BIOLOGICAL CONTROL OF *PHYTOPHTHORA* SPP. CAUSING ROOT ROT OF POMELO USING *CHAETOMIUM* SPP.

PHUNG MANH HUNG

A THESIS SUBMITED IN FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN AGRICULTURE

FACULTY OF AGRICULTURAL TECHNOLOGY
KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

2016
KMITL-2016-AG-D-064-005

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Thesis:	Biological Control of *Phytophthora* spp. Causing Root Rot of Pomelo Using *Chaetomium* spp.
Student:	Mr. Phung Manh Hung
Student ID:	56604006
Degree:	Doctor of Philosophy
Program:	Agriculture
Year:	2016
Thesis Advisor:	Asst. Prof. Dr. Wattanachai Pongnak
Thesis Co-advisor:	Assoc. Prof. Dr. Kasem Soytong
	Asst. Prof. Dr. Supattra Poeaim

ABSTRACT

Phytophthora root rots are being major problem of citrus industry in Thailand. In this research, six isolates were found and divided into 2 groups, 3 slow-growing and 3 fast-growing isolates of *Phytophthora* spp. from samples in Chachoengsao province and Bangkok, Thailand, respectively. Based on morphological characteristics and ITS ribosomal DNA sequence analysis, the slow-growing isolates were identified as *Phytophthora palmivora*. Meanwhile, the fast-growing isolates were identified as *Phytophthora nicotianae*. All obtained isolates showed high virulence for pomelo seedlings in pathogenicity test.

Isolates *P. palmivora* PHY02 and *P. nicotianae* KA1 were tested against *Chaetomium* spp. as biological control agents. *Chaetomium globosum* CG05, *Chaetomium lucknowense* CL01 and *Chaetomium cupreum* CC3003 inhibited 50 – 61% of colony growth, 92 – 99% of spore production of *P. palmivora* PHY02, and 50 – 56% of colony growth of *P. nicotianae* KA1, in bi-culture tests. Especially, all the tested antagonists grew over, parasitized hyphae and degraded colony of the tested

pathogens. Furthermore, all crude extracts of the tested *Chaetomium* spp. exhibited antifungal activity on mycelial growth (effective dose ED_{50} values of 26.5 – 2,495 µg/mL) and spore formation (ED_{50}: 2.3 – 307.9 µg/mL) of *P. palmivora* PHY02, *in vitro*. The crude extracts even showed more strongly antifungal activity on mycelial growth of *P. nicotianae* KA1with ED_{50} values of 2.6 – 101.4 µg/mL. It is suggested that both antibiosis and mycoparasitism mechanisms involved in bioactivities of the tested *Chaetomium* spp. against *P. palmivora* PHY02 and *P. nicotianae* KA1.

Moreover, *Chaetomium* spp. and their crude extracts reduced 38 – 66% root rot, and increased 27 – 91% dry weight of plants inoculated with *P. palmivora* PHY02, compared to the inoculated control seedlings. In cases of seedlings inoculated with *P. nicotianae* KA1and treated with *Chaetomium* spp. and their crude extracts, root rot rates were reduced by 66 – 73% and dry plant weight increased by 72 – 85%. More interestingly, in most cases during the *in vivo* trials, the spores of tested antagonists gave the same or even better positive effects in controlling *P. nicotianae* KA1and *P. palmivora* PHY02 than their crude extracts. Thus, using spores of these antagonists to control *Phytophthora* pomelo root rots may provide a simple and economical approach, and may be easier to use in large-scale applications than crude extracts as fungicides.

It is particularly noble that *C. lucknowense* is reported for the first time as an effective antagonist against *Phytophthora* root rots of citrus, in this study. The effectiveness of the tested *Chaetomium* strains and their crude extracts in the control of *P. palmivora* and *P. nicotianae* in this study provides a convincing reason to promote the applications of these strains to control *Phytophthora* root rot in citrus.

ACKNOWLEDGEMENT

I would like to express my deepest thankfulness to my advisor Asst. Prof. Dr Wattanachai Pongnak, for his valuable advices, encouragement, support and help throughout my thesis.

My specially gratefulness to my co-advisors, Assoc. Prof. Dr. Kasem Soytong and Asst. Prof. Dr. Supattra Poeaim for all their important suggestions, comments, valuable advices, kind help and constant encouragement during my study in Thailand.

I would like to acknowledge the King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand for giving me scholarship for my doctoral study; and the Faculty of Agricultural Technology (KMITL) for providing research fund.

I would like to thank to Northern Mountainous Agriculture and Forestry Science Institute (NOMAFSI), Phu Tho, Vietnam for giving me this great chance to study in doctoral degree. My great thank also conveys to my colleagues at NOMAFSI (Vietnam) for their kind assistance and encouragement.

