ฤทธิ์ต้านเชื้อราของอนุพันธ์กรดเบนโซอิกและกรดซินนามิกต่อโรคยอดเน่าสับปะรด

นางสาวรัชยาวรรณ์ มงคล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIFUNGAL ACTIVITY OF BENZOIC ACID AND CINNAMIC ACID DERIVATIVES AGAINST PINEAPPLE HEART ROT DISEASE

Miss Rachsawan Mongkol

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	ANTIFUNGAL ACTIVITY OF BENZOIC ACID AND CINNAMIC ACID DERIVATIVES AGAINST PINEAPPLE HEART ROT DISEASE
By	Miss Rachsawan Mongkol
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Warinthorn Chavasiri, Ph.D
Thesis Co-advisor	Assistant Professor Jittra Piaphukiew, Ph.D

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Professor Udom Kokpol, Ph.D.)

..... Thesis Advisor
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

..... Thesis Co-advisor
(Assistant Professor Jittra Piaphukiew, Ph.D.)

..... External Member
(Panpim Wornkhorporn, Ph.D.)

..... Member
(Associate Professor Surachai Pornpakakul, Ph.D.)

รัชยาวรรณ์ มงคล : ฤทธิ์ต้านเชื้อราของอนุพันธ์กรดเบนโซอิกและกรดซินนามิกต่อโรคยอดเน่าสับปะรด. (ANTIFUNGAL ACTIVITY OF BENZOIC ACID AND CINNAMIC ACID DERIVATIVES AGAINST PINEAPPLE HEART ROT DISEASE) อ.ที่ปรึกษา : ผศ.ดร.วรินทร ชวศิริ, อ.ที่ปรึกษาร่วม : ผศ.ดร.จิตตรา เพียภูเขียว 80 หน้า.

เชื้อรา 15 ไอโซเลตซึ่งแยกมาจากใบสับปะรดที่มีอาการของโรคยอดเน่าจากแปลงปลูกบริเวณอำเภอชะอำ จังหวัดเพชรบุรี เมื่อทดสอบความสามารถในการก่อให้เกิดโรคยอดเน่าในหน่อสับปะรดที่แข็งแรงของเชื้อทุกไอโซเลตพบว่า เชื้อราสามไอโซเลตคือ RM1, RK1 และ *Phytophthora* สามารถทำให้หน่อสับปะรดเป็นโรคยอดเน่าได้ และเมื่อพิสูจน์ชนิดของเชื้อตามลักษณะสัณฐานวิทยาและ DNA พบว่า ไอโซเลต RM1 คือ *Fusarium oxysporum f. sp. vasinfectum* ไอโซเลต RK1 คือ *Lasiodiplodia theobromae* และ *Phytophthora* คือ *Phytophthora parasitica*

การทดสอบประสิทธิภาพของอนุพันธ์กรดเบนโซอิกและกรดซินนามิก 12 ชนิด ที่ความเข้มข้น 100 ppm ในการต้านเชื้อ *F. oxysporum, L. theobromae* และ *P. parasitica* เพื่อคัดเลือกสารที่สามารถยับยั้งเชื้อราได้ดีที่สุด พบว่า *n*-butyl 4-hydroxybenzoate ให้ผลการยับยั้งการเจริญของเส้นใยบนอาหารแข็งดีที่สุดโดยสามารถยับยั้งเชื้อ *F. oxysporum, L. theobromae* และ *P. parasitica* ได้ 73, 87 และ 80 เปอร์เซ็นต์ตามลำดับ และ *n*-butyl 4-hydroxybenzoate สามารถยับยั้งการเจริญของเส้นใยของทุกเชื้อได้อย่างสมบูรณ์ที่ความเข้มข้น 250 ppm เมื่อเลี้ยงในอาหารเหลวและสามารถยับยั้งการงอกของสปอร์ได้สมบูรณ์ที่ความเข้มข้น 100 และ 250 ppm สำหรับเชื้อ *P. parasitica* และ *F. oxysporum* ตามลำดับ นอกจากนี้เมื่อทดสอบ *n*-butyl 4-hydroxybenzoate ในใบสับปะรด พบว่าที่ 3,000 ppm สามารถควบคุมการเกิดโรคได้ และเมื่อทดสอบในหน่อพบว่าที่ความเข้มข้นเดียวกันสามารถป้องกันการเกิดโรคได้มากกว่าการรักษาโรคยอดเน่าที่เกิดจากเชื้อ *P. parasitica* ผลของการใช้เมตาแลคซิลร่วมกับ *n*-butyl 4-hydroxybenzoate ที่ความเข้มข้น EC_{50} 1.4 ppm อัตราส่วน 4:1 สามารถยับยั้งการเจริญของเส้นใยได้ไม่ต่างจากการใช้เมตาแลคซิล

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อ.....รัชยาวรรณ์ มงคล.....

ปีการศึกษา.....2550.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....จิตตรา เพียภูเขียว.....

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RACHSAWAN MONGKOL: ANTIFUNGAL ACTIVITY OF BENZOIC ACID AND CINNAMIC ACID DERIVATIVES AGAINST PINEAPPLE HEART ROT DISEASE. THESIS ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D. THESIS COADVISOR: ASST. PROF. JITTRA PIAPUKIEW, Ph.D. 80 pp.

Fifteen phytopathogenic fungi isolates were isolated from pineapple leaves showing symptoms of heart rot, collected at Cha-Am district, Phetchaburi province. All isolates were subjected to pathogenicity test on healthy suckers of pineapple. Three isolates as RM1, RK1 and *Phytophthora* caused heart rot disease. Based on fungal morphology and analysis of DNA sequencing, those fungi were identified as *Fusarium oxysporum f. sp. vasinfectum*, *Lasiodiplodia theobromae* and *Phytophthora parasitica*. The screening of antifungal activity of twelve compounds of benzoic acid and cinnamic acid derivatives at 100 ppm against three isolates was conducted. *n*-Butyl 4-hydroxybenzoate revealed the best activity of mycelial growth on agar plate assay against *F. oxysporum, L. theobromae* and *P. parasitica* with %inhibition of 73, 87 and 80%, respectively. *n*-Butyl 4-hydroxybenzoate at 250 ppm completely inhibited mycelial growth of all fungi in broth and completely inhibited zoospore germination at 100 and 250 ppm for *P. parasitica* and *F. oxysporum*, respectively. *n*-Butyl 4-hydroxybenzoate at 3,000 ppm completely inhibited pineapple heart rot disease caused by *P. parasitica* when tested by detached leaf method and as preventative of the disease on suckers in laboratory. Synergistic at EC_{50} 1.4 ppm of metalaxyl and a mixture of metalaxyl and *n*-butyl 4-hydroxybenzoate at the ratio of 4:1 showed inhibition of mycelial growth not different from that observed from solely metalaxyl.

Field of study.....Biotechnology.....Student's signature...Rachsawan Mongkol.....
Academic year.....2007.....Advisor's signature.....W. Chavasiri.....
Co-advisor's signature.....Jittra Piapukiew.....

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LIST OF ABBREVIATIONS

°C	degree Celsius
CA	carrot agar
CB	carrot broth
cm	centimeter
conc.	concentrated
$CHCl_3$	chloroform
CTAB	cetyltrimethylammonium bromide
DMSO	dimethylsulfoxide
EtOH	ethanol
g	gram
h	hour
^1H NMR	proton nuclear magnetic resonance
M	molar
mg	milligram
min	minute
mL	milliliter
mm	millimeter
NaOCl	sodium hypochlorite
No.	number
PDA	potato dextrose agar
PDB	potato dextrose broth
ppm	part per million
RT	room temperature
sp.	species
µL	microliter
µm	micrometer
UV	ultraviolet
v/v	volume by volume

CHAPTER I

INTRODUCTION

Pineapple has been a very popular fruits throughout the world. According to FAO online database, more than 80 countries produce nearly 14 million tonnes of pineapple annually. It is also estimated that of all the pineapple produced, two thirds are traded in the international market as fresh fruits or processed products. Pineapple has contributed to the economies of many producing countries in the form of fresh fruit, juice and canned pineapple (Anonymous, 2006). Pineapple production is an important economic fruit of Thailand. Besides being consumed fresh, it is processed and the present value of export is over a billion baths annually. Thailand is a word leader in pineapple production and has been the word's number one export for many year (Aupunt, 2003; Anonymous, 2001).

1.1 The pineapple

1.1.1 History and morphology

Christopher Columbus came to the Caribbeans in 1493, he and his crew ate the unknown fruit which found there. They thought the fruit looked like a pine cone, so they called it the "Pine of the Indies". Later on, they added the word "apple" because they thought it had associated with another delicious fruit. So, the name "pineapple" was born (Levins, 2004).

As pineapple cultivation in European greenhouses expanded during the 18th and 19th centuries, many varieties were imported. The now famous variety Cayenne Lisse (Smooth Cayenne) was introduced from French Guiana in 1819. The Spaniards and Portuguese dispersed other varieties, including Singapore Spanish, to Africa and Asia during the great voyages of the 16th and 17th centuries. Commercial processing of pineapple started in Hawaii at the end of the 19th century. The invention and refinement of the automatic peeling and coring machine by Henry Ginaca, a Hawaiian Pineapple Company (Dole) employee, between 1911 and 1919 allowed the development of a large scale economically viable canning industry. Major pineapple products of international trade are canned slices, chunks, crush and juice and fresh fruit (Fig 1.1).

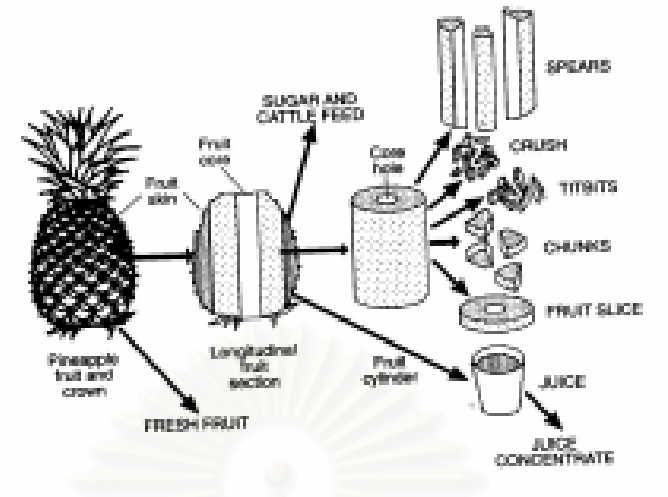


Fig 1.1 Pineapple canned production (Rohrbach, 2003).

Thailand is the world's largest producer of pineapple. In 1999 was the year in which there had been a significant increase in terms of both areas of planting and production yield in Thailand. This could partly be as a result of the high price of pineapple during 1997 to 1998 which inspired the farmer to try to expand and optimize their growing area. The canned pineapple industry is still rather competitive with strong challengers from countries like Philippines and Indonesia . The leading pineapple producing countries follow by Thailand are the Philippines with 1.5 million tonnes, Brazil with 1.4 million tonnes, China with 1.4 million tonnes and India with 1 million tones (Rohrbach and Eeckenbrugge, 2003) (Table 1.1).

Table 1.1 Pineapple production in the world.

Country	Production (t)	Harvested (ha)	Yield (t ha^{-1})
Thailand	2,300,000	97,300	24
Philippines	1,571,904	45,000	35
Brazil	1,442,300	59,238	24
China	1,284,000	57,700	22
India	1,100,000	80,000	14
Nigeria	881,000	115,000	8
Mexico	535,000	12,500	43
Costa Rica	475,000	12,000	40
Colombia	360,000	9,000	40
Indonesia	300,000	42,000	7
Venezuela	300,000	15,000	20
USA	293,000	8,130	36
Kenya	280,000	8,500	33
Côte d'Ivoire	225,675	5,200	43
South Africa	145,441	6,200	23
Australia	140,000	3,000	47
Dominican Republic	136,862	5,500	25
Malaysia	130,000	7,000	19
Guatemala	101,287	3,710	27
Honduras	70,000	3,900	18
Cameroon	42,000	4,000	11
Martinique	20,800	484	43
Swaziland	19,680	600	33
Cuba	19,000	400	48
Cambodia	16,500	1,600	10
Puerto Rico	15,000	500	30

Sourse: The Pineapple (Rohrbach, 2003)

Pineapple has the sciencetific name of *Ananas comosus* (L.) Merr. in the member of the family Bromeliaceae which embraces about 2,000 species, mostly epiphytic and many strikingly ornamental (Anonymous, 2003). There are 5 major pineapple groups grown throughout the world. Two of these, Cayenne and Queen, are widely cultivated in South Africa. The Smooth Cayenne cultivar (Fig 1.2) is used for canning (75% of which is exported), because of its high sugar content and unsuitable canning qualities, and Queen is cultivated only for fresh consumption. However, because production of the Queen pineapple is more costly, fresh consumption is shifting towards the Cayenne.Cayenne plants and the fruit are normally larger than that of the Queen, with succulent yellow fruit. Queen fruit has a golden yellow color and is less juicy.

Fig 1.2 Pineapple fruits “Smooth Cayenne” at field, Cha-Am district, Phetchaburi province.

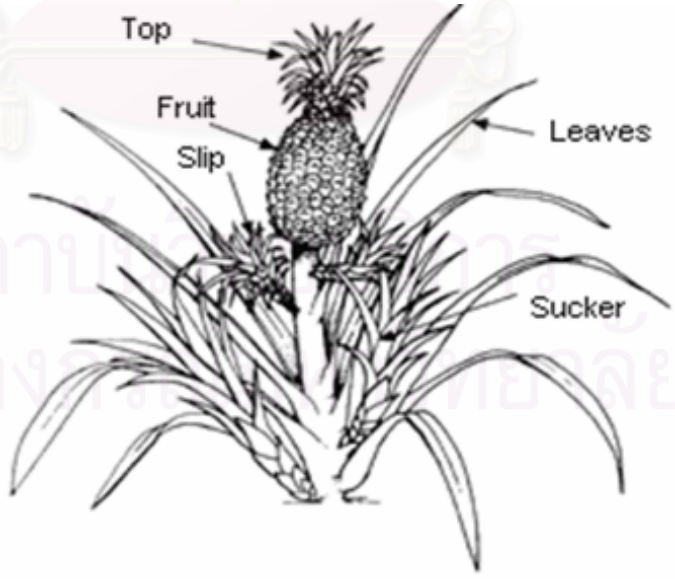


Fig 1.3 Morphology of Pineapple.

Pineapple is a perennial plant that requires a functional root system to produce multiple fruitings. Economic production of 'Smooth Cayenne', the dominant pineapple cultivar grown commercial has been base on a two or three fruit crop cycle requiring approximately 32 or 46 months, respectively for completion. A field newly planted with crowns requires approximately 18 months after planting before the first fruiting, referred to as the 'plant crop' is harvested. Two subsequent fruitings, referred to as 'ratoon crop' are produced from vegetative suckers (also call shoots) on the plant. Fruits are harvested year round for fresh market and canning operations. Generally, production levels and fruit quality are highest during the summer (Rohrbach, 2002). It grows to 50-100 cm tall. It has narrow, tapering, pointed leaves up to 100 cm long arranged in a spiral rosette, crowded on and tightly clasping a central stem. The leaf margins are usually but not always spiny. The inflorescence consists of 100-200 flowers arranged in a compact spiral cluster. The flowers are perfect. The fruit is a terminal cylindrical, compound structure at the apex of the stem. At the apex, the fruit bears a compressed, leafy shoot called a crown (Fig 3.13). The typically yellow fruit flesh is best eaten when sweet and moderately acid; it may contain from 10 to 18 percent sugar and from 0.5 to 1.6 percent titratable acidity (Evan *et al*, 2002).

Pineapple can grow well in soils of medium fertility and soil acidity at pH of 4.5-5.5. Soil pH greater than 7.0 should be avoided. The soil must be meticulously prepared to ensure proper drainage, which will minimize heart rot and root rot. The soil is plowed, left exposed to the sunlight and plant waste and debris removed. To ensure that the crop receives adequate nutrients and leaves no nitrate residues in the fruit, soil should be analyzed and nutrient recommendations for each area followed rigorously. Good soil drainage is a necessity. Pineapple plant growth occurs between 21 and 35ºC and the plant' phenotype varies depending on the production area's mean temperature and rainfall of 1,000-1,500 mm annually. Similar sized suckers or crowns are selected for planting on the same field to ease the care and maintenance of the crop as well to ensure that the fruit is harvested at the same time. The aims in the layout of a pineapple land are to control water runoff, facilitate good drainage and prevent root and heart rot, uniform distribution of sunlight to all plants. Planting is done by hand, with or without the aid of a planting machine. Plant pineapple between July and December because rapid growth, uniform stand. Do not plant between February and April because the temperatures become progressively lower and

retarded growth (Anonymous, 2005). Commercial production consists of a series of fruit cycles the number depending on the effectiveness of pest and disease management.

Important growing provinces are Prachuab Khirikhan, Phetchburi, Chonburi, Rayong, Chachoengsao, Chantaburi and Trat where large areas are devoted to growing pineapple for canneries. The harvested area, production and yield per rai of pineapple from various provinces in Thailand, Prachuap Khiri Khan province is the most harvested area (276,587 rai from the whole kingdom is 613,800 rai) and 824,828 tons from 2.2 millions tons of the whole production (Table 1.2).

.

Table 1.2 Pineapple of Thailand: Area, production and yield per rai by province, 2003-2005.

Harvested area (Rai)			Production (Tons)			Yield per rai (Kgs.)			Province
2546 2003	2547 2004	2548 2005	2546 2003	2547 2004	2548 2005	2546 2003	2547 2004	2548 2005	
508,877	**556,275**	**613,800**	**1,899,424**	**2,100,979**	**2,183,280**	**3,733**	**3,777**	**3,557**	**Whole Kingdom**
11,468	12,577	13,505	45,631	48,202	51,468	3,979	3,833	3,811	Lampang
5,462	17,860	26,971	20,483	88,255	109,647	3,750	4,941	4,065	Phitsanulok
-	1,032	1,111	-	4,003	3,664	-	3,879	3,298	Uttaradit
-	130	180	-	496	566	-	3,815	3,144	Kamphaeng Phet
25,305	20,039	21,355	101,878	81,859	84,433	4,026	4,085	3,954	Uthai Thani
6,146	1,026	1,085	21,757	3,549	3,639	3,540	3,459	3,354	Loei
14,320	14,350	14,637	58,955	59,623	48,625	4,110	4,155	3,322	Nong Khai
9,412	5,927	6,840	32,716	19,446	23,261	3,476	3,281	3,401	Nakhon Phanom
4,465	4,002	4,402	15,628	13,411	13,338	3,500	3,351	3,030	Suphan Buri
10,662	10,579	11,826	62,970	61,032	66,072	5,906	5,769	5,587	Chachoengsao
5,658	5,350	6,442	26,676	24,877	27,639	4,715	4,650	4,290	Chanthaburi
14,573	18,577	25,273	49,825	63,570	84,841	3,419	3,422	3,357	Trat
53,919	56,377	58,524	268,056	315,770	309,416	4,971	5,601	5,287	Rayong
27,350	30,290	31,980	170,445	205,298	191,429	6,232	6,778	6,005	Chon Buri
30,052	30,563	32,267	95,798	95,156	93,533	2,855	3,113	2,899	Kanchanaburi
28,660	30,367	31,482	90,680	92,647	86,799	3,164	3,051	2,757	Ratchaburi
30,005	34,974	36,503	101,117	111,190	108,500	3,370	3,179	2,972	Phetchaburi
216,358	251,055	276,587	673,090	764,503	824,828	3,111	3,045	2,982	Prachuap Khiri Khan
15,062	11,200	12,930	73,819	48,092	51,582	4,901	4,294	3,989	Chumphon

Source: Center for Agricultural Statistics, Office of Agricultural Economics, Ministry of Agricultural & Co-Operatives

1.1.2 Diseases

Diseases of pineapple include plant as well as fruit problems. A heart and root rot is caused by *Phytophthora parasitica*, *P. cinnamomi*. Some root rots are caused by *Pythium* spp. Fruitlet core rot, incited by *Penicillium funiculosum* and *Fusarium moniliforme var. subglutinans*, is problematic sporadically. Butt rot caused by *Thielaviopsis paradoxa*. Black rot of pineapple is caused by *Ceratocystis paradoxa*. Pineapple wilt is a serious disease, occurs only in association with a mealybug and a closterovirus (PMWaV). Yellow spot virus (tomato spotted wilt virus) also occurs in pineapple but is not usually a severe problem.

Bacterial diseases caused by *Erwinia carotovora* and *E. chrysanthemi* can be problems of the fruit. The disease affects the quality of the fruit and is detected after reaching the consumer. Pink disease of the fruit is caused by *Acetomonas* spp. and is only a problem in canned fruit. While some losses due to fruit diseases occur, they are not large enough to justify the costs of control even though annual losses may reach a few million dollars.

1.1.3 Pineapple heart rot disease

Heart rot, which is also known as top rot in Australia, was first described in the 1920s. Worldwide losses are highly variable, and are caused by plant mortality rather than reduced fruit size or quality. Heart rot has been reported in Australia, Hawaii, the Philippines, South Africa, Taiwan and Thailand. In Thailand, a heart rot disease was first reported by Leelasetkul in 1972 (Takaya et al, 1980). Heart rot is caused by *Phytophthora parasiticai*, *P. cinnamomi* and *P. palmivora*; *P. parasitica* and *P. cinnamomi* are most common (Ploetz, 2003) and caused by *Fusarium* sp., *Pythium aphanidermatum* (Andrew and Stephen, 2005). This disease is generally found in newly planted pineapple and occasionally in developing plants and complete rotting of the central portion of the stem. In the initial stages, the leaves turn yellowish and white decaying areas which have brownish margins further rotting produce specific odor. The sudden death of plants after heavy rains is a characteristic of the disease (Singh, 2000). The rot symptom is usually spreads from lower part of plant to root. Dissemination, transmission by planting of infected suckers, infecting and spreading well at higher temperature (25-30ºC) on dry and highly alkaline soil (Anonymous, 2003).

