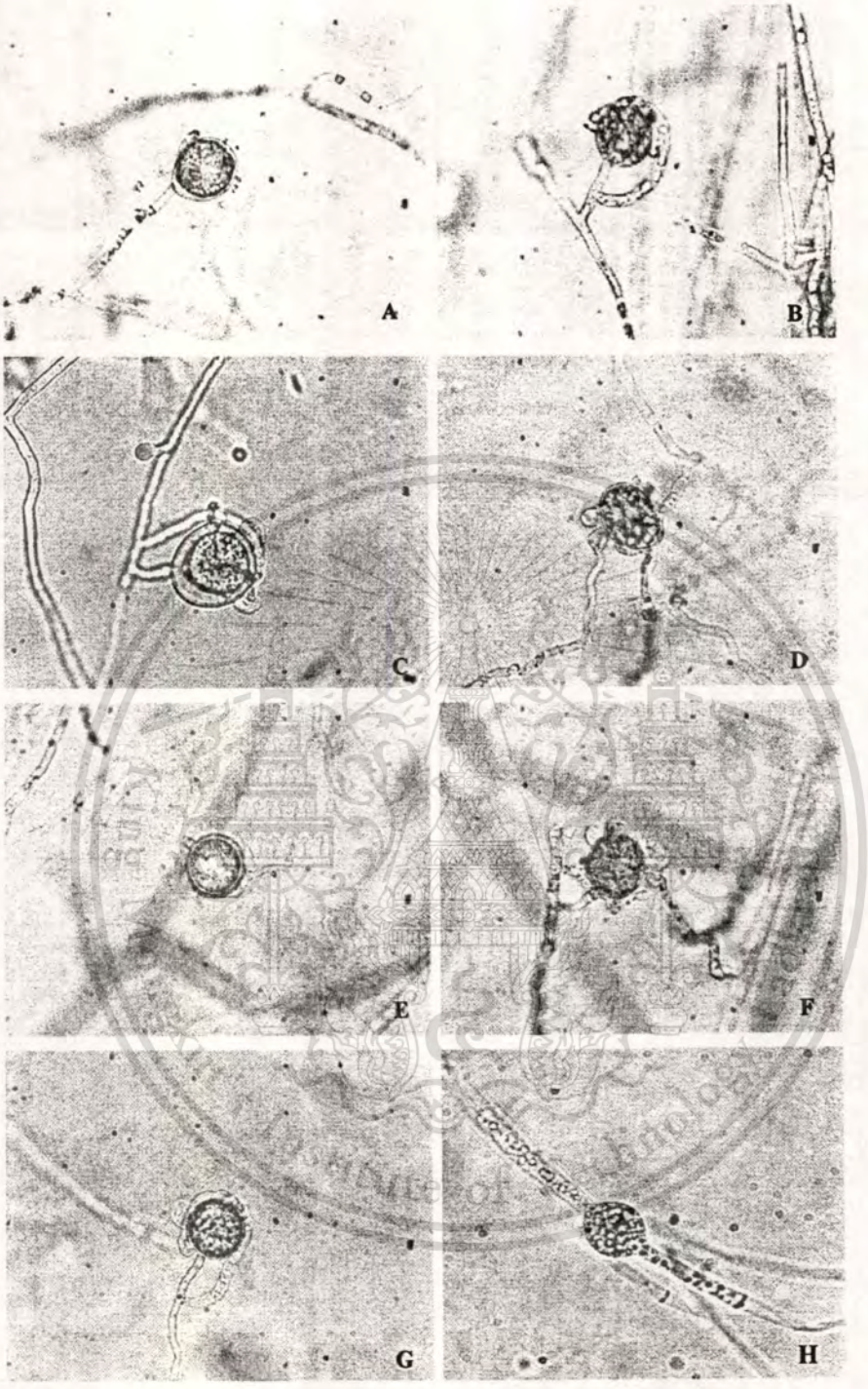

Fig. 2. Morphological and reproductive structures of *Pythium* sp. A-G oogonium and antheridium wrapping around the oogonium, H hyphal swelling. Bar= 30 µm

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1   GGATGCGGAAGGATCATTACCACAC
26  CATAAAACTTTCCACGTGAACCGTT
51  ACAATTATGTTCTGTGCTGTCTCTCG
77  GGATGGCTGAACGAAGGTGGGCTGC
102ATGTAT----GTGTAG--TCTGCCGATG
124TACTTTTCAAACCCATT-ACTAAATA
149CTGAACTATACTCCGAGAACGAAA
173GTTTTTGGTTTTAATCCATAACAAC
198TTTCAGCAGTGGATGTCTAGGCTCG
223CACATCGATGAAGAACGCTGCGAA
247CTGCGATACGTAATGCGAATTGCA
271GAATTCAGTGAGTCATCGAAATTT
295TGAACGCACATTGCACTTTCGGGA
320TATTCCTGGAAGTATGCTTGTATCA
345GTGTCCGTACATCAAACTTGCCTTTC
371TTTTTTTTGTGTAGTCAAGGAGAGAA
396ATGGCAGAATGTGAGGTGTCTCGCT
420GGCTCCCTCTTCGGAGGAGAAGACG
446CGAGTCCCTTTAAATGTACGTTCGC
473TCTTTCTTGTGTCGAAGTAGAAGTG
498TGACTATCGAACGCAGTGGTCTGTT
522TGGATCGTTTTGCGCGAGTTGGCGA
547CTTCGGTTAGGACATTAAAGGAAGC
575AACCTCTATTGGCGGTATGTTAGGC
600TTCGGCCCGACTTTGCAGCTGACAG
625TGTGTAGTTTTCTGTTCTTTCCTTGA
651GGTGTACCTGT-TTGTGTGAGGCAAT
676GGTCTAGGCAAATGGTTATTGTGTA
701GTAGGTGGTTGCTGCTCTTTGGCGCC
727C-----TCTCG-AG---GGTAAAGGAGGC
745AACACCAATTTGGGATTAGTCTGTG
770GA----------TTTATTC-----ATGGGCGCT
788TTTCAATTTGGACCTGATATCAAGT
813AA
```

Fig. 3. ITS region of the nuclear ribosomal DNA of *Pythium* sp.

ACKNOWLEDGMENTS

The author wish to acknowledge Prof.Dr.Fu-Cheng Lin, Dr. Hong-Kai Wang and Assoc. Prof.Dr. Kasem Soytong for useful advices and for supporting this useful research during six months and express my sincerely thanks to the members in the College of Agriculture and Biotechnology Institute, Zhejiang University, Hangzhou, P.R.China.

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Proceedings of The International Conference on Integration of Science & Technology for Sustainable Development (ICIST)

"Biological Diversity, Food and Agricultural Technology"

26 - 27 April, 2007

Held at KMITL, Bangkok, Thailand.

International Conference on Integration of Science & Technology
for Sustainable Development, Bangkok, Thailand. 26 – 27 April 2007, 129-133.

Morphology and molecular phylogeny of *Pythium sylvaticum* isolated from pineapple roots

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ABSTRACT

Pythium sylvaticum was isolated from soil samples taken in the rhizosphere of pineapple. This species is characterized by its smooth-walled, spherical sporangia and usually formed sexual structures. The antheridial branches wrap around the oogonium and soon disappear after fertilization. Random amplified polymorphic DNA (RAPD) revealed genetic dissimilarity among isolates of *P. sylvaticum*.

Keywords

P. sylvaticum, oogonium, RAPD, phylogeny

1. INTRODUCTION

The genus *Pythium* belongs to Oomycetes which was discovered by Pringsheim in 1858. These fungi are phenotypically true fungi having coenocytic branched mycelia. This genus is often known as phytopathogen causing root rot and damping-off disease. Most of these fungi can live as saprophytes, while others live as facultative saprophytes, becoming plant pathogen from time to time and causing extensive damage to economically important crops worldwide[1]. Plant diseases caused by oomycetes are often encountered under water-logged or wet soil conditions as found in littoral ecosystems. Species were usually identified by the study of their morphological and reproductive characters. Morphological characteristics and the taxonomy of the genus *Pythium* have described by Middleton[2], Waterhouse[3] and Plaats-Niterink[4]. *P. sylvaticum* was isolated and identified from soil and root samples taken in a pineapple plantation. The morphological and reproductive details of this fungus, together with their phylogeny is presented in this paper.

2. MATERIALS AND METHODS

2.1 Source of Isolates

All of the isolates were obtained from soil collected from the rhizosphere of pineapple showing symptom of root rot and brought to the laboratory at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand for isolation to pure culture.

2.2 Fungal isolation

The soil samples were mixed with sterile water (1/4 w/v) and baited with leaflets of pineapples (1x1 cm) floated on the surface of the water. After 2-3 days incubation at room temperature, discolured baits were blotted on sterile paper towel to remove excess water and plated onto selective medium modified from Tsao (1983)[5] which contains Potato dextrose agar (PDA) amended with 25 mg l^{-1}benomyl, 10 mgl^{-1} rifampicin, 200 mg l^{-1} ampicillin, 0.05 ml l^{-1} nystatin. Colonies growing from the baits were transferred to PDA, corn meal agar (CMA) and Potato-Carrot agar (PCA) for studying their morphology. Cultures were stored on PDA slant with sterile parafin oil.

2.3 Morphological characterization

The morphological characters were examined dimension as follows: oogonia and antheridia. Isolates were pre-grown on PDA plates at room temperature, then agar disks were taken from actively

growing colony margins and inoculated onto PCA and CMA plates. Cultures were incubated for 3 to 4 d at room temperature for morphological observation. At least 30 structures were examined for each isolate for all the characters studied. The identification was made according to the keys of Van der Plaats-Niterink[4]

2.4 DNA extraction

The isolates were grown in 50 ml test tubes containing 20 ml PDB (Potato dextrose broth) at 28°C on an arbital shaker (180 rpm) for 3-10 d. The mycelium was harvested by filtration. Excess water was removed from mycelium by pressing in a paper towel. A mycelial mat was placed in a prechilled mortar, frozen with liquid nitrogen, and ground to fine powder. Mycelial powder was suspended in 600 µl CTAB buffer (cetyltri-methyl-ammonium bromide), vortex and incubated at 65°C for 30 min, and 600 µl CIA (chloroform: isoamyl alcohol, 24:1 (v/v)) was added. The solution was incubated for 25 min on a shaking platform and centrifuged at 7,000 rpm for 5 min at 4°C. The aqueous phase (top) was transferred to a new microcentrifuge tube and repeat CIA extraction. After the second CIA was washed 300 µl isopropanal was added and mixed by inverting the tube several times. The tube was stored at room temperature, and then was centrifuged at 10,000 rpm for 10 min, the supernatant was decanted and drained on a paper towel for 30 min, the pellet was resuspended with 50 ml sterile double distill water.

2.5 RAPD analysis

PCR was carried out in 25 µl, of reaction mix containing 2.5 µl of 10 x PCR buffer, 2.5 µl $MgCl_2$, 0.5 dNTP, 0.4 µl *Taq* polymerase, 2 µl primer and 1 µl template DNA. The thermocycler was programmed for one cycle of 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 35°C,and 1 min at 72°C,and one cycle of 10 min at 72°C. The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide (0.5 µg/ml). and photographed under UV lighting. After preliminary of 3 isolates with 112 arbitrary 10-base oligonucleotide primers, 8 primers were selected and used to evaluate all 32 *P. sylvaticum*.isolates. Comparisons of each banding profile for each primer were conducted on the basic of presence or absence (1/0) of RAPD products of the same size. Matrixes were generated and analyzed using NTSYSpc version 2.2, SAHN and UPGMA clustering method to produce a similarity dendrogram.

Fig 1 A pineapple showing symptom of root rot, B 3 d-old culture of *Pythiu sylvaticum* T14 on PDA.

