MATERIALS AND METHODS

Materials

1. Isolation and identification of *Talaromyces*

1.1 Materials for collected soil samples

1.1.1 shovel

1.1.2 permanent marker

1.1.3 plastic bags

1.1.4 rubber band

1.1.5 camera

1.1.6 note book

1.2 Laboratory Materials

1.2.1 permanent marker

1.2.2 forceps

1.2.3 fine needle s

1.2.4 Petri dishes

1.2.5 test tube s

1.2.5 beakers

1.2.6 agar media

1.2.7 electric scale

1.2.8 hot air oven

1.2.9 autoclave

1.2.10 alcohol lamp

1.2.11 65%, 70% and 95% ethyl alcohol

1.2.12 slide s and coverslips

1.2.13 distilled water

1.2.14 lactophenol

1.2.15 stereo microscope (SZ-PT Olympus)

1.2.16 light microscope (BH-2 Olympus)

1.2.17 camera lucida

1.2.18 Scanning Electron Microscope (JEOL JSM 6400)

1.2.19 thermometer

1.2.20 oil emersion

2. Preservation

2.1 sterilized soil

2.2 sterilized filter paper Whatman No.1

2.3 liquid paraffin

2.4 aluminum foil

2.5 paper bags

2.6 plastic bags

2.7 vials, size 1 dram.

2.8 Petri dishes

2.9 forceps

2.10 dessicator, electronic dry cabinet (WEIFO)

3. Media (Appendix)

3.1 Glucose Ammonium Nitrate Agar (GAN)

3.2 Cornmeal Agar (CMA)

3.3 25% Glycerol Nitrate Agar (G25N)

3.4 Potato Dextrose Agar (PDA)

3.5 Malt Extract Agar (MEA)

3.6 Czapek's Agar (CZA)

3.7 Czapek Yeast Autolysate Agar (CYA)

3.8 Oatmeal Agar (OMA)

3.9 Water agar

4. Phylogeny study of *Talaromyces*

4.1 Centrifuge

4.2 Hot water bath

4.3 Spatulas

4.4 Pipettes 1,000, 200, 100, 20, 2 µl

4.5 Pipette tips

4.6 Mortar and Pestle

4.7 Liquid nitrogen

4.8 Micro centrifuge tubes

5. Glasshouse Material

5.1 pots 10 cm

5.2 trays

5.3 sterilized soil

5.4 mungbean seeds

6. Isolation and purification of the secondary metabolite from *Talaromyces*

6.1 cultivating medium

6.2 Petri dishes

6.3 cork borer

6.4 Erlenmeyer flask 250, 500,1,000 and 2,000 ml

6.5 fitrate pump

6.6 paper filtrate Whatman No. 1

6.7 rotary evaporator

6.8 column chromatography

6.9 TLC aluminium sheets 20 x 20 cm silica gel 60 F_{254}, Merck

6.10 silica gel 60 F_{254} (0.063–0.200 mm), Merck for column chromatography

6.11 silica gel 60 F_{254} (0.063–0.200 mm), Merck for thin layer chromatography

6.12 20 x 20 cm glass plates

6.13 sea sand

6.14 cotton

6.15 ethyl acetate (EtOAc)

6.16 chloroform ($CHCl_3$)

6.17 acetone ($(CH_3)_2CO$)

6.18 petroleum ether (Petrol)

6.19 methanol (CH_3OH)

6.20 formic acid (HCOOH)

6.21 distilled water

6.22 microcapillary pipettes, calibrated size 10 µl

6.23 vials, 4 dram

6.24 volumetric flask

6.25 hot plate

6.26 UV detector

6.27 ultrasonic machines

6.28 aluminum foil

6.29 tank chamber

7. Stucture elucidation of the compounds

7.1 Proton Nuclear Magnetic Resonance (^{1}H NMR)

7.2 Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR)

7.3 Correlation Spectroscopy (COSY)

7.4 Heteronuclear Single Quantum Coherence (HSQC)

7.5 Heteronuclear Multiple Bond Correlation (HMBC)

7.6 High Resolution Mass Spectrometry (HRMS)

Methods

1. Isolation and identification of *Talaromyces*

1.1 Soil samples collection

Forty-five soil samples were collected from agricultural fields, unagricultural fields, forest and along the roadside (Table 3, Figure 4). Labelled with locations, dates, and names of the collecter and brought to the laboratory for isolated this fungus.

2. Isolation of *Talaromyces*

2.1 Soil plate method (A modification of Warcup, 1950)

A small amount of soil (0.005-0.015 g) was placed onto a sterile Petri dish. About 10 ml of warm GAN containing rose bengal and streptomycin was added and the Petri dish was gently rotated to disperse the soil particles before the agar solidified. The plates were, then placed in covered boxes for incubation in darkess at room temperature. Hyphal tips were transferred onto PDA and maintained as pure cultures for identification.

2.2 The dilution plate method (Barron 1968)

A 10 g of soil samples was added to 100 ml of sterile distilled water. Suspensions were vigorously shaken until thoroughly mixed and 10 ml the suspension was mixed with 90 ml of sterile distilled water in a flask. Ten ml samples were then transferred through a succession of 90 ml sterile distilled water blanks until the desired dilution was reached. One-ml aliquots of the selected dilution (usually 10^{-2}, 10^{-3}, 10^{-4}) were pipetted into Petri dishes for each selected dilution. The same procedures described in the previous method were followed.