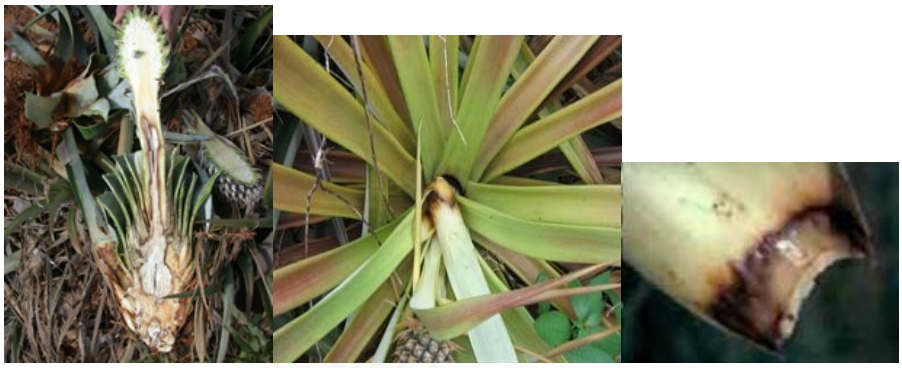


Fig 1.4 Signs and symptoms of the pineapple heart rot disease.

1.1.4 Morphology and epidemiology of *Phytophthora parasitica*

Hyphae of *P. parasitica* typically are 3-9 µm in width. The terminal or sometimes intercalary or lateral, ovoid to globose sporangia, 20-42 x 25-50 µm are borne on sporangiophore. Zoospores are 5-8 x 8-12 µm. *P. parasitica* are heterothallic and generally from oospores only when the A1 and A2 mating types are present. Chlamydospores germinate in moist soil to produce hyphae that can infect roots or immature leaf and stem tissue directly, or terminate in the formation of sporangia. In turn, zoospores are the important infective propagules. Since their production and release, and infection and disease development are all favoured by high soil moisture, heart rot is especially prevalent in poorly drained soils.

Free water is required for sporangium formation and zoospore release. Infection by zoospores is through leaf axils during the first 3-4 months following planting. It grows up the root, through the older part of the stem and eventually to the stem apex, causing the heart rot symptom. Little evidence exists for secondary spread from crowns (Ploetz, 2003).

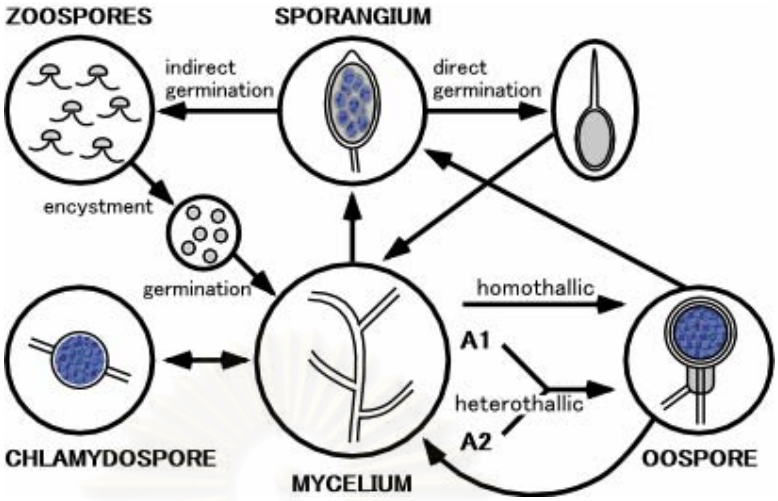


Fig 1.5 Life cycle of *Phytophthora* sp.

Souse: http://upload.wikimedia.org/wikipedia

1.1.5 Control of disease

Good soil drainage and use of healthy planting material at helps in minimizing the spread of the disease. Soil fumigation with chloropicrin is a satisfactory control for disinfestation of small planting areas or soil fumigating with 1,3-dichloropropene. Dipping slips before planting in Bordeaux mixture has been shown to provide partial control in replanted fields, and captafol, as a preplant dip and drench suckers intended for planting in fungicides such as fosetyl-Al or development of resistance in the fungus against fosetyl-Al can be avoided by using it in combination with mancozeb at a total of 50 g/20 l of water, every 3-6 months.

There is no evidence of natural resistance to *P. parasitica* in the above genera, although some interspecific hybrids may be resistant to this species and *P. cinnamomi* (Anonymous, 2003) or applied at planting and again after one and two months by ridomil (0.8 kg a.i. ha^{-1}) and aliette (8 kg a.i. ha^{-1}) controlled *P. cinnamomi* better than the standard difolatan (11 kg a.i. ha^{-1}) (Allen *et al*, 2005). Pre-plant dips and post-plant foliar applications of fosetyl-Al are also effective. Metalaxyl is effective, but is registered only as a pre-plant dip. Tolerance to metalaxyl can develop after prolong use. '59-656', a cultivar that was developed at the Pineapple Research Institute of Hawaii, is resistant to both *P. cinnamomi*, *P. nicotinanae*. It has fruit characteristics, quality and yield potential that are similar to the those for 'Smooth Cayenne' (Ploetz 2003). Rodriguez *et al* (2002) studies in vitro bioassay to differentiate pineapple plant resistance levels to *P. parasitica* (heart rot disease).

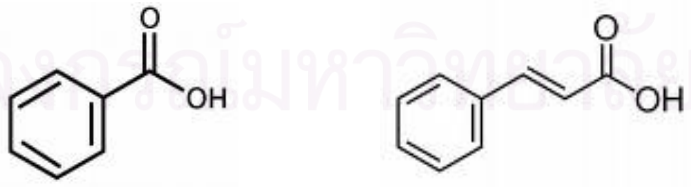
Because of that, using the same types of fungicide or single type of fungicide especially metalaxyl to encouragement the fungi, then it can adapt themselves to resistance against metalaxyl fungicide (Mukalazi *et al*, 2001).

1.2 Literature search on the antimicrobial activity of benzoic acid derivatives

Plants have defended themselves against potential pathogenic microorganisms to ensure survival, growth and development. Because of that, plants have evolved complex, integrated defenses system against pathogenic microorganisms. These mechanisms include biochemical pathways for the synthesis of different compounds.

Phenolic acids are compounds diverse in structure but characterized by hydroxylate aromatic rings such as many benzoic acid derivatives. Further, they form a large group including the widely distributed hydroxybenzoic and hydroxycinnamic acids. It has been shown the biocidal action of phenolic acids in vitro against bacteria and fungi. In vitro evaluation of the phenolic acid effect either one-by-one and in a mixture of phenolic over *Alternalia alternata* spore germination was done with various concentrations.

Benzoic acid was discovered in the 16th century. The dry distillation of gum benzoin was first described by Nostradamus (1556). In 1875 Salkowski discovered the antifungal abilities of benzoic acid, which were used for a long time in the preservation of benzoate containing fruits. Benzoic acid and its salts are used as a food preservative. Benzoic acid inhibits the growth of mold, yeast and some bacteria. It is either added directly or created from reactions with sodium, potassium or calcium salt. The mechanism starts with the absorption of benzoic acid in to the cell. If the intracellular pH changes to 5 or lower (Anonymous, 2007).



A B

Fig 1.6 Structure of benzoic acid (A), cinnamic acid (B).

It was found infected tomato fruits showed significantly higher concentrations of vanilic acid in the epicarp. Chlogenic acid and the mixture of phenolic acid inhibit spore germination by 30%, where as caffeic acid and vanilic acid inhibit 16% at 500 mM (Ruelas *et al*, 2006). The research of phenolic compounds accumulated in infected tissues, He *et al* (2007) reported about the contents of free salicylic acid and conjugated salicylic acid were determined by using TLC and HPLC at different time after attack by *Tetranychus urticae Koch* in the first true leaf of frijole (*Phaseolus vulgaris.* L) plant. A maximal nine fold increase in the free salicylic acid content in the attacked leaves was observed. The content of free salicylic acid in the systemic leaves increased about 500% while the content of conjugated salicylic acid in the systemic leaves increased.

The biologically active constituents isolated from Galla rhois were characterized as the phenolics methyl gallate and gallic acid by spectroscopic analyses. Methyl gallate was highly suppressive to *Magnaporthe grisea, Botrytis cinerea* and *Puccinia recondita*, where as gallic acid exhibited good antifungal activity to *M. grisea* and *Erysiphe graminis*. These two compounds were ineffective against rice sheath blight caused by *Rhizoctonia solani*. Methyl gallate did not adversely affect conidial germination (94%) but significantly inhibited appressorium fotmation (7%) of *M. grisea* (Ahn *et al*, 2005).

Benzoic acid derivatives have been from dichloromethane extract of the leaves of *Piper dilatatum*. Four of the benzoic acid derivatives (genuine acid, taboganic acid, methyl ester of 2,2-dimethyl-6-carboxychroman-4-one, and methyl ester of 2,2-dimethyl-3-hydroxy-6-carboxychroman) displayed antifungal properties against *Cladosporium cucumerinum* in direct bioautography on TLC plates (Terreaux *et al*, 1998). Vinyl monomers with phenol and benzoic acid as pendant groups were synthesized, and their antimicrobial activities were examined on equal weight basis using the halo zone test. For both bacteria and fungi, the halo zone diameter decreased in the order of *p*-hydroxyphenyl acrylate > allyl *p*-hydroxyphenyl acetate ≈ *p*-2-propenoxphenol. Polymerization of the monomers decrease their antimicrobial activity significantly. Antimicrobial polymers could find a successful application such as coating on glassy polymers (Park *et al*, 2001).

2,5-Dimethocybenzoic (DMB) acid was tested as an antifungal compound to control the postharvest decay pathogens of strawberry fruits. The compound completely inhibited in vitro spore germination and mycelial growth of *Botrytis cinerea* and *Rhizopus stolonifer* at a concentration of 5×10^{-3} M. The effect of DMB acid treatments on the decay of strawberry fruits, stored at 20°C or at 3°C plus a period of simulated shelf-life storage, was investigated. Its practical use on berriws under field conditions has been also tested. The best results were obtained when fruits were dipped for 1 min 10-2 DMB acid in combination with 0.05% (v/v) Tween 20 (Lattanzio *et al*, 1996).

Sivakumar *et al* (2002) to study of investigate the effect of the volatile GRAS compound cinnamaldehyde on postharvest diseases and overall quality of rambutan. Laboratory experiments demonstrated that 30 ppm cinnamaldehyde completely inhibited mycelial growth and germination of conidia of *Botryodiplodia theobromae, Colletotrichum gloeosporioides* and *Gliocephalotrichum microchlamydosporum* which cause the postharvest disease, stem-end rot, anthracnose and brown spot, respectively. When cinnamaldehyde (30 ppm) impregnated blotting sheets were used in commercial packaging, incidence and severity of all three postharvest diseases decreased. Treated fruits retained color, overall quality and sensory characters at 13.5°C and 95% RH for 14 days.

The essential oils isolated from nine geographical provenances of indigenous cinnamon (*Cinnaminmum osmophloeum* Kaneh.) leaves were examined by GC-MS and their chemical constituents were compared. According to GC-MS and cluster analyses the leaf essential oils of the nine provenances and their relative contents were classified into six chemotype; cinnamaldehyde type, cinnamaldehyde/cinnamyl acetate type, cinnamyl acetate type, camphor type and mix type. Result from the antifungal tests demonstrated that the leaf essential oils of cinnamaldehyde type and cinnamaldehyde/cinnamyl acetate type had an excellent inhibitory effect against white-rot fungi, *Trametes versicolor* and *Lenzites betulina* and brown-rot fungi, *Laetiporus sulphureus*. Cinnamaldehyde possessed the strongest antifungal activity in comparison with other constitue (Cheng *et al*, 2006).

The study describes inhibitory properties of combinations of oil of fennel, oil of anise or oil of basil with either benzoic acid or methyl paraben against *Listeria monocytogenes* and *Salmonella enteriditis*. Micro-organisms were cultured at 37°C in

broth and viable counts measured over a 48-h period. *S. enteriditis* was particularly sensitive to inhibition by a combination of oil of anise, fennel or basil with methyl paraben after 1 h *L. monocytogenes* was less sensitive to inhibition by each combination however there was a significant reduction in growth by combinations of all oils and methyl paraben at 8, 24 and 48 h. Synergistic inhibition by one or more combinations was evident against each micro-organism (Fyfe *et al*, 1998).

Post-harvest potato diseases are responsible for significant economic loss. Tuber infection may occur naturally through lenticels and eyes or mechanically through wounds incurred during harvest. There are few fungicides approved for use on human foodstuffs and there is necessity to screen the efficacy of alternative compounds against pathogens responsible for post-harvest disease in potatoes. In vitro trials were conducted to evaluate the effect of several organic and inorganic salt compounds and two commercial fungicides on mycelial growth, sporulation and spore germination of *Alternaria alternate, Botrytis cinerea, Fusarium solani* var. *coeruleum, Phytophthora erythroseptica, P.infestan, Verticillium albo-atrum* and *V. dahliae.*

The effects of various salt compounds on these fungi were evaluated at three concentrations: 0.002, 0.02 and 0.2 M. Overall, mycelium growth and spore germination of all pathogens were strongly inhibited by sodium metabisulfite and propyl-paraben. Spore germination in most pathogens was consistently inhibited by the aluminum compounds (aluminum chloride, aluminum acetate and alum) and the commercial fungicides mancozeb and copper sulfate (Mills *et al*, 2004). Furthermore, the several salt compounds were tested in vitro as inhibitors of *Erwinia carotovora* subsp. *atroseptica* and *E.carotovora* subsp. *carotovora*, causal agents of bacteria soft rot of potato. At the concentrations 0.002, 0.02 and 0.2 M with sodium metabisulfate, propyl-paraben, alum, potassium sorbate, calcium propionate and copper sulfate pentahydrate were completely inhibitory at the lowest concentration (0.002 M) (Mills *et al*, 2006).

The effects of cinnamic acid and its derivatives on the activity of mushroom tyrosinase. Results showed that cinnamic acid, 4-hydroxycinnamic acid and 4-methoxycinnamic acid stringly inhibited the diphenolase activity of mushroom tyrosinase and the inhibition was reversible. The IC_{50} values were estimated to be 2.10, 0.50 and 0.42 mM (Shi *et al*, 2005).

Two types of fungicides are used for the control of potato late blight; contact fungicide (more properly defined as non-systemics, also called residual fungicide) and systemic fungicide. Cinnamic acid derivatives are systemic fungicide. Dimethomorph is one of the most recently developed fungicides. Commercialized since 1990, it is basically a transluminar fungicide that is reported to have excellent antisporulating activity, preventing sporangium and oospore production. Dimethomorph affects the formatin of the cell wall by promoting its lysis and cell death. It is considered as a low-risk aget for promoting the development of resistance to it in the pathogen (Enrique *et al*, 2000).

From these literature research show that benzoic acid derivatives may control in the plant disease resistance. From the reasons above, fungi caused the pineapple heart rot disease can adapt themselves to resistance metalaxyl fungicide so that the chemical in benzoic acid derivatives should be alternative to control the pineapple heart rot disease in pineapple field of Thailand.

1.3 Objectives of this research

The objectives of this research were to isolate and identification pathogenic of pineapple heart rot disease and to find the evaluation activity of benzoic acid and cinnamic acid derivatives against pineapple heart rot disease.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

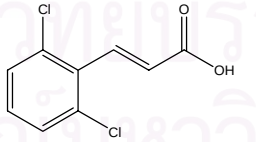
Benzoic acid derivatives and related compounds, and Tween 80 were purchased from Fluka. Cinnamic acid derivatives were received as gifts from Ms. Sujittra Deesamer (Faculty of Science, Chulalongkorn University). Dimethylsulfoxide (DMSO) was purchased from Merck. 2,6-Dichlorocinnamic acid was prepared according to Deesamer (2000).

2.2 Equipments

Polytetrafluoroethylene (PTFE) membrane 13 mm diameter syringe filter (0.2 µm pore size) was purchased from Chrom Tech. The ^{1}H-NMR spectra were determined with a Varian nuclear magnetic resonance spectrometer model Mercury 400.

2.3 Synthesis of 2,6-dichlorocinnamic acid

Followed by that described by Ms. Deesamer (2000), malonic acid 3.12 g (0.03 mol) and 2,6-dichlorobenzaldehyde 5.20 mL (0.03 mol) were dissolved in 5.20 mL of anhydrous pyridine and 0.28 mL of piperidine. The mixture was refluxed for 1.5 h, then cooled to room temperature (RT) and poured into 10 g of ice containing 8 mL of conc. HCl and 26 mL of H_2O. The colorless solid was precipitated, filtered and washed with ice-water and recrystallized with 95%EtOH.



2,6-dichlorocinnamic acid: pale yellow needle crystal, m.p. 195-196 °C, R_f 0.66 (ethanol); ^{1}H-NMR ($CDCl_3$) δ (ppm): 6.82 (1H, d, *J*=16.37 Hz, CH=C**H**-CO_2H), 7.23 (1H, m, Ar**H**), 7.37 (2H, d, *J*=8.06 Hz, Ar**H**), 7.92 (1H, d, *J*=16.37 Hz, ArC**H**=CH); ^{13}C-NMR (DMSO) δ (ppm):170.8 (-**C**OOH), 135.1, 131.6 (2x1C), 130.1 and 128.9 (2x1C) (aromatic carbons), 140.4 and 159.0 (olefinic carbons).

2.4 Sample collection

The pineapple leaves showing symptoms of heart rot were collected from 5 pineapple fields at Cha-Am district, Phetchaburi province in October 2005 and December, 2006. All leaf samples were kept in paper bags and processed within 24 hours after collection. Furthermore, the equipment and selective media were necessary to isolate the pathogenic fungi *in situ*.

2.5 Isolation of the plant pathogens

2.5.1 Fungal isolation

Fungi were isolated using plant tissue transplanting methods as described by Agrios (1997). The pineapple leaves showing heart rot collected from the fields were cut into small sections (0.5x0.5 cm) from the margins of lesion regions to contain both diseased and healthy tissue. Then, sections were placed in surface sterilized 1.5% sodium hypochlorite (NaOCl) for 5 mins, rinsed once in sterile distilled water and blotted dry on sterile paper towels to remove excess NaOCl. The sterile leaf pieces were then placed onto potato dextrose agar (PDA) in Petri dishes, usually three to five pieces per dish. Petri dishes were incubated in the dark at RT (28-30°C) for 3-4 days. Following incubation, hyphal tips from the margins of actively growing cultures were removed with a sterile needle and plated onto fresh PDA plate.

2.5.2 Isolation of *Phytophthora* species

The infected leaf sections were cleaned with sterile paper towels and then plated onto selective RNV medium plates. Plates were incubated in the dark at RT for 3-4 days. The hyphal tips from the leaf section during the incubation period were placed on carrot agar plates (CA) (Puphaiboon, 2006).

2.6 Pathogenicity test

All fungi isolates and *Phytophthora* isolates were determined pathogenicity of disease on pineapple under laboratory using postulation methods and modified Koch's methods (Agrios, 1997). The healthy suckers of pineapple were surface sterilized by dipping in 1.5% NaOCl for 5 mins followed by rinsing with sterilized distilled water. The suckers were then allowed to air dry. After air drying, each leaf of suckers was slightly wounded with sterile knife.

The zoospore suspension of *Phytophthora* species was obtained by temperature shift method described by Takaya *et al*. (1980). Briefly, colonies of *Phytophthora* on CA medium were submerged in sterilized distill water. The colonies were incubated at RT for 3-4 days. The sporangia were produced, after that moved to 4°C for 30 min and then removed to RT. In 30-60 min, many zoospores swarm out from zoosporangia. Final concentration of zoospore suspension (Fig 2.1A) used for pathogenicity test was 10^7spore/mL.

Spore suspensions were obtained by flooding 5-7 days old mycelial disk (8 mm diameter) of spore producing fungal isolates with sterile distilled water and shaking. The suspension was filtered through sterile muslin cloth. The final concentration of spore suspension was 10^7 spore/mL (Fig 2.1B).

Mycelial suspensions were obtained by flooding colonies of non spore producing fungal isolates and agitating the surface with a glass rod.

The artificial inoculations by wounded suckers were dipped directly in the spore suspensions or mycelial suspensions. The suckers were placed in plastic bags containing wet cotton wool for humidity maintaining. The suckers in plastic bags were incubated at RT for 5-7 days. Following inoculation, the incidence of infected suckers showing symptoms of heart rot disease was recorded. The pathogenic isolates were reisolated to be pure culture. The morphological characteristics of the culture obtained from the second isolation which was the same as that obtained in the first isolation were used for further study.

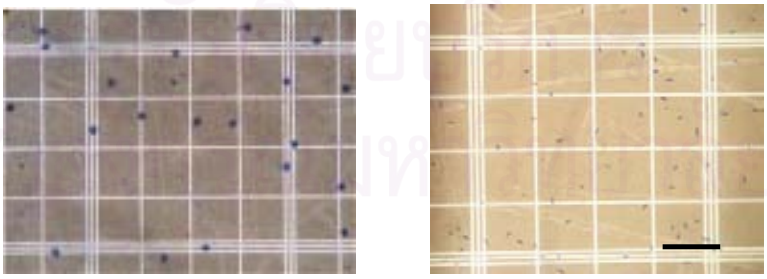


Fig 2.1 Microscopic field of haemacytometer slide with zoospores of *Phytophthora* sp. (A) and spore suspension of *Fusarium* sp. (B) (bar = 10 µm)

2.7 Identification and classification

Identification was conducted followed by Waterhouse (1970), Erwin and Ribeiro (1996) for *Phytophthora* species. Indentification of *Fusarium* species were followed by Burgess *et al.* (1994), Leslie and Summerell (2006). The methods and modified were described as below.