My sincere gratitude also conveys to Asst. Prof. Dr. Ammorn (from KMITL,Thailand), Prof. Dr. Cynthia C. Divina (from Central Luzon State University, Philippines), and Dr. Somdej Kanokmedhakul (from Khon Kaen University, Thailand), whom gave me helpful advices and kindly shared their knowledge.

I would like to thanks to my friends, whom I met in Ph.D. Research Lab at Biocontrol Unit and Mycological Research Section in Department of Plant Production Technology, Faculty of Agricultural Technology, KMITL for their friendship and sharing with me the remarkable time during my study.

Most of all, I would like to express my deep gratitude to my family for all their love, encouragement and sacrifices.

Phung Manh Hung

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CHAPTER 1

INTRODUCTION

1.1. Statement and significance

Pomelo (*Citrus maxima)* is known as a primary species of genus *Citrus*, belonging to *Rutaceae* (Mabberley. 1997). Pomelo has been widely grown for commercial purpose in Thailand and other countries in Southeast Asia where it originated (TFNet. 2013). However, under wet climatic conditions of the region, *Phytophthora* diseases are being major problems of citrus industry in general and of pomelo in particular. All over different citrus species, the yield losses due to *Phytophthora* diseases were estimated to be 6 – 12% per year. Among diseases of citrus causing by *Phytophthora* spp., root rots are the most prominent in all citrus types (Drenth and Guest. 2004).

Disease symptoms that caused by *Phytophthora* spp. are often confused with damages from other pathogens and abiotic agents. Thus, the heavy losses of citrus and other crops due to *Phytophthora* are often resulted to delay in recognition of the organisms as causal agents under investigation. Isolation and identification of *Phytophthora* spp. are the only accurate method of early detection the pathogens, although that are always difficult (Erwin and Ribiero. 1996).

Current practices for controlling *Phytophthora* diseases are largely based on cultivation management in fields and application of synthetic fungicides (Cacciola and San Lio. 2008; Graham *et al.* 2014). Intensive use of chemical fungicides in control of plant pathogens may lead to harmful to the environment and human health. Moreover, fungicide resistance has become a serious problem in chemical control of *Phytophthora* diseases in citrus and other crops (Erwin and Ribiero. 1996; Timmer *et al.* 1998; Gisi and Sierotzki. 2008).

The need to reduce the use of noxious synthetic fungicides in agriculture production has led to a search for biological control agents against plant pathogens, which are safe for both the environment and human consumption. Biological control, using microorganisms to suppress plant disease, provides a high potential alternative to the use of synthetic chemicals, is the most cost effective and safety to the environment (Sáenz-de-Cabezón. 2010; Narayanasamy. 2013).

Chaetomium Kunze is a large genus of saprophytic ascomycetes with more than 350 recognized species (Zhang *et al.* 2012). Relying on lytic enzymes, they decompose cellulose and other organic materials (Sun *et al.* 2006; Longoni *et al.* 2012). Some *Chaetomium* species are reported to act as antagonists against various plant pathogens. Even commercial bio-product has been developed from potent strains of *Chaetomium* spp. (Soytong *et al.* 2001). Furthermore, over 200 metabolites with a wide range of bioactivities have been found from the genus *Chaetomium* and many of which exhibited antifungal activity against plant pathogens (Zhang *et al.* 2012). Inadequate understandings of the modes and abilities of *Chaetomium* species in controlling *Phytophthora* species, however, are major reason of the limited application of these high potential biological control agents in citrus production.

1.2. Objectives

- To isolate and identify *Phytophthora* spp. causing root rots of pomelo, using morphology and molecular phylogeny.
- To prove pathogenicity of *Phytophthora* isolates.
- To control *Phytophthora* spp. causing root rots of pomelo using *Chaetomium* spp. and their crude extracts.

1.3. Scope, place and time of study

This study covered from collection soil and diseased samples, isolation and identification of *Phytophthora* spp. causing root rots of pomelo in Thailand. Biological

control of pomelo root rots was investigated by using *Chaetomium* spp. and their metabolites.

The experiments were conducted at Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Ladkrabang, Bangkok, Thailand. In addition, for molecular identifications of pathogens, DNA(s) of isolates were extracted and amplified at Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL). Sequencing of cloned DNA(s) was performed at First Base Laboratory, Selangnor, Malaysia. The study was started from June 2013 until January 2016.

CHAPTER 2

LITERATURE REVIEW

2.1. Current status of pomelo production

Pomelo is believed to be a native plant species in Southeast Asia, and the plants have been widely spread and commercially grown in many other regions in the world. According to the Food and Agricultural Organization of the United Nations Statistics Division, in 2013, the world harvested areas of pomelo and grapefruit was estimated at roughly 328,000 ha, resulting in production of 8.4 million tons with the gross value was 3.88 billion USD. In which, the Asian countries have accounted for approximately 58% of the harvested areas and 65% of production. Still, China (main land) is the biggest producer of the world with the harvested area covered on 78,000 ha, resulting in production of more than 3.7 million tons (FAOSTAT. 2013).