2.3 Alcohol treatment method (A modification of Warcup and Baker, 1963)

0.03 g of soil samples was placed in 65% ethanol for 10-20 min. The liquid was decanted, bits of the treated soil were dispensed into several sterile Petri dishes, and the plates were immediately poured with GAN. The same procedures described in the previous method were follwed.

2.4 Heat treatment method (A modified of Warcup and Baker, 1963)

One g of soil samples was placed in a sterile test tube in a water bath at 60-80°C for 20-30 min. Excess water was drained off and soil particle were placed into Petri dishes. The same procedures (2.1-2.3) described previously were followed.

2.5 Single ascospore isolation (Intana, 2003)

PDA plus 100 ppm streptomycin was poured in a Petri dish. A sterile glass rod was used to spread 0.5 ml ascospores suspension (10^3 ascospores / ml) on a solidified agar media and incubated for 24 h at room temperature. Ascospore germination was examined under a light or compound microscopes and a piece of agar containing a single ascospore was transferred to slant PDA.

3 Identification of *Talaromyces* species (Stolk and Samson, 1972; Ramirez, 1982; Manoch *et al.*, 2004)

3.1 Macroscopic examination

Morphological characteristics of colonies were determined as growth pattern, color, texture on different media, such as CZA, MEA, CYA, CMA, OMA and G25N agar for 7 to 14 days, at 25°C, 28°C and 37°C (Pitt, 1979a). Diameters of

colonies were measured in milimetres, most effectively by transmitted light and from the reverse side.

Colony characteristics were examined under a stereoscopic microscope and naked eyes. The microscope was used for assessing texture of colonies and the appearance of penicilli and conidial chains. For judgement of conidial and colony colours, Rayner's "A Mycological Colour Chart" (Rayner, 1970) has been employed.

3.2 Microscopic examination

Microscopic characteristics were examined on a slide preparation using sterile distilled water and lactophenol as mounting media and examined under a light microscope (Olympus BH-2 with Normaski Interference Contrast). Camera lucida drawings were employed. Photomicrographs of fungal structure were taken under stereo, light and scanning electron microscopes.

Study on ornamentation of ascospore was conducted using Scanning Electron Microscopy. Matured ascomata and ascospores of *Talaromyces* from dry culture agar media were transferred with a fine needle and placed onto double-strick scotch tape on aluminium stubs. The specimens were coated with gold for 5-7 min. and examined in a JEOL JSM 6400 scanning electron microscope (Manoch *et al.*, 2004).

Identification was based on morphological characteristics examination under a stereo, light and scanning electron microscopes. *Talaromyces* were identified following the researches done in previous reports (Stolk and Samson, 1972; Pitt, 1979a; Takada and Udagawa, 1988; Yaguchi *et al.*, 1992; Yaguchi *et al.*, 1993a, b; Udagawa, 1993; Yaguchi *et al.*, 1994a, b; Yuguchi *et al.*, 1996; Udagawa *et al.*, 1993; Udagawa and Suzuki, 1994).