3. RESULTS AND DISCUSSION

A total of 70 soil samples from the rhizosphere of pineapple showing symptom of root rot (Fig 1) were collected by random. Thirty-two isolates were *P. sylvaticum*. Its presence within the rhizospere of pineapple roots indicates its parasitic and perhaps saprophytic activity towards this plant. Colonies on PCA and CMA are submerged, on PDA showed a rosette pattern (Fig 1) Main hyphae up to 11 µm wide. Hyphal swellings frequent. Oogonia,18-21 µm diam abundantly produced in single culture,globose,smooth-walled, and borne terminally or intercalary. Antheridia usually monoclinous, often also diclinous, 1-4 per oogonium, always wrapping around the oogonium (Fig 2). Morphologically these isolates come close to species like *P rhizosaccharum*. However, it is different from *P rhizosaccharum* in its oogonia having two oospores[6] *P. sylvaticum* is the first known

heterothallic species of *Pythium*[7]. Fresh isolates usually show no sexual organs in single culture, but after long maintenance oogonia can be produced in single cultures[4]. RAPD patterns of eight primers (Table 1) were demonstrated differences between the isolates (Fig 3, 4)

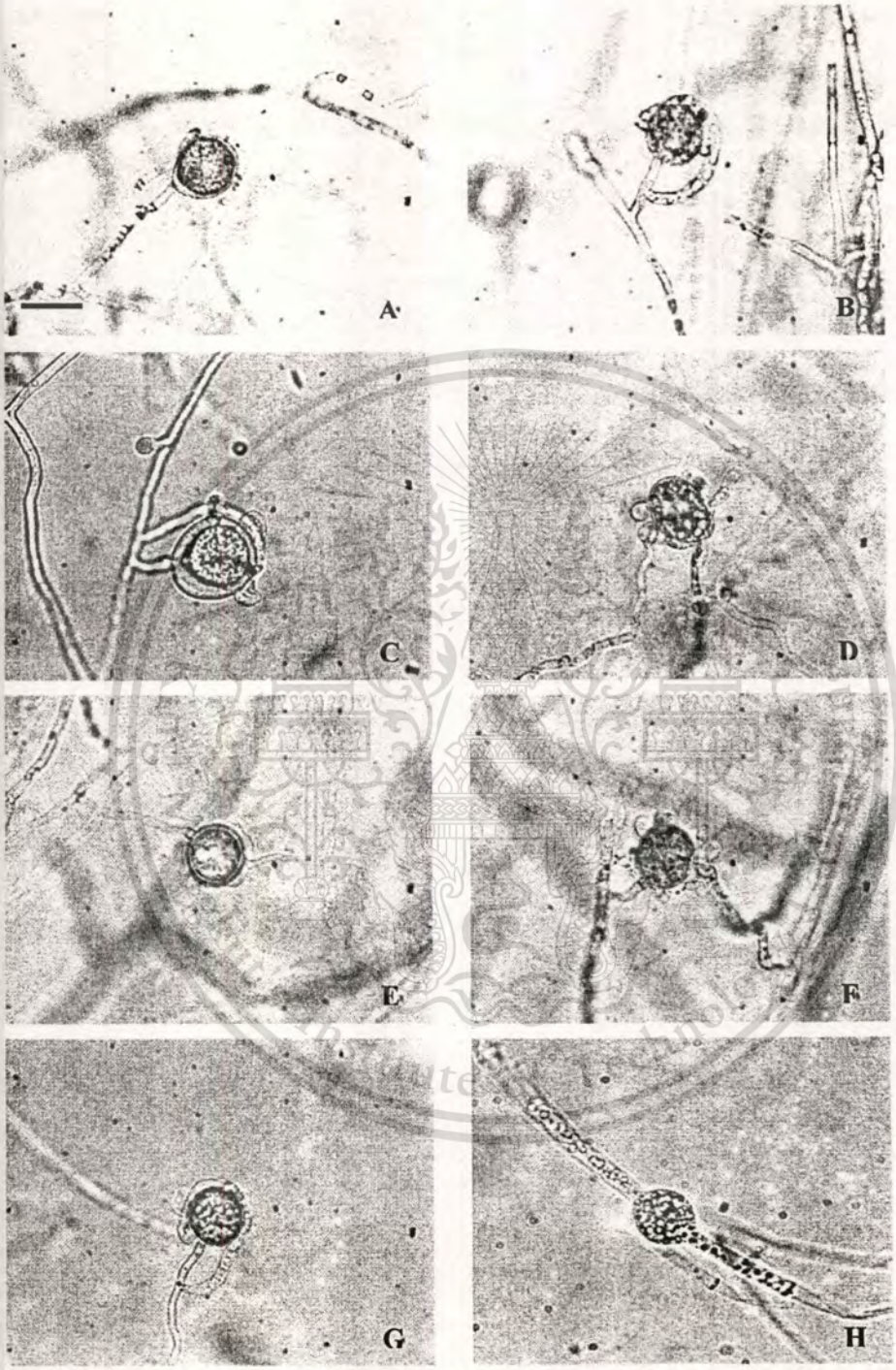

Fig 2 Morphological and reproductive structures of *Pythium sylvaticum* a pineapple showing root rot, A-G oogonium and antheridium wrapping around the oogonium,H hyphal swelling. Bar= 20 μm

Table 1 primer sequences producing clear and reproducible bands of *Pythium sylvaticum* in the RAPD analysis.

Primer code	Base sequence from 5' to 3'
S1224	GTCTTGGGCA
S1170	TGGGTGATCC
S1168	ACCCCCACAC
S1286	CCCGAGATCC
S1398	TGGTCCAGCC
S1372	GATGGGCCTG
S1028	AAGCCCCCCA
S2102	GACACACTCC

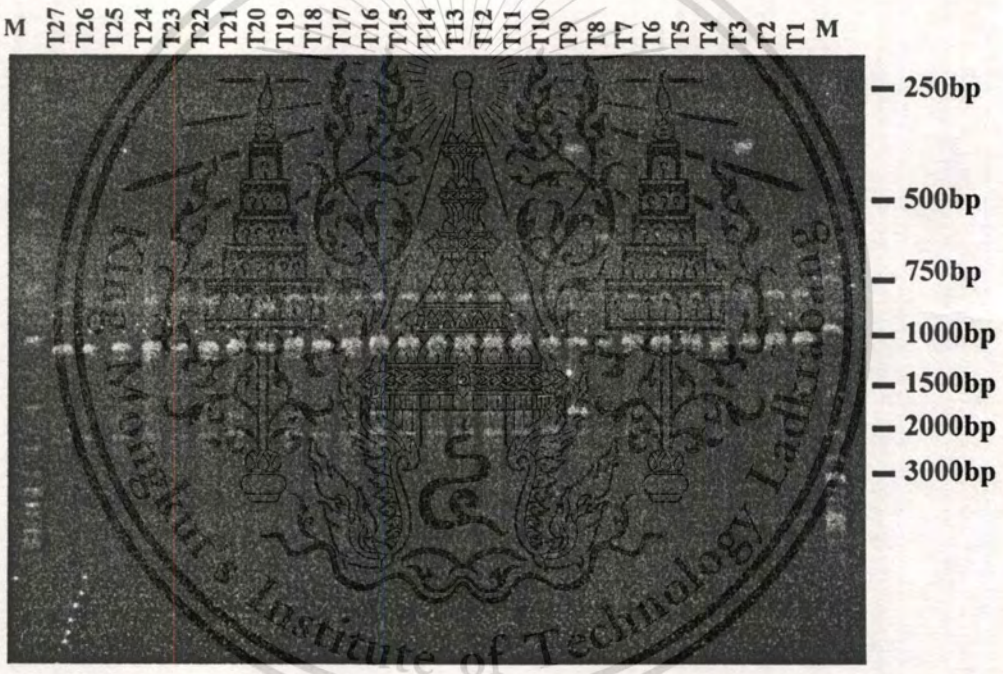

Fig 3 RAPD banding patterns of 27 *Pythium* isolates obtained by amplification of primer s1398. Markers (1kb DNA Ladder) appear on both sides

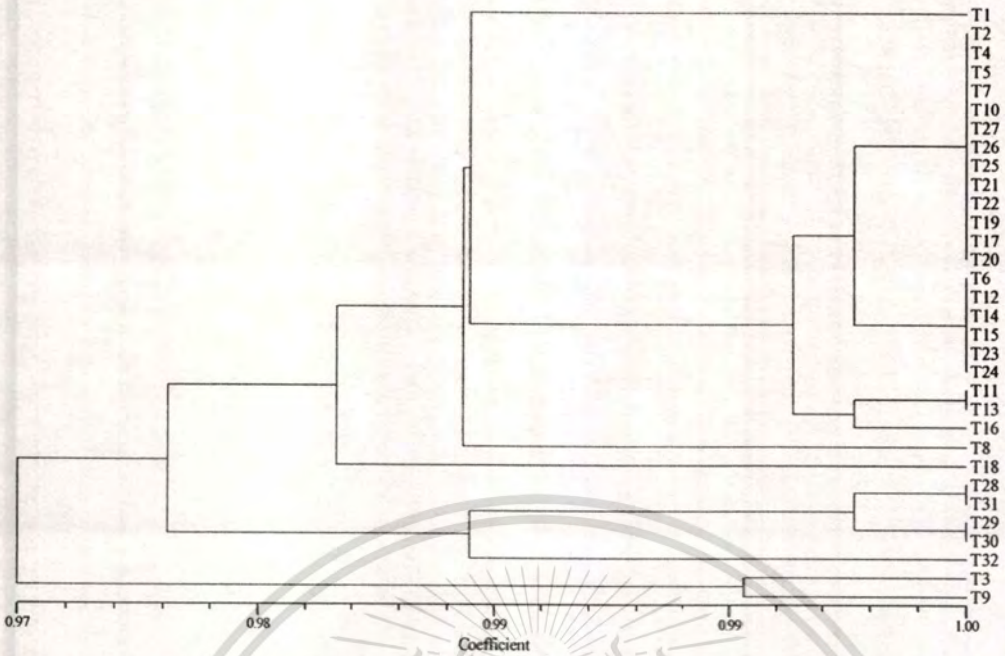

Fig 4 Dendrogram of 32 *Pythium sylvaticum* isolates based on dissimilarity from RAPD and generated by UPGMA cluster analysis.

4. ACKNOWLEDGMENTS

The author wish to acknowledge Prof.Dr.Fu-Cheng Lin, Dr. Hong-Kai Wang and Assoc. Prof.Dr. Kasem Soytong for useful advices and for supporting this useful research during six months and express my sincerely thanks to the members in the College of Agriculture and Biotechnology Institute, Zhejiang University, Hangzhou, P.R.China.

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The 2nd Annual Meeting of Thai Mycological Association and
Mycology Conference in Thailand. 23 June 2007, p. 8.

Pythium spp. Isolated from Rhizosphere of Pineapple Rot: Morphology and ITS Region of rDNA

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***Abstract*:** *Pythium* sp. was isolated from soil samples taken from a pineapple plantation in Phattalung province, Thailand during December 2005-March 2006. This fungus is characterized by its smooth-walled, spherical sporangia and usually formed sexual structures. The antheridial 1-5 per oogonium, stalks usually monoclinous, often also diclinous are presented. The ITS region of the ribosomal nuclear DNA was amplified and sequenced for further identify to confirm the species.

Key words: *Pythium*, Antheridia, Oogonia, ITS region, Ribosomal DNA

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Journal of Agricultural Technology

First report of pineapple root rot caused by *Pythium graminicola* in Thailand

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Pornsuriya, C., Wang, H.K., Lin, F.C. and Soytong, K. (2008). First report of pineapple root rot caused by *Pythium graminicola*. Journal of Agricultural Technology 4(1): 139-150.

Isolates of *Pythium graminicola* Subramaniam were isolated from soil samples taken from the rhizosphere of pineapple cv. Pattavia (*Ananas comosus* Merr) showing symptom of root rot from pineapple plantations in Thailand. They were characterized by filamentous, inflated sporangia and plerotic oospores with usually 1-7 antheridia. The ITS region of the ribosomal nuclear DNA was amplified and sequenced for identification and building a phylogenetic tree to confirm the species. *P. graminicola* isolate T25 was proved to be pathogenic isolate of pineapple root rot. This is the first report of *P. graminicola* causing pineapple root rot in Thailand.

Key words: *Pythium graminicola*, pineapple root rot, ITS region, Phylogenetic tree, Pathogenicity

Introduction

Pineapple (*Ananas comosus* Merr.) is one of the most important economic plants in Thailand. It is grown mainly for fresh, canned fruits and juice, and is the only source of bromelain, an enzyme used in pharmaceuticals. Thailand is one of the ten leading exporters of processed pineapples. In 2006, Thailand exported fresh fruits and processed pineapple to Europe, America and Japan over 800 thousand tons, the export value was about 20 billion baht (National Food Institute. 2007). The growth area of pineapple in Thailand has been expanded because the increased worldwide demand for pineapple products has greatly stimulated plantings. Root rot symptoms are commonly found in the fields and become major losses in pineapple plantations. Root rot of pineapple in Thailand has been firstly reported by Leelasetthakul (1972) which caused by *Phytophthora parasitica* and also reported by other researchers

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(Kueprakone *et al.*,1977; Silayoy, 1987; Department of Agriculture, 2008). But in 2008, Department of Agriculture, Ministry of Agriculture and cooperatives, Thailand reported that pineapple root rot caused by *Pythium* spp. but it was not identified into species level. However, *Pythium graminicola* was reported to be pathogenic to pineapple root rot in Hawaii (van der Plaats-Niterink, 1981) and *Pythium arrhenomanas* has been reported for the most common species that infected to pineapple (Rohrbach and Apt, 1993).

The genus *Pythium* belongs to Oomycetes which was discovered by Pringsheim in 1858 (Martin, 1991). It is often known as a pathogen causing root rot and damping-off disease. The taxonomy of the genus *Pythium* is mainly based on the morphological descriptions and the keys provided by Middleton (1943), Waterhouse (1968) and van der Plaats-Niterink (1981). However, morphological observations are now being supplemented with molecular techniques such as PCR and sequencing. The internal transcribed region (ITS) of the ribosomal nuclear DNA and the nucleotide sequence of this region has become a useful tool in fungal taxonomy and it is currently used to identify different species of *Pythium* (Singh *et al.*, 2003; Paul, 2001; Nechwatal *et al.*, 2005).

The objective of this study was to identify and confirm *Pythium graminicola* causing root rot of pineapple cv. Pattavia based on morphology and molecular phylogeny.

Materials and Methods

Isolation and morphological study

Soil samples were collected from the rhizosphere of pineapple plants showing typical symptoms of root rot during the rainy season. The samples were kept in individual clean plastic bags, brought to the laboratory at Department of Plant Pest Management Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand for isolation to pure culture.

Isolation method was modified from Nechwatal *et al.* (2005). Soil samples were mixed with sterile water (1/4 w/v) and baited with leaflets of pineapples (1x1 cm) floated on the surface of the water. After 2-3 days incubation at room temperature, discoloured baits were blotted on sterile paper towel to remove excess water and plated onto water agar (WA). Two days later, hyphal tips growing from the baits were transferred to Potato dextrose agar (PDA) and isolated to pure culture. Cultures were stored on PDA slant with sterile paraffin oil for further identification and maintenance.