Thailand is the second producer of pomelo in Asia, just behind China. In 2013, the harvested area in Thailand was estimated at 32,500 ha increasing by 500 ha in 2012. The fruit production reached to 305,000 tons that accounted for about 25% total citrus production of this country, increasing by 5,000 tons in 2012. The gross value of production was estimated at 312 million USD that increased by 32 million USD in 2012 (FAOSTAT. 2013).

The demand for pomelo is predicted to increase from East consumers, especially in Chinese communities and Vietnamese during Lunar New Year (TFNet. 2013). Meanwhile, European countries are still the biggest importers of pomelo and grapefruit more than 752,374 tones, valued at 726.45 million USD in 2012 (FAOSTAT. 2013). Furthermore, the demand from these countries is predicted to continue increasing in future. The increasing world demand for pomelo will encourage larger production for export and domestic consumption.

2.2. History, taxonomy and biology of Phytophthora

Plant destruction caused by *Phytophthora* may be most early recorded to damage citrus before the organisms were discovered in real scientific terms. In 1876, 31years after causing late blight of potato that lead to the famous potato famine of Ireland in 1845, the organism was first time described and got the name "Phytophthora" means " Plant destroyer" by Anton de Bary (Erwin and Ribiero. 1996). However, even before the Irish famine time, during 1832 – 1836, *Phytophthora* epidemic erased 200-year-old citrus plants in Azores (Portugal). Later the epidemic spread throughout Europe, which killed all lemon (*Citrus × limon*) and citron trees (*Citrus medica*) in Italy during 1855 – 1889 and in Greece during 1869 – 1880 (Naqvi. 2004).

The genus *Phytophthora*, along with *Pythium*, belongs to the family Pythiaceae, order Peronosporales within the Oomycota, Stramenopile (Fig 2.1) (Beakes *et al.* 2012; Thines, 2014). Until now, more than 120 species within the *Phytophthora* genus have been detected and officially described, and most are plant pathogens (Phytophthora Database, 2015). Some *Phytophthora* species are narrow host range but some are very wide host range. For instance, *Phytophthora fragariae* infects a single host (Kennedy and Duncan. 1995), while some species such as *Phytophthora nicotianae*, *Phytophthora palmivora* and *Phytophthora cinnamoni* are able to infect several hundred to thousand different plant species, and the others infect from some to hundreds hosts (Erwin and Ribiero. 1996).

Although Oomycetes share many characteristics of ecology and life history with the true fungi such as Basidiomycetes and Ascomycetes, they are clearly distinguished from these fungi by their genetics, cell wall compositions, and biochemical pathways. Most parts of life history of Oomycetes are diploid whereas in the fungi are haploid. Cell walls of Oomycetes are composed of β-glucans but not chitin, which is common cell wall component of fungi. Unlike fungi, members of

which passes through a cycle of dispersal and encystment before germinating. Some species, such as *P. cinnamomi*, *P. palmivora*, *P. nicotianae*, also produce asexual chlamydospores from the mycelium. Meanwhile, oospores are resulted from sexual reproduction. All these spore types are potentially infective. In addition, chlamydospores and oospores also have function as overwintering or resting structures. All species of *Phytophthora* have a soil-borne resting stage. Most species are dispersed primarily in the soil, via the release of zoospores from infected plant material. By producing caducous (deciduous) sporangia, however, some species can disperse aerially such as *P. palmivora*, *P. infestans*, (Erwin and Ribiero. 1996).