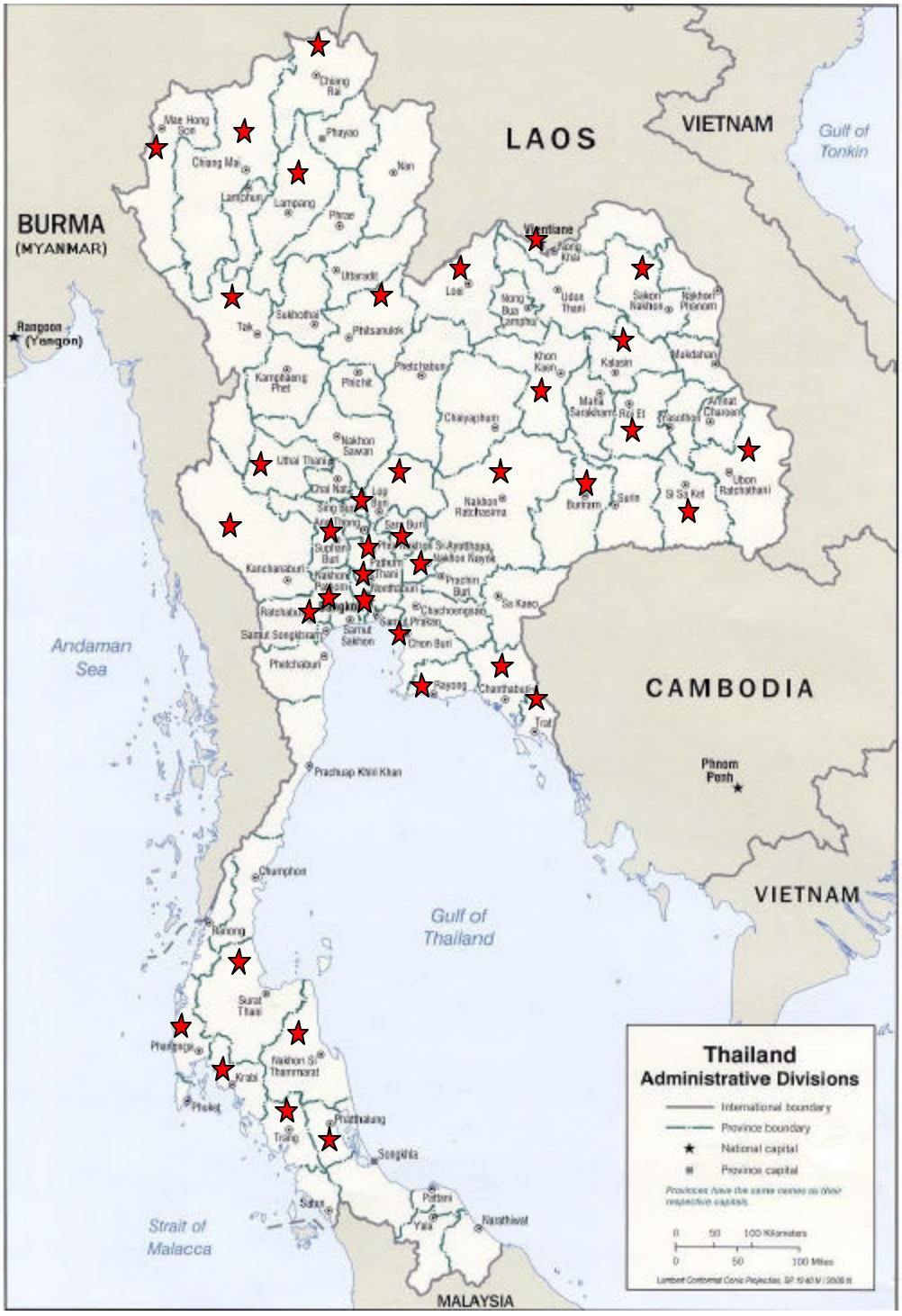

Figure 4 Map of Thailand indicating the collection sites of the soil samples from 38 provinces

Table 3 Forty-five soil samples were collected from various locations in Thailand

Part and Province	Location	Collecting date
North		
Chiang Mai	Agricultural soil, Queen Sirikit Botanic Garden	11 June 2003
	Agricultural soil, Amphur Mae Tang	17 July 2004
	Nonagricultural soil, Amphur Mae Sa	29 January 2002
	Forest soil, Mok Fa water fall	17 December 2004
Chiang Rai	Agricultural soil, Horticulture Research Institute	16 January 2004
Lumpang	Agricultural soil, Amphur Maung	15 December 2004
Mae Hong Son	Forest soil, Amphur Maung	3 December 2003
Tak	Nonagricultural soil, Amphur Maung	5 November 2003
North-East		
Buri Rum	Nonagricultural soil, Amphur Chareamprakient	15 January 2002
Pitsanulok	Forest soil, Pu Kin Rang Kha	1 January 2002
Kalasin	Nonagricultural soil, Amphur Maung	16 April 2003
Khon Kaen	Nonagricultural soil, Amphur Chumpae	2 July 2003
Loei	Forest soil, Pu Kra Doug	17 November 2001
Ubon Ratchathani	Agricultural soil, Amphur Maung	29 November 2001
Nakhon Ratchasima	Agricultural soil, Amphur Pak Thong Chai	25 October 2002
	Agricultural soil, Amphur Dan Kun Tod	19 August 2003
Nong Khai	Forest soil, Amphur Maung	11 September 2004
Roi Et	Nonagricultural soil, Amphur Maung	6 October 2003
Sakon Nakhon	Forest soil, Amphur Pupan	16 September 2001
	Nonagricultural soil, Amphur Kum Ta Kra	21 January 2000
Si Sa Ket	Nonagricultural soil, Amphur Kantharak	27 October 2001
Central		
Ang Thong	Agricultural soil, Amphur Maung	29 November 2004
Phra Nakhon Si Ayutthaya	Agricultural soil, Amphur Wangnoi	18 October 2003
Bangkok	Agricultural soil, Kasetsart Univ., Bang Khan	11 July 2003
	Nonagricultural soil, Kasetsart Univ., Bang Khan	5 June 2004
Kanchanaburi	Agricultural soil, Tong Pa Poom	30 June 2002
Lop Buri	Nonagricultural soil, Amphur Maung	11 May 2003
Uthai Thani	Nonagricultural soil, Amphur Maung	19 May 2004
Nakhon Pathom	Agricultural soil, Amphur Kumpangsan	4 February 2002
Nonthaburi	Agricultural soil, Amphur Maung	9 May 2003
Ratchaburi	Nonagricultural soil, Amphur Nong Po	10 October 2001
Saraburi	Nonagricultural soil, Amphur Maung	19 May 2004
Sing Buri	Nonagricultural soil, Amphur Maung	27 April 2004
Suphan Buri	Agricultural soil, Amphur Dam Bang Nang Buon	5 January 2002
East		
Chanthaburi	Agricultural soil, Amphur Ta Mai	24 November 2004
Chon Buri	Agricultural soil, Tambol Bangsarai, Amphur Sattahip	2 January 2002
Rayong	Nonagricultural soil, Amphur Ban Phe	15 August 2003
Trat	Forest soil, Ko Koh	9 August 2003
South		
Krabi	Nonagricultural soil, Ko Lanta	10 August 2003
Nakhon Si Thammarat	Nonagricultural soil, Walairak Univ., Amphur Maung	14 March 2004
Phatthalung	Agricultural soil, Amphur Bang Kaew	25 March 2005
Phang Nga	Nonagricultural soil, Amphur Maung	22 July 2003
Surat Thani	Nonagricultural soil, Amphur Maung	8 December 2003
Trang	Nonagricultural soil, Amphur Maung	10 December 2003

4 Preservation

Pure cultures were maintained on slant PDA covered with liquid paraffin, filter paper and sterilized soil at the culture collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok.

4.1 PDA slant method (Smith and Onions, 1994)

Pure cultures of *Talaromyces* spp. were maintained on PDA slants at 28°C. Subculturing was carried out every 6 months.