The assessment of growth rates for the isolates of *Pythium*.spp. were grown on PDA, V8A and Potato-Carrot agar (PCA) (van der Plaats-Niterink, 1981) in 90 mm Petri dishes, and incubated at room temperature (28-30°C). Hyphal growth was recorded every 24 hr for 3 days. Colony morphology was observed after incubation for 7 days at room temperature. Investigation on sporangial development was made on agar discs which cut from the edge of actively growing colony on PDA, and floated in sterile distilled water for 24 hr at room temperature. Oogonia, antheridia and oospore characteristics were determined after 3 days of incubation at room temperature on V8A and PCA. At least 30 mature oogonia/oospores were chosen at random for recording at 400 magnification with the light microscope. The morphological identification was based on the work of Waterhouse (1968) and van der Plaats-Niterink (1981).

Sequence analysis

Sequence analysis of the ITS regions of the rDNA repeats were performed to determine the phylogenetic relationship.

For DNA extraction, 33 isolates of *P. graminicola* were grown in 50 ml test tubes containing 20 ml PDB (Potato dextrose broth) at 28°C on an arbital shaker (180 rpm) for 3-10 days. The mycelium was harvested by filtration. Excess water was removed from mycelium by pressing in a paper towel. The DNA was extracted by following the protocols of Lee and Taylor (1990) with some modifications. A mycelial mat was placed in a prechilled mortar, frozen with liquid nitrogen, and ground to fine powder. Mycelial powder was suspended in 600 µl CTAB buffer (cetyltri-methyl-ammonium bromide), vortex and incubated at 65°C for 30 min, and 600 µl CIA (chloroform: isoamyl alcohol, 24:1 (v/v)) was added. The solution was incubated for 25 min on a shaking platform and centrifuged at 7,000 rpm for 5 min at 4°C. The aqueous phase (top) was transferred to a new microcentrifuge tube and repeat CIA extraction. After the second CIA was washed 300 µl isopropanal was added and mixed by inverting the tube several times. The tube was stored at room temperature, and then was centrifuged at 10,000 rpm for 10 min, the supernatant was decanted and drained on a paper towel for 30 min, the pellet was resuspended with 50 ml sterile ddH_2O.

ITS rDNA

Polymerase chain reaction (PCR) amplification of ITS1, 5.8S and ITS2 regions was performed with universal primers ITS1(TCC GTA GGT GAA

CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (White *et al.*, 1990). The reaction was carried out in 50 µl volumes containing 1 µl genomic DNA, 3 µl $MgCl_2$, 1 µl dNTPs, o.4 µl of each primer and 1 µl *Taq* DNA polymerase in 5 µl PCR buffer. Amplifications were done with the following temperature cycling parameters: denaturation at 94°C for 3 min for first cycle and 1 min each for subsequent cycles, annealing for 30 sec at 55°C, and elongation for 1 min at 72°C. To assess the efficiency of the amplification, 5 µl PCR products were electrophoresed in a 1% agarose gel in 1xTAE buffer. The remaining volumes of the PCR amplicons were purified for DNA sequencing. Purified template DNA was sequenced by Shanghai Sangon Biological Engineering Technology & Services Co.,Ltd (Shanghai, P.R China).

In order to determine the phylogenetic relationship, sequence analysis of the ITS regions of the rDNA repeats were performed and data compared to related species retreived from GenBank (Table 1.). Isolates of *Pythium volutum* were used as outgroup. All sequence editing and aligning were carried out using BioEdit, version 7.0.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data were analysed and neighbour-joining phylogenetic analyses conducted using ClustalW, version 1.83. Phylogenetic analysis was performed using PAUP* 4.0b8 (Swofford, 2001). Trees were drawn using Treeview.

***Pathogenicity* test**

All isolates of *P. graminicola* were tested for pathogenicity by Koch's Postulate on detached leaves and roots of pineapple cv. Pattavia.

Pathogenicity tests on detached leaves

Pathogenicity test was carried out as described by Soytong *et al.* (2005) with some modifications. Six-month-old pot-grown pineapple was used for the assessment of the pathogenicity. Each isolate, pineapple leaves of approximately the same age (the same position on the plant) were collected, clipped on base and apex (length *ca* 10 cm), and surface-sterilized by soaking in 10% ethanol for 3 min and then each pineapple leaf was wounded with needle-pricking method (0.5 cm in length and 1 min in depth from the surface). The wounded leaves were inoculated with a plug (5 mm diameter) of tested fungal isolate which taken from the margin of an actively growing colony on PDA. The control was treated with a plug of PDA. The inoculated leaves were kept in sealed plastic boxes containing moist paper towels at room temperature (28-30°C). The lesion diameter was recorded and confirmed by re-isolation after 5 days of incubation. The experiments were designed as completely

randomized design (CRD) with four replicates and data was subjected to statistical analysis, and the variance of lesion diameter was computed, then treatment means were compared using the Duncan's Multiple Range Test (DMRT) at P=0.01.

Table 1. *Pythium* sequences from GenBank used in this study.

Pythium species*	Host/substrate	Locality	ITS GenBank accession No.
P. aphanidermatum	-	-	AY598622
P. aristosporum	*Triticum aestivum*	Canada	AY598627
P. aristosporum	-	-	AB160843
P. aristosporum	-	-	AB095042
P. arrhenomanes	-	-	AF330183
P. arrhenomanes	-	-	AF330181
P. arrhenomanes	-	-	AF330182
P. arrhenomanes	-	-	AJ233444
P. arrhenomanes	*Zea mays*	USA	AY858635
P. arrhenomanes	-	-	AB095039
P. arrhenomanes	-	-	AF330180
P. arrhenomanes	-	-	AF330179
P. arrhenomanes	-	-	AJ233439
P. arrhenomanes	-	-	AF330174
P. arrhenomanes	-	-	AF330178
P. graminicola	-	-	AF330173
P. graminicola	*Saccharum officinarum*	Jamaica	AY598625
P. graminicola	-	-	AY243091
P. graminicola	-	-	AF330165
P. graminicola	-	-	AF330165
P. phragmitis	*Phragmites australis*	Germay	AY594259
P. torulosum	-	-	AB095046
P. torulosum	-	-	AB160846
P. torulosum	-	-	AF330194
P. vanterpoolii	*Triticum sativum*	UK	AY598685
P. vanterpoolii	-	-	AB095043
P. vanterpoolii	-	-	AJ233461
P. volutum	-	-	AJ233464
P. volutum	*Triticum* sp. and *Hordeum* sp.	Janpan	AY598686

* ITS sequences of *Pythium* species were retrieved from GenBank

Pathogenicity tests on pineapple roots

The pathogenicity test was done by using completely randomized design (CRD) with four replicates. The experiment was carried out using the young suckers of pineapple cv.Pattavia. All isolates of *P. graminicola* were grown on

PDA in 9-cm-diameter Petri dishes. The inoculum was prepared by the method of Shang *et al.* (1999) with modifications. Seven-to ten-day-old culture was flooded with 20 ml sterile distilled water for 48 hrs and a flame sterilized glass spreader was used to rub the colony surface to dislodge the sporangia into sterile distilled water. Sporangial suspension was incubated at 20°C for 1 hr to allow the sporangia to release their zoospores. Zoospores suspension was adjusted to $1x10^6$ zoospores/ml (Chern *et al.*, 1998). Pineapple suckers were stripped off the lower leaves and placed in 8 cm diameter of clear glass containing 100 ml distilled water. After 2 weeks, the plants were removed from plastic cup, and 10 roots end per plant were cut (3 mm) before inoculation. The root system of each tested plant was placed in sterile distilled water inoculated with 100 ml zoospore suspension ($1x10^6$ zoospores/ml). Control plant was placed with 100 ml sterile distilled water without inoculum. All tested plants were maintained indoor near a sunny window until root rot occurs approximately 1 week, and then removed from plastic cup. Disease severity index (DSI) was recorded by modified following scale of Ahmed *et al.* (1999) as follows: 1 = no root rot, 2= 1-25% root rot, 3=26-50% root rot, 4=51-75% root rot and 5= >76-100% root rot.

Results and Discussion

Isolation and morphological study

The 33 isolates of *P. graminicola* were isolated from rhizosphere soils of pineapple cv. Pattavia which causing root rot. All isolates were studied for their growth rate and morphology for identification.

With this, the growth rate and morphology of 10 selected isolates of *P. graminicola* were reported in this paper. They were recorded morphological characteristics by comparison to the work of van der Plaats-Niterink (1981) that is presented in Table 2. In general, colonies appeared like rosetted on PDA, radiated pattern on PCA and V8A. Appressoria commonly produced and sporangia are not observed on solid agar, but readily produced in water, showing toruliod sporangia (Fig. 1). Oogonia produced abundantly in single culture, strictly globose, smooth-walled, terminally and intercalary borne. Antheridia are usually monoclinous, often also diclinous, usually crook-necked, 1-7 per oogonia. Oospores are single, plerotic, completely filling the oogonia (Fig. 2).

Our research finding has been found that the pineapple root rot is caused by *P. graminicola* which van der Plaats-Niterink (1981) reported as the same species. But it is contradicted to previously reports of Leelasetthakul (1972), Kueprakone *et al.* (1977), Silayoy (1987) and Department of Agriculture (2008) who stated that pineapple root rot in Thailand caused by *P. parasitica*

Fig 1. Appressoria and sporangia of *Pythium graminicola*.
A = Appressoria, B and C = toruloid sporangia, Bar = 30 µm.

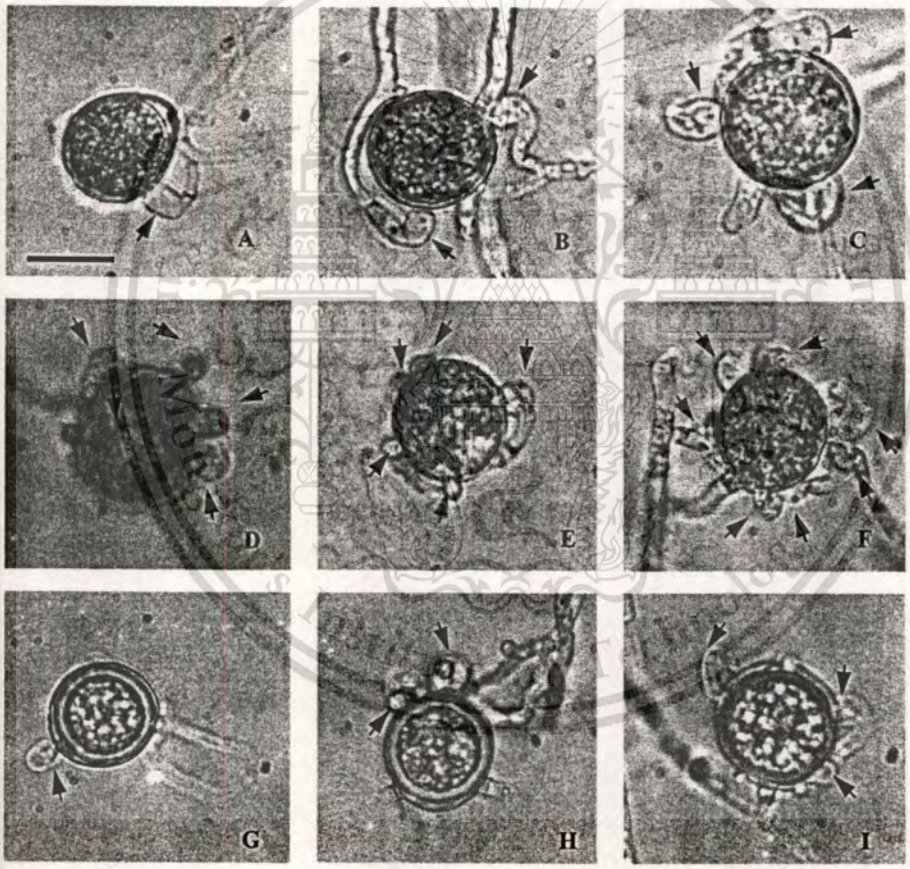

Fig. 2. Oogonia, oospores and antheridia (arrow heads) of *Pythium graminicola*.
A = Oogonia with single, monoclinous antheridia, B-F = Oogonia with two mono- or diclinous antheridia.
G-I = Oospores with one or more antheridium,. Bar=20 µm.

according to morphological studies. With this, the causing agents of pineapple root rot may possible caused by both species, *P. parasitica* and *P. graminicola*, which may depends on different in ecological diversity.

Sequence analysis

Thirty-three isolates of *Pythium* spp. were identified by morphological characters as *P. graminicola*. The isolates were studied to confirm morphological identification by using ITS sequences with the length of the complete ITS1, 5.8S and ITS2. The BLAST searches which indicated the species of *P. graminicola* that can be seen in Fig. 3. It is clearly demonstrated to identify and confirm to be *P. graminicola* as a valid identification.

Although, *P. graminicola* has been difficulty considered to separate from *P. arrhenomanas* in the past due to overlapping of morphological characters, but it could be clearly distinguished from this species by molecular evidence (Chen and Hoy, 1993). As our result, *P. graminicola* from the related species, *P. arrhenomanas*, *P. aristosporum*, *P. arrhenomanes*, *P. vanterpoolii* and *P. torulosum* which showing in Fig. 3. *Pythium* spp. isolate T3, T25 and T27 were grouped in the species of *P. graminicola*.