2.7.1 Maccroscopic characteridtics

Phytopathogenic fungi were cultured in media namely PDA and CA. The colony characteristics of each isolate were observed; for example, shape, size, color, margin, and pigment.

2.7.2 Microscopic characteristics

The specimens for light microscopy were mounted in lacto phenol blue for observation the characteristic mycelial, spore arrangements and other characteristics necessary for a definitive identification on an Olympus CH2 research microscope. Examine the preparation microscopically, first under the low-power (10X) objective and the under high-power (40X), or under oil immersion (100X) if suspicious fungal structures were seen. The slide culture preparing was described by Mat-arhin (2006) as below.

1. A piece of filter paper was placed into a sterile Petri dish, then a pair of thin glass rods were placed to serve as supports for a glass slide.

2. Place a 1x1 mm block or plug of PDA or CA on the surface of the microscope slide. The margins of the agar plug in four places were inoculated with small mycelia of the studies colony using the tip of a needle. Gently heated a cover slip by passing it quickly through the flame and immediately or autoclaved cover slip, placed it directly on the surface of the inoculated agar block.

3. Added sterilized water in the Petri dish to moist filter paper. Then placed the lid on the Petri dish and incubated at RT for 3 to 5 days.

4. When growth visually appears to be mature. Prepared slide by drop lacto phenol blue on then put the cover slip from Petri dish to the slide with a pair of forceps. After the cover slip had been removed from the agar block, lower agar slide was prepared again by removing agar block and stained with lacto phenol blue. The mount can be prepared for further study by rimming the outside margins of the cover slip with clear fingernail polish.

2.7.3 Molecular identification

2.7.3.1 DNA extraction

DNA extraction was carried out using cetyltrimethylammonium bromide (CTAB) described by Tansuwan (2006). 50 mg of mycelium were homogenized in 1,000 µL of washing buffer (0.1 M Tris-HCl (pH 8.0), 2% 2-mercaptoethanol, 1% polyvinylpyrrolidone and 0.05 M ascorbic acid) with a pestle in a mortar. Then the sample was transferred to 1.5 mL microcentrifuged tube and centrifuged the sample in washing buffer at 15,000 g for 3 min. After removal of the supernatant, the pellet was washed 4-5 times with the washing buffer and centrifuged at 15,000 g for 3 min. DNA was then extracted from the washed pellet respectively, by adding 700 µL of 2X CTAB solution and incubation at 65°C for 1 h, extraction with $CHCl_3$: isoamyl alcohol (24:1, v/v) and centrifugation the suspension at 15,000 g for 8 min. The supernatant was extracted using the same $CHCl_3$: isoamyl alcohol (24:1, v/v) centrifuged the suspension at 15,000 g for 8 min and transferred the supernatant 500 µL into a new microcentrifuged tube.

After that, nucleic acid was precipitated by adding 500 µL isopropanol into the supernatant cooled in ice water for 30 min and then centrifuged at 8,000 g for 10 min. The supernatant was poured into waste beaker and then suspended the pellet by adding 500 µL of 70% EtOH. After centrifugulation at 8,000 g for 5 min, the DNAs was dried at RT. 100 µL of TE buffer was added to dissolve the fungal DNA and the solution was kept at -20°C until use.

2.7.3.2 PCR amplification and electrophoresis

The ITS region of DNAs was amplified with the primers ITS_{1F} and ITS_4 (Duggal *et al*, 1997). PCR amplification was performed in 10 µL in reaction mixtures as presented in Table 2.1.

Table 2.1 Component of the mixture used in Polymerase Chain Reaction (PCR).

Compound	Final concentration	Volume (μL)
10X PCR buffer without $MgCl_2$	1X	1.0
2 mM dNTP mixed	0.2 mM	1.0
5 u/μL *Taq* DNA polymerase	0.5 units/10μL	0.1
25 mM $MgCl_2$	2.5 mM	1.0
20 μM Primer I (ITS1 F)	1 μM	0.5
20 μM Primer II (ITS4)	1 μM	0.5
DNA template	-	1.0
Sterilized distilled water	-	4.9
Total volume		10

The amplified reactions were performed in a Thermal cycler. The conditions of reaction were described as below.

Initial denaturation	94°C	5 min	
Amplification			
Denaturation	94°C	1 min	38 Cycles
Annealing	51°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	5 min	
Holding	4°C		

PCR products were loaded into 1.5% agarose gel in TBE buffer setting power supply at about 100 volt for 0 min after staining with ethidium bromide, band patterns were visualized on a UV transilluminator using 312 nm wavelengths.

2.3.7.3 DNA sequencing

The DNA sequencing was analyzed at Macrogen, Inc. Seoul, South Korea. ITS sequences obtain from Genbank DNA database (http://www.ncbi.nlm.nih.gov) using BLAST program.

2.8 Antifungal activity of benzoic acid and cinnamic acid derivatives against radial growth on agar plate assay

The bioassay was conducted using the agar medium assay (Thedpitak, 2005). Certain compounds were chosen from the former research on anti-phytopathogenic fungal activity of benzoic acid and cinnamic acid derivatives. Those included benzoic acid, *p*-anisic acid, salicylaldehyde, cinnamaldehyde and two esters namely *n*-propyl 4-hydroxybenzoate and *n*-butyl 4-hydroxybenzoate. The compounds in a series of cinnamic acid derivatives included 4-phenoxycinnamic acid, 4-nitrocinnamic acid, 4-*tert*-butyl cinnamic acid, 4-trifluoromethylcinnamic acid, 2,6-dichlorocinnamic acid and α-methyl-*trans*-cinnamaldehyde. All selected compounds were dissolved in DMSO or Tween 80 and then applied to a 13 mm diameter PTEF membrane syringe filter (0.2 µm pore size) before being added to sterile PDA (sterilization at 15 psi and 121 ºC) to obtain the experimental concentration.

The effect on mycelial growth was examined by 8 mm diameter of mycelial disk, taking from the margin of stock cultures (2 days old for rapid fungi, 5 days old for *Phytophthora parasitica* and 7 days old for another fungi) using the sterilized cork borer to remove mycelial disk and then placed at the center of Petri dish (CA for *Phytophthora parasitica*, Czapek agar for rapid fungi and PDA for another fungi). The Petri dish containing the medium supplemented with 100 ppm of benzoic acid and cinnamic acid derivatives for screening the compound that shown the best result. A control plate contained a medium plus DMSO or Tween 80. The plates were incubated at RT. Radial measurements of growth were taken when fungi of the control plate reached the edge of the control plate. The colony diameter was measured in millimeter and calculated for percent inhibition.

The growth of the colonies in control sets was compared with that of various treatments and the difference was converted into percent inhibition. Each test was replicated 5 times, and the data was averaged. Fosetyl-Al and metalaxyl, two commercially available fungicides were employed as reference compounds. The growth number of the colonies in control sets was compared with that of various treatments and the difference was converted into percent inhibition.

Percentage inhibition of spore germination = [(C-T) × 100/C]

C: mean number of colonies of control plate

T: mean number of colonies of treatment plate

2.9 Antifungal activity of *n*-butyl 4-hydroxybenzoate at various concentrations against mycelial growth in broth

The mentioned compound that exhibited the best result of the inhibition of mycelial growth at 100 ppm on agar plate assay was selected and then tested with mycelial growth in carrot broth (CB) (for *Phytophthora parasitica*), czapek's broth (for rapid growth fungi) and potato dextrose broth (PDB) at various concentrations (0, 25, 50, 100, 250 ppm). The mycelial disks of the pathogenic fungi were taken from the edges of stock culture plate and then moved into the 250 mL flask containing 100 mL medium broth and supplement with *n*-butyl 4-hydroxybenzoate at various concentrations as described above and compared with fresh medium without treating as control flask.

The inoculated flasks were incubated at RT for 14 days. The mycelium of stationary cultures were harvested at the following time intervals: 0, 2, 4, 6, 8, 10, 12 and 14 days by filtration through Whatman No1 filter paper and then removed the filtrated mycelium into an incubator at 60°C for 3 times (3 days) and dry weights were measured until values being stable. Each treatment was replicated for 3 times.

2.10 Antifungal activity of *n*-butyl 4-hydroxybenzoate at various concentrations against spore germination

Spore suspensions were prepared under aseptic conditions according to a modified procedure described by Chang *et al.* (2007). Fungus spores of *Fusarium* sp. were grown on Petri plate containing PDA. Following 10 days of incubation at RT, fungal spores were removed using steriled water. The resulting suspension was filtered aseptically through a sterilized muslin cloth. The filtrate which was adjusted using sterilized water to a concentration of 10^4 spore/mL was estimated with haemacytometer slide (depth 0.1 mm, 1/400 mm^2) under microscope. The 1 mL (10^4 spore/mL) suspension was removed to each tube containing 9 mL of *n*-butyl 4-hydroxybenzoate at various final concentrations (0, 25, 50, 100, 250 and 500 ppm), which the final concentration of spore suspension was 10^3 spore/mL. These tubes

were incubated at RT at different times (0 and 1 h) and then 100 µL of the suspensions (10^3 spore/mL) was streaked aseptically on fresh PDA plates, 90 mm in diameter. The plates were incubated at RT for 48 h. The fungal colonies originated from germinated spores were recorded compared with DMSO plate and evaluate the percent inhibition of spore germination. The IC_{50} values (the concentration in ppm that inhibition 50% of the mycelium of fungi growth) were calculated by probit analysis (Cheng *et al*., 2006). The percentages of spore germination were calculated as follows:

Percentage inhibition of spore germination = [(C-T) × 100/C]

C: mean fungal colonies of control plate (mm)

T: mean fungal colonies of treatment (mm)

2.11 Antifungal activity of *n*-butyl 4-hydroxybenzoate against heart rot disease by detached pineapple leaves methods

The pineapple leave (Fig 2.2 A) was inoculated for providing experimental materials. Inoculation was carried out as follows: the healthy leaves of pineapple were pulled out from the intermediate part of the suckers (from leaf order 5-7 to 15-17, counting from top leaf) and then the surface was sterilized with 1.5% NaOCl and re-rinsed with sterilized water, air dried for 30 min. Following by washing and drying, pineapple leaves were wounded on the upper side using cork borer No. 2 (0.7 cm diameter) and sterilized by dipping in 90% EtOH between each wounding event. The leaves were wounded about 3-4 cm high from the basal of leave (nearly white and green color) on the upper surface and five leaves of pineapple for each treatment.

The preventative effect of the treated compounds, 20 µL of *n*-butyl 4-hydroxybenzoate at various concentrations (250, 500, 1000, 2000, 3000 and 5000 ppm) was dropping on the wounded leaves and then air dried for 30 min at ambient RT. The leaves were inoculated by pipetting 20 µL of spore suspension (10^7spore/mL) into the wound and covered with moisture cotton wool. Five leaves per treatment were placed in a plastic bag. The bags were sealed and then incubated at RT for 3-7 days. The lesion of disease incidence was recorded and compared with the control group that treated by sterilized distilled water. The experiments were repeated for 3 times.

2.12 Antifungal activity of *n*-butyl 4-hydroxybenzoate against heart rot disease on sucker in laboratory

The experimental technique is similar to that described above, with a few modifications. The healthy suckers (Fig 2.2B) about 10-15 cm (picked up from the fruits abnormally sprouting crowns, 10-20 crowns on a fruits) 2 months age, were used for the assay in laboratory. The healthy suckers were surface sterilized with 1.5% NaOCl and re-rinsed with sterilized water, air dried and wounded with knife. Two sections were generated.

The first part as a preventative test: the suckers were dipping directly into the solution of *n*-butyl 4-hydroxybenzoate at various concentrations (250, 500, 1000, 2000, 3000 and 5000 ppm) with 0.2% Tween 80 and then air dried for 30 min before dipping in spore or zoospore suspension (10^7spore/mL). The suckers were placed into a plastic bag (5 suckers per treatment per bag) containing moist cotton wool, bound the plastic bag and incubated at RT for 3-7 days. The disease incidences were checked by the number of suckers that showed the heart rot disease and compared with those dipped in sterilized water before dipped in spores or zoospore suspension. The experiments were repeated for 3 times.

The second part as a curative test: the suckers were infected with spore or zoospore suspension and incubated for 1 day. Infected suckers were dipped into the solution of *n*-butyl 4-hydroxybenzoate at various concentrations and then kept in plastic bag (5 suckers per treatment per bag) containing a moist cotton wool for maintaining moist condition. The disease incidences on the sucker were checked by the number of suckers that showed heart rot disease and compared with suckers dipped into the sterilized water before dipped in spores or zoospore suspension. The experiments were repeated for 3 times (Takaya *et al.*, 1980).

2.13 Synergistic study of metalaxyl and *n*-butyl 4-hydroxybenzoate

Synergist is a chemical which, when mixed with pesticide, may increase the pesticidal activity (Johnson and Cole, 2001). For this experiment, synergistic effect was performed by combination of metalaxyl with butyl 4-hydroxybebzoate at concentration of EC_{50} of metalaxyl and varied the ratio of metalaxyl:*n*-butyl 4-hydroxybenzoate at 0:1, 1:4,1:1, 4:1 and 1:0 v/v. The synergist compounds, 1 mL of each ratio was added to 9 mL of cooled carrot agar media after autoclaving. After

addition of the synergist, about 10 mL of medium was poured per 9 cm diameter Petri plate. Agar disks (8 mm in diameter) were cut from the margin of *P. parasitica* growing on CA for 5 days at RT. The disk was transferred to the center of an agar plate. Control plates contained only CA. There were three replicates per ratio. Mycelial growth was recorded to the mm along the longest radius after 5 days incubation in the dark at RT.

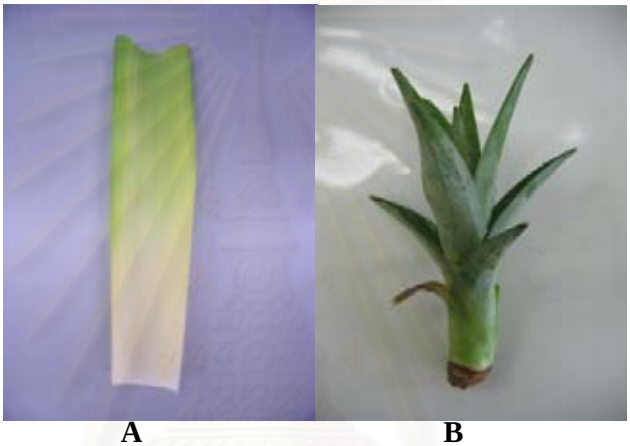


Fig 2.2 Pineapple leaf (A) and sucker (B) were used in this experiment.

CHAPTER III

RESULTS AND DISCUSSION

The pathogenic fungi from pineapple leaves collected from the field at Cha-Am district, Phetchaburi province were isolated. Koch's postulation method was used to prove which fungi caused the pineapple heart rot disease. *In vitro* antifungal activity against phytopathogenic fungi caused pineapple heart rot disease of benzoic acid and cinnamic acid derivatives were thoroughly evaluated. Antifungal activity was performed by agar medium assay according to the procedure described in Chapter II. Top compounds that expressed good antifungal activity against pineapple heart rot disease from agar medium plate assay at 100 ppm were collected for further study. The variation of the concentrations of selected compounds was carried out in broth medium. Antifungal activity on pineapple suckers which showed good activity at high concentration was also performed in laboratory. Synergistic combination of a selected compound with metalaxyl was evaluated.

3.1 Sample collection

A preliminary field survey was carried out at Cha-Am district, Phetchaburi province in October 2005 and December 2006 with the aim to observe the disease incidence in pineapple fields and to collect samples (Fig 3.1A). It was however difficult to find newly infected plants in older pineapple fields (Fig 3.1B). In some young pineapple fields, on the other hand very severe occurrence of the disease was observed. The disease was found not only in flat fields but also in the fields located on slopes of hillsides. Generally, the disease patches were formed at the lower part of the fields and caused heavier damages. Another interesting point was that there was an instance of the disease occurring along pathway of rain water runoff which indicated that inocula might be spread out by rain water.

A **B**

Fig 3.1 Pineapple field at Cha-am district, Phetchaburi province with heart rot disease (A), disease incidence in older pineapples (B).

In some fields with the evidence of being trace of earlier flooding, the disease plants were observed scatterly. The way to collect the pineapple samples with heart rot symptoms was the recognition of the earliest symptom at basal end of central leaves if the leaves were pulled out rightly. The affected plants did sometime not show any abnormality on their appearance, but the central leaves could be readily pulled out from their points of attachment displaying transparent water soaked soft rot, light brown margin at leaf bases and yellowing of upper parts of leaves. Another diagnostic sign of this disease was the specific odor of putrefaction that was invariably emitted but not odor occurred by bacteria (Fig 3.2).

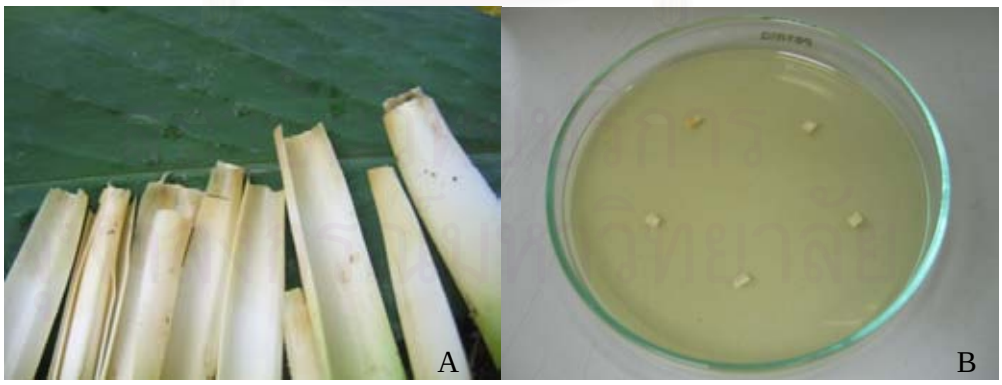


Fig 3.2 Sampling collection pineapple leaves with heart rot lesion (A) and isolation on RNV medium (B).

3.2 Isolation of phytopathogenic fungi

Phytopathogenic fungi were isolated from pineapple leaves that showed heart rot disease symptom (Fig 3.3). Fifteen pure cultures of fungi were placed on PDA or CA. The fungi were incubated at RT until they were fully grew on Petri dish, then the characteristics of fungi including their colony, colors, pigment and spore were carefully observed (Table 3.1). The characteristics and morphology of fungi are presented in Fig 3.4. The specimen of sample collection was important if that specimen was collected from the pineapple leaves nearly decayed or exhibited dark brown margin at the basal or bad smell caused by bacteria. When these specimens were brought to the isolation step, many bacteria and saprophytes were emitted from the specimen that made the isolation of true causal pathogenic fungi become difficult. The specimen ought to be fresh disease incidence with water soak only no dark brown margin, even though the pineapple leaves were still green color and not showed anything abnormally when the leaves were pulled out easily from intermediate part of pineapple sucker (Fig 3.2).

A **B**

Fig 3.3 Pathogenic fungal isolation on RNV medium for *Phytophthora* sp. (A), isolation on PDA for fungi (B).

Table 3.1 Characteristics of fungi isolated from pineapple leaves showing heart rot disease.

No	Isolate	Colony color (aerial mycelium)	Color pigment production on the PDA	Sporulation on PDA
1	RM1	White	Red	*Fusarium* sp.
2	RM2	White	Not produce	*Mycelia sterilia*
3	RM3	White	Not produce	*Mycelia sterilia*
4	RM4	Quit gray	Black	*Mycelia sterilia*
5	RT1	Light yellow	Yellow	*Fusarium* sp.
6	RT2	White	Purple	*Fusarium* sp.
7	RT3	White	Not produce	*Mycelia sterilia*
8	RK1	Gray	Gray	*Mycelia sterilia*
9	RK2	Heavy gray	Gray	*Mycelia sterilia*
10	RK3	Black	Black	*Curvulalia* sp.
11	RK4	White	Light brown	*Mycelia sterilia*
12	Rm1	White	Red	*Mycelia sterilia*
13	Rm2	White	Not produce	*Mycelia sterilia*
14	Rm3	White	Not produce	*Mycelia sterilia*
15	*Phytophthora*	White	Not produce	*Phytophthora* sp.