4.2 Liquid paraffin method (Smith and Onions, 1994)

Pure cultures were maintained on PDA agar slant in a small vial (1 drams). Liquid paraffin was placed in a vial and autoclaved three times. Covering the pure culture on agar with sterile liquid paraffin about 2/3 of a vial and stored at 28 °C in order to prevent dehydration and slow down metabolic activity and growth through reduced oxygen tension.

4.3 Filter paper method (Fong *et al.*, 2000)

Fifteen pieces (0.5 x 0.5 cm^2) of sterile filter paper Whatman No. 1 were placed on PDA in sterile Petri dish. The mycelia were transferred on PDA and incubated for 7-14 days depend on the species. The filter papers with fungal mycelium were transferred to new sterile Petri dish by using sterile forcep and placed in a dessicator or electric dessicator (35°C) for 7-10 days. Dried filter papers covered mycelium mass and ascomata were kept in an aluminum foil, labeled and placed in a box for storage at -20 °C.

4.4 Soil Culture (Smith and Onions, 1994)

Loamy soil was placed in a vial about 2/3 full and autoclaved twice at 121°C for 15 min. One ml of spore suspension in sterile water was added The soil cultures were left to grow at room temperature and then left to dry while stored in a refrigerator at 4-10°C.

5. The molecular study of *Talaromyces* species

5.1 Cultivation of *Talaromyces* strains for DNA extraction using CTAB method

Twenty-one fungi were selected for this studied (Table 4). The preparative for DNA extraction of fungal mycelium, the fungi were grown in 250 ml flask containing 100 ml of PDB and inoculated with two mycelial plugs taken from stock culture of each fungal grown on PDA. Culture flasks were incubated at 28-30°C on rotary shakers at 150 rpm for 2-3 days. The mycelia were harvested from broth through filter paper (Whatman No. 1) by vacuum filtration.

5.2 Methods for DNA preparation

5.2.1 DNA extraction from fungal material

- Preheat extraction CTAB (2% w/v CTAB, 20mM EDTA, 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl) buffer at 65°C.

- Grind 1-2 g of mycelial by adding liquid nitrogen in a mortar and pestle until the tissues are powdery.

- Add 500 µl of warm extraction buffer to the powdered tissue. Mix and incubate in waterbath at 65°C for 10 min.

- Add 500 µl Chloroform-Isoamyl alcohol (24:1). Mix and spin at 12,000-14,000 rpm for 5 min.

- Transfer aqueous phase into a clean microtube and add 0.8 vol. of isopropanol.

- Mix carefully and spin at 5,000 rpm for 2-3 min. (repeat if top phase is not clear) and discard aqueous phase.

- Add 600-700 µl cold absolute ethanol.

- Centrifuge, remove ethanol and wash with 70% ethanol. Air dried the pellet and resuspend in Rnase. Store DNA samples at 4°C or -20°C for longer periods.

Table 4 Taxa and selected isolates used for studying molecular phylogeny

Taxon	**Strains**
Genus *Talaromyces*	
Section *Talaromyces*	
Series *Flavi*	
T. flavus	KUFC 3381
T. helicus var. major	KUFC 3598
T. indigoticus	KUFC 3366
T. macrosporus	KUFC 3381
T. stipitatus	KUFC 3594
Series *Lutei*	
T. austrocalifornicus	KUFC 3401
T. luteus	KUFC 3331
T. rotundus	KUFC 3359, KUFC 3446
T. wortmannii	KUFC 3333
Talaromyces sp. 1	KUFC 3399, KUFC 3631
Talaromyces spp.	KUFC 3352, KUFC 3370, KUFC 3470
Series *Trachyspermus*	
T. trachyspermus	KUFC 3355
Talaromyces sp. 2	KUFC 3383
Section Emersonii	
T. bacillisporus	KUFC 3350
Genus *Byssochlamys*	
B. fulva	KUFC 2849
Class Trichocomaceae	
Acremonium anamorph	KUFC 3645
Penicillium anamorph	KUFC 3580

5.3 PCR conditions for amplification of microsettalites genes

The SSR regions were amplified by the polymerase chain reaction (PCR) using the respective primer combination (Table 5). DNA amplification was performed in final volume of 50 µl containing 1X buffer (10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM each deoxynucleoside triphosphate, 50 pmole of each primer, 2.5 U/µl of *Taq* DNA polymerase, and 50 ng of genomic DNA from each fungi. The DNA was amplified by thermocycler (Perkin-Elmer 9600/ Applied Biosystem) and the following parameters: 5 min of denaturation at 94 ^{o}C, followed by 30 cycles of amplication with denaturation at 94 ^{o}C for 45 sec, annealing at melting temperature of each primer pairs for 45 sec as shown in table 5, DNA elongation at 72 ^{o}C for 30 sec and a final elongation for 7 min at 72 ^{o}C. A negative control lacking template DNA was included for each set of reactions. Ten microliters from each PCR reaction were electrophoresed on 3 % agarose gel electrophoresis in 0.5X Tris-acetate buffer. DNA in gels were stained with ethidium bromide (0.5 ug per ml) and viewed under UV- transilluminator.