Therefore, morphological observation and molecular analysis should be used for identify different species of *Pythium*. This agrees with Singh *et al.*(2003), Paul (2001), Lévesque and De Cock, (2004) and Nechwatal *et al.*(2005) who have used morphology and molecular analysis for identify the different species of *Pythium*.

Pathogenicity test

Ten isolates of *P. graminicola* were proved to be pathogenic to pineapple root rot. Lesion lengths on leaves after 5 days inoculation of *P. graminicola* isolate T3, T5, T7, T9, T11, T12, T18, T25, T26 and T27 were 1.00, 0.79, 0.93, 0.98, 0.83, 1.02, 0.94, 1.40, 0.81 and 0.98, respectively. The Disease severity index on root test were 4, 2, 3, 3, 2, 4, 5, 2, 4 and 1, respectively. The isolate of *P. graminicola*, T25 was high significantly different at P=0.01 and lesion was larger than the other isolates on detached leaves. Moreover, the tested roots were faster infection in the inoculated plants. Therefore, *P. graminicola*, T25 was the most aggressive isolate both on detached leaf and root tests which disease severity index showed over 76-100% root rot (Table 3.and Fig. 4).

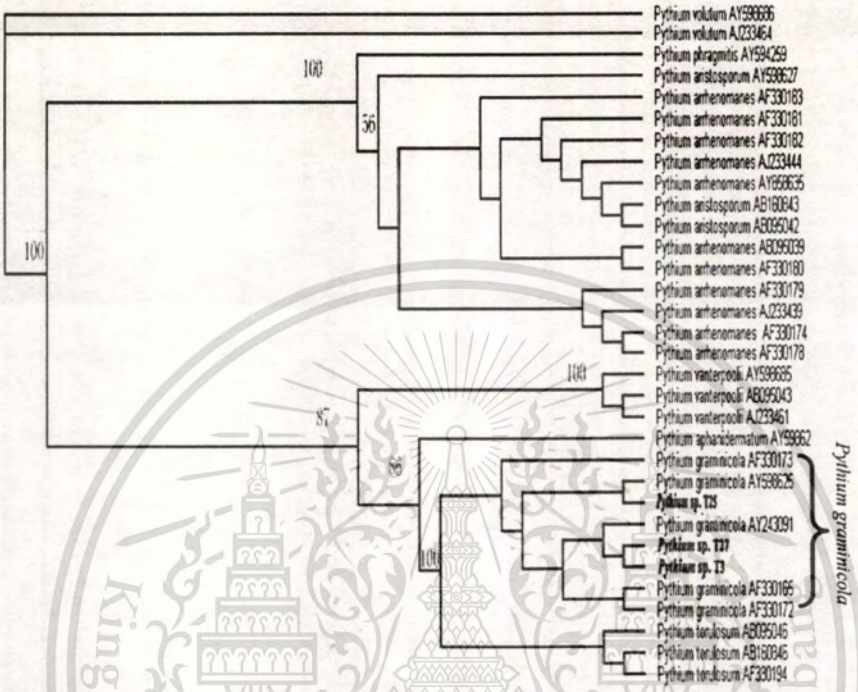

Fig. 3. Phylogenetic tree of *Pythium* species including the related species from GenBank that constructed after distance-based analysis of ITS1, 5.8S and ITS2 regions of rDNA. Numbers at the branches indicate the percentage of bootstrap values after 1,000 replications (values below 50% not shown). *Pythium volutum* was used as an outgroup.

Table 3. Virulence of *Pythium graminicola* isolates causing pineapple root rot.

Isolates	lesion length (cm)[1/]	disease severity index (DSI)[2/]
P. graminicola T3	1.00^{bc}	4
P. graminicola T5	0.79^{d}	2
P. graminicola T7	0.93^{c}	3
P. graminicola T9	0.98^{bc}	3
P. graminicola T11	0.83^{d}	2
P. graminicola T12	1.02^{b}	4
P. graminicola T18	0.94^{bc}	3
P. graminicola T25	1.40^{a}	5
P. graminicola T26	0.81^{d}	2
P. graminicola T27	0.98^{bc}	4
control	0^{e}	1

[1/] Average of four replications. Means of the lesion length of leaves followed by a common letter were not significantly different (P=0.01) by DMRT.

[2/] disease severity index of pineapple root rot, 1 = no root rot, 2= 1-25% root rot, 3=26-50% root rot, 4=51-75% root rot and 5= >76-100% root rot(modified from Ahmed *et al.*,1999).

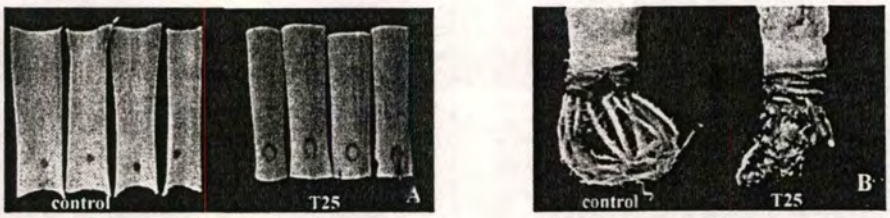

Fig. 4. Pathogenicity test on detached leaves and roots of *Pythium graminicola* T25.
A = The lesion on detached leaves after 5 days of inoculation.
B = Root rot after 1 week of inoculation.

Conclusion

P. graminicola was firstly proved to be pathogenic to pineapple cv. Pattavia causing root rot in Thailand. It was confirmed by morphology under microscopic observation and molecular phylogeny as a valid confirmation.

Acknowledgements

We would like to thank Mr. Tong-bao Liu and Mr. Boli Hu for his technical help and the part of molecular work has been supported by college of Agriculture and Biotechnology, Biotechnology Institute, Zhejiang University, Hangzhou, P.R. China.

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(Received 14 January 2008; accepted 12 May 2008)

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Abstract: Isolates of *Pythium graminicola* Subramaniam were isolated from soil samples taken from the rhizosphere of pineapple cv. Pattavia (*Ananas comosus* Merr) showing symptom of root rot from pineapple plantations in Thailand. They were characterized by filamentous, inflated sporangia and plerotic oospores with usually 1-7 antheridia. The ITS region of the ribosomal nuclear DNA was amplified and sequenced for identification and building a phylogenetic tree to confirm the species. *P. graminicola* isolate T25 was proved to be pathogenic isolate of pineapple root rot. This is the first report of *P. graminicola* causing pineapple root rot in Thailand.

Key words: *Pythium graminicola*, pineapple root rot, ITS region, Phylogenetic tree, Pathogenicity

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New record of *Chaetomium* species isolated from soil under pineapple plantation in Thailand

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Pornsuriya, C., Lin, F.C., Kanokmedhakul, S. and Soytong, K. (2008). New record of *Chaetomium* species isolated from soil under pineapple plantation in Thailand. Journal of Agricultural Technology 4(2): 91-103.

Chaetomium species were isolated from soil in pineapple plantations in Phatthalung and Rayong provinces by soil plate and baiting techniques. Taxonomic study was based on available dichotomously keys and monograph of the genus. Five species are recorded as follows: *C. aureum*, *C. bostrychodes*, *C. cochliodes*, *C. cupreum* and *C. gracile*. Another four species are reported to be new records in Thailand as follows: *C. carinthiacum*, *C. flavigenum*, *C. perlucidum* and *C. succineum*.

Key words: *Chaetomium*, Taxonomic study

Introduction

Chaetomium is a fungus belonging to Ascomycota of the family Chaetomiaceae which established by Kunze in 1817 (von Arx *et al.*, 1986). *Chaetomium* Kunze is one of the largest genera of saprophytic ascomycetes which comprise more than 300 species worldwide (von Arx *et al.*, 1986; Soytong and Quimio, 1989; Decock and Hennebert, 1997; Udagawa *et al.*, 1997; Rodríguez *et al.*, 2002). Approximately 20 species have been recorded in Thailand (Table 1). *Chaetomium* species are well known as coprophilous, seed and soil fungi (Somrithipol, 2004; Somrithipol *et al.*, 2004), and also found in organic compost (Soytong, 1990). They degrade cellulose and other organic material and act as antagonist against plant fungal pathogens (Soytong, 2001). *C. globosum* is reported by several researchers to be a strong cellulose decomposer

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(Umikalsom, *et al.*, 1997; Umikalsom, *et al.*, 1998) and expressed a very effective antagonist of various soil microorganisms (Aggarwal, *et al.*, 2004; Dhingra, *et al.*, 2003; Soytong *et al.*, 2001). In Thailand *Chaetomiun* species were screened for using as antagonist in 1989 (Soytong *et al.*, 2001). It has also been reported that some isolates of *C. globosum* produce antibiotics that can suppress damping-off of sugar beet caused by *Pythium ultimum* (Di-Pietro *et al.*, 1991). *C. cupreum* and *C. globosum* have been reported to reduce leaf spot disease of corn caused by *Curvularia lunata*, rice blast caused by *Pyricularia oryzae*, sheath blight of rice caused by *Rhizoctonia oryzae* and tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* (Soytong, 1992a, 1992b).

Moreover, *Chaetomium* species are noted for their secondary metabolite content with biological activities. Several types of compounds have been investigated from *Chaetomium* spp. e.g. benzoquinone derivatives (Brewer *et al.*, 1986), a new anthraquinone-chromanone compound named chaetomanone and seven known compounds, ergosterol, ergosteryl palmitate, chrysophanol, chaetoglobosin C, alternariol monomethyl ether, echinuline and isochaetoglobosin D were found from *C. globosum* KMITL-N0802 and also reported that chaetomanone and echinulin showed activity towards *Mycobacterium tuberculosis* (Kanokmedhakul *et al.*, 2001). Three new azaphilones named rotiorinols A-C, two new stereoisomers, (-)-rotiorin and *epi*-isochromophilone II and a known compound, rubrorotiorin, were isolated from the fungus *C. cupreum* CC3003 of which compounds, rotiorinols A, rotiorinols C, (-)-rotiorin and rubrorotiorin act as antifungal activity against *Candida albicans* (Kanokmedhakul *et al.*, 2006). Four new dimeric spiro-azaplilones, cochliodones A-D, two new azaphiliones, chaetoviridines E and F, a new epi-chaetoviridin A, and known compounds, chaetoviridin A, ergosterol, chaetochalasin A were isolated from *C. cochliodes* VTh 01 and *C. cochliodes* CTh 05. Chaetoviridines E and chaetochalasin A exhibited antimalarial activity against *Plasmodium falcipulum* while cochliodones C, chaetoviridines E and F, chaetochalasin A expressed antimycobacterial activity against *M. tuberculosis*. Furthermore, *C. cochliodes* VTh 01 and *C. cochliodes* CTh 05 were reported to be antagonistic to *Fusarium oxysporum* f sp *lycopersici* causing tomato wilt (Phonkerd *et al.*, 2008), Chaetominedione is reported as a new tyrosine kinase inhibitor isolated from the algicolous marine fungus *Chaetomium* sp.(Abdel-Lateff, 2008) etc.

Chaetomium species are traditionally identified by morphological data, the type of terminal hair and lateral hairs or ascomatal hairs (straight, hooked, spiral, coiled etc.) covering the ascomata, the shape and size of asci and ascospores according to von Arx *et al.* (1986) and Seth (1970).

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The objective of this research was to investigate the species of *Chaetomium* isolated from soil in pineapple plantations from Phatthalung and Rayong provinces, Thailand.

Table 1. List of *Chaetomium* species in Thailand.

Species	Reference
C. ampullare Chivers	Soytong, 1991
C. apiculatum Lodha	Udagawa, 1973
C. aureum Chivers	Soytong, 1991; Petcharat and Soytong, 1991
C. bostrychodes zopf	Soytong, 1991
C. cocholiodes Palliser	Soytong, 1991
C. cupreum Ames	Soytong, 1991; Petcharat and Soytong, 1991; Somrithipol, 2004
C. deceptivum Malloch & Benny	Soytong, 1991
C. floriforme Gené & Guarro	Gené and Guarro, 1996
C. fusiforme Chivers	Petcharat and Soytong, 1991
C. globosum Kunze	Soytong, 1991; Petcharat and Soytong, 1991; Somrithipol, 2004; Somrithipol *et al.*, 2004
C. gracile Udagawa	Petcharat and Soytong, 1991
C. hamadae (Udagawa) v. Arx	Soytong, 1991
C. homopilatum Omvik	Soytong, 1991
C. indicum Corda	Somrithipol *et al.*, 2004
C. longicolleum Krezm. & Badura	Soytong, 1991
C. lucknowense Rai & Tewari	Soytong, 1991; Petcharat and Soytong, 1991
C. malaysiense v. Arx	Soytong, 1991
C. megasporum Sorgel	Soytong, 1991
C. seminudum Ames	Soytong, 1991
C. thermophilum La Touche	Somrithipol, 2004
C. tortile Bainier	Somrithipol *et al.*, 2004
C. variosporum Udagawa et Horie	Udagawa, 1973
C. venezuelense Ames	Udagawa, 1973
C. vitellinum Carter	Soytong, 1991

Materials and methods

Source of isolates

Soil samples for the recovery of *Chaetomium* spp. were collected from pineapple plantations in Phatthalung and Rayong provinces, Thailand, during August to November 2007. Soil samples were kept in clean plastic bags, brought to the laboratory at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Isolation and identification

Chaetomium species were originally isolated by soil plate technique and baiting technique according to the method described by Soytong (1989).