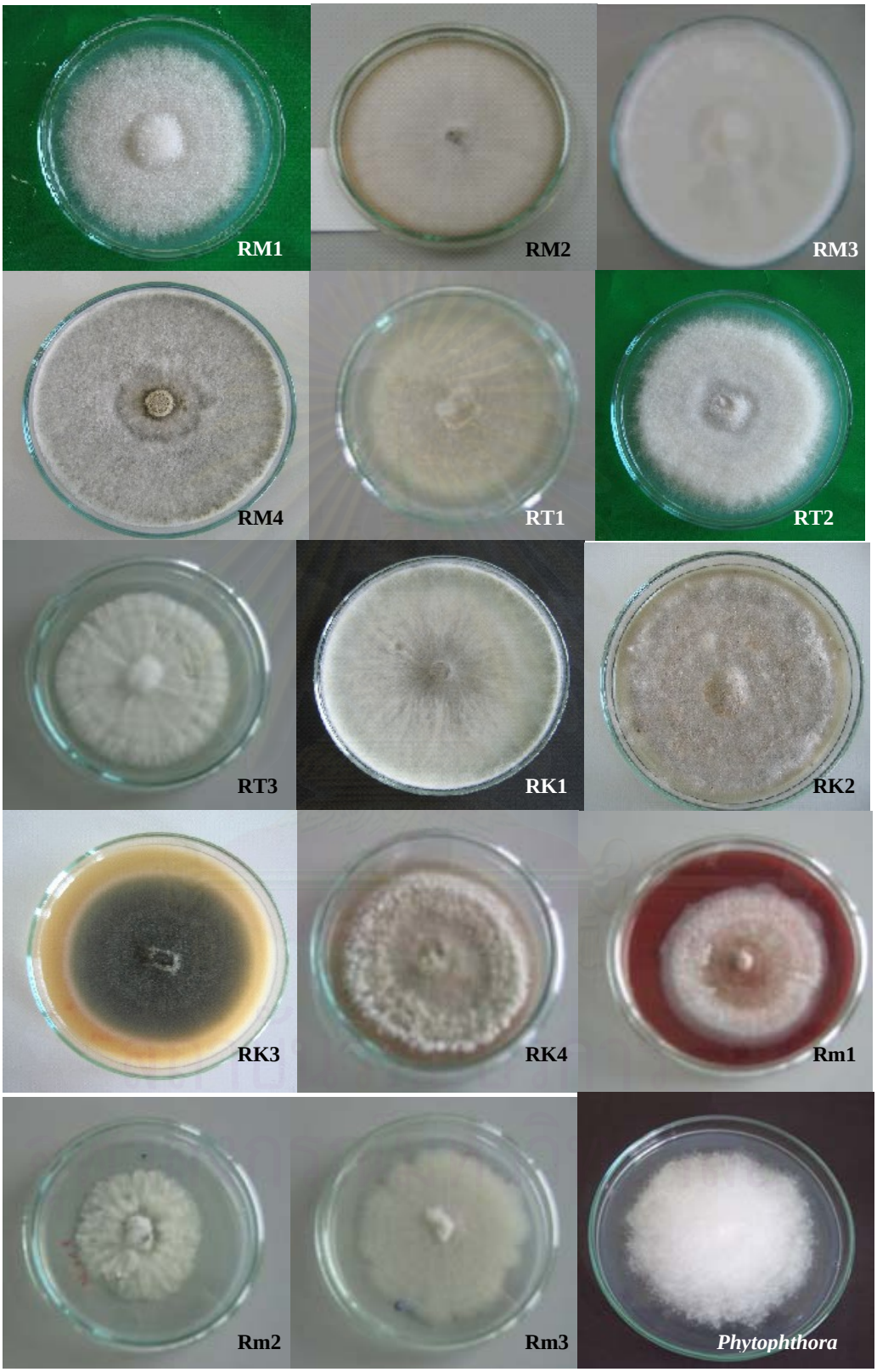


Fig 3.4 The fungi isolated from the pineapple leaves.

3.3 Pathogenicity test

In order to prove the etiology role of a microorganism in a particular disease, a strict set of procedure, known as Koch's postulates needed to perform with healthy suckers in laboratory. After inoculation of pineapple suckers with suspension, the suckers were kept in a plastic bag in the presence of wet cotton to maintain moist conditions. After inoculation for 5-7 days, all suckers were pulled out from the plastic bag and checked for the heart rot disease incidence. The results of Koch's postulate are shown in Table 3.2 and Fig 3.5.

Table 3.2 Phytopathogenic fungi isolated from pineapple leaves disease tissue proved by Koch's postulation methods.

No.	Isolates	symptom of heart rot disease*
1	RM1	+
2	RM2	-
3	RM3	-
4	RM4	-
5	RT1	-
6	RT2	-
7	RT3	-
8	RK1	+
9	RK2	-
10	RK3	-
11	RK4	-
12	Rm1	-
13	Rm2	-
14	Rm3	-
15	*Phytophthora*	+

* Appearance of pineapple heart rot disease

\+ : show heart rot disease

\- : not show heart rot disease

Fig 3.5 The suckers with and without heart rot disease when inoculated by suspension of fungi that isolated from the pineapple field.

Inoculation with isolate RM1 (A), Inoculation with isolate RM2 (B), Inoculation with isolate RM3 (C), Inoculation with isolate RM4 (D), Inoculation with isolate RT1 (E), Inoculation with isolate RT2 (F), Inoculation with isolate RT3 (G), Inoculation with isolate RK1 (H), Inoculation with isolate RK2 (I), Inoculation with isolate RK3 (J), Inoculation with isolate RK4 (K), Inoculation with isolate Rm1 (L), Inoculation with isolate Rm2 (M), Inoculation with isolate Rm3 (N), Inoculation with isolate *Phytophthora* (O).

The isolate RM1, RK1 and *Phytophthora* species were obtained from the inoculation of the heart rot disease to the healthy sucker which showed typical symptoms of disease such as complete soft rot of tissue and water soak. Isolate RM1 was the one that checked disease incidences for 7 days after inoculation. All suckers inoculation showed the heart rot disease symptom, white mycelial of fungi was covered all the suckers and dark brown leaf and water soak soft rot tissue and gave out specific odor of putrefaction, and cotton in the basal having red and purple color. The isolate RK1 and *Phytophthora* exhibited the symptom after inoculation for 5 days. The suckers were covered with fluffy white aerial mycelium growth of fungi. Although, the pineapple leaf was still green but the suckers were water soaked lesions, which had spongy like soft rot tissue appearance. The leaves were easily pulled out from the stem and gave out of specific odor but not like bad smell occurred from bacteria.

The other isolate: RM2, RM3, RM4, RT1, RT2, RT3, RK2, RK3, RK4, Rm1, Rm2 and Rm3 could not be destroyed the suckers to appear the heart rot disease after inoculation with suspension. The suckers were also healthy but some lesions on pineapple leave were noticed such as dark brown tissue and wilt or water soak at some lower leaf part of the sucker stem and the wound was brown, not different from the control. The suckers that inoculated with suspension of isolate RM2, RM3, RM4, RT1, RT2, RT3, RK2, RK3, RK4, Rm1, Rm2 and Rm3 were not only healthy green leaf, but they also grew and produced a new root. These isolates may thrive as a secondary infectious, which infected the plant when occurred disease. In causal organisms of heart rot and root rot of pineapple, *P. parasitica, P. cinnamomi, P. palmivora, P. meadii* and *P. melongenae* were reported from Hawaii and Australia (Rohrbach *et al*. 2002). Suzui *et al.* (1976) isolated the pathogenic fungi from pineapple and some economic plants, beside *Phytophthora* sp. and detected *Fusarium, Rhizoctonia, Pythium* and others. Only *P. parasitica* was isolated from the heart rot and root rot of pineapple in the laboratory level and the results of cross inoculation indicated that only *P. parasitica* isolated from pineapple, citrus and orchid caused heart rot in pineapple. It was suggested that *P. parasitica* be the dominant species of *Phytophthora* in pineapple in Thailand. Beside that Kueprakone *et al.* (1973) recognized that *P. palmivora* from durian was capable of infecting pineapple in an inoculation test in Thailand.

In this study, beside *Phytophthora* species, it was disclosed that the isolate RM1 and PK1 could cause pineapple heart rot disease. The isolates that were capable of making healthy suckers to express symptoms of heart rot disease would be identified. For developing chemical control method of a plant disease, epidemiological and ecological information about the disease and pathogen should naturally be taken into considerations.

3.4 Identification of caused pathogenic fungi

3.4.1 Identification of isolate *Phytophthora* sp.

Phytophthora isolated from infected leaf tissues of pineapple from the filed at Cha-Am district, Phetchaburi province was identified according to the protocol reported by Waterhouse (1970) and Erwin *et al.* (1996). Oomycetes exhibited filamentous growth habit, and were often inaccurately referred to be as fungi. Modern biochemical analyses as well as phylogenetic analyses based on sequences of ribosomal and mitochondrial genes suggested that oomycetes share little taxonomic affinity to filamentous fungi, but are more closely related to golden brown algae in the Kingdom Stramenopila. Therefore, oomycetes included a unique group of eukaryotic plant pathogens, which developed the ability to infect plants independently from true fungi. This suggested that oomycetes have distinct genetic and biochemical mechanisms for interacting with plants (Kamoun, 2000).

Phytophthora referred to as a fungal-like organism but it was classified under a different kingdom altogether: Stramenopila (previously named Chromista). This was a good example of convergent evolution: *Phytophthora* was morphologically very similar to true fungi yet its evolutionary history was quite distinct. In contrast to fungi, stramenopiles were more closely related to plants than animals. Whereas fungal cell walls were made primarily of chitin, stramenopile cell walls were constructed mostly of cellulose (Erwin *et al*., 1996).

These obtained results were recoded including stripe, spore, sporangiophore, shape, size, color, margin and pigment. Firstly, the characteristics of *Phytophthora* were cultured on PDA for 5 days and then prepared the fungi to study morphology of *Phytophthora*. The characteristics of colony like chrysanthemum flower and the mycelia silky sticky like spider web, zonate, abundant aerial mycelium fluffy white

color on PDA under the scattered light condition at RT (Fig 3.6). The culture mature (7 days) on PDA was rarely produced the sporangial compared with those on CA and sterilized water was poured into the plate until 5-10 mm above. The mycelium growth was then conducted under sterilized water which was incubated the mycelial on CA plate at RT for 3-4 days. When the mycelium was observed under light microscopic, the abundant of produced sporangial could be easily noticed. These CA plates were then kept at 18°C for 30 min and then to RT. Within 30-60 min, many zoospores swarm out from zoosporangia.

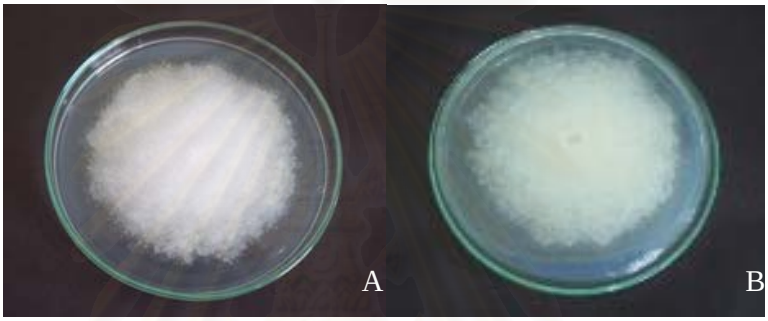


Fig 3.6 Colony type of *Phytophthora* species growth on PDA, up side (A) and down side (B).

The slide culture specimens for light microscopy were mounted in lacto phenol blue to observe the characteristics of mycelium, sporangium and others shown in Fig 3.7. The smooth walled and 3-4 µm (100X) wide of hyphae could be easily visualized and the mycelium was generally coenocytic with no septa or with a few septa when the culture was very old. Sporangiophores were slendered with long smooth sympodially branching 1-2 µm wide. Sporangia were produced at terminal, sometimes intercalary or lateral (Fig 3.7 A, B). Typical shape of the sporangia was ovoid to nearly global shape of 14-18 x 18-25 µm and length-breadth ratio (L/B) ~ 1.3. In addition, the characteristics of these sporangia were honey-color, smooth and thick cell wall, and of hyphaline marked as papillate. Zoospore spherical to oval released from sporangium (Fig 3.7 C, D).

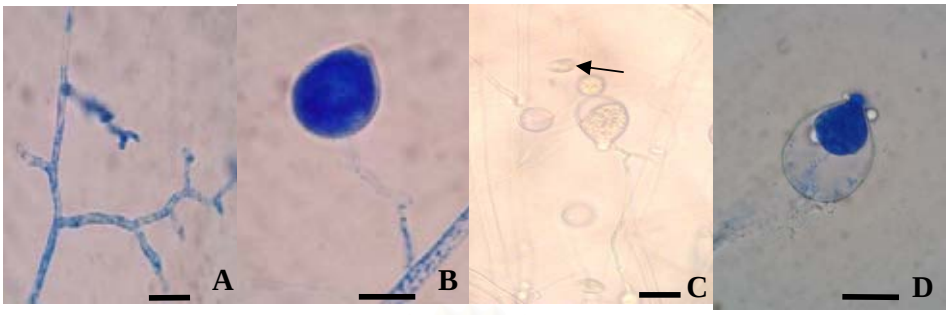


Fig 3.7 *Phytophthora* sp. under light micrographs; mycelia (A), sporangia (B), zoospore (arrow) (C) and sporangia releasing zoospore (D). The bar = 10 µm

The results of the morphology of *Phytophthora* with macroscopic and microscopic characteristics were expanded and identified using the key reported by Ho *et al*. (1995). The taxonomy of *Phytophthora* was identified according to "A Dichotomous Key to Taiwan Species of *Phytophthora*" (Ho *et al*., 1995). The fungus can be cultured on agar media, sex organ not produced in single culture, sporangia papillate to semipapillate, nondeciduous and good growth on agar medium at 35°C; mycelium tufted. The results clearly confirmed this pathogen as *Phytophthora parasitica.*

Phytophthora parasitica taxonomy scientific classification

Domain	Eukaryota
Kingdom	Stramenopila (Chromista)
Phylum	Oomycota
Class	Oomycetes
Order	Peronosporales
Family	Pythiaceae
Genus	*Phytophthora*
Species	*P. parasitica*

3.4.2 Identification of fungal isolate RM1

The morphology of RM1 is shown in Fig 3.8. A pure culture, obtained from hyphal tip isolation. The aerial mycelium observed was extensive cotton-like in culture with fluffy white color vegetative hyphae and floccose edge of colony. The

pigment produced by the mycelium on PDA was pale orange to red. For the old cultures, the darkened color of purple was visualized (Fig 3.8). The mycelium had septate. This fungi was produced both macro and microconidia from slender phialides. The macroconidia were short to medium in length (11-20 µm), 3-5 µm in wide. They were 3-4 septate, thin-walled, smooth, and sickle (canoe) to almost cylindrical shaped. The apical cell was short blunt to slightly hooked apical cell and notched basal cell (Fig 3.9C). The microconidia was non septate and oval (Fig 3.9D). The chlamydoconidia was absent when cultured on PDA. On the basis of the macroscopic features, the abundant production of macroconidia and microconidial morphology this case strain tentatively identified as *Fusarium* sp.

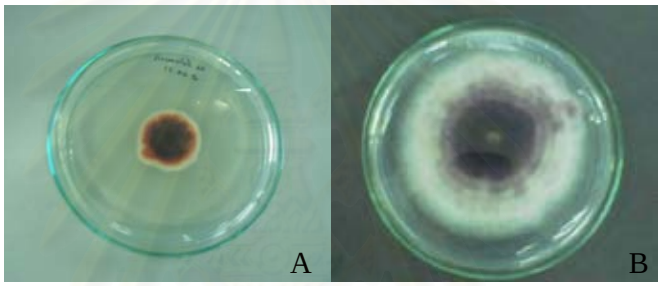


Fig 3.8 Colony characteristic of fungal isolate RM1 on PDA at 3 days (A) and 7 days (B).

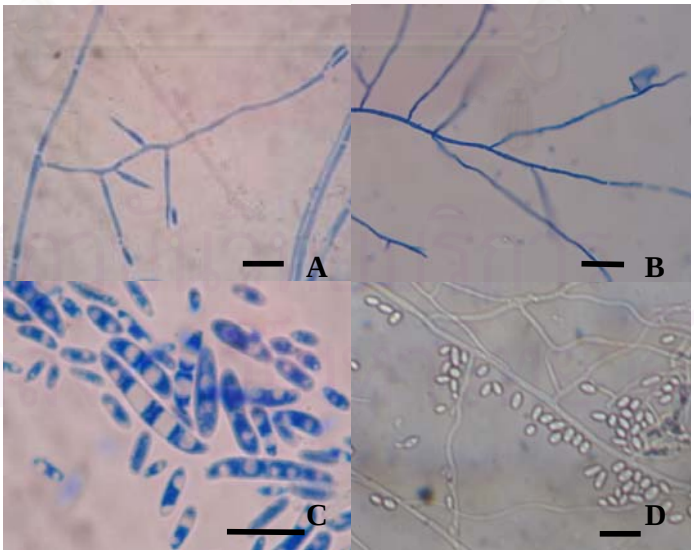


Fig 3.9 Characteristics under light microscope of isolate RM1. Sporangiophore (A), sympodium branching (B), macrospore (C) and microspore (D). The bar = 10 µm

RM1 was sent for identification by molecular methods at the Macrogen, Seoul, South Korea. The rDNA ITS region of isolate RM1 was amplified with the conserved fungal primer ITS_{1F} and ITS_4. The length of corresponding ITS fragment of isolate RM1 was 580 bp, containing a part of the 18S, ITS1, 5.8S, and 28S rDNA is shown in Fig 3.10.

```
5′  CTTGGTCCAT TTAGAGGAAG TAAAAGTCGT AACAAGGTCT
    CCGTTGGTGA ACCAGCGGAG GGATCATTAC CGAGTTTACA
    CTCCCAACCC CTGTGAACAT ACCACTTGTT GCCTCGGCGG
    ATCAGCCCGC TCCCGGTAAA ACGGGACGGC CCGCCAGAGG
    ACCCCTAAAC TCTGTTTCTA TATGTAACTT CTGAGTAAAA
    CCATAAATAA ATCAAAACTT TCAACAACGG ATCTCTTGGT
    TCTGGCATCG ATGAAGAACG CAGCAAAATG CGATAAGTAA
    TGTGAATTGC AGAATTCAGT GAATCATCGA ATCTTTGAAC
    GCACATTGCG CCCGCCAGTA TTCTGGCGGG CATGCCTGTT
    CGAGCGTCAT TTCAACCCTC AAGCACAGCT TGGTGTTGGG
    ACTCGCGTTA ATTCGCGTTC CCCAAATTGA TTGGCGGTCA
    CGTCGAGCTT CCATAGCGTA GTAGTAAAAC CCTCGTTACT
    GGTAATCGTC GCGGCCACGC CGTTAAACCC CAACTTCTGA
    ATGTTGACCT CGGATCAGGT AGGAATACCC GCTGAACTTA
    AGCATATCAA TAGCGGAGGA 3′
```

Fig 3.10 Nucleotide sequences of partial 18S region, complete ITS region of the isolate RM1.

A blast search was performed to find a similar sequence to ITS region of fungal isolate RM1 in the GenBank DNA database, available from: http://www.ncbi.nlm.nih.gov. The results revealed that ITS region of isolate RM1 had 99% identity to *Fusarium oxysporum f. sp. vasinfectum* alignment data of ITS region of isolates RM1 was showed in Appendix D.

Fusarium oxysporum taxonomy scientific classification

Domain	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Class	Sordariomycetes
Order	Hypocreales
Family	Nectriaceae
Genus	*Fusarium*
Species	*F. oxysporum f. sp. vasinfectum*

F. oxysporum species complex gathers diverse soil-born and that infect a variety of hosts causing various diseases, vascular wilt, corm rot, root rot, and damping-off (Gonsalves and Ferreira, 2008). Moreover, *F. oxysporum* is a pathogen that causes tulip bulb rot and also reported to infect mustard, rice, spinach, banana and tomato (Kuwaju, 2004). In pineapple, *Fusarium* sp. was reported as causes stem rot disease and heart rot of pineapple in Australia (Rohrbach and Johnson, 2003). Nonetheless, in Thailand there was no report on pineapple heart rot caused by *Fusarium* sp. Thus, this is the first report regarding to this matter.

3.4.3 Identification of fungal isolate RK1

RK1 was identified base on analysis of the DNA sequence of the ITS region. Primers ITS_{1F} and ITS_4 were used to amplify the ITS1-5.8S-ITS2 region from total DNA extract. Sequence analysed of the DNA sequence of the ITS region.

The ITS fragment length of fungal isolate RK1 was 580 bp fragment as shown in Fig 3.11. A blast search was performed to find a similar sequence to ITS region of fungal isolate RK1 in the GenBank DNA database, available from: http://www.ncbi.nlm.nih.gov. The ITS region of isolate RK1 had 99% identify to *Botryodiplodia theobromae* but for this research the isolate RK1 not show sexual stage. So this reason, isolate RK1 was identify to *Lasiodiplodia theobromae*, which was anamoph of *Botryodiplodia theobromae*. The alignment data of ITS region of isolate RK1 was showed in Appendix D.

```
5′   CTTGGTCCAT TTAGAGGAAG TAAAAGTCGT AACAAGGTTT
     CCGTAGGTGA ACCTGCGGAG GATCATTACC GAGTTTTCGA
     GCTCCGGCTC GACTCTCCCA CCCTTTGTGA ACGTACCTCT
     GTTGCTTTGG CGGCTCCGGC CGCCAAAGGA CCTTCAAACT
     CCAGTCAGTA AACGCAGACG TCTGATAAAC AAGTTAATAA
     ACTAAAACTT TCAACAACGG ATCTCTTGGT TCTGGCATCG
     ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
     AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
     CCCCTTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
     TACAACCCTC AAGCTCTGCT TGGAATTGGG CACCGTCCTC
     ACTGCGGACG CGCCTCAAAG ACCTCGGCGG TGGCTGTTCA
     GCCCTCAAGC GTAGTAGAAT ACACCTCGCT TTGGAGCGGT
     TGGCGTCGCC CGCCGGACGA ACCTTCTGAA CTTTTCTCAA
     GGTTGACCTC GGATCAGGTA GGGATACCCG CTGAACTTAA
     GCATATCAAT AGCCGGAGGA 3′
```

Fig 3.11 Nucleotide sequences of partial 18S region, complete ITS region of the isolate RK1.