Table 5 List of primer sequences of 10 microsatellite loci

Primer	Primer sequence (5'-3')	Tm (^{0}C)
MG 13	5' CAC-GTG-TCA-AGT-CAT-AAT-AAA-TAG 3'	56.3
MG 14	5' AAT-CTG-CTG-CCA-ATA-GTC-AT 3'	56.3
PM1F	5' CCT-GTT-TGT-CTT-TTG-TGC-TG 3'	52.7
PM1R	5' GTA-CGG-GCT-AGC-TGT-CAG-TG 3'	52.7
PM2F	5' TTA-CTC-GAT-ACG-GCA-GTT-GG 3'	52.7
PM2R	5' TGT-TAC-GAT-AAC-CGC-GTC-TG 3'	52.7
PM7F	5' TCC-CTC-ACA-TGC-TAA-TGA-TG 3'	52.7
PM7R	5' ACG-ACT-CGG-AGG-AAT-TGA-GA 3'	52.7
PM12F	5' GCC-CAC-ACT-GAC-ACA-CTA-TG 3'	52.7
PM12R	5' ATA-TCT-TGG-TGC-CAC-CTG-AC 3'	52.7

Source: Fisher *et al.* (2004a)

5.4 Data analysis

For each bands of DNA in gels were assigned a number that fragments of the same size from different strains have the same number. Numbered fragments were treated as characters with two states, present (=1) or absent (=0), and their distribution was tabulated for all the fungi studied. For molecular phylogeny analysis: The data was aligned by TreeCon Software for the neighbor-joining analysis (Nei and Li, 1979). A bootstrap analysis was conducted with 100 replications.

6. Test of antagonism against plant pathogenic fungi by *Talaromyces*

Talaromyces spp. were subcultured on PDA for 14-21 days or until ascospores became mature. Ascospore suspension was prepared by Petri dish flooding the culture surface with sterile water and then ascomata of *Talaromyces* spp. were scraped from medium surface by sterile spatula. The concentration was determined with haematocytometer before adjusted with sterile water to 10^6 ascospore / ml for antagonistic test in this study (Intana , 2003).

6.1 *In vitro* growth inhibition and overgrowing of mycelia of plant pathogen

Twenty isolates of *T. flavus*, each isolate of *T. bacillisporus* and *Talaromyces* sp. KUFC 3399 were selected to test for antagonistic activity against 15 species of plant pathogenic fungi (Tables 6-7). The young mycelium from the colony margin of *Talaromyces* spp. and the specific plant pathogenic fungus were cut with sterile cork borer (0.8 cm diam) and placed as a dual culture on PDA, 7 cm apart. All Petri dishes were incubated at room temperature (28 ^{o}C) for 14 days. The inhibition levels were calculated by using the formula: G_1-G_2 / G_1 x 100 where G_1 = colony radius of plant pathogenic fungi in the control and G_2 = colony radius of plant pathogenic fungi in the dual culture test (Intana, 2003). Each treatment was performed with three replicates.

6.2 Antagonistic tests of *Talaromyces flavus* in greenhouse

A modification of Madi *et al.*, (1997) method for antagonistic tests of *T. flavus* in the greenhouse was conducted. Dried mungbeen seeds (*Vigna radiata* (L.)R. Wilczek) were surface disinfested with 0.525% sodium hyperclorite for 5 min, rinsed 3 times with sterile water and immersed in ascospore suspension (10^6 ml) of *T. flavus* strains for 24 hr. Three 10 cm plastic pots were filled with 400 g of garden soil. Ten bean seeds were placed on the soil surface in each pot and two sclerotia of *Sclerotium rolfsii* were placed 0.5-1 cm apart from each bean seed. Seeds and sclerotia were covered with 150 g of soil, and the pots were incubated in the greenhouse at temperature ranging from 28 to 30°C. Disease symptoms were recorded at 7 and 14 days after planting. There were three treatments in the experiment: mungbean + *S. rolfsii* + *Trichoderma harzianum* (Unigreen®), mungbean + distilled water and mungbean + *S. rolfsii*. Disease reduction by *T. flavus* treatment against plant pathogenic fungi was described previously by Madi *et al.*, (1997). The experiment was conducted three times, with 3 replicates per treatment. Each pot was served as a replicate, and the data were pooled for analysis.

Table 6 Isolates of *Talaromyces flavus*, *T. bacillsporus* and *Talaromyces* sp. 1 (KUFC 3399) from various locations, used for antagonistic test against plant pathogenic fungi

T. flavus strains KUFC	Location	Methods
3334	Forest soil, Mae Hong Son	Heat treatment
3363	Watermelon field soil, Chon Buri	Alcohol treatment
3381	Chili field soil, Kasetsart Univ., Bangkok	Heat treatment
3388	Mango field soil, Chiang Mai	Soil plate technique
3395	Paddy soil, Suphan Buri	Soil plate technique
3397	Cucumber field soil, Nonthaburi	Heat treatment
3400	Corn field soil, Chiang Mai	Alcohol treatment
3485	Mungbean field soil, Nakhon Ratchasima	Dilution plate technique
3446	Paddy soil, Chiang Mai	Alcohol treatment
3450	Longan field soil, Chiang Rai	Heat treatment
3473	Nonagricultural soil, Krabi	Alcohol treatment
3483	Tomato field soil, Nonthaburi	Heat treatment
3501	Cucumber field soil, Chiang Mai	Alcohol treatment
3506	Paddy soil, Bangkok	Heat treatment
3508	Cabbage field soil, Chiang Mai	Soil plate technique
3523	Cucumber field soil, Kanchanaburi	Soil plate technique
3525	Kale field soil, Nonthaburi	Heat treatment
3528	Mungbean field soil, Chon Buri	Heat treatment
3530	Durian field soil, Chanthaburi	Dilution plate technique
3550	Forest soil, Sakon Nakhon	Soil plate technique
T. bacillisporus	Nonagricultural soil, Kasetsart Univ., Bangkok	Heat treatment
Talaromyces sp. KUFC 3399	Forest soil, Trat	Heat treatment

Table 7 Fifteen species of plant pathogenic fungi from various diseased plants used for testing of antagonistic activity of *Talaromyces* spp.