Soil plate technique, soil samples were dried and ground to fine particles; 0.005-0.015 g of each soil sample were placed to sterilized Petri dishes and then overlaid with glucose-ammonium nitrate agar (GANA) medium (10 g glucose, 1 g NH_4NO_3, 1 g Difco bacto yeast extract, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 20 g agar, 0.06 g rose bengal, 0.03 g streptomycin, 1,000 ml distilled water). After 2-7 d incubation at room temperature in the dark, *Chaetomium* spp. were observed under stereo microscope and isolated into pure culture by single spore isolation.

Baiting technique, each soil sample (*ca* 10 g) were placed to sterilized Petri dishes and moistened with sterile distilled water before baited with small pieces of sterilized straws, filter paper, tissue paper and pineapple leaves. After 21 d incubation at room temperature, *Chaetomium* spp. on baits were daily observed and picked their ascomata to glass slide with small amount of sterilized water before spread on water agar (WA) in a 9-cm-diameter Petri dish. The WA plates were incubated for 12 h at room temperature, then single colony was transferred onto PDA plates and isolated into pure culture. All isolates were kept in culture collection, Herbarium of Thai Mycological Association (H-TMA) at King Mongkut's Institute of Technology Ladkrabang, Thailand.

Results and discussion

Isolation and identification

Thirty isolates of *Chaetomium* were obtained in pure culture and identified into 9 species as presented in Table 1 and Fig.1-9. *C. cupreum* was the most common species which was found in soil from pineapple plantations taken from both Phatthalung and Rayong provinces. The most isolates were obtained by baiting technique, except *C. cupreum* S1 which was found by soil plate technique.

Identification of *Chaetomium* species are usually considered morphological characters (Arx *et al.*, 1986; Seth, 1970; Soytong and Quimio, 1989; Gené and Guarro, 1996; Rodríguez *et al.*, 2002) and molecular methods were used in the taxonomy of *Chaetomium* by Lee and Hanlin (1999). In the GenBank database (2008) sequences of 29 identified and of 44 unidentified *Chaetomium* species are now deposited. It is needed to do more identification

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work both morphological and molecular data to confirm species in the near future.

Table 2. *Chaetomium* species isolated from soil in pineapple plantation at different locations in Thailand.

Methods	Species	Isolates	
		Phatthalung	Rayong
soil plate	*C. cupeum*	S1	-
	C. aureum	MB601, MB608, MB603, MB103	RY102
	C. bostrychodes	PR1, PR2, PR3, NB701	-
	C. carinthiacum	NB501	-
	C. cochliodes	-	RY301
baiting	*C. cupreum*	NB201, MB303, MB301, V4B1,	RY201, RY202, RY203, RY204
	C. flavigenum	MB607, MB402, MB606, MB611, MB604	-
	C. gracile	NB401, MB605	-
	C. perlucidum	NB202, NB501	-
	C. succineum	MB305, NB304	-

Five species are recorded as follows:-

Chaetomium aureum Chivers. Proc.Am.Arts Sci. 48: 87 (1942).

Young colonies usually are white by aerial mycelium. Mature colonies become red by a red pigment exudate. Ascomata are pale green, ovate in shape, 78.5-142.6 x 90.6-180.3 µm. Ascomatal hairs arcuate, septate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are elliptical, 5.0-7.7 x 8.5-12.5 µm, with two apical germ pores (Fig.1).
Isolate examined: MB607.

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

Colonies are rapidly growing, young colonies usually are white by aerial mycelium, occasionally with a purple pigment exudate. Mature colonies become green to brown with ascomata. Ascomata are olivaceous, maturing within 10-14 days, dark green to brown when old, ovate in shape, 190.2-349.8 x 272-419.8 µm. Ascomatal hairs usually spirally coiled. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are lemoniform, 7.5-9.9 x 8.6-11.3 µm, with an apical germ pore (Fig.2).
Isolate examined: PR1.

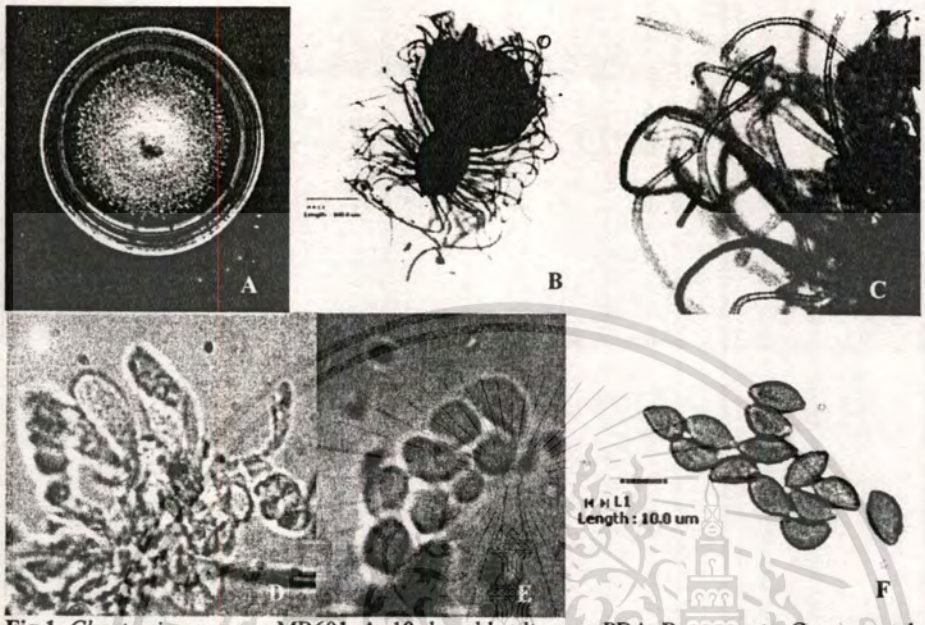

Fig.1. *Chaetomium aureum* MB601. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores.

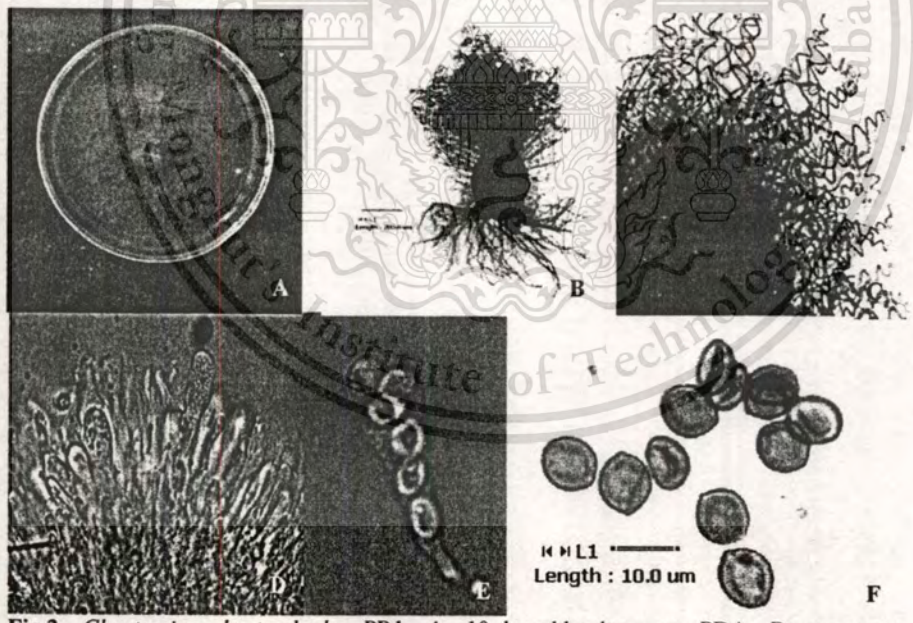

Fig.2. *Chaetomium bostrychodes* PR1. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores.

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

Colonies are rapidly growing, young colonies usually are white by aerial mycelium, occasionally with a purple pigment exudate. Mature colonies become green to brown with ascomata. Ascomata are olivaceous, maturing within 10-14 days, dark green to brown when old, ovate in shape, 107.1-143.1x122.0-209.2 µm. Ascomatal hairs usually irregularly sinuous. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are broadly ovate to lemon-shaped, 4.2-5.9x7.1-10.0 µm, with an apical germ pore (Fig.3).
Isolate examined: RY301.

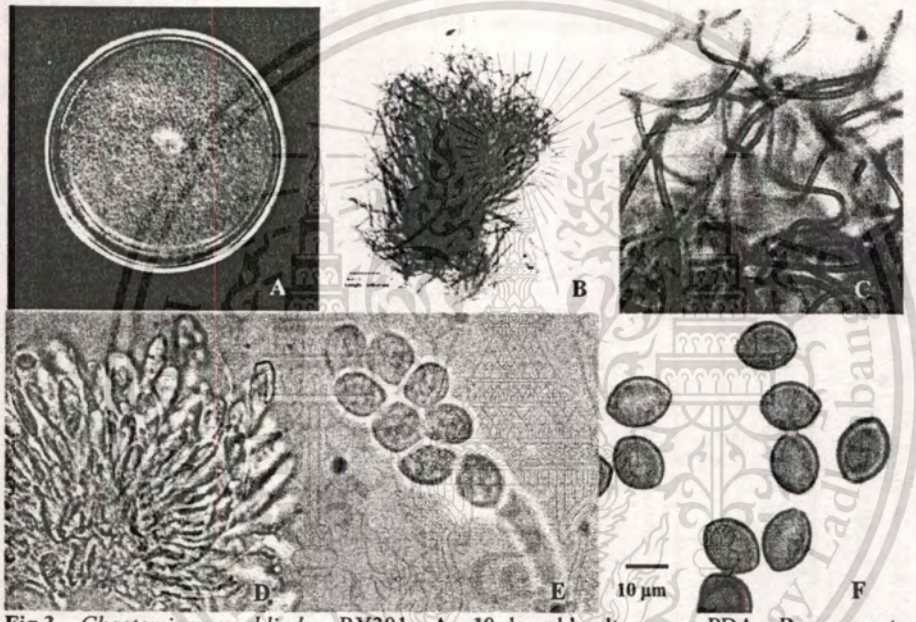

Fig.3. *Chaetomium cochliodes* RY301. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores.

Chaetomium cupreum Ames. Mycologia 41(6): 642 (1950)

Colonies usually are red due to a red pigment exudate. Ascomata are red, maturing within 10-14 days, ovate in shape, 79.7-142.7 x 94.7-151.5 µm. Ascomatal hairs arcuate, apically circinate or coiled, septate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are reniform, 4.7-6.7 x 6.7-10.0 µm, with a single apical germ pore (Fig.4).
Isolate examined: RY202

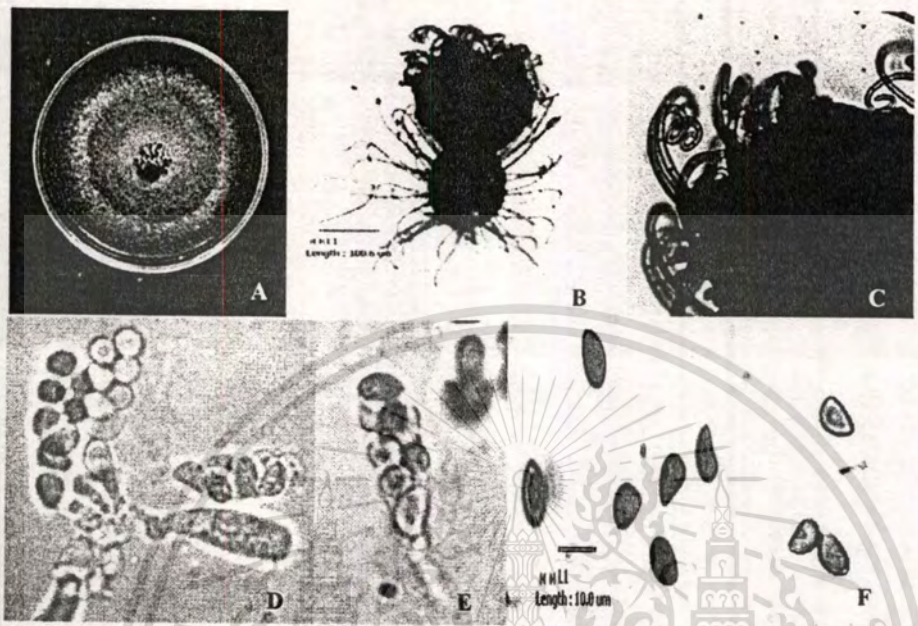

Fig.4. *Chaetomium cupreum* RY202. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores.

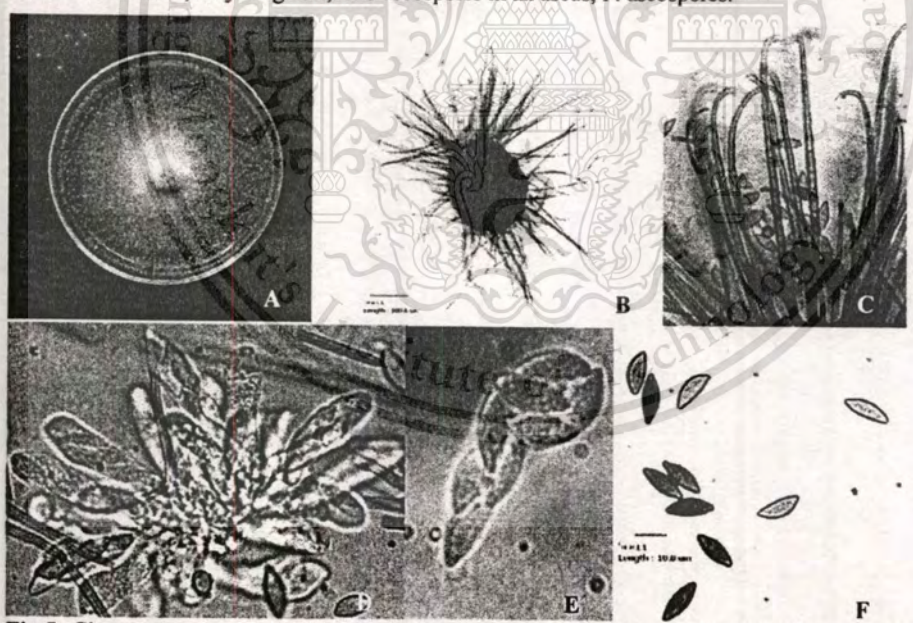

Fig.5. *Chaetomium gracile* NB401. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores.