Lasiodiplodia theobromae taxonomy scientific classification

Domain	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Class	Dothideomycetes
Order	Botryosphaeriales
Family	Botryosphaeriaceae
Genus	*Botryosphaeria* (anamorph: *Lasiodiplodia*)
Species	*L. theobromae*

Lasiodiplodia theobromae causes a post-harvest rot of taro corms. It was frequently isolated in decayed corm tissues behind advancing rots caused by *Phytophthora colocasiae* and *Pythium splendens*. Even in the absence of other fungi, it entered corms through wounds made at harvest. This fungus causes a spongy rot, occasionally becoming dry and powdery, white or cream at first and rapidly becoming

dark blue to black with an indistinct margin between healthy and diseased tissue (Wright and Jackson, 2007). Furthermore, B. *theobromae* or *L. theobromae* caused pineapple heart disease in Ghana (Bijlmakers, 1996). In Thailand, there was no report concerning the pineapple disease caused by B. *theobromae* or *L. theobromae*. Therefore, this was the first issue involving this fungus that showed the harmfulness of this disease on the healthy sucker of pineapple.

3.5 Antifungal activity of benzoic acid and cinnamic acid derivatives against radial growth on agar plate assay

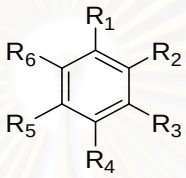
The antifungal activity of twelve benzoic acid and cinnamic acid derivatives (Table 3.3) at a concentration of 100 ppm against mycelial growth of pathogenic fungi is presented in Figs 3.10-3.13.

The screening for mycelial growth inhibition activity against *F. oxysporum* revealed that the growth inhibition against this fungus of *n*-butyl 4-hydroxybenzoate was 73% which was the most potent among tested compounds, followed by *n*-propyl 4-hydroxybenzoate with 61% inhibition (Figs 3.12 and 3.13). 2,6-Dichlorocinnamic acid, *α*-methyl-*trans*-cinnamaldehyde and 4-phenoxycinnamic acid displayed the inhibitory activity more than 50% against *F. oxysporum* with percentage inhibition of 59, 57 and 54%, respectively. While 4-*tert*-butylcinnamic acid, 4-trifluoromethylcinnamic acid, *p*-anisic acid, benzoic acid and 4-nitrocinnamic acid exhibited the activity against *F. oxysporum* less than 50% with percentage inhibition of 47, 45, 30, 25 and 24%, respectively. The rest compounds including cinnamaldehyde and salicylaldehyde expressed the percent inhibition of 14 and 12%, respectively. Metalaxyl and fosetyl-al were less effective in controlling the disease caused by *F. oxysporum*. Benomyl, a standard fungicide revealed the complete inhibition against *F. oxysporum*. All compounds were significantly reduced the mycelial growth compared with the sterilized water control plate.

The antifungal activity of benzoic and cinnamic acids derivatives against *P. parasitica* was determined (Figs 3.12 and 3.14). *n*-butyl 4-hydroxybenzoate and *n*-propyl 4-hydroxybenzoate showed the highest percentage of inhibition as 80 and 78% respectively. From Table 3.3, three compounds namely 4-phenoxycinnamic acid, 4-*tert*-butylcinnamic acid and 2,6-dichlorocinnamic acid (entries 7, 9 and 12) exhibited medium mycelial growth inhibition more than 50% while nine compounds (entries 1-

6, 8, 10 and 11) displayed slight inhibition less than 50%. The above experimental results of all tested compounds revealed significantly different compared with control plates. Metalaxyl showed high inhibition effect against *P. parasitica,* but not complete inhibition (87%). In contrast, fosetyl-al displayed slight inhibition only 12%.

Table 3.3 Benzoic and cinnamic acid derivatives and related compounds used in this research



No	R_1	Compound	R_2	R_3	R_4	R_5	R_6
1	COOH	Benzoic acid	H	H	**H**	H	H
2	COOH	*p*-anisic acid	H	H	**OCH_3**	H	H
3	CHO	salicylaldehyde	OH	H	**H**	H	H
4	CH=CH-COH	cinnamaldehyde	H	H	**H**	H	H
5	$COOCH_2CH_2CH_3$	*n*-propyl 4-hydroxybenzoate	H	H	**OH**	H	H
6	$COOCH_2CH_2CH_2CH_3$	*n*-butyl 4-hydroxybenzoate	H	H	**OH**	H	H
7	CH=CH-COOH	4-phenoxycinnamic acid	H	H	**OPh**	H	H
8	CH=CH-COOH	4-nitrocinnamic acid	H	H	**NO_2**	H	H
9	CH=CH-COOH	4-*tert*-butylcinnamic acid	H	H	**$C(CH_3)_3$**	H	H
10	CH=CH-COOH	4-trifluoromethylcinnamic acid	H	H	**CF_3**	H	H
11	$CH=C(CH_3)$-COH	α-methyl-*trans*-cinnamaldehyde	H	H	**H**	H	H
12	CH=CH-COOH	2,6-dichlorocinnamic acid	Cl	H	**H**	H	Cl

Cinnamaldehyde in this research displayed the fungal growth inhibition against *F. oxysporum* 14%, *P. parasitica* 21% and *L. theobromae* 50%. According to the literature, Thedpitak (2005) reported the antifungal activity of cinnamaldehyde against *P. parasitica* and *F. oxysporum*. Cinnamaldehyde exhibited complete inhibition on the mycelial growth of *P. parasitica* at 1.0 mM. At the same concentration of this compound, a slight mycelial inhibition (40%) was observed in the case of *F. oxysporum*. Another study revealed that 30 ppm of cinnamaldehyde indeed completely inhibited mycelial growth and germination of the conidia of *Botryodiplodia theobromae, Collectotrichum goleosporioides* and *Gliocephalotrichum microchlamydosporum* (Sivakumar *et al*., 2002).

Lattanzio *et al.* (1994) addressed *in vitro* antifungal activity of phenolic compounds. Certain benzoic acid derivatives were active against pathogens commonly found during the storage of fruits and vegetables caused by *Botrytis cinerea, Sclerotinia sclerotiorum* and *Gloeosporium album.*

n-Butyl 4-hydroxybenzoate expressed the best activity among twelve compounds investigated at the concentration of 100 ppm against fungal mycelial growth of *L. theobromae* in agar plate assay (Figs 3.12 and 3.15). This compound exhibited 87% inhibition. Other four compounds (Table 3.3, entries 5, 7, 11 and 12) displayed strong mycelial growth inhibition (more than 70%), while three compounds (entries 4, 9 and 10) revealed moderate inhibition (more than 50%). Furthermore, four tested compounds (entries 1-3 and 8) showed slight mycelial growth suppression (less than 50%).

Following this screening, *n*-butyl 4-hydroxybenzoate exhibited the highest antifungal activity against all three pathogenic fungi examined. *n*-Butyl 4-hydroxybenzoate was therefore selected to use in the following study.

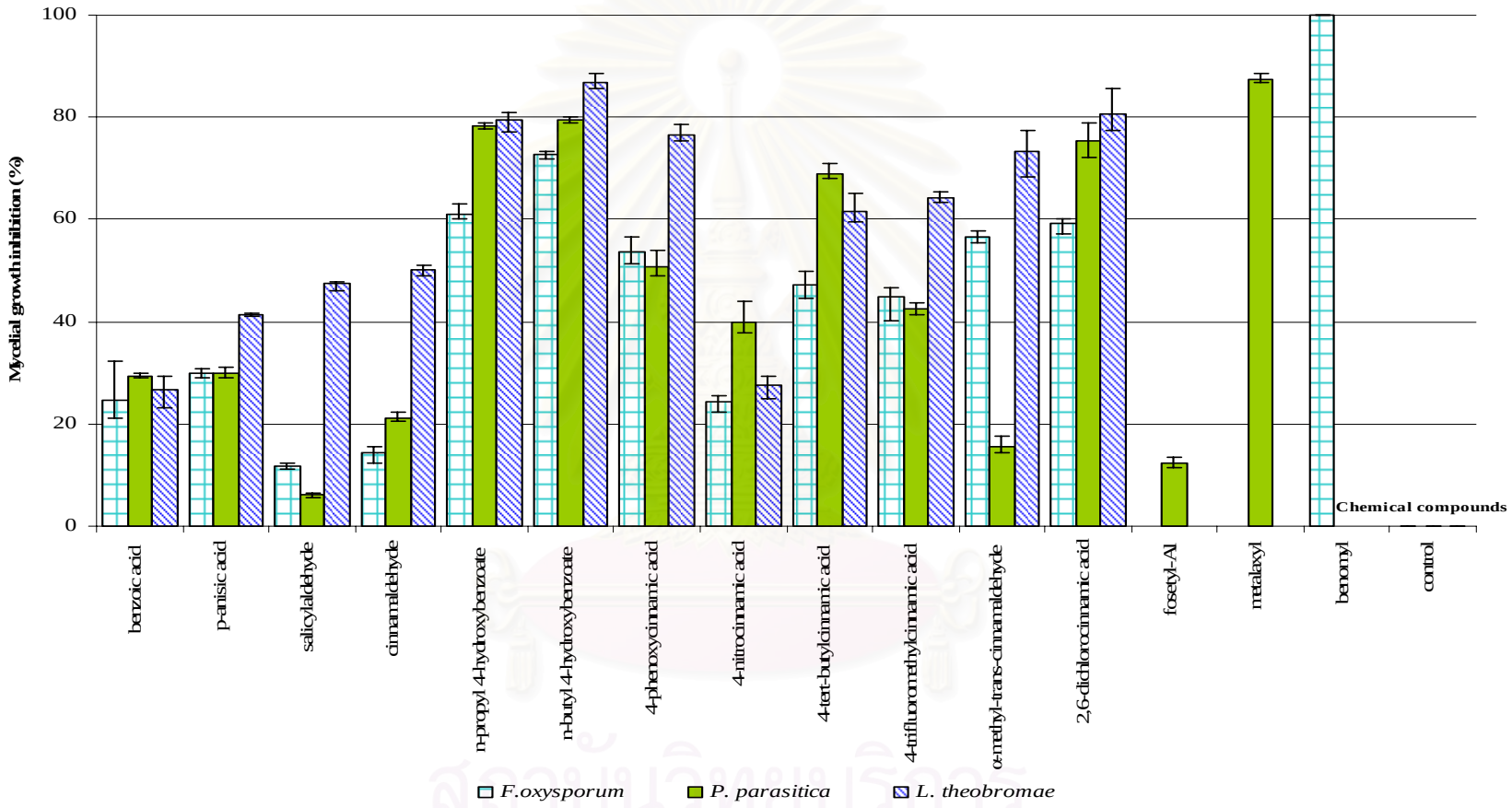


Fig 3.12 Effect of benzoic acid and cinnamic acid derivatives at 100 ppm against mycelial growth of *F. oxysporum*, *L. theobromae* and *P. parasitica*.

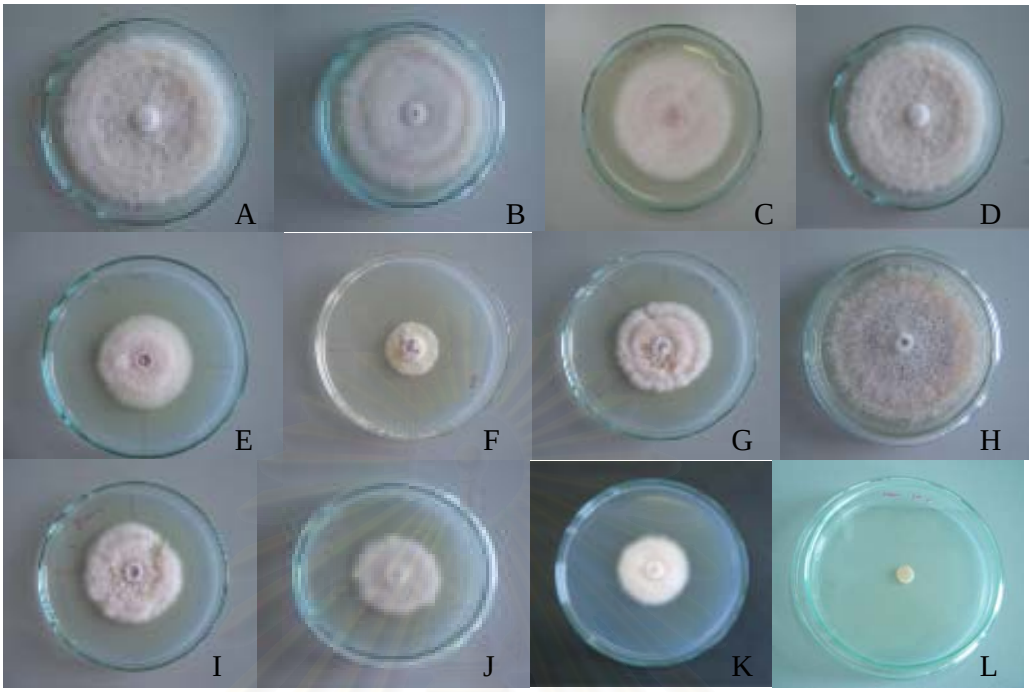


Fig 3.13 Effect of benzoic acid and cinnamic acid derivatives at 100 ppm against *Fusarium oxysporum* on PDA. Treated with DMSO (A) , benzoic acid (B), *p*-anisic acid (C), cinnamaldehyde (D), *n*-propyl 4-hydroxybenzoate (E), *n*-butyl 4-hydroxybenzoate (F), 4-phenoxycinnamic acid (G), 4-nitrocinnamic acid (H), 4-trifluoromethylcinnamic acid (I), α-methyl-*trans*-cinnamaldehyde (J), 2,6-dichlorocinnamic acid (K) and benomyl (L).

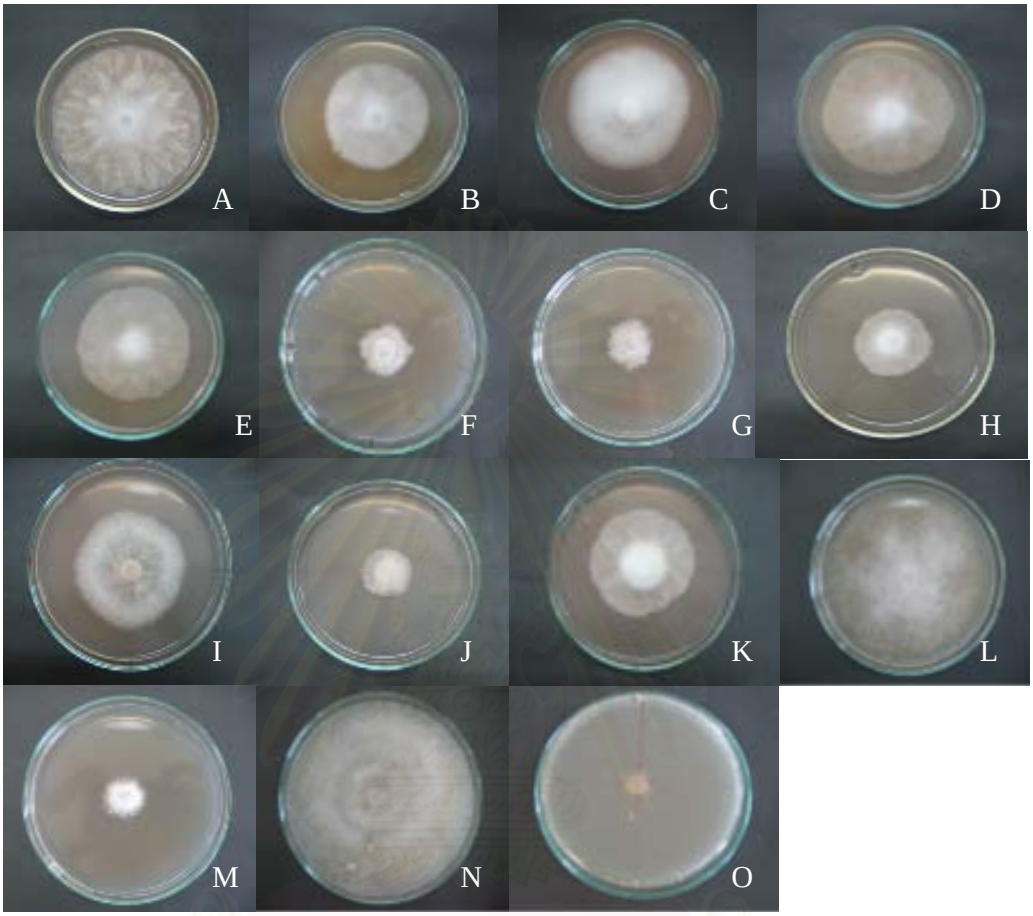


Fig 3.14 Effect of benzoic and cinnamic acid derivatives against *P. parasitica* on carrot agar. DMSO (A), benzoic acid (B), *p*-anisic acid (C), salicylaldehyde (D), cinnamaldehyde (E), *n*-propyl 4-hydroxybenzoate (F), *n*-butyl 4-hydroxybenzoate (G), 4-phenoxycinnamic acid (H), 4-nitrocinnamic acid (I), 4-*tert*-buthylcinnamic acid (J), 4-trifluoromethylcinnamic acid (K), α-methyl-*trans*-cinnamaldehyde (L), 2,6-dichloro-cinnamic acid (M), fosetyl-Al (N) and metalaxyl (O).

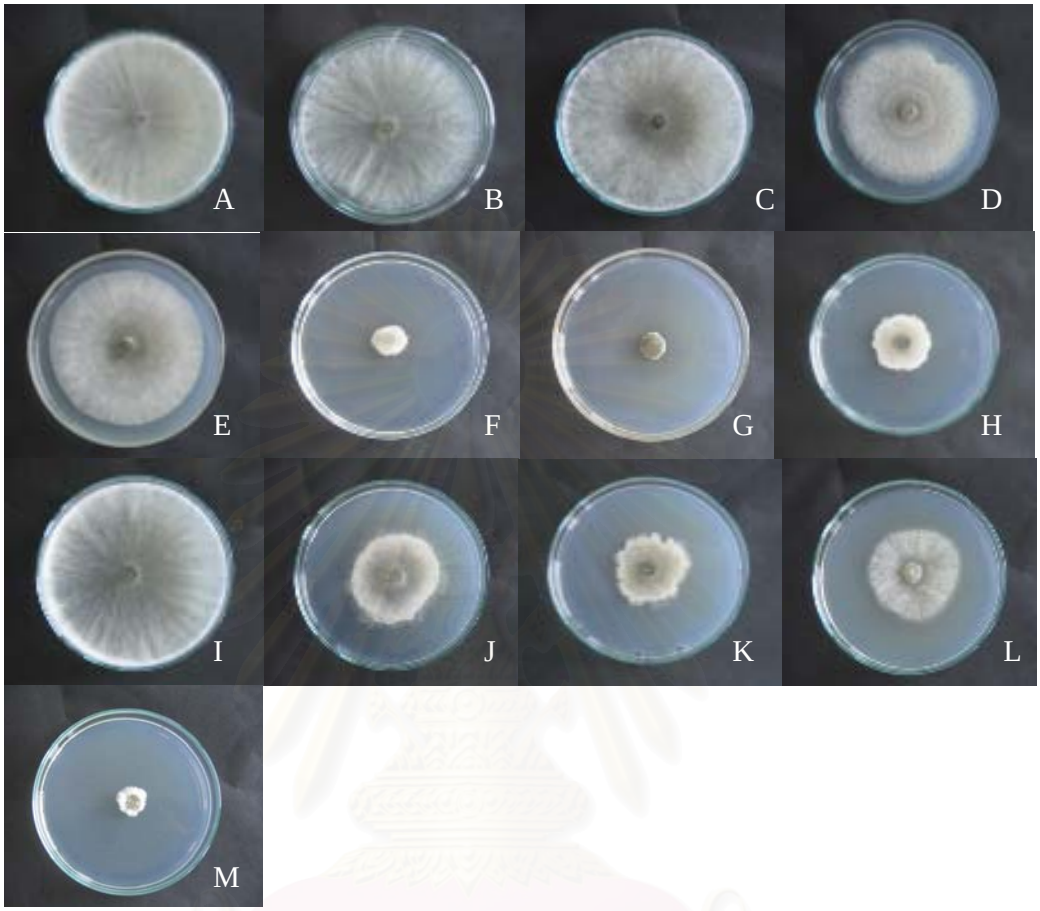


Fig 3.15 Effect of benzoic acid and cinnamic acid derivatives against *L. theobromae* on Capek's agar. DMSO (A), benzoic acid (B), *p*-anisic acid (C), salicyaldehyde (D), cinnamaldehyde (E), *n*-propyl 4-hydroxybenzoate (F), *n*-butyl 4-hydroxybenzoate (G), 4-phenoxycinnamic acid (H), 4-nitrocinnamic acid (I), 4-trifluoromethylcinnamic acid (J), 4-*tert*-buthylcinnamic acid (K), *α*-methyl-*trans*-cinna-maldehyde (L) and 2,6-dichlorocinnamic acid (M)**.**

3.6 Antifungal activity of *n*-butyl 4-hydroxybenzoate against mycelial growth in broth.

The effectiveness of *n*-butyl 4-hydroxybenzoate in aforementioned experiments revealed the best activity against all tested pathogenic fungi (*F. oxysporum*, *P. parasitica* and *L. theobromae*) which caused pineapple heart rot disease in agar plate assay. *n*-Butyl 4-hydroxybenzoate was thus selected for further study. The study protocol involved the variation of the concentrations of a tested compound as 25, 50, 100 and 250 ppm and the treatment in broth medium against mycelial growth of pathogenic fungi to confirm the observed activity and to explore the least concentration of *n*-butyl 4-hydroxybenzoate that still revealed the complete inhibition of mycelial growth of all caused pathogenic fungi.