Plant pathogenic fungi	Host plant	Class
Phytophthora palmivora	*Durio zibethinus* (durian)	Oomycetes
Phytophthora parasitica	*Citrus reticulata* (orange)	Oomycetes
Peronophythora litchii	*Litchi chinensis* (litchi)	Oomycetes
Pythium aphanidermatum	*Cucumis sativus* (cucumber)	Oomycetes
Colletotrichum capsici	*Capsicum annuum* (chili)	Coelomycetes
Colletotrichum gloeosporioides	*Pyrus pyrifolia* (pear)	Coelomycetes
Lasiodiplodia theobromae	*Garcinia mangostana* (mangosteen)	Coelomycetes
Pestalotiopsis guepinii	*Psidium guajava* (guava)	Coelomycetes
Phyllosticta sp.	*Pyrus pyrifolia* (pear)	Coelomycetes
Curvularia lunata	*Zea mays* (corn)	Hyphomycetes
Fusarium oxysporum f.sp. *lycopersici*	*Lycopersicon esculentum* (tomato)	Hyphomycetes
Helminthosporium maydis	*Zea mays* (corn)	Hyphomycetes
Helminthosporium oryzae	*Oryza sativa* (rice)	Hyphomycetes
Rhizoctonia solani	*Oryza sativa* (rice)	Agonomycetes
Sclerotium rolfsii	*Vigna radiata* (mungbean)	Agonomycetes

7 Analytical secondary metabolites of *Talaromyces bacillisporus* and *Talaromyces* sp.1 (KUFC 3399)

7.1 General Experimental

- Merck Si gel 60 (0.2-0.5 mm; 70-230 mesh) was used for column chromatography
- Analytical and preparative TLC was performed on silica gel-60 (GF_{254}; Merck), 0.25 thickness. The plates were activated at 110^{o}C in the oven for 1 hour. All TLC plates were visualized under UV 254 nm or developed with iodine vapor.
- Melting points were recorded on a Bock Monoscope and are uncorrected.
- Rotations were determined on a Polax-2L instrument.
- ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature in DMSO on a Bruker DRX instrument operating at 500 and 125 MHz respectively, ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectra were measured on a Bruker CxP spectrometer. The solvents used were deuterated chloroform (Merck) or hexadeuterated dimethylsulfoxide (Merck).
- EI mass spectra were measures on a Hitachi Perkin-Elmer RMV-GM instrument. For HR mass spectra were measured on CONCEPT II, 2 sectors mass spectrometer. The accelerating voltage was 8 KV.
- X-ray diffraction studies were performed with a Stoe IPOS image plant equipped with Mo Kα radiation. The structure was solved using SHELX 597 and refined with SHELXL 97. A perspective view of the molecule was obtained with ORTEP.
- The solvents used were commercial grade of Vidrolab 2 which were distilled prior to use and analytical reagent grade of brand Merck and Lab-Scan.
- All solvents were evaporated either by reduced pressure using " Buchi evaporator" or nitrogen gas.
- The weight was measured on the balance Mettler AE 200.

7.2 Isolation and Purification of the Secondary Metabolites from *Talaromyces bacillisporus*

7.2.1 Fungus material

Talaromyces bacillisporus C.R. Benjamin was isolated from a soil sample collected on the campus of Kasetsart University, Bangkok, Thailand in July 2003 and identified by Assoc. Prof. Dr. Leka Manoch on the basis of the description in Pitt (1979a) and with standard tests. A sample with accession number KPFC 3350 has been deposited in the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University.

7.2.2 Extraction and isolation of the constituents

7.2.2.1 Preparation of the crude extract

Twenty-five 1,000 mL Erlenmeyer flasks each containing 200 g of rice and 100 mL of H_2O were autoclaved at 121°C for 15 min., inoculated with three mycelium plugs of *Talaromyces bacillisporus* KPFC 3350 culture and incubated at 28 °C for 30 days. To each flask containing the moldy rice was added 400 ml of EtOAc, after which the contents were left to macerate for 3 days and then filtered using filter paper (Whatman No. 1). Evaporation of the combined filtrates to a volume of 1,000 ml at reduced pressure followed by addition of anhydrous sodium solephate, filtration and evaporation of the filtrate at reduced pressure furnished 105 g of dark brown crude EtOAc extract which was extract with $CHCl_3$ (3 x 500 ml). The $CHCl_3$ extracts were combined and concentrated at reduced pressure to afford 85 g of a brown viscous mass.