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Chaetomium gracile Udagawa. J.gen.appl.Microbiol. 6: 235 (1960).

Colonies usually yellow due to yellow pigment exudates. Ascomata are olivaceous grey, maturing within 10-14 days, ovate in shape, 75.4-161.4x110.2-202.8 µm. Ascomatal hairs arcuate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are fusiform, 5.1-6.9x12.7-17.7 µm, with two apical germ pores (Fig.5).
Isolate examined: NB401.

Four species are reported to be new records in Thailand as follows:-

Chaetomium carinthiacum Sőrgel. Arch. Mikrobiol 40: 393 (1961).

Colonies usually are white by aerial mycelium, without a pigment exudate. Mature colonies become green to brown with ascomata. Ascomata are olivaceous, maturing within 10-14 days, dark green when old, ovate in shape, 96.1-146.4x101.8-153.2 µm. Ascomatal hairs irregularly sinuous with roughened hairs. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are elliptical, 4.3-6.2x8.4-11.6 µm, with an apical germ pore (Fig.6).
Isolate examined: NB501.

Chaetomium flavigenum van Warmelo. Mycologia. 58: 847 (1966).

Colonies usually are white by aerial mycelium, becoming red or orange due to a red pigment exudate. Mature colonies become green to brown with ascomata. Ascomata are olivaceous to brown, maturing within 10-14 days, dark grey-green when old, ovate in shape, 92.5-134.9 x 113.2-190.3 µm. Ascomatal hairs arcuate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are fusiform, 4.0-6.3 x 6.6-11.2 µm, with two apical germ pores (Fig.7).
Isolate examined: MB601.

Chaetomium perlucidum Sergejeva. Notulae Syst. Sect. Crypt. Inst. Bot. Acad. Sci. U.S.S.R. 11: 108 (1956).

Colonies usually are white or greylish by aerial mycelium, without a pigment exudate. Mature colonies dark grey to black with ascomata. Ascomata are grey, ovate in shape, 91.9-145.5x120.1-190.6 µm. Ascomatal hairs undulate and irregularly sinuous. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are elliptical, 3.4-5.5x6.7-9.2 µm, with an apical germ pore (Fig.8).
Isolate examined: NB202.

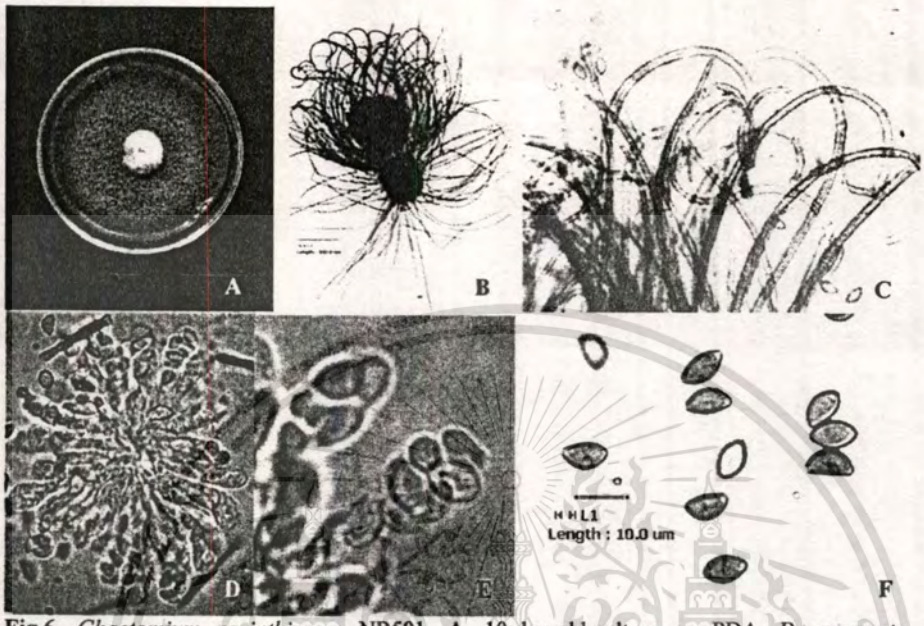

Fig.6. *Chaetomium carinthiacum* NB501. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores.

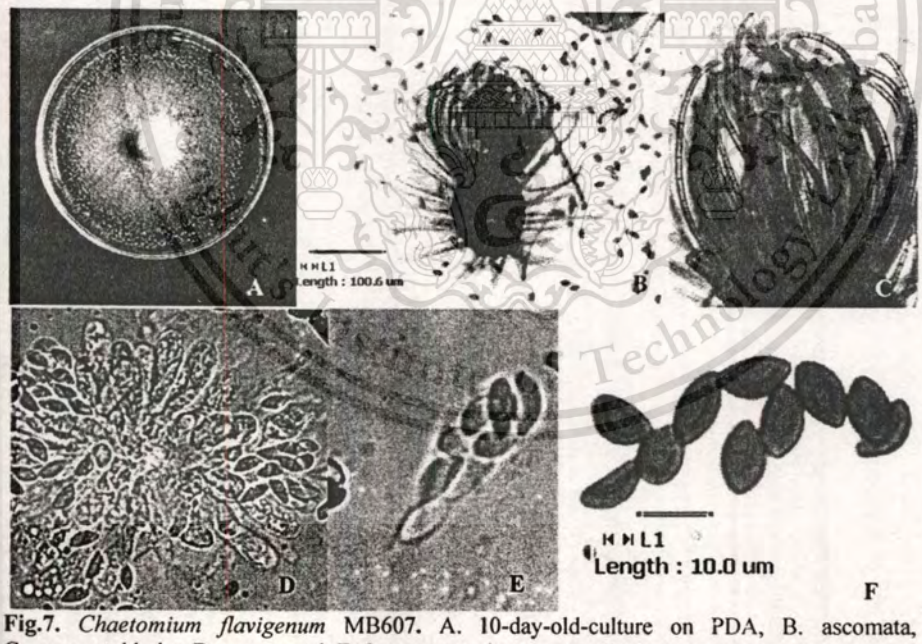

Fig.7. *Chaetomium flavigenum* MB607. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores.

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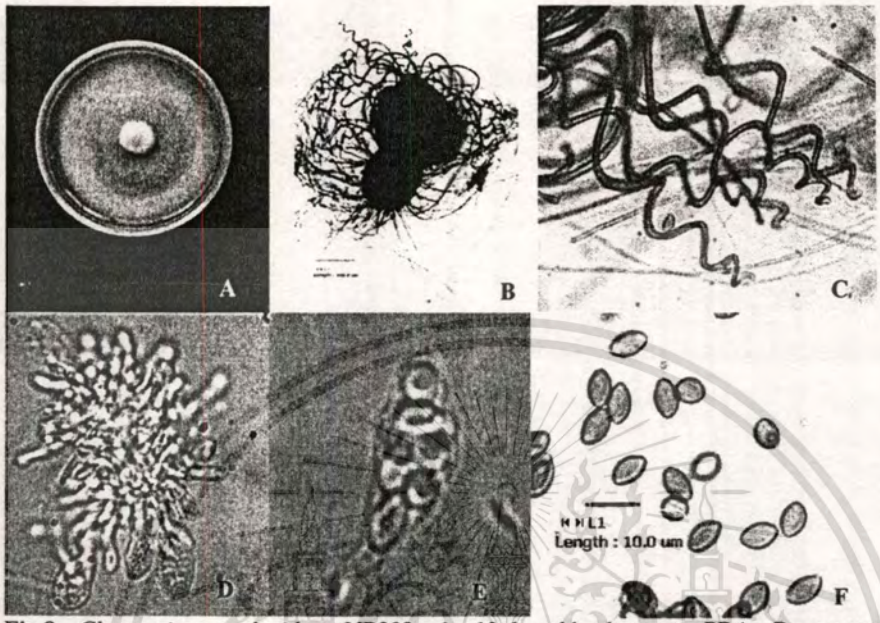

Fig.8. *Chaetomium perlucidum* NB202. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores.

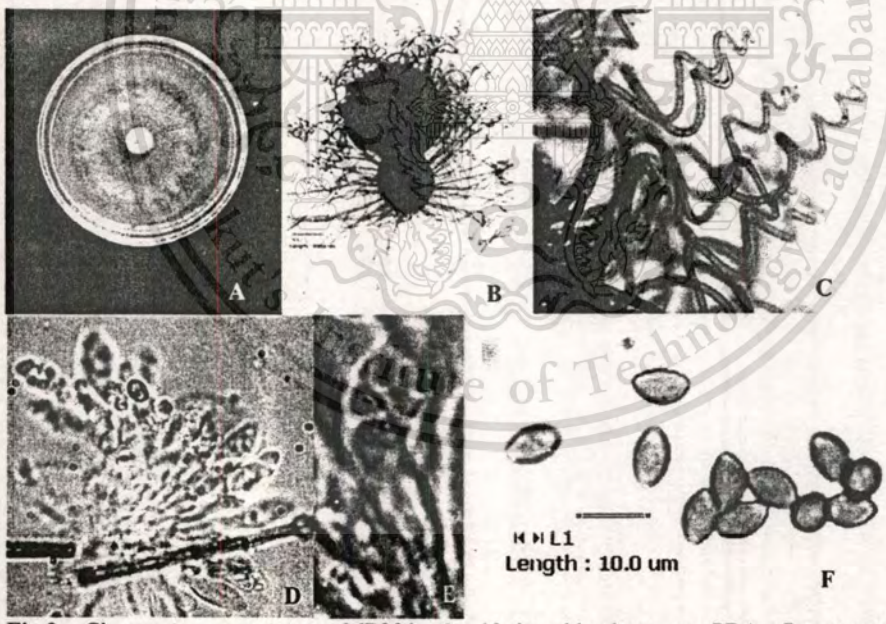

Fig.9. *Chaetomium succineum* MB304. A. 10-day-old-culture on PDA, B. ascomata, C. ascomatal hairs, D. young asci, E. 8 ascospores in an ascus, F. ascospores.

Chaetomium succineum Ames. Mycologia. 41: 445 (1949).

Colonies usually are dark green or greylish by aerial mycelium, without a pigment exudate. Mature colonies dark grey to black with ascomata. Ascomata are grey, ovate in shape, 107.1-143.1x122.0-209.2 μm. Ascomatal hairs loosely hairs. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are elliptical, 4.2-5.9x7.1-10.0 μm, with an apical germ pore (Fig.9).
Isolate examined: MB304.

Acknowledgements

We would like to thank Mr. Sommart Pornsuriya and Ms. Chamaiporn Charoenporn for helping to collect soil samples. This research project is reported as a part of Ph.D.thesis.

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(Received 14 August 2008; accepted 23 October 2008)

The 4th Annual Meeting of Thai Mycological Association and
Mycology Conference in Thailand. 24 October 2009

Biological control of pineapple root rot

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Abstract: Root rot of pineapple (*Ananas comosus* (L.) Merr.) var. Pattavia was found in the fields at Petchaburi, Phatthalung, Prachuap Khiri Khan and Rayong provinces. Forty-four isolates of pathogen were encountered by baiting technique. All of them were proved to be pathogenic to pineapple by detached leaves and root test. The isolate RY803 from Rayong provinces gave more virulent than any others. All 44 isolates were identified using the morphological characters and confirmed by their ITS region of the nuclear rDNA. Fifteen isolates of *Pythium aphanidermatum* and 29 isolates of *P. graminicola* were described. *P. aphanidermatum* RY803 was the most virulent while *P. graminicola* was the first report causing root rot of pineapple in Thailand. Therefore, *P. aphanidermatum* RY803 was used for screening antagonistic fungi by bi-culture plate and crude extract test.

Forty-two isolates of soil fungi were isolated by baiting and soil plate techniques for screening antagonists against *P. aphanidermatum* RY803 All promising antagonists were tested for their ability to control *P. aphanidermatum* RY803 in bi-culture plate. Bi-culture test for antagonism showed that *C. aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocadium* sp. RY102, *Gliocadium* sp. RY111, *T. harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112 could inhibit the growth and oospore formation to *P. aphanidermatum* RY803 over 80%. The crude Ethyl acetate extracts of *C. cochliodes* RY301 at the concentration of 1,000 µg/ml gave significantly highest inhibition of mycelial growth and oospores formation ($P<0.01$). Moreover, the hyphae, oogonia and oospores of *P. aphanidermatum* RY803 became abnormal protoplasm in cell and demonstrated uncommon shape.

Mycofungicide produced from *C. cochliodes* RY301 in oil form significantly reduced root rot severity and gave greater plant growth than non-treated plants control in pot experiment. This implies that *C. cochliodes* RY301 could act as both mycofungicide and plant growth stimulant.