The potency of *n*-butyl 4-hydroxybenzoate on mycelial growth of pathogenic fungi could be clearly observed from the protocol described in Chapter II. *n*-Butyl 4-hydroxybenzoate could significantly reduce the dry weight of mycelial growth of all tested fungi and all concentrations compared with distilled water sterilized control plate. Increasing the concentration of *n*-butyl 4-hydroxybenzoate reduced the mycelial growth. The mycelial growth was completely inhibited at the concentration of 250 ppm. The results of the dry weight of pathogenic fungi are shown in Table 3.4 and Figs 3.16-3.19.

Table 3.4 Dry weight (g) of pathogenic fungi treated with *n*-butyl 4-hydroxybenzoate at various concentrations in broth medium incubated for 14 days.

Pathogenic tested	Concentration of *n*-butyl 4-hydroxybenzoate (ppm)±SD*				
	0	25	50	100	250
F. oxysporum	0.91±0.02^{d}	0.88±0.01^{c}	0.7±0.02^{b}	0.67±0.03^{b}	0.08±0.0^{a}
P. parasitica	0.39±0.01^{d}	0.3±0.01^{c}	0.24±0.01^{b}	0.03±0.0^{a}	0.01±0.0^{a}
L. theobromae	1.2±0.02^{e}	1.09±0.02^{d}	1.03±0.03^{c}	0.09±0.003^{b}	0.03±0.0^{a}

*Values followed by different letters are significantly different (P=0.05) according to Duncan's multiple rang test.

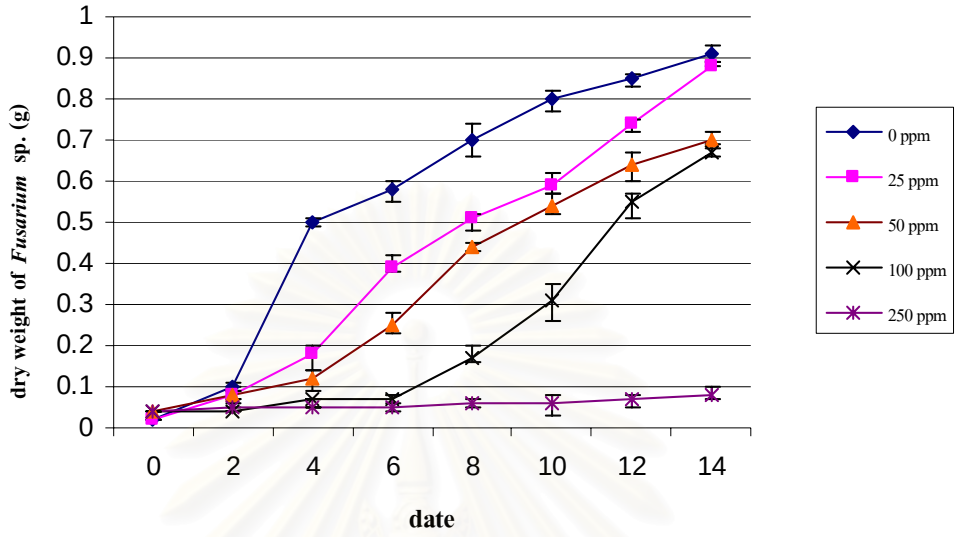


Fig 3.16 Effect of *n*-butyl 4-hydroxybenzoate at various concentrations against mycelial growth of *F. oxysporum* in potato dextrose broth.

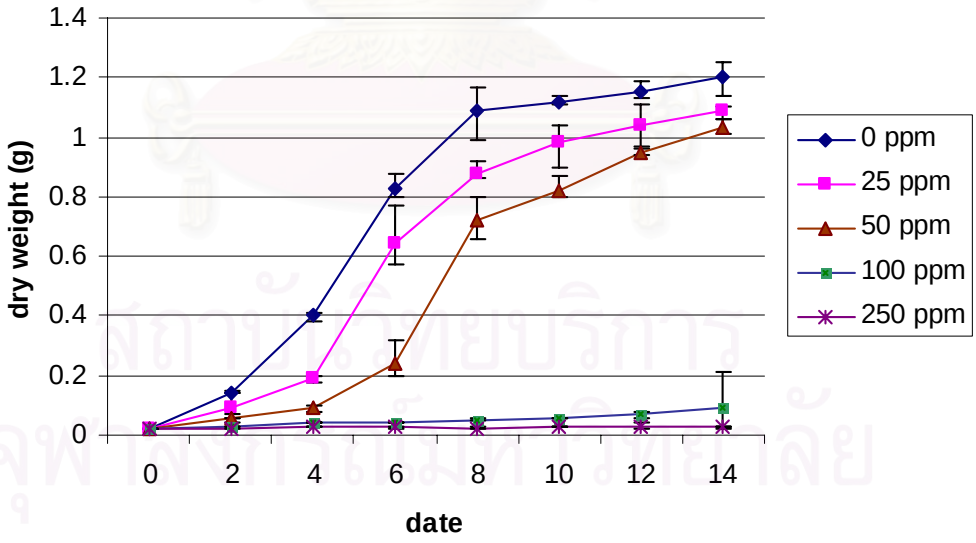


Fig 3.17 Effect of *n*-butyl 4-hydroxybenzoate at various concentrations against mycelial growth fungal isolate of *L. theobromae* in Czapek broth.

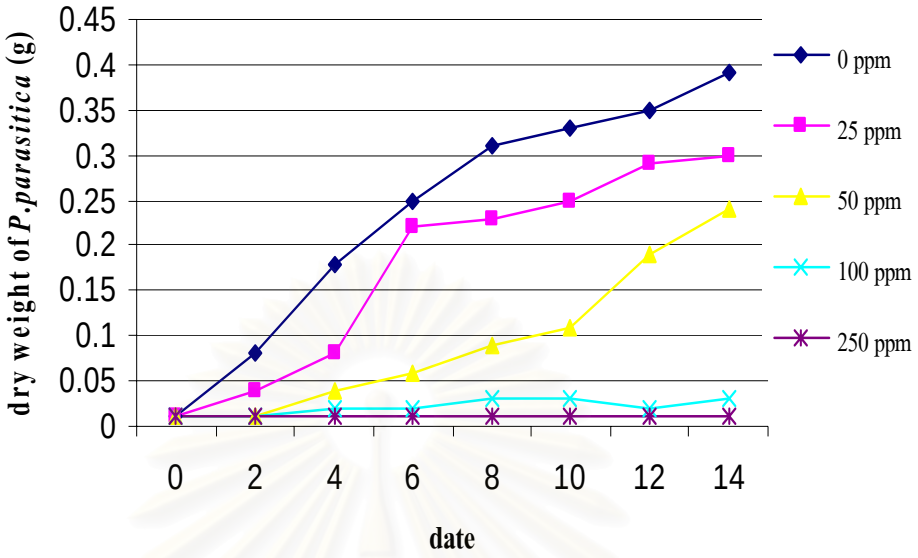


Fig 3.18 Effect of *n*-butyl 4-hydroxybenzoate at various concentrations against mycelial growth of *P. parasitica* in carrot broth.

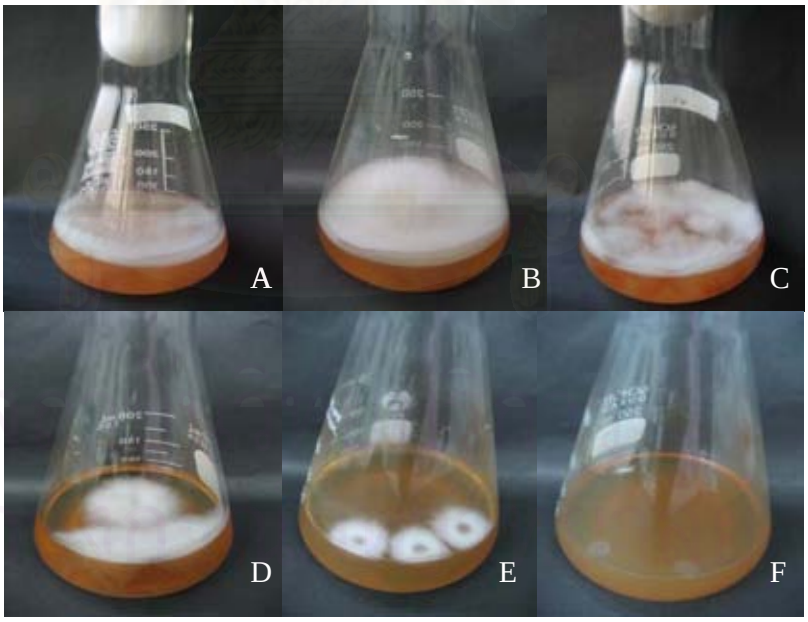


Fig 3.19 Mycelial of *P. parasitica* (14 days) growth in carrot broth supplement with *n*-butyl 4-hydroxybenzoate at various concentrations; 0 (A), DMSO (B), 25 (C), 50 (D), 100 (E) and 250 (F) ppm.

The growth rate of all fungi in the initially stage was slow because of slow adaptation themselves to broth medium utilized, which supplements with chemicals at different concentrations. When the incubation was up to 6 days, the fungus can increasingly grow. At the concentration of *n*-butyl 4-hydroxybenzoate of 25 and 50 ppm, the pathogenic fungi can adapt themselves to the medium used and could grow normally when time passed (Figs 3.16-3.19).

3.7 Antifungal activity of *n*-butyl 4-hydroxybenzoate at various concentrations against spore germination

The antifungal activity of mycelial growth and spore germination greatly affects the costs and benefits of fungal disease control. If *n*-butyl 4-hydroxybenzoate was active to inhibit both mycelial growth and spore germination, it would then be able to control all the subsequent steps in the disease cycle. Therefore, it was rationalized to further evaluate *n*-butyl 4-hydroxybenzoate for its efficiency against spore germination of *F. oxysporum* and *P. parasitica*.

To test spore germination, 100 µl of the suspensions were spread aseptically on PDA plates supplemented with different concentrations of *n*-butyl 4-hydroxybenzoate (0, 25, 50, 100, 250 and 500 ppm). The plates were incubated at RT for 48 h. Following the incubation, fungal colonies originated from germinated spores were enumerated in order to evaluate the percentage of inhibition of spore germination. The results are shown in Table 3.5.

Table 3.5 Effect of *n*-butyl 4-hydroxybenzoate on the inhibition of spore germination

Pathogenic tested	Concentration (ppm) ± SD*						IC_{50} (ppm)
	0	25	50	100	250	500	
F. oxysporum	0 ± 0^{d}	2.7 ± 0.05^{c}	21.4 ± 0.2^{b}	33.0 ± 0.3^{b}	100 ± 0^{a}	100 ± 0^{a}	130.63
P. parasitica	0 ± 0^{d}	5.9 ± 0.07^{c}	21.3 ± 0.1^{b}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}	61.37

*Values followed by different letters are significantly different (P=0.05) according to Duncan's multiple rang test.

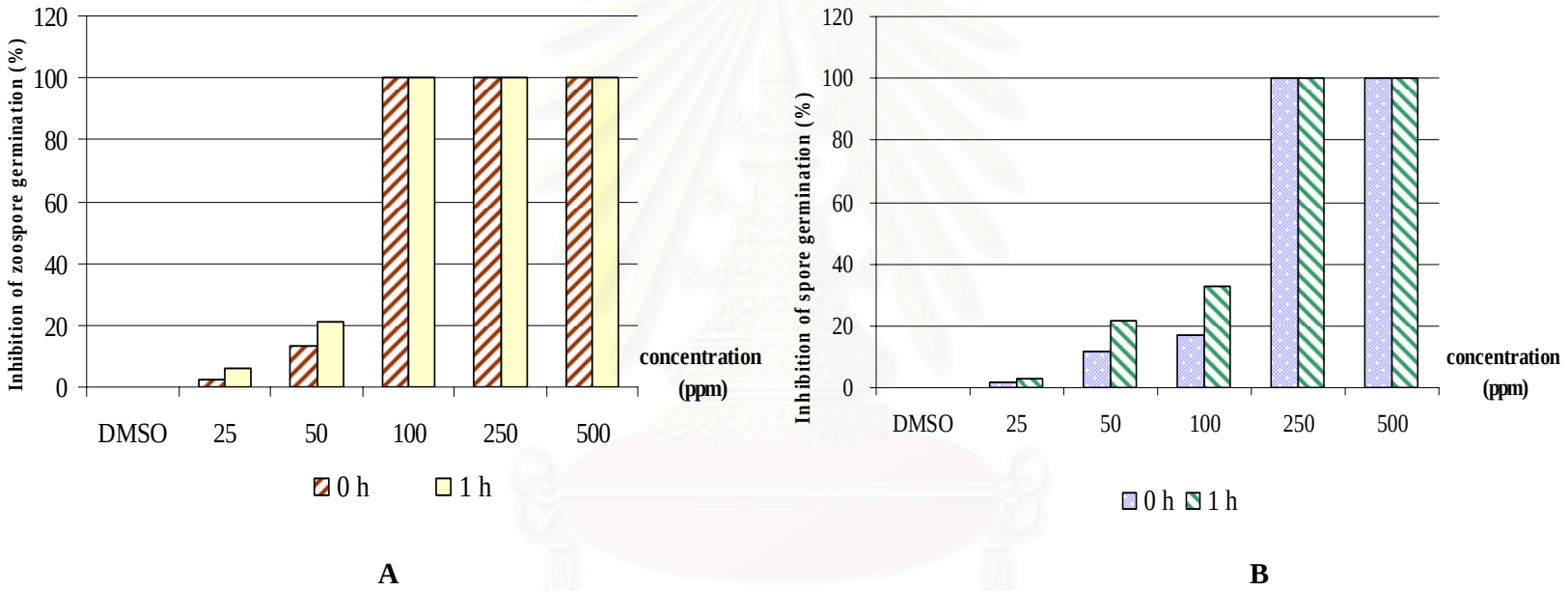


Fig 3.20 Effect of *n*-butyl4-hydroxybenzoate at various concentrations against spore germination at 0 and 1 h.

A: *P. parasitica*, **B**: *F. oxysporum*

According to the results obtained in Table 3.5 and Fig 3.18, *n*-butyl 4-hydroxybenzoate significantly completely inhibited the spore germination of *Fusarium* sp. from a concentration of 100 ppm and complete by inhibited zoospore germination of *P. parasitica* at 100 ppm with differently significant form 50 ppm and not significant at concentration higher than 100 ppm. The results at 0 and 1 h (Fig 3.20) after incubation did not show different percent inhibition of spore germination. This study clearly discovered that *n*-butyl 4-hydroxybenzoate inhibited *in vitro* both spore germination and mycelial growth of *F. oxysporum* and *P. parasitica*.

Zoospores were sensitive to chemicals more than mycelia. This was because zoospores lack of the cell wall, thus had the lowest average sensitivity towards the chemicals (Jonghe *et al.*, 2007). Similar responses were found for the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) on the zoospore structures of *Pythium* spp. (de Souza *et al.*, 2003).

3.8 Antifungal activity of *n*-butyl 4-hydroxybenzoate against pineapple heart rot disease on detached leaves of pineapple in laboratory

Since *F. oxysporum* and fugi isolate *L. theobromae* have lost their activity to cause pineapple heart rot disease during the study, maybe kept its for long time so that these two fungi were excluded from the test on pineapple leaf.

The antifungal activity of *n*-butyl 4-hydroxybenzoate was studied by treating on pineapple leaves with heart rot disease. After the artificial inoculation and incubation for 3 days, all leaves were pulled out and measured the lesion of disease incidence (Table 3.6). The pineapple leaves which were treated by *n*-butyl 4-hydroxybenzoate at the concentrations of 100 to 2500 ppm showed water soaked lesion and soft rot tissue. Comparing the results gaining from the study on the effect of *n*-butyl 4-hydroxybenzoate at 100 to 500 ppm with control, there was no significant different. At 1000 to 2500 ppm of *n*-butyl 4-hydroxybenzoate the invasion of lesion was inhibited; however, not to prevent the disease occurrences. Up to 3000 ppm, this compound expressed the completed inhibition incidences on heart rot disease with its phytotoxicity on plant tissue.

Table 3.6 Lesion diameter of heart rot disease on pineapple leaf.

concentration of *n*-butly 4-hydroxybenzoate (ppm)	Lesion diameter (cm)
	mean±SD*
0	6.3±0.67 [f]
100	6.2±0.76 [ef]
250	6.2±0.57 [ef]
500	5.8±0.67 [def]
1000	5.4±0.42 [cde]
1500	5.3±0.27 [cd]
2000	4.70±0.97 [c]
2500	4.57±0.87 [c]
3000	1.40±0.1 [a]
5000	1.32±0.1 [a]
metalaxyl 250 ppm	0.80±0.0 [a]
Fosetyl-Al 250 ppm	2.34±1.15 [b]

*Values followed by different letters are significantly different (*P*=0.05) according to Duncan's multiple rang test.

Metalaxyl revealed completely inhibition on the developed disease lesion at 250 ppm, while fosetyl-al decreased the lesion of disease, but could not control the disease incidences.

The degradation of plant cell wall was likely to be a component of virulence of *Phytophthora*, particularly during penetration of plant epidermis and establishment of haustoria. The degradative enzymes were important virulence factors for *Phytophthora* (Kamoun, 2000). In general, from the *in vitro* experiments, it could be concluded that mycelium was the most resistant structure, followed by sporangia and zoospores. The high sensitivity of the zoospores may be explained by the absence of protective cell wall. This was also found by Oros *et al*. (1999) who tested the direct effect of eleven sulfosuccinic acid ester surfactants against the asexual spores of *Plasmopara halstedii*.

3.9 Antifungal activity of *n*-butyl 4-hydroxybenzoate against pineapple heart rot disease on sucker in laboratory

The antifungal activity of *n*-butyl 4-hydroxybenzoate against pineapple heart rot disease was treated by artificial inoculation with *P. parasitica* isolated from pineapple heart rot disease in field. Various concentrations of *n*-butyl 4-hydroxybenzoate were applied to suckers against pineapple heart rot disease caused by *P. parasitica*. Zoospores of the causal organisms played the most important role in fields (Ahonsi *et al*, 2007) therefore, the zoospore suspension was used for artificial inoculums on suckers.

Pineapple suckers without growing any new root were used in these experiments and were inoculated with zoospore. Five suckers were initially inoculated in each treatment. After inoculation, suckers were kept in plastic bags with moist condition for 3 days. For preventative disease, the concentration of *n*-butyl 4-hydroxybenzoate at 250-2500 ppm could not control the heart rot disease, the color of suckers was changed to pale green, mycelial were observed and water soaked spongy like. At 3000 and 5000 ppm, the preventative disease was observed with some side effects from the treated chemical (Fig 3.21). Phytotoxicity of wounded suckers might occur by an excess DMSO, because the control group which was treated with the dilution of DMSO (5%) revealed some blight on the wounded leaves. Another series of experiments was thus conducted. The non-wounded suckers were prepared and tested. The results revealed that the suckers remained healthy and did not show any side effects. It should be noted that an excess DMSO was destroyed the fresh cut tissue of pineapple.

n-Butyl 4-hydroxybenzoate at high concentration was disclosed as a preventative of pineapple heart rot disease. Compared with metalaxyl, the dose of 3000 ppm of *n*-butyl 4-hydroxybenzoate must be used. The suckers which were dipped in metalaxyl and fosetyl-Al were not infected by *P. parasitica*. Fosetyl-Al was however less effective than metalaxyl and showed significant difference from *n*-butyl 4-hydroxybenzoate. The dose of 250 ppm of metalaxyl completely prevented the disease and did not show phytotoxicity on pineapple fresh-cut tissue.

For curative experiments, all concentrations of *n*-butyl 4-hydroxybenzoate could not curative heart rot disease. All suckers inoculated became heart rot. This same trend was observed for metalaxyl. Suckers were completely decayed. The results are presented in Table 3.7.

Table 3.7 Effect of *n*-butyl 4-hydroxybenzoate against heart rot disease treated on suckers in laboratory.

conc. of butyl 4-hdroxybenzoate (ppm)	**No. of suckers with disease**	
	preventative	**curative**
DMSO	5	5
0	5	5
250	5	5
500	5	5
1000	5	5
1500	5	5
2000	5	5
2500	5	5
3000	0	5
5000	0	5
Metalaxyl 250 ppm	0	5
Fosetyl-al 250 ppm	5	5

From the experiment of testing antifungal activity *in vitro*, it was found that the concentration of *n*-butyl 4-hydroxybenzoate used was less than *in vivo*. Mill *et al*. (2006) reported the salts of sorbic, propionic and benzoic acids were collectively known as the classical food preservatives. Those compounds showed a highly significant effect on bacterial growth *in vitro*; however, these effects were less pronounced *in vivo* experiments.