7.2.2.2 Fractionation of the crude extract

The crude $CHCl_3$ extract was applied to a silica gel column (200 g), and eluted with petrol-$CHCl_3$, $CHCl_3$ and $CHCl_3$-acetone, 300-500 ml / fractions being collected as follows:

Fractions	Eluents
1-142	$CHCl_3$-petrol (1:1)
143- 218	$CHCl_3$-petrol (7 : 3)
219- 286	$CHCl_3$-petrol (9 : 1)
287- 315	$CHCl_3$-acetone (9 : 1)
316- 343	$CHCl_3$-acetone (4 : 1)
344- 365	$CHCl_3$-acetone (7 : 3)

The fractions were analyzed by analytical TLC and combined, according to their composition, as follows:

Fractions	Isolated compounds
1-6 (1,038.9 mg)	not purified
7-19 (1,638.2 mg)	not purified
20-22 (447.2 mg)	not purified
23-28 (600 mg)	**bacillisporin A** (**4**, 300 mg)
	duclauxin (**50**, 20 mg)
29-111 (2,000 mg)	**bacillisporin A** (**4**, 1,250 mg)
	bacillisporin D (**69**, 9 mg)
	duclauxin (**50**, 27 mg)
112-122 (276.6 mg)	not purified
123-132 (244.5 mg)	not purified
133-142 (224.4 mg)	not purified
143-160 (2,000 mg)	**bacillisporin E** (**70**, 22 mg)
	bacillisporin C (**6**, 600 mg)
161-174 (795 mg)	not purified
175-185 (424.6 mg)	not purified
186-190 (165.3 mg)	not purified
191-201 (317.1 mg)	not purified
202-210 (404.4 mg)	not purified
211-215 (280 mg)	**bacillisporin B** (**4**, 200 mg)
216-225 (570.8 mg)	not purified

Fractions	Isolated compounds
226-256 (656.8 mg)	not purified
257-267 (795 mg)	not purified
268-271 (20.8 mg)	not purified
272-279 (66.2 mg)	not purified
280-286 (47.4 mg)	not purified
287-293 (623.2 mg)	not purified
294-306 (299.4 mg)	not purified
307-315 (177.6 mg)	not purified
316-343 (604.4 mg)	not purified
344-365 (400.1 mg)	not purified

7.2.2.3 Isolation and Purification of the compounds

Fractions 23-28 (600 mg) were combined and recrystallized from $CHCl_3$-petrol to give bacillisporin A (**4**) as a pale yellow solid (300 mg).

TLC of the mother liquor (silica gel, $CHCl_3$-acetone-HCO_2H, 95:5:1) gave 35 mg of duclauxin (**50**) as a yellow solid.

Fractions 29-111 (2 g) were combined, applied to a silica gel column (50 g) and eluted with $CHCl_3$-petrol and $CHCl_3$-acetone, 100 ml subfractions being collected as follows:

Fractions	Eluents
1-22	$CHCl_3$-petrol (7 : 3)
23-64	$CHCl_3$-petrol (9 : 1)
65- 98	$CHCl_3$-acetone (9 : 1)

Subfractions 3-5 were combined (800 mg) and recrystallized from $CHCl_3$-petrol to give more bacillisporin A (**4**, 250 mg).

Purification of the mother liquor by TLC (silica gel, $CHCl_3$-acetone-HCO_2H, 95:5:1) gave 27 mg of duclauxin (**50**) as a yellow solid.

Subfractions 43-64 (30 mg) were combined and purification by TLC (silica gel, $CHCl_3$-acetone-HCO_2H, 85:15:1) to give 9 mg of bacillisporin D (**69**) as a yellow solid.

Fractions 143-160 (2 g) were combined and recrystallized from $CHCl_3$-acetone to give 480 mg of bacillisporin E (**70**). The mother liquor was chromatographed over silica gel (20 g) and eluted with $CHCl_3$-petrol and $CHCl_3$-acetone as follows using 100 mL/ subfractions.

Fractions	**Eluents**
1-61	$CHCl_3$-petrol (7 : 3)
62-72	$CHCl_3$-petrol (9 : 1)
73-87	$CHCl_3$-acetone (9 : 1)
88-100	$CHCl_3$-acetone (4 : 1)

Subfraction 16-21 (200 mg) were combined and purified by TLC (silica gel, $CHCl_3$-acetone-HCO_2H, 85: 15: 1) to give 22 mg of bacillisporin E (**70**).

Combination of fractions 161-180 (1.5 g) and recrystallization from $CHCl_3$-acetone afforded 600 mg of bacillisporin C (**6**). Combination of fractions 211-215 (180 mg) and recrystalization from $CHCl_3$-acetone afforded 200 mg of bacillisporin B (**5**).

7.3 Isolation and purification of the secondary metabolites from *Talaromyces* sp. 1 (KUFC 3399)

7.3.1 Fungal material

The fungus was isolated from a soil sample collected at Trat Province, Easthern Thailand in August 2003. The strain was deposited at the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University with the accession number KPFC 3399.

7.3.2 Method of culture and extraction of the constituents

7.3.2.1 Preparation of the crude extract

Fifty 1000 ml Erlenmeyer flasks containing 200 g of rice and 100 ml of water, autoclaved at 121ºC for 15 min, were inoculated with ten mycelium plugs from the *Talaromyces* sp. 1 (KUFC 3399) culture and incubated at 28ºC for 30 days. Each flask with the moulded rice was added 400 ml of ethyl acetate and the content was left to macerate for 3 days. The content of the flasks was filtered by filter paper and the filtrate was evaporated under reduced pressure to give 3 litres of the solution and then anhydrous sodium solephate was added and filtered. The ethyl acetate solution was evaporated under reduced pressure to give 79.2 g of dark brown viscous mass of a crude ethyl acetate extract which was extracted by $CHCl_3$ (3x500 ml). The chloroform extracts were combined and evaporated under reduced pressure to give a brown viscous mass of crude chloroform extract (51.4 g).