Key words: biological control, *Chaetomium*, *Pythium aphanidermatum*, pineapple root rot

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Journal of Agricultural Technology 2010 Vol.6(2): 299-308
Available online http://www.ijat-rmutto.com
ISSN 1686-9141

Efficacy of antifungal metabolites from some antagonistic fungi against *Pythium aphanidermatum*

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Pornsuriya, C., Soytong, K., Kanokmedhakul, S. and Lin, F.C. (2010). Efficacy of antifungal metabolites from some antagonistic fungi against *Pythium aphanidermatum*. Journal of Agricultural Technology 6(2): 299-308.

Antifungal metabolites from *Chaetomium aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocladium catenulatum* RY102, *G. catenulatum* RY111, *Trichoderma harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112 extracted by hexane, EtOAc and MeOH were tested for inhibition of *Pythium aphanidermatum* RY803 causing pineapple root rot. Crude EtOAc extract of *C. cochliodes* strain RY301 showed the highest antifungal activity against *P. aphanidermatum* RY803. Treatment with crude EtOAc extract of *C. cochliodes* strain RY301 at 1,000 µg/ml inhibited mycelial growth and oospore formation against *P. aphanidermatum* RY803 by 71.00 and 88.95%, respectively. It also inhibited oospore formation with effective dose (ED_{50}) value of 64 µg/ml. Moreover, *P. aphanidermatum* RY803 on PDA added with crude EtOAc extract of *C. cochliodes* RY301 showed abnormal features of hyphae, oogonia and oospores. It was implied that the antagonistic mechanism of *C. cochliodes* RY301 was lysis and antibiosis.

Key words: Antifungal metabolites, *Chaetomium cochliodes*, *Pythium aphanidermatum*

Introduction

Pineapple (*Ananas comosus* Merr.) is one of the most important economic plants in Thailand. It is grown mainly for fresh, canned fruits and juice, and is the only source of bromelain, an enzyme used in pharmaceuticals. The growth area of pineapple in Thailand has been expanded because the increased worldwide demand for pineapple products has greatly stimulated

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plantings. Root rot symptoms are commonly found in the fields and become major losses in pineapple plantations. The disease caused by *Pythium aphanidermatum* (Edson) Fitzp. It is a ubiquitous phytopathogen with a wide host range and an aggressive species of *Pythium*, causing serious root rot and damping-off in various economic plant crops (Parker, 2010).

The sustainable agricultural practices rely on the integration of biotechnology with traditional agricultural practices (Haggag and Mohamed, 2007). Most sustainable and environmentally acceptable control may be used biocontrol agents for reducing the use of agricultural chemicals and their residues in the environment. Biological control using antagonists to control root rot of several plants caused by pathogenic fungi has been extensively studied, and several examples of successful disease control exist. The used antagonistic fungi for controlling root rot have been reported such as *Aspergillus* spp., *Chaetomium cupreum*, *C. globosum*, *C. cochliodes*, *Gliocadium virens*, *Penicillium* spp., *Trichoderma harzianum*, *Trichoderma viride* (Ezziyyani *et al.* 2007; Abdelzaher. 2003; Soytong *et al.* 2001; Galland and Paul. 2001; Naseby *et al.* 2000; Ahmed *et al.* 1999).

Biological control of plant diseases by using antagonistic microorganisms involved in mechanisms as antibiosis, competition suppression, direct parasitism, induced resistance, hypovirulence and predation (Haggag and Mohamed, 2007). The antagonistic activity against plant pathogens has often been associated with secondary metabolite production (Aggarwal *et al.*, 2003; Park *et al.*, 2005; Eziashi *et al.*, 2006; Kishore *et al.*, 2007) This paper attempted to screen for promising antagonists producing antifungal metabolites and investigated efficacy of antifungal metabolites in inhibiting mycelial growth and oospore formation of *P. aphanidermatum* RY803.

Materials and methods

Promising antagonistic fungi and pathogen

Ten promising antagonistic fungi *Chaetomium aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *Gliocladium catenulatum* RY102, *G. catenulatum* RY111, *Trichoderma harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112 were isolated from soil under pineapple plantation in Phatthalung and Rayong provinces. Fungal pathogen *Pythium aphanidermatum* RY803 used in this study was isolated from rhizosphere soil of pineapples showing root rot symptom. The isolate was proved to be the highest virulence causing root rot of pineapple according to previous report (Pornsuriya *et al.*, 2009). All promising

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antagonistic fungi were tested to be potential antagonists against *P. aphanidermatum* RY803 by bi-culture plate method. Cultures were maintained on potato dextrose agar slants.

Fungal growth and extraction of crude extracts

The mycelial plugs of each promising antagonist was transferred into potato dextrose broth (PDB) and incubated in static state at room temperature (28-30°C) for 4 weeks. Fungal mycelia were removed from liquid by cheesecloth filtration and dried over night at 28-32°C. Subsequently, the extraction of each promising antagonist was performed by the method described by Kanokmedhakul *et al.* (2006). Each air-dried mycelial mat was ground and extracted with hexane (1:1 vol/vol) and incubated by shaking for 24 hrs at room temperature. The solvent was separated out of the marc by filtration through filter paper (Whatman No.4). The marc from hexane extraction was further extracted with ethyl acetate (EtOAc) and followed with methanol (MeOH) using the same procedure as hexane. The solvents were separately evaporated in vacuo to yield crude hexane, EtOAc and MeOH extracts, respectively. Each crude extract was weighed (Table 1), and then kept in refrigerator (4°C) until use for testing antifungal metabolites against *P. aphanidermatum* RY803.

***In vitro antifungal metabolites against Pythium aphanidermatum* RY803**

Each crude extract from antagonists was dissolved with 2% dimethyl sulfoxide (DMSO) and then tested for inhibitory activity against mycelial growth and oospore formation of *P. aphanidermatum* RY803 on potato dextrose agar (PDA) at concentrations of 0, 10, 50, 100, 500 and 1000 µg/ml. Agar plug (3 mm diameter) of *P. aphanidermatum* RY803 was cut from the margin of the 3-d-old colony and transferred to the middle of PDA containing each concentration of crude extract and incubated at room temperature for 2-10 days depended on its activity. The experiment was done by using Completely Randomized Design (CRD) with four replications. Data were collected as colony diameter (cm) and oospore formation. The statistical analysis of variance (ANOVA) was computed. Treatment means were compared using the Duncan's multiple range test (DMRT) at P=0.01. The effective dose of ED_{50} values was computed using probit analysis.

Results and discussion

Fungal growth and extraction of crude extracts

Yields of crude extracts from 10 antagonistic fungi were recorded as shown in Table 4.1. Crude MeOH extract from *Trichoderma harzianum* RY104 gave the highest yield (4.113 g) followed by crude MeOH extract from *Chaetomium bostrychodes* PR101, crude EtOAc extract from *C. aureum* MB601, crude EtOAc extract from *C. cupreum* NB201 and crude hexane extract from *T. harzianum* RY104 that gave yield of crude extract by 3.492, 3.004, 2.032 and 2.331 g, respectively. The results indicated that the yields of crude extracts varied according to species of fungi, number of mycelial mats and kind of solvents.

Table 4.1. Yields of mycelial mats and crude extracts from 10 antagonistic fungi.

Fungi	Air-dried mycelial mats (g)	Yields of crude extracts (g)		
		hexane	EtOAc	MeOH
Chaetomium aureum MB601	49.93	1.335	3.004	1.892
Chaetomium bostrychodes PR101	36.49	0.625	1.098	3.492
Chaetomium cochliodes RY301	37.09	0.430	1.352	1.503
Chaetomium cupreum NB201	49.92	0.927	2.032	1.327
Chaetomium cupreum RY202	48.54	1.012	1.083	2.316
Gliocladium catenulatum RY102	45.38	0.343	0.257	1.322
Gliocladium catenulatum RY111	38.65	0.415	0.916	1.234
Trichoderma harzianum RY 101	40.20	0.996	0.450	1.207
Trichoderma harzianum RY 104	70.27	2.331	1.333	4.113
Trichoderma harzianum RY 112	42.37	1.172	0.453	1.365

In vitro antifungal metabolites against Pythium aphanidermatum RY803

Antifungal activities of crude extracts on mycelial growth of *P. aphanidermatum* RY803 were recorded at 2 days (Fig. 1 and Table 2). Crude EtOAc extract from *Chaetomium cochliodes* RY301 at concentration of 100, 500 and 1,000 µg/ml and *Trichoderma harzianum* RY112 at concentration of 500 and 1,000 µg/ml gave colony diameter of *P. aphanidermatum* RY803 by 3.23, 1.83, 1.45, 2.75 and 1.48 cm, respectively (Table 2). The data of colony diameter were transformed into percent of mycelial growth inhibition. The results indicated that crude EtOAc extract from *C. cochliodes* RY301 gave the greatest inhibition on mycelial growth of *P. aphanidermatum* RY803 by 63.40 and 71% at concentration of 500 and 1,000 µg/ml, respectively (Fig. 1) while crude EtOAc extract from *T. harzianum* RY112 could inhibit the mycelial growth of *P. aphanidermatum* RY803 by 70.04% at concentration of 1,000 µg/ml, and the other crude extracts inhibited mycelial growth of *P. aphanidermatum* RY803 less than 50% at all tested concentrations.

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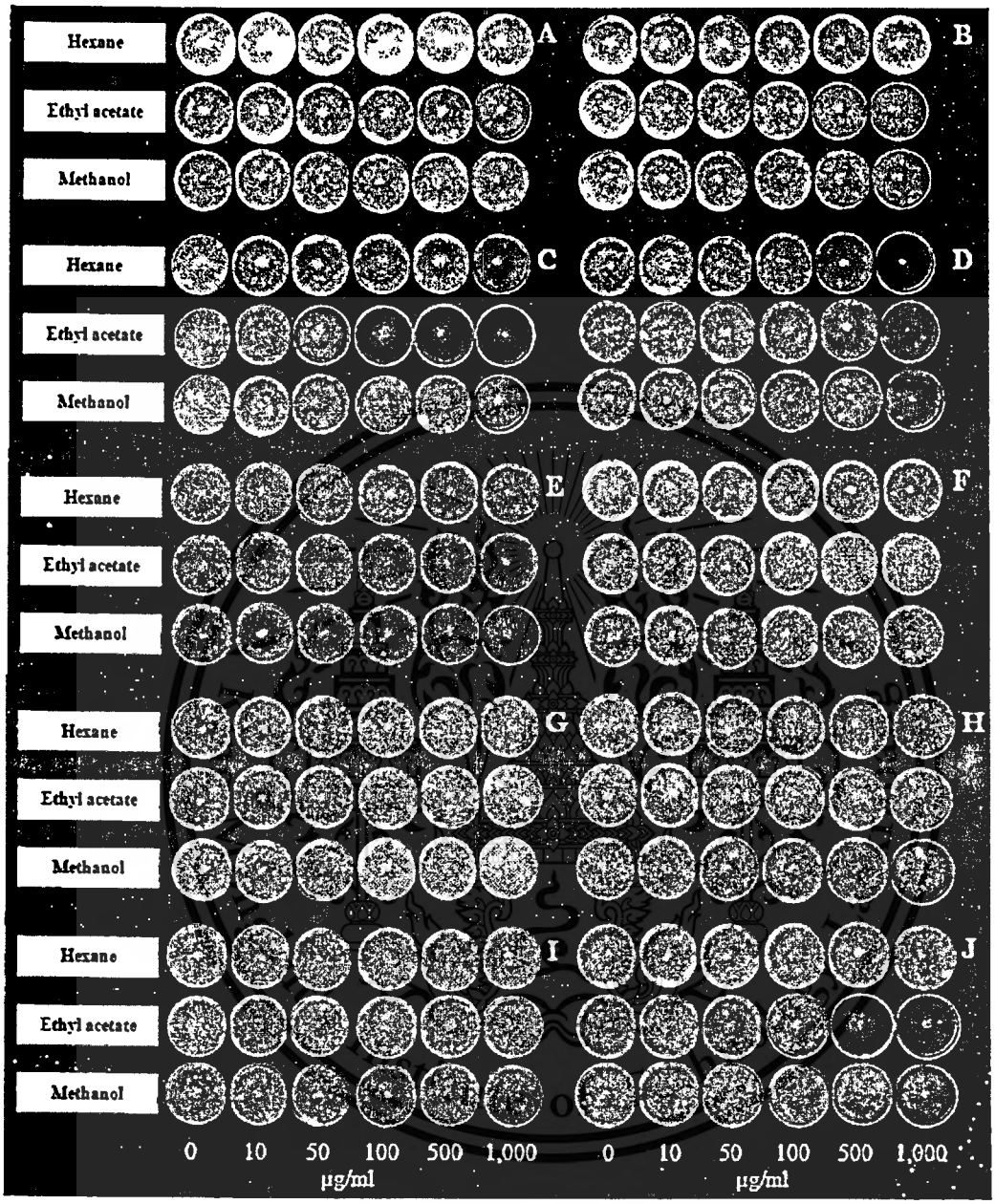

Fig.1. Colony growth of *Pythium aphanidermatum* RY803 on PDA containing crude hexane, EtOAc and MeOH extracts from promising antagonistic fungi at 0, 10, 50, 100, 500 and 1,000 µg/ml concentrations. A. *Chaetomium aureum* MB601, B. *Chaetomium bostrychodes* PR101, C. *Chaetomium cochliodes* RY301, D. *Chaetomium cupreum* NB201, E. *Chaetomium cupreum* RY202, F. *Gliocladium catenulatum* RY102, G. *Gliocladium catenulatum* RY111, H. *Trichoderma harzianum* RY101, I. *Trichoderma harzianum* RY104 and J. *Trichoderma harzianum* RY112.