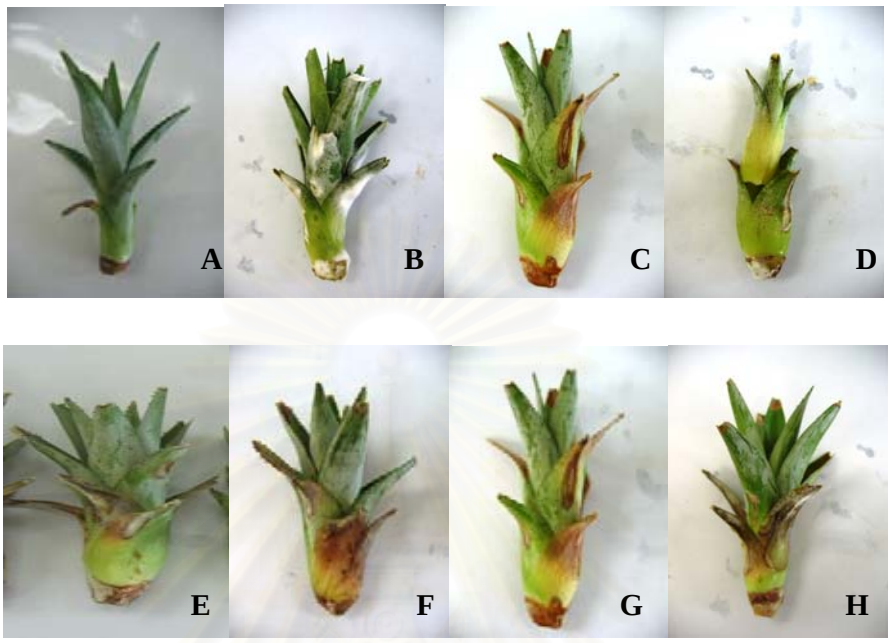


Fig 3.21 Signs of heart rot disease on pineapple suckers.

Control groups; no inoculation (A), inoculation with zoospore suspension (B), treated with 5% DMSO and no inoculation (C), treated with 5% DMSO and inoculation with zoospore suspension (D).

Treatment groups; treated with 3000 ppm and no inoculation (E), treated with 3000 ppm and inoculation with zoospore (F), treated with 5000 ppm and no inoculation (G), treated with 5000 ppm and inoculation with zoospore (H).

The application of metalaxyl and fosetyl-Al as a reference fungicide was tested in sucker. Metalaxyl showed activity to control disease more than fosetyl-Al. Meanwhile, Thomides and Elena (2001) applied this fungicide against peach stem canker against stem canker disease caused by *P. cactorum* and was found that fosetyl-Al was less effective than metalaxyl. Timmer and Castle (1985) studied the effect of metalaxyl and fosetyl-al against *P. parasitica* on Sweet Orange. Metalaxyl was highly effective for *Phytophthra* infection of citrus and best applied as a soil drench or trunk paint. Rohrbach and Schenck (1985) was showed metalaxyl and fosetyl-Al could controll pineapple heart rot caused by *P. parasitica* and *P. cinnamomi*. Preplant suckers material dipped with metalaxyl at 600 ppm and fosetyl-al at 1,200 ppm resulted in significant heart rot control and increased plant growth. Both fungicides were highly effective in controlling pineapple heart rot when applied as preplant sucker dips. This outcome was similar to that observed from this research that metalaxyl displayed the effect to control heart rot disease more than that of fosetyl-Al.

In contrast, the experiment of Jenaksorn (1982) and Phatpakorn (1983) revealed that fosetyl-Al exhibited the most efficacy to control heart rot of pineapple compared with metalaxyl plus captafol, which was treated by dipping the sucker in fungicide before planting.

These results confirmed the previous reports that metalaxyl could be employed to control the preventative disease more than the curative ones detected in the pineapple. Compared with metalaxyl, the effective dose of fosetyl-al needed to be used in higher concentration. For *n*-butyl 4-hydroxybenzoate, it was observed that this compound could also be employed to prevent heart rot disease more than curative. For this reason, it is interesting to develop the formulation of butyl 4-hydroxbenzoate as fungicide.

3.10 Synergistic study of metalaxyl and *n*-butyl 4-hydroxybenzoate

The efficiency of metalaxyl at various concentration (0, 0.1, 0.5, 1, 2.5, 3, 5, 10, 50 and 100 ppm) against *P. parasitica* is presented in Table 3.8. Mean mycelial inhibition was increased with increasing metalaxyl concentration from 0.1 to 10 ppm, but mycelial growth inhibition was observed not significant at 10, 50 and 100 ppm. *In vitro* inhibition mycelial growth response of *P. parasitica* var. *nicotianae* to metalaxyl was increased by increasing the concentrations from 0.1 to 10 ppm, but similar at 10

and 100 ppm and the inhibition of some isolates decreased with increasing concentrations (Shew, 1984) that concurred with this research.

Metalaxyl at concentrations of 0.1, 0.5, 1.0, 2.5, 3, 5 and 10 ppm was to calculated by binomial equation and gave EC_{50} about 1.4 ppm. After that, to indicates the potential of synergistic combination of *n*-butyl 4-hydroxybenzoate with metalaxyl at various ratios followed by Chapter II. Antifungal activity of synergistic combination against mycelial growth of *P. parasitica* was showed in Table 3.9. When the ratio of metalaxyl increased, the percentage of mycelial inhibition was increased. Although percent inhibition of individual metalaxyl was better than synergist combination with *n*-butyl 4-hydroxybenzoate, the combination of metalaxyl with *n*-butyl 4-hydroxybenzoate at ratio of 4:1 displayed 59% inhibition which was close to the use single metalaxyl (62%).

According to the previous reports on the mechanisms of metalaxyl fungitoxicity and resistance to metalaxyl. *Phytophthora* was found to resistance to metalaxyl but the mechanism remained obscure (Fisher and Hayes, 1984). Davidse *et al.* (1988) studied antifungal modes of action of metalaxyl and several fungicides to resistant strains of *Phytophthora* sp. The resistant stains were found not affect at concentration as high as 100 ppm and found endogenous nuclear RNA polymerase activity of resistance appeared less than sensitive stains.

Chang and Ko (1990) investigated the mutants of *P. parasitica* Dustur that was resistant to either metalaxyl or chloroneb and found that these mutants conferred by a single dominant gene in the heterozygous condition. Furthermore, Gu and Ko (2000) studied metalaxyl and chloroneb resistant protoplasts of *P.parasitica* Dustur and tested with 25 ppm metalaxyl and 200 ppm chloroneb. The pathogens were resistant to either metalaxyl or chloroneb; but not to a mixture of both fungicides.

Tolerance to metalaxyl can develop after the prolong use or using the same or single types of fungicide especially metalaxyl to encourage the fungi adapt themselves to resistant of metalaxyl fungicide.

Table 3.8 Mycelial growth inhibition of *P. parasitica* by metalaxyl at various concentrations.

Concentration of metalaxyl (ppm)	Mycelial growth inhibition±SD (%)*
0	0±0[e]
0.1	30±0.7[d]
0.5	35.56±0.5[c]
1	41.11±0.2[c]
2.5	67.78±0.5[b]
3	75.56±0.6[ab]
5	80±0.1[a]
10	84±0.2[a]
50	84±0.1[a]
100	83±0.1[a]

* Values followed by different letters are significantly different (P=0.05) according to Duncan's multiple rang test.

Table 3.9 Mycelial growth inhibition of *P. parasitica* at various ratios of metalaxyl:*n*-butyl 4-hydroxybenzoate.

Ratio[A]	Mycelial inhibition (%)±SD*
0	0±0[c]
0:1	0±0[c]
1:4	37.78±0.3[b]
1:1	38.89±0.2[b]
4:1	58.89±0.07[a]
1:0	62.22±0.2[a]

[A] Metalaxyl: *n*-butyl 4-hydroxybenzoate

* Values followed by different letters are significantly different (P=0.05) according to Duncan's multiple rang test.

Synergistic and antagonistic interactions of fungicides against *Pythium aphanidrmatum* were studied on perennial ryegrass. The combination of mancozeb and metalaxyl could be used to control the resistant stain. The level of disease control provided by the synergistic combinations was equal to or greater than that provided by each of the components applied singly at their full label rate (Couch and Smith, 1991). The combined treatment of *Enterobacter aerogenes* and metalaxyl to control *Phytophthora* crown and root rot caused by *P. cactorum* in apple tree was studied. That combination was reduced the expression of visual symptoms of crown and root infected tree (Levesque *et al*., 1993). Fyfe *et al.* (1998) reported the combinations for oil of anise with methyl paraben and oil of fennel with benzoic acid were better inhibitor than each compound against *Listeria monocytogenes* and *Salmonella enteriditis*. Thus, a mixture of certain compounds may act synergistically to augment the inhibition of disease development and prevent the pathogenic fungi resistant to fungicide.

From this research, it was disclosed that the amount of metalaxyl could be reduced and certain amount could be replaced with *n*-butyl 4-hydroxybenzoate. Beside that *n*-butyl 4-hydroxybenzoate had a lower price (20 bath per g, Dr.Ehrenstorfer®, Science plus Ltd.) compared with metalaxyl (6,700 bath per g, Fluka®; A.C.S. Xenon Ltd.). Furthermore, *n*-butyl 4-hydroxybenzoate or butyl paraben was friendly to environmental and human. The LD_{50} of metalaxyl was 669 mg/kg and LD_{50} of *n*-butyl 4-hydroxybenzoate was 13,200 mg/kg (http://msds.chem.ox.ac.uk). Moreover, *n*-butyl 4-hydroxybenzoate should be developed for the formulation to obtain excellent activity to control diseases.

CHAPTER IV

CONCLUSION

Fifteen pathogenic fungi isolates which caused heart rot disease were isolated from pineapple leaves derived from the pineapple filed at Cha-Am district, Phetchaburi province. All fifteen isolates were subjected to pathogenicity test and found that three isolates (RM1, RK1 and *Phytophthora*) could make healthy sucker become heart rot symptom. Those three isolates were identified as *Fusarium oxysporum f. sp. vasinfectum*, *Lasiodiplodia theobromae* and *Phytophthora parasitica*.

The antifungal activity of benzoic acid and cinnamic acid derivatives against pineapple heart rot disease was screened *in vitro* as inhibitors of three pathogenic fungi. All compounds were mixed with medium agar to concentration of 100 ppm. The results revealed that among all tested compounds, *n*-butyl 4-hydroxybenzoate exhibited the highest level of percentage inhibition of *in vitro* mycelial growth. The examination on antifungal activity at various concentrations in broth medium against mycelium growth was thoroughly performed. After 14 days of incubation at RT, dry weights were recorded, *n*-butyl 4-hydroxybenzoate at all concentrations (25, 50, 100, 250 ppm) significantly decreased dry weight of mycelial growth compared with controlled plate. At 250 ppm, the inhibition of mycelium growth of all pathogenic fungi was completely observed.

In vivo examination was conducted on pineapple leaves and suckers in laboratory. The examination on the preventative and curative effects of the effective compound was artificial infected using the suspension of *P. parasitica*. *n*-Butyl 4-hydroxybenzoate could be utilized as preventative for pineapple heart rot disease at high concentration (3000 ppm), but for curative purposes, all concentrations of *n*-butyl 4-hydroxybenzoate could not be used.

Synergistic combination of metalaxyl with *n*-butyl 4-hydroxybenzoate in the ratio of 4:1 at concentration of EC_{50} 1.4 reduced the mycelial growth and gave percent inhibition nearly mycelial growth in plate containing only metalaxyl. *n*-Butyl 4-hydroxybenzoate should be developed and formulated as a fungicide to control disease and to test in greenhouse or pineapple field.

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APPENDICES

APPENDIX A

Media

1. Potato Dextrose Agar (PDA)

Potato	200	g
Dextrose	20	g
Agar	15	g
Water	1,000	ml

The potato are peeled, washing, diced and boiled until soft (actual time varied with the size of the potato). Filter the boiled potatoes through a single layer of cheesecloth, which leaves some sediment in the broth.

2. Potato Dextrose Broth (PDB)

Potato	200	g
Dextrose	20	g
Water	1,000	ml

The similar preparing in PDB but not add agar in broth.

3. Czapek's Agar

$NaNO_3$	2	g
K_2HPO_4	1	g
$MgSO_4$	0.5	g
KCl_2	0.5	g
$FeSO_4$	0.01	g
Sucrose	30	g
Agar	15	g
Water	1,000	ml

4. Czapek's Broth

$NaNO_3$	2	g
K_2HPO_4	1	g
$MgSO_4$	0.5	g
KCl_2	0.5	g
$FeSO_4$	0.01	g
Sucrose	30	g
Water	1,000	ml

5. Carrot Agar

Carrot	200	g
Agar	15	g
Water	1,000	ml

The medium is prepared by washing peeling and dicing 200 g of fresh carrots. Place the carrots and some water in a blender until the mixture appears smooth and with no large lumps. Boiled the carrots and then filtrate with cheesecloth. Add the water to 1000 ml and 15 g of agar in carrot broth.

6. RNV

Benlate (Benomyl 50%)	0.12	g
Mycostatin (Nystatin $1x10^5$ unit/ml)	3	ml
PCNB (Pentachloronitrobenzene)	0.16	g
Rifadin	0.06	g
Ampicillin (500 mg/capsule)	12	capsule
Water	600	ml

15 ml of RNV solution add to 150 ml PDA media.

APPENDIX B

Table B1. Effect of benzoic and cinnamic acid derivatives at 100 ppm on the mycelial growth of phytopathogenic fungi on agar medium assay.

Compounds	[A]mycelial sensitivity response (%)±SD		
	F. oxysporum	*L. theobromae*	*P. parasitica*
benzoic acid	24.67±7[g]	26.64±2.54[g]	29.33±0.61[g*]
p-anisic acid	29.78±0.93[f]	41.33±1.45[f]	29.78±0.93[g]
salicylaldehyde	11.78±0.61[h]	47.56±3.39[f]	6.23±0.61[j]
cinnamaldehyde	14.45±1.58[h]	50.11±0.82[f]	21.22±0.82[h]
propyl 4-hydroxybenzoate	61±0.83[c]	79.62±2.14[b]	78.22±0.61[b]
butyl 4-hydroxybenzoate	72.55±0.74[b]	86.83±1.83[a]	79.56±0.67[b]
4-phenoxycinnamic acid	53.55±1.99[d]	76.64±1.45[b]	50.89±2.14[d]
4-nitrocinnamic acid	24.44±1.36[g]	27.44±1.68[cd]	39.78±2.76[f]
4-*tert*-buthylcinnamic acid	47.11±2.02[f]	61.67±8.22[e]	68.89±1.11[c]
4-trifluoromethylcinnamic acid	44.89±2.79[f]	64.33± 0.78[cd]	42.44±1.45[e]
α-methyl-*trans*-cinnamaldehyde	56.67±0.78[c]	73.33 ± 3.96[e]	15.55±1.58[i]
2,6-dichlorocinnamic acid	59.33±2.56[c]	80.51 ± 5.12[g]	70±2.87[c]
fosetyl-Al	12.89±0.99[h]	-	12±0.93[i]
metalaxyl	13.11±1.99[h]	-	87.45±0.74[a]
benomyl	100.00[a]	-	-
DMSO	0±0.00[i]	0±0.00[h]	0±0.00[k]
Tween 80	0±0.00[i]	0±0.00[h]	0±0.00[k]
PDA	0±0.00[i]	0±0.00[h]	0±0.00[k]

[A] Values are the mean of experiments, each with five replicates.

*Values followed by different letters are significantly different (P=0.05) according to Duncan's multiple rang test.

APPENDIX C

Sequences producing significant alignments:

Accession	Description	score (Bits)	E value
AF322076.1	Fusarium oxysporum f. sp. vasinfectum strain Ag149-III 18S rib.....	1037	0.0
AF322075.1	Fusarium oxysporum f. sp. vasinfectum strain Ag149-I 18S rib.....	1037	0.0
AF322074.1	Fusarium oxysporum f. sp. vasinfectum strain Ag149 18S ribo.....	1037	0.0
DQ420797.1	Uncultured soil fungus clone 115-62 18S ribosomal RNA gene.....	1035	0.0
DQ420790.1	Uncultured soil fungus clone 53-17 18S ribosomal RNA gene.....	1035	0.0
DQ420779.1	Uncultured soil fungus clone 138-37 18S ribosomal RNA gene.....	1035	0.0
DQ420774.1	Uncultured soil fungus clone 137-16 18S ribosomal RNA gene.....	1035	0.0
AM260916.1	Uncultured fungus 18S rRNA gene (partial), ITS1, 5.8S rRNA gene.....	1031	0.0
EF090502.1	Uncultured fungus clone PN7A.1 18S ribosomal RNA gene.....	1024	0.0
EU326216.1	Fusarium oxysporum isolate XSD-78 18S ribosomal RNA gene.....	1022	0.0
DQ682576.1	Fusarium sp. IBL 03152 18S ribosomal RNA gene, partial sequence.....	1022	0.0
AF443071.1	Fusarium oxysporum 18S ribosomal RNA gene, partial sequence.....	1022	0.0
EU364866.1	Fusarium oxysporum strain F-G.1.5-031017-01 18S ribosomal RNA.....	1016	0.0
EU364853.1	Fusarium oxysporum strain F-T2.1.1-030616-15 18S ribosomal RNA.....	1016	0.0
EU364852.1	Fusarium oxysporum strain F-T2.1.1-030616-01 18S ribosomal RNA.....	1016	0.0
EU364844.1	Fusarium oxysporum strain F-H.6.5-030318-01 18S ribosomal RNA.....	1016	0.0
EU326215.1	Fusarium oxysporum isolate XSD-73 18S ribosomal RNA gene.....	1014	0.0
AY123744.1	Uncultured soil fungus isolate 5 small subunit ribosomal RNA.....	1014	0.0
AY123742.1	Uncultured soil fungus isolate 3 small subunit ribosomal RNA.....	1014	0.0
EU364854.1	Fusarium oxysporum strain F-T.1.7-040427-1 18S ribosomal RNA.....	1011	0.0
EU364850.1	Fusarium oxysporum strain F-T.1.7-030514-31 18S ribosomal RNA.....	1011	0.0
EU364842.1	Fusarium oxysporum strain F-H.6.5-030318-02 18S ribosomal RNA.....	1011	0.0
EU326194.1	Fusarium oxysporum isolate XSD-142 18S ribosomal RNA gene.....	1011	0.0
EU273520.1	Fusarium oxysporum isolate XSD-40 18S ribosomal RNA gene.....	1011	0.0
EF577235.1	Fusarium proliferatum small subunit ribosomal RNA gene.....	1011	0.0
AM901902.1	Uncultured ascomycete ITS region including 18S rRNA gene, ITS1.....	1009	0.0
DQ535184.1	Fusarium oxysporum f. sp. melonis strain HKU-956 18S ribosomal.....	1009	0.0
AY669124.1	Fusarium oxysporum strain F-H.6.5-030318-J1 18S ribosomal RNA.....	1009	0.0
AY123745.1	Uncultured soil fungus isolate 6 small subunit ribosomal RNA.....	1009	0.0
AY123743.1	Uncultured soil fungus isolate 4 small subunit ribosomal RNA.....	1009	0.0
EU364863.1	Fusarium oxysporum strain F-X.1.7-030527-12 18S ribosomal RNA.....	1007	0.0
EU364862.1	Fusarium oxysporum strain F-X.1.7-030527-11 18S ribosomal RNA.....	1007	0.0
EU364860.1	Fusarium oxysporum strain F-X.1.7-030520-13 18S ribosomal RNA.....	1007	0.0
EU364859.1	Fusarium oxysporum strain F-X.1.7-030520-12 18S ribosomal RNA.....	1007	0.0
EU152473.1	Fusarium oxysporum strain Y2 18S ribosomal RNA gene, partial.....	1007	0.0
AY462579.1	Fusarium oxysporum f. sp. vasinfectum isolate PA1 18S ribosomal.....	1007	0.0
AY669122.1	Fusarium oxysporum strain F-W.6.2-030304 18S ribosomal RNA.....	1005	0.0
EF060710.1	Hypocreales sp. LM398 18S ribosomal RNA gene, partial sequence.....	1003	0.0
AM260843.1	Uncultured fungus 18S rRNA gene (partial), ITS1, 5.8S rRNA.....	1002	0.0
AY462580.1	Fusarium oxysporum f. sp. vasinfectum isolate PA3 18S ribosomal.....	1002	0.0
AY147369.1	Fusarium oxysporum 18S ribosomal RNA gene, partial sequence.....	1002	0.0
EU364857.1	Fusarium oxysporum strain F-X.1.7-030520-03 18S ribosomal RNA.....	1000	0.0
EU364849.1	Fusarium oxysporum strain F-T.1.7-030514-21 18S ribosomal RNA.....	1000	0.0
EU364848.1	Fusarium oxysporum strain F-T.1.7-030514-12 18S ribosomal RNA.....	1000	0.0
AY669120.1	Fusarium oxysporum strain F-T.1.7-030520 18S ribosomal RNA.....	1000	0.0
AY533376.1	Gibberella moniliformis small subunit ribosomal RNA gene.....	1000	0.0
EF060712.1	Hypocreales sp. LM400 18S ribosomal RNA gene, partial sequence.....	998	0.0
AY043468.1	Fusarium oxysporum strain 17 internal transcribed spacer 1, 5.8S.....	998	0.0
DQ421283.1	Uncultured soil fungus clone 265-40 18S ribosomal RNA gene.....	996	0.0
AY667489.1	Fusarium oxysporum isolate 12-152 internal transcribed spacer.....	996	0.0
AY123741.1	Uncultured soil fungus isolate 2 small subunit ribosomal RNA.....	994	0.0

Fig C1. Alignment data of ITS region of isolate RM1

Sequences producing significant alignments:

Accession	Description	score (Bits)	E value
AY669125.1	Fusarium oxysporum strain F-X.1.7-030520-12 18S ribosomal RNA.....	987	0.0
EU563518.1	Fungal sp. 144C2AJ 18S ribosomal RNA gene, partial sequence.....	985	0.0
AY729069.1	Fusarium sp. 448 small subunit ribosomal RNA gene.....	985	0.0
AY729056.1	Fusarium sp. 415 small subunit ribosomal RNA gene.....	985	0.0
AF455450.1	Gibberella sacchari isolate wb395 small subunit ribosomal RNA.....	985	0.0
DQ369912.1	Zea mays cultivar line T66 internal transcribed spacer 1.....	985	0.0
AY970527.1	Fusarium oxysporum isolate 471 small subunit ribosomal RNA gene.....	985	0.0
EF505450.1	Uncultured endophytic fungus clone 15-13-01 18S ribosomal RNA.....	979	0.0
AB331950.1	Fungal sp. TT-F2 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2.....	979	0.0
AY729071.1	Fusarium sp. 463 small subunit ribosomal RNA gene, partial.....	979	0.0
AY729055.1	Fusarium sp. 414 small subunit ribosomal RNA gene, partial.....	979	0.0
AY729053.1	Fusarium sp. 412 small subunit ribosomal RNA gene, partial.....	979	0.0
EU563522.1	Fungal sp. 144c2f 18S ribosomal RNA gene, partial sequence.....	977	0.0
DQ420787.1	Uncultured soil fungus clone 265-15 18S ribosomal RNA gene.....	977	0.0
X94173.1	Fusarium oxysporum 18S rRNA gene (partial), 5.8S rRNA gene, 28S.....	977	0.0
EU563517.1	Fungal sp. 144c2a 18S ribosomal RNA gene, partial sequence.....	976	0.0
EU219559.1	Fungal sp. ZZF51 18S ribosomal RNA gene, partial sequence.....	976	0.0
EU326203.1	Fusarium oxysporum isolate XSD-76 18S ribosomal RNA gene.....	974	0.0
AY729075.1	Fusarium sp. 471 small subunit ribosomal RNA gene, partial.....	974	0.0
AY729070.1	Fusarium sp. 459 small subunit ribosomal RNA gene, partial.....	974	0.0
AY729064.1	Fusarium sp. 424 small subunit ribosomal RNA gene, partial.....	974	0.0
AY729059.1	Fusarium sp. 418 small subunit ribosomal RNA gene, partial.....	974	0.0
AY729057.1	Fusarium sp. 416 small subunit ribosomal RNA gene, partial.....	974	0.0
AY729052.1	Fusarium sp. 411 small subunit ribosomal RNA gene, partial.....	974	0.0
EU543261.1	Fusarium sp. Dzf18 18S ribosomal RNA gene, partial sequence.....	972	0.0
EU285554.1	Fusarium oxysporum isolate GXF-6 18S ribosomal RNA gene, partial.....	972	0.0
AY729068.1	Fusarium sp. 447 small subunit ribosomal RNA gene, partial sequence...	972	0.0
DQ166550.1	Fusarium sp. JJ002 18S ribosomal RNA gene, partial sequence.....	972	0.0
X78260.1	F.oxysporum (f.sp.vasinfectum, BIE) 5.8S rRNA gene.....	972	0.0
EF505455.1	Uncultured endophytic fungus clone 15-17-77 18S ribosomal RNA.....	970	0.0
X78259.1	F.oxysporum (f.sp.vasinfectum, Cuanza Bul) 5.8S rRNA gene.....	968	0.0
X78258.1	F.oxysporum (f.sp.vasinfectum, Cuanza Norte) 5.8S rRNA gene.....	968	0.0
EU363511.1	Fusarium sp. CPCC 480097 18S ribosomal RNA gene, partial sequence.....	966	0.0
EU285553.1	Fusarium oxysporum isolate GXF-5 18S ribosomal RNA gene, partial.....	966	0.0
EU285552.1	Fusarium oxysporum isolate GXF-2 18S ribosomal RNA gene, partial.....	966	0.0
EF590328.1	Fusarium oxysporum f. cubense strain ATCC 96285 18S ribosomal RNA.....	966	0.0
EF495237.1	Fusarium oxysporum strain Ppf15 18S ribosomal RNA gene, partial.....	966	0.0
EF495230.1	Fusarium oxysporum strain Ppf16 18S ribosomal RNA gene, partial.....	966	0.0
DQ459007.1	Fusarium oxysporum 18S ribosomal RNA gene, partial sequence.....	966	0.0

Fig C1. (continued)

gb|AF322076.1|AF322076 *Fusarium oxysporum* f. sp. *vasinfectum* strain Ag149-III 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Length=616

Score = 1037 bits (561), Expect = 0.0

Identities = 574/580 (98%), Gaps = 3/580 (0%)

Strand=Plus/Plus

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Query  4    GGTCCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGA  63
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Sbjct  38   GGT-CATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGA  96

Query  64   TCATTACCGAGTTTAC-ACTCCC-AACCCCTGTGAACATACCACTTGTTGCCTCGGCGGA 121
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Sbjct  97   TCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGCCTCGGCGGA 156

Query  122  TCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTAT 181
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Sbjct  157  TCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTAT 216

Query  182  ATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTT 241
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Sbjct  217  ATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTT 276

Query  242  CTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG 301
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Sbjct  277  CTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG 336

Query  302  AATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTC 361
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Sbjct  337  AATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTC 396

Query  362  GAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCC 421
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Sbjct  397  GAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCC 456

Query  422  CCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTG 481
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Sbjct  457  TCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTG 516
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Fig C1. (continued)

Sequences producing significant alignments:

Accession	Description	Score (Bits)	E value
EF423547.1	Botryosphaeria rhodina isolate P130 internal transcribed spacer 1.....	1027	0.0
EF423533.1	Botryosphaeria rhodina isolate P054 internal transcribed spacer 1.....	1007	0.0
EF564146.1	Botryosphaeria rhodina isolate M35 18S ribosomal RNA gene, partial.....	1003	0.0
EU563652.1	Fungal sp. 604C2K 18S ribosomal RNA gene, partial sequence; internal.....	998	0.0
EF060575.1	Botryosphaeriaceae sp. LM241 18S ribosomal RNA gene, partial sequence....	990	0.0
EU563649.1	Fungal sp. 4221c4a 18S ribosomal RNA gene, partial sequence; internal....	989	0.0
EU563614.1	Fungal sp. 231c2a 18S ribosomal RNA gene, partial sequence; internal.....	989	0.0
AB297716.1	Lasiodiplodia sp. RM2 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S.....	989	0.0
EU563647.1	Fungal sp. 4221c3a 18S ribosomal RNA gene, partial sequence; internal....	983	0.0
EF622074.1	Lasiodiplodia theobromae strain CBS111530 18S ribosomal RNA gene.....	983	0.0
EF622072.1	Lasiodiplodia theobromae strain CBS339.90 18S ribosomal RNA gene.....	983	0.0
EF622071.1	Lasiodiplodia theobromae strain CBS306.58 18S ribosomal RNA gene.....	983	0.0
EF622069.1	Lasiodiplodia theobromae strain CBS287.47 18S ribosomal RNA gene.....	983	0.0
EF622067.1	Lasiodiplodia theobromae strain CBS175.26 18S ribosomal RNA gene.....	983	0.0
EU407235.1	Botryosphaeria rhodina isolate B-013 internal transcribed spacer 1.....	983	0.0
DQ458891.1	Botryosphaeria rhodina strain CAA006 18S ribosomal RNA gene, partial.....	983	0.0
DQ458890.1	Botryosphaeria rhodina strain CBS124.13 18S ribosomal RNA gene, partial..	983	0.0
DQ212767.1	Rhizoctonia bataticola isolate 1 18S ribosomal RNA gene.....	983	0.0
AY640255.1	Botryosphaeria rhodina strain CBS 164.96 18S ribosomal RNA gene, partial.	983	0.0
EF622084.1	Lasiodiplodia parva strain CBS494.78 18S ribosomal RNA gene, partial.....	977	0.0
EF622083.1	Lasiodiplodia parva strain CBS456.78 18S ribosomal RNA gene, partial.....	977	0.0
EF622082.1	Lasiodiplodia parva strain CBS356.59 18S ribosomal RNA gene, partial.....	977	0.0
EF622070.1	Lasiodiplodia theobromae strain CBS289.56 18S ribosomal RNA gene.....	977	0.0
EF622085.1	Lasiodiplodia parva strain CBS495.78 18S ribosomal RNA gene, partial.....	972	0.0
EF622073.1	Lasiodiplodia theobromae strain CBS559.70 18S ribosomal RNA gene.....	972	0.0
EF622068.1	Lasiodiplodia theobromae strain CBS190.73 18S ribosomal RNA gene.....	972	0.0
EF110920.1	Botryosphaeria rhodina strain Br1 18S ribosomal RNA gene, partial.....	970	0.0
AY601898.1	Fungal endophyte sp. J1 internal transcribed spacer 1, partial sequence..	968	0.0
DQ222238.1	Rhizoctonia bataticola isolate 14 18S ribosomal RNA gene, partial.....	966	0.0
EU563646.1	Fungal sp. 4221C2e 18S ribosomal RNA gene, partial sequence; internal....	965	0.0
EU563644.1	Fungal sp. 4221c2b 18S ribosomal RNA gene, partial sequence; internal....	965	0.0
EU563635.1	Fungal sp. 263c1a 18S ribosomal RNA gene, partial sequence; internal.....	965	0.0
DQ222240.1	Rhizoctonia bataticola isolate Rb18 18S ribosomal RNA gene, partial.....	963	0.0
EU012372.1	Botryosphaeria rhodina strain UCD1012BC 18S ribosomal RNA gene.....	961	0.0
EU012371.1	Botryosphaeria rhodina strain UCD923SN 18S ribosomal RNA gene, partial...	961	0.0
EU012370.1	Botryosphaeria rhodina strain UCD921SN 18S ribosomal RNA gene, partial...	961	0.0
EU012369.1	Botryosphaeria rhodina strain UCD919SN 18S ribosomal RNA gene, partial...	961	0.0
EU012368.1	Botryosphaeria rhodina strain UCD918SN 18S ribosomal RNA gene, partial...	961	0.0
EU012367.1	Botryosphaeria rhodina strain UCD917SN 18S ribosomal RNA gene, partial...	961	0.0
EU012366.1	Botryosphaeria rhodina strain UCD916SN 18S ribosomal RNA gene, partial...	961	0.0
EU012365.1	Botryosphaeria rhodina strain UCD914SN 18S ribosomal RNA gene, partial...	961	0.0
EU012364.1	Botryosphaeria rhodina strain UCD881SN 18S ribosomal RNA gene, partial...	961	0.0
EU012363.1	Botryosphaeria rhodina strain UCD810SN 18S ribosomal RNA gene, partial...	961	0.0
EF622081.1	Lasiodiplodia pseudotheobromae strain CBS447.62 18S ribosomal RNA gene...	961	0.0
EF622080.1	Lasiodiplodia pseudotheobromae strain CBS374.54 18S ribosomal RNA gene...	961	0.0
EF622079.1	Lasiodiplodia pseudotheobromae strain CBS304.79 18S ribosomal RNA gene...	961	0.0
EF622078.1	Lasiodiplodia pseudotheobromae strain CBS116460 18S ribosomal RNA gene...	961	0.0
EF622077.1	Lasiodiplodia pseudotheobromae strain CBS116459 18S ribosomal RNA gene...	961	0.0
EF622076.1	Lasiodiplodia theobromae strain CBS113520 18S ribosomal RNA gene.....	961	0.0
EF622075.1	Lasiodiplodia theobromae strain CBS112874 18S ribosomal RNA gene.....	961	0.0
DQ008312.1	Botryosphaeria rhodina strain UCD220Co internal transcribed spacer 1.....	961	0.0
DQ008311.1	Botryosphaeria rhodina strain UCD206Co internal transcribed spacer 1.....	961	0.0
DQ008310.1	Botryosphaeria rhodina strain UCD205Co internal transcribed spacer 1.....	961	0.0
DQ008308.1	Botryosphaeria rhodina strain UCD191Co internal transcribed spacer 1.....	961	0.0
DQ233596.1	Botryosphaeria rhodina strain UCD1814Md 18S ribosomal RNA gene, partial..	955	0.0
DQ233594.1	Botryosphaeria rhodina strain UCD256Ma 18S ribosomal RNA gene, partial...	955	0.0
DQ008309.1	Botryosphaeria rhodina strain UCD202Co internal transcribed spacer 1.....	955	0.0

Fig C2. Alignment data of ITS region of isolate RK1

Sequences producing significant alignments:

Accession	Description	Score (Bits)	E value
EF564147.1	Botryosphaeria rhodina isolate M114 18S ribosomal RNA gene, partial.....	953	0.0
AY662402.1	Botryosphaeria rhodina isolate GL3-8B 18S ribosomal RNA gene, partial....	952	0.0
EU563626.1	Fungal sp. 261C1AN 18S ribosomal RNA gene, partial sequence; internal....	950	0.0
DQ233597.1	Botryosphaeria rhodina strain UCD1962SB 18S ribosomal RNA gene, partial..	950	0.0
DQ233595.1	Botryosphaeria rhodina strain UCD526Kr 18S ribosomal RNA gene, partial...	950	0.0
DQ233593.1	Botryosphaeria rhodina strain UCD197Co 18S ribosomal RNA gene, partial...	950	0.0
DQ233592.1	Botryosphaeria rhodina strain UCD196Co 18S ribosomal RNA gene, partial...	950	0.0
EU563627.1	Fungal sp. 261C1Q 18S ribosomal RNA gene, partial sequence; internal.....	948	0.0
AF502851.1	Leaf litter ascomycete strain its345 isolate 1000059760 internal.....	946	0.0
EU563653.1	Fungal sp. 604c4a 18S ribosomal RNA gene, partial sequence; internal.....	942	0.0
EU012376.1	Botryosphaeria rhodina strain UCD1060BC 18S ribosomal RNA gene, partial..	939	0.0
EU012375.1	Botryosphaeria rhodina strain UCD1030BC 18S ribosomal RNA gene, partial..	939	0.0
EU012373.1	Botryosphaeria rhodina strain UCD1014BC 18S ribosomal RNA gene, partial..	939	0.0
DQ458892.1	Lasiodiplodia gonubiensis strain CBS115812 18S ribosomal RNA gene.....	939	0.0
AY753995.1	Ascomycete sp. CM48 internal transcribed spacer 1, 5.8S ribosomal RNA....	939	0.0
AY942180.1	Botryosphaeria rhodina 18S ribosomal RNA gene, partial sequence.....	937	0.0
EF622017.1	Botryosphaeria rhodina 18S ribosomal RNA gene, partial sequence.....	933	0.0
AY753997.1	Ascomycete sp. CM50 internal transcribed spacer 1, 5.8S ribosomal RNA....	933	0.0
EU563631.1	Fungal sp. 261C2AP 18S ribosomal RNA gene, partial sequence; internal....	931	0.0
EU012374.1	Botryosphaeria rhodina strain UCD1028BC 18S ribosomal RNA gene, partial..	928	0.0
AY568635.1	Botryosphaeria rhodina isolate 31-M-Mexico 18S ribosomal RNA gene.....	928	0.0
DQ307677.1	Botryosphaeria rhodina 18S ribosomal RNA gene, partial sequence.....	926	0.0
AY754000.1	Ascomycete sp. CM53 internal transcribed spacer 1, 5.8S ribosomal.....	926	0.0
AY754009.1	Ascomycete sp. CM64 internal transcribed spacer 1, 5.8S ribosomal.....	918	0.0
EU563650.1	Fungal sp. 4221C4AA 18S ribosomal RNA gene, partial sequence; internal...	917	0.0
EU563640.1	Fungal sp. 298C3 18S ribosomal RNA gene, partial sequence; internal.....	915	0.0
EU564805.1	Lasiodiplodia theobromae 18S ribosomal RNA gene, partial sequence.....	915	0.0
EU563622.1	Fungal sp. 2552c2c 18S ribosomal RNA gene, partial sequence; internal....	913	0.0
AY753993.1	Ascomycete sp. CM46 internal transcribed spacer 1, 5.8S ribosomal RNA....	913	0.0
AY160201.1	Lasiodiplodia theobromae isolate L.theo1 18S ribosomal RNA gene.....	913	0.0
EU563634.1	Fungal sp. 261c3a 18S ribosomal RNA gene, partial sequence; internal.....	911	0.0
EF445362.1	Lasiodiplodia plurivora strain STE-U 5803 18S ribosomal RNA gene.....	911	0.0
AY612337.1	Botryosphaeria rhodina isolate MAMB 05 18S ribosomal RNA gene, partial...	911	0.0
AY754004.1	Ascomycete sp. CM58 internal transcribed spacer 1, 5.8S ribosomal RNA....	907	0.0
EF641303.1	Botryosphaeria rhodina strain FSN2 18S ribosomal RNA gene, partial.....	904	0.0
AY746000.1	Ascomycete sp. CM 27 18S ribosomal RNA gene, partial sequence.....	902	0.0
EU563641.1	Fungal sp. 298C4Z 18S ribosomal RNA gene, partial sequence.....	898	0.0
AY754002.1	Botryosphaeria rhodina strain CM55 internal transcribed spacer 1, 5.8S...	898	0.0
AF243400.1	Botryosphaeria rhodina isolate 96-172 18S ribosomal RNA gene, partial....	898	0.0
EF622086.1	Lasiodiplodia crassispora strain CBS110492 18S ribosomal RNA gene.....	893	0.0
EU331086.1	Botryosphaeria rhodina culture-collection ATCC:MYA-4195 18S ribosomal....	893	0.0
AY753991.1	Ascomycete sp. CM42 internal transcribed spacer 1, 5.8S ribosomal RNA....	887	0.0
EU563617.1	Fungal sp. 231C2I 18S ribosomal RNA gene, partial sequence.....	885	0.0

Fig C2. (continued)

gb|EF622074.1| *Lasiodiplodia theobromae* strain CBS111530 18S ribosomal RNA gene,
partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Length=542

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 Identities = 540/543 (99%), Gaps = 3/543 (0%)
 Strand=Plus/Plus

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Query  99   CACCCTTTGTGAACGTACCTCTGTTGCTTTGGCGGCTCCGGCCGCCAAAGGACCTTCAAA 158
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Query  159  CTCCAGTCAGTAAACGCAGACGTCTGATAAACAAGTTAATAAACTAAAACTTTCAACAAC 218
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Sbjct  181  GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT 240

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Sbjct  421  ATACACCTCGCTTTGGAGCGGTTGGCGTCGCCCGCCGGACGAACCTTCTGAACTTTTCTC 480

Query  519  AAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA-GCCGGA 577
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Sbjct  540  GGA  542
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Fig C2. (continued)

APPENDIX D

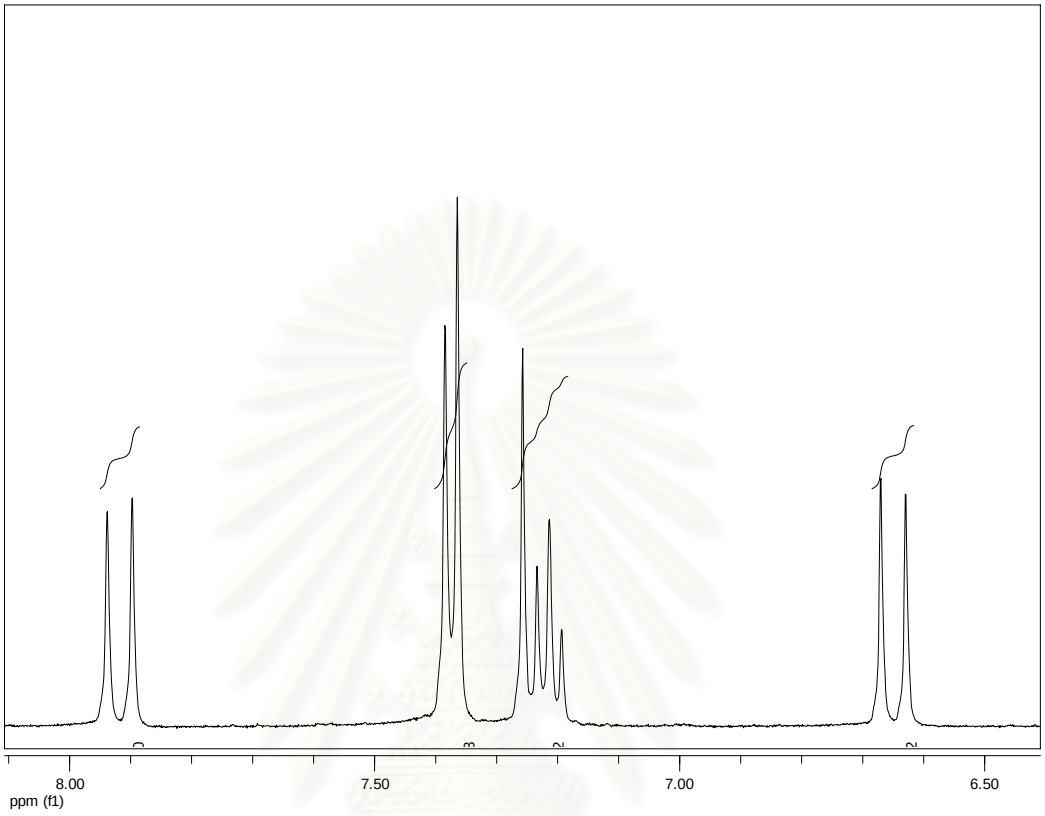

Fig D1 ^{1}H-NMR spectrum of 2,6-dichlorocinnamic acid

VITAE

Miss Rachsawan Mongkol was born in January 7, 1982 in Cha-Am district, Phetchaburi province, Thailand. She graduated with Bachelor Degree of Science from Faculty of Animal Science and Agricultural Technology, Silpakorn University, Nakornprathom, Thailand in 2004. She graduated in Master of Science in Biotechnology in 2007 from the Program in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand. During the couse of study, she obtained financial support from Graduate School Chulalongkorn University.