7.3.2.2 Fractionation of the crude extract

The crude $CHCl_3$ extract was chromatographed over silica gel column (200 g), and eluted with Petrol-$CHCl_3$, $CHCl_3$ and $CHCl_3$-acetone, 300-500 ml / fractions being collected as follows;

Fractions	Eluents
1- 202	$CHCl_3$-petrol (1 : 1)
201-265	$CHCl_3$-petrol (7 : 3)
266- 285	$CHCl_3$-petrol (9 : 1)
286- 316	$CHCl_3$-acetone (9 : 1)
317- 343	$CHCl_3$-acetone (4 : 1)
344- 368	$CHCl_3$-acetone (7 : 3)

All collected fractions were analyzed by analytical TLC and combined, according to their composition, as follows;

Fractions	Isolated compounds
1-4 (52 mg)	not purified
5-15 (8 mg)	not purified
16-24(3 mg)	not purified
25-27 (2 mg)	not purified
28-37 (443 mg)	not purified
38-46 (273 mg)	not purified
47-64 (273 mg)	not purified
65-76 (273 mg)	***N*-benzoylphenylalanyl-*N*-benzoylphenylalaninate** (**77**, 22 mg)
77-90 (123 mg)	**vermistatin** (**75**, 59 mg)
91-92 (145 mg)	**vermistatin** (**75**, 20 mg) **thailandolide B** (**72**, 11.3 mg)
93-95 (56 mg)	**vermistatin** (**75**, 15 mg)
96 (28.5 mg)	not purified
97-107 (244 mg)	**thailandolide A** (**71**, 200 mg)

Fractions	Isolated compounds
108-110 (58.6 mg)	not purified
111-130 (570 mg)	**penisimplicissin** (**74**, 15.4 mg)
31-144 (275.3 mg)	not purified
145- 149 (121 mg)	**2-glyceryl palmitate** (**78**, 11.7 mg)
150-162 (387 mg)	not purified
163-179 (336 mg)	not purified
180 -202 (517 mg)	not purified
203 -220 (756 mg)	not purified
221-280 (1,190 mg)	**hydroxydihydrovermistatin** (**76**, 37 mg)
281-291(1,270 mg)	not purified
292-297 (542 mg)	not purified
298-303 (302 mg)	not purified
304-320 (498 mg)	not purified
321-339 (521 mg)	not purified
340-355 (569 mg)	not purified
356-368 (671 mg)	not purified

7.3.4 Isolation and Purification of the compounds

Fractions 65-75 were combined (345 mg) and recrystallized from a mixture of $CHCl_3$ and petrol to give white solid of ***N*-benzoylphenylalanyl-*N*-benzoylphenylalaninate** (**77**, 200 mg).

Fractions 77-90 and 93-95 were combined (179 mg) and recrystallized from a mixture of $CHCl_3$ and petrol to give white solid of **thailandolide B** (**72**, 59 mg).

Fractions 91 and 92 were combined (45 mg) and purified with TLC (silica gel, $CHCl_3$-Me_2O-HCO_2H; 95:5:1) to give **thailandolide B** (**72**, 11.3 mg) and **vermistatin** (**75**, 20 mg).

Fractions 97-107 were combined (244 mg) followed by recrystallized from a mixture of $CHCl_3$ and petrol to give white solid of **thailandolide A** (**71**, 200 mg).

Fractions 111-130 were combined (570 mg) and recrystallized from $CHCl_3$ and petrol gave yellow solid (45 mg), which was further purified by TLC (silica gel, $CHCl_3$-Me_2O-HCO_2H; 95:5:1) to give **penisimplicissin** (**74**, 15.4 mg).

Fractions 145-159 were combined (121 mg) and purified by TLC (silica gel, $CHCl_3$-Me_2O-EtOAc: HCO_2H; 85:10:5:1) to give **2-glyceryl palmitate** (**78**, 11.7 mg).

Fractions 221-280 were combined (1.19 g) and applied on a silica gel column (10 g) and eluted with $CHCl_3$-petrol, 100 ml / subfractions were collected as follows:

Fractions	**Eluents**
1- 50	$CHCl_3$-petrol (1 : 1)
51- 74	$CHCl_3$-petrol (7 : 3)
75- 80	$CHCl_3$-acetone (9 : 1)

Subfractions 28-32 (127 mg) were combined and purified by TLC (silica gel, $CHCl_3$-Me_2O-HCO_2H; 4:1:0.1) to give ***O*-methylated derivative** (**73**, 37 mg).

Fractions 286-313 were combined (2.2 g) and applied on a silica gel column (12 g) and eluted with $CHCl_3$-petrol, 100 ml sfrs were collected as follows: Sfrs 1-25 ($CHCl_3$-petrol, 7:3), 26-50 ($CHCl_3$-petrol, 9:1). Sfrs 16-24 were combined (220 mg) and purified by TLC (silica gel, $CHCl_3$-Me_2O-HCO_2H; 4:1:0.1) to give **hydroxydihydrovermistatin** (**76**, 46 mg).

7.4 Structure elucidation of the compounds

The structure of the compounds were established by spectroscopic methods (^{1}H, ^{13}C NMR, COSY, HSQC, HMBC, HRMS) as well as comparison of their NMR data with those in the literatures.

8. Place

The experiments of taxonomic study and antagonistic test were conducted at Mycology Labolatory, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok. For scanning electron photomicrographs were examined at Scientific Equipment Centre, Biomolecular Sciences, Kasetsart University. The isolation, purification and structure elucidation of the secondary metabolites were conducted at Instituto de Ciencias Biomedicas de Abel Salazar (ICBAS), Universidade do Porto, Portugal.

9. Duration

The study was carried out during October 2002 to October 2006.