Table 2. Colony diameter of *Pythium aphanidermatum* RY803.

Crude extracts of promising antagonistic fungi	Colony diameter of *Pythium aphanidermatum* RY803 at each concentration (cm)					
	0	10	50	100	500	1,000
Chaetomium aureum MB601						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium bostrychodes PR101						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium cochliodes RY301						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	3.23 b	1.83 c	1.45 d
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium cupreum NB201						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Chaetomium cupreum RY202						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Gliocladium catenulatum RY102						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Gliocladium catenulatum RY111						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Trichoderma harzianum RY101						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Trichoderma harzianum RY104						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	5 a	5 a
MeOH	5 a	5 a	5 a	5 a	5 a	5 a
Trichoderma harzianum RY112						
Hexane	5 a	5 a	5 a	5 a	5 a	5 a
EtOAc	5 a	5 a	5 a	5 a	2.75 b	1.48 c
MeOH	5 a	5 a	5 a	5 a	5 a	5 a

[1]Average of four replications. Means followed by a common letter in each promising antagonistic fungus are not significantly different at P=0.01 by Duncan's Multiple Range Test.

Antifungal activities of crude extracts on oospore formation of *P. aphanidermatum* RY803 were recorded at 10 days (Tables 3). All tested crude extracts significantly inhibited oospore formation of *P. aphanidermatum* RY803. Particularly, crude EtOAc extract of *C. cochliodes* RY301 at 1,000 µg/ml gave the most inhibition effect on oospore formation of *P. aphanidermatum* RY803 by an average of 88.95%. The data of oospore inhibition were computed into effective dose (ED_{50}) values at each crude extract. Crude EtOAc, MeOH and hexane extract from *C. cochliodes* RY301 gave the greatest ED_{50} at 64, 84 and

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178 µg/ml, respectively followed by crude EtOAc extract from *C. aureum* MB601, crude EtOAc extract from *T. harzianum* RY101 gave the greatest ED_{50} at 368 and 473 µg/ml, respectively.

Besides, mycelial growth and oospore formation of *P. aphanidermatum* RY803 on PDA added with crude EtOAc extract from *C. cochliodes* RY301 showed abnormal features. Hyphae, oogonia and oospores formed abnormal protoplasm in cell and demonstrated uncommon shape (Fig. 2).

Table 3. Percent inhibition of oospore formation of *Pythium aphanidermatum* RY803

Crude extracts of promising antagonistic fungi	Inhibition of oospore formation[1] (%) of *Pythium aphanidermatum* RY803 at each concentration (µg/ml)						ED_{50}
	0	10	50	100	500	1,000	(µg/ml)
Chaetomium aureum MB601							
Hexane	0 k	8.35 j	9.71 i	12.54 h	12.83 h	12.83 h	NF
EtOAc	0 k	17.85 g	33.92 d	41.59 c	49.33 b	58.27 a	368
MeOH	0 k	8.13 j	8.53 j	29.66 f	30.59 ef	31.51 e	6,582
Chaetomium bostrychodes PR101							
Hexane	0 e	0.72 e	5.12 d	26.27 c	29.46 b	31.40 a	2,744
EtOAc	0 d	9.50 c	34.88 b	35.13 b	36.51 b	55.04 a	753
MeOH	0 e	15.20 d	36.16 c	37.19 b	37.23 b	57.98 a	662
Chaetomium cochliodes RY301							
Hexane	0 j	26.33 h	38.77 f	56.83 e	57.12 e	57.69 e	178
EtOAc	0 j	25.11 h	32.96 g	61.93 d	82.72 b	88.95 a	64
MeOH	0 j	20.38 i	27.63 h	57.72 e	61.91 d	70.10 c	84
Chaetomium cupreum NB201							
Hexane	0 j	1.90 j	19.00 h	35.00 e	37.08 e	61.93 a	575
EtOAc	0 j	1.34 j	26.28 f	50.60 c	58.17 b	58.20 b	297
MeOH	0 j	8.32 i	23.08 g	45.61 d	52.21 c	57.86 b	543
Chaetomium cupreum RY202							
Hexane	0 d	0.66 d	8.43 c	28.49 b	33.21 b	47.50 a	976
EtOAc	0 f	10.09 e	18.95 d	28.09 c	33.55 b	56.49 a	994
MeOH	0 d	13.58 c	21.78 c	36.09 b	37.13 b	58.22 a	762
Gliocladium catenulatum RY102							
Hexane	0 k	7.78 ij	10.96 hi	13.92 fgh	21.04 e	25.14 d	45,615
EtOAc	0 k	8.67 ij	11.66 ghi	18.18 f	34.66 ab	36.94 ab	3,515
MeOH	0 k	5.03 j	15.05 fg	29.70 c	33.87 b	38.08 a	2,218
Gliocladium catenulatum RY111							
Hexane	0 j	8.28 hi	11.32 gh	13.56 fgh	20.70 d	27.32 c	40,815
EtOAc	0 j	12.60 gh	14.18 efgh	19.60 def	33.84 ab	38.73 a	4,888
MeOH	0 j	3.66 ij	14.55 defgh	16.10 defg	20.27 de	31.90 bc	8,701
Trichoderma harzianum RY101							
Hexane	0 m	5.29 l	9.24 k	11.43 j	13.75 i	15.83 h	NF
EtOAc	0 m	18.44 g	33.73 e	43.46 c	49.75 b	54.07 a	473
MeOH	0 m	8.61 k	16.72 h	30.52 f	33.12 e	37.46 d	3,422
Trichoderma harzianum RY104							
Hexane	0 d	8.91 c	11.74 c	14.12 bc	21.56 ab	24.58 a	NF
EtOAc	0 d	11.15 c	13.90 bc	19.53 b	35.77 a	38.23 a	3,515
MeOH	0 h	6.24 g	14.47 ef	29.89 bc	34.06 ab	38.47 a	2136
Trichoderma harzianum RY112							
Hexane	0 j	7.05 hi	10.97 ghi	13.47 fgh	20.50 de	24.41 d	NF
EtOAc	0 j	10.03 ghi	13.18 fgh	18.94 def	45.18 b	61.87 a	617
MeOH	0 j	5.77 ij	14.64 efg	29.88 c	37.99 b	40.19 b	2,034

[1]Average of four replications. Means followed by a common letter in each promising antagonistic fungus were not significantly different at P=0.01 by Duncan's Multiple Range Test. NF = no effect.

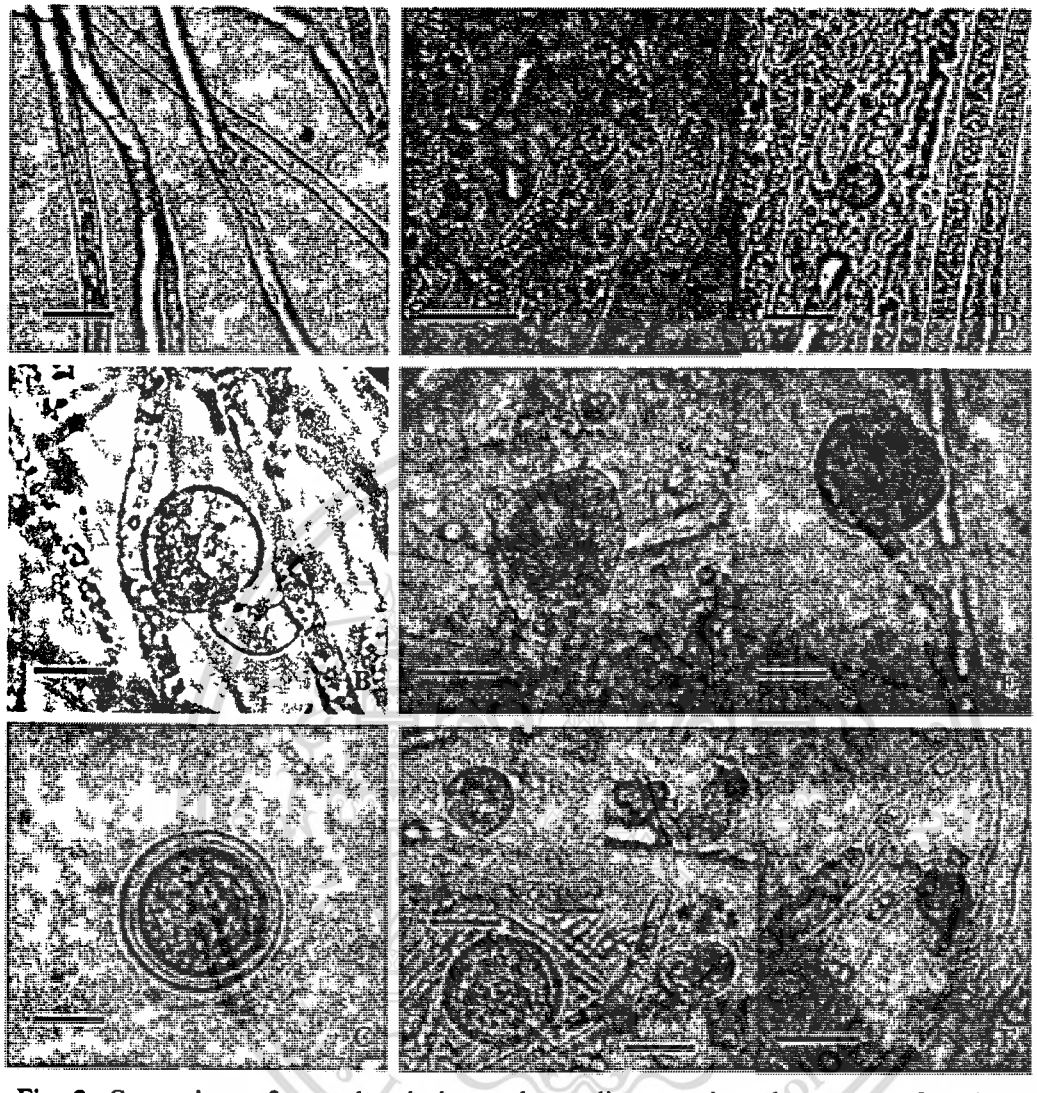

Fig. 2. Comparison of normal and abnormal mycelia, oogonia and oospores of *Pythium aphanidermatum* RY803 on PDA added with crude EtOAc extract from *Chaetomium cochliodes* RY301. A, B and C showed normal mycelia, oogonium and oospore, respectively. D, E and F showed abnormal mycelia, oogonia and oospores, respectively. Bars. = 10 μm.

Crude hexane, EtOAc and MeOH extracts from 10 promising antagonistic fungi *C. aureum* MB601, *C bostrychodes* PR101, *C. cochliodes* RY301, *C. cupreum* NB201, *C. cupreum* RY202, *G. catenulatum* RY102, *G. catenulatum* RY111, *T. harzianum* RY 101, *T. harzianum* RY 104 and *T. harzianum* RY 112 were tested to find out the most efficient antagonist for controlling pineapple root rot caused by *P. aphanidermatum* RY803. *C. cupreum*, *G. catenulatum* and *T. harzianum* have well known to be antagonist for plant disease control (Soytong,

1992a, 1992b; Biren *et al.*; 1999; Ezziyyani *et al.*, 2007). *C. cupreum* has been reported to reduce leaf spot disease of corn caused by *Curvularia lunata*, rice blast caused by *Pyricularia oryzae*, sheath blight of rice caused by *Rhizoctonia oryzae* and tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* (Soytong, 1992a; 1992b). *G. catenulatum* was a mycoparasite of several fungal genera including *Aspergillus flavus* and *Sclerotium cepivorum* (Biren *et al.*; 1999; Tsigbey *et al.*, 1999). *T. harzianum* has been reported as biocontrol agent for controlling *Phytophthora capsici* (Ezziyyani *et al.*, 2007) and *Pythium ultimum* (Naseby *et al.*, 2000). In this study *C. cochliodes* RY301 was found to be the greatest antagonist against *P. aphanidermatum* RY803 causing root rot of pineapple. The results implied that the antagonistic mechanism of *C. cochliodes* RY301 was lysis and antibiosis due to it produce some metabolites that could inhibit mycelial growth, oospore formation and formed abnormal of hyphae, oogonia and oospores. This result was supported by previous report (Phonkerd *et al.*, 2008) that *C. cochliodes* VTh01 and *C. cochliodes* CTh05 could produce four new dimeric spiro-azaplilones, cochliodones A-D, two new azaphiliones, chaetoviridines E and F, a new epi-chaetoviridin A, and known compounds, chaetoviridin A, ergosterol, chaetochalasin A. Chaetoviridines E and chaetochalasin A exhibited antimalarial activity against *Plasmodium falcipulum* while cochliodones C, chaetoviridines E and F, chaetochalasin A expressed antimycobacterial activity against *Mycobacterium tuberculosis*. Furthermore, *C. cochliodes* VTh01 and *C. cochliodes* CTh05 were reported to be antagonistic to *Fusarium oxysporum* f. Sp. *lycopersici* causing tomato wilt (Phonkerd *et al.*, 2008). Therefore it was possible that *C. cochliodes* RY301 might have the similar active compounds that could be antagonist against *P. aphanidermatum* RY803. In this study, it was pointed out that *C. cochliodes* RY301 could exhibit inhibitory activity against *P. aphanidermatum* RY803.

Acknowledgements

We would like to thank the Biocontrol research unit at King Mongkut's Institute of Technology Ladkrabang, Bangkok for providing necessary facilities in this research. This research project is reported as a part of Ph.D.thesis.

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(Received 12 January 2010; accepted 15 February 2010)

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