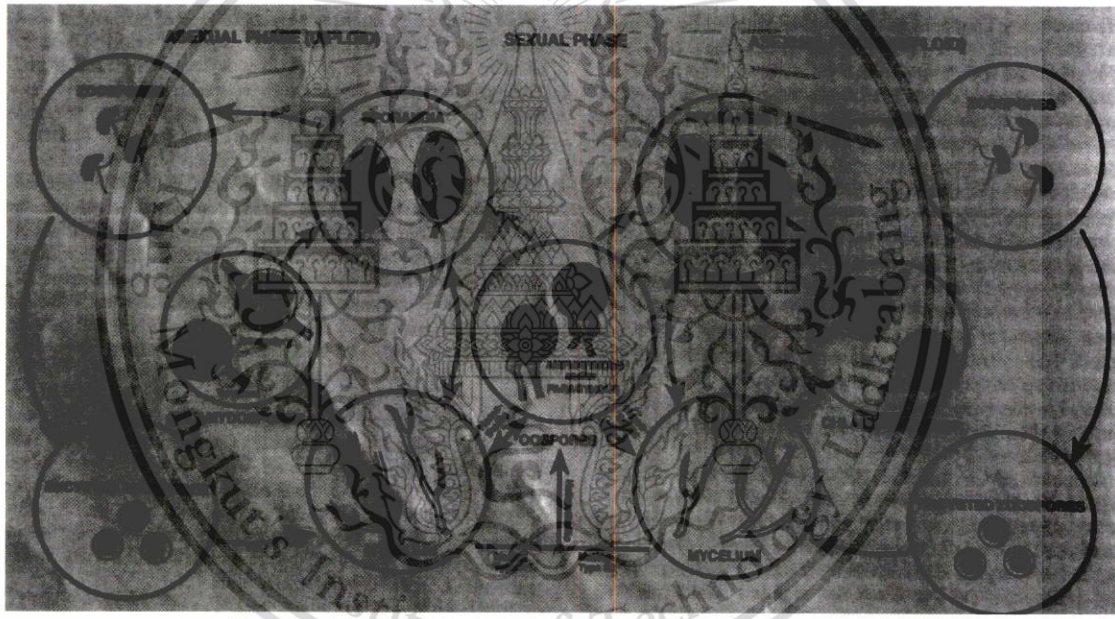


Figure 2.2. Life history of *Phytophthora* species (Source: Erwin and Ribiero. 1996).

2.3. *Phytophthora* diseases and their economic impacts on citrus industry

2.3.1. *Phytophthora* diseases in citrus

The most serious diseases of citrus plants caused by *Phytophthora* spp. are foot rot, gummosis and fibrous root rot (Graham and Timmer. 1992; Graham and Menge. 1999; Graham and Feichtenberger. 2015).

Phytophthora spp. attacks to natural wounds around the base of trunk such as bark cracks. The bark cracks ooze gum known as gummosis. In case of resistant rootstocks, the lesions may extend toward up bud-union. The infection may spread to the soil line and causes rot of trunk know as foot rot or collar rot, if rootstocks are susceptible. The trunks are partially girdled, resulting in defoliation of canopy, or death of trees in severe cases (Naqvi. 2004).

When *Phytophthora* species infect the feeder roots (fibrous roots), they cause rot of root cortex that called fibrous root rot. The cortex becomes soft, discolored, and then may be sloughed from the roots, leaving only the white stele (Graham and Timmer. 2003). The root rots due to *Phytophthora* is usually unrecognized by the growers until the symptoms of chlorosis or gummosis appear on canopy and trunk. The first visible symptom of Phytophthora root rots is when leaves of plant become chlorotic, in which, veins and leaf tissue bordering them turn yellow. This symptom is easy to confuse with symptom of nitrogen deficiency (Gisi and Sierotzki. 2008; Erwin and Ribiero. 1996; Savita *et al.* 2012). The production of new roots cannot compensate for the numbers of damaged roots. The root system, therefore, is unable to provide adequate water and nutrient for the tree, thus, resulting in the reduction of yield, defoliation and twig dieback of canopy (Graham and Timmer. 2003; Graham and Timmer. 2008).

In the citrus growing areas, the most important *Phytophthora* spp. are *P. nicotianae*, *P. citrophthora*, and *P. palmivora* (Graham and Timmer. 1992; Graham and Menge. 1999; Graham and Feichtenberger. 2015). Of which, *P. nicotianae* is most

common subtropical and tropical areas and causes root rot, foot rot, crown rot, gummosis. Occasionally, it attacks aerial parts of the tree causing brown rot of fruits, but it usually does not infect far above the ground. While *P. citrophthora* is common in Mediterranean climates with cool wet winters, *P. palmivora* is common in humid subtropical and tropical climates. Both species cause root rot, gummosis, and brown rot of fruit (Graham and Timmer. 1992, 1998; Timmer *et al.* 2000). The *P. palmivora* is known more virulence and aggressive, can damage larger rots than *P. nicotianae* (Zitko *et al.* 1991; Zitko and Timmer. 1994). Citrus root rot can be especially severe when rootstocks are susceptible to *Phytophthora*. Larger roots can be damaged when *P. palmivora* or *P. citrophthora* are presence. At some locations, the complex of root weevil (*Diaprepes abbreviatus*) damage and *Phytophthora* infection complex makes the disease situation and tree decline faster and graver (Graham *et al.* 2003).

2.3.2. Disease cycle and epidemics

The disease cycle of *Phytophthora* root rot and foot rot in citrus has been described (Graham and Timmer. 1992; Graham and Menge. 1999). As showing in the Fig 2.3, zoospores that released by sporangia can swim a short distance in water or spread out by rain or water irrigation. These zoospores infect to the wounds on roots and the elongated zones of root tips that are usually attract them. After contacting the roots, zoospores can germinate and produce sporangia rapidly on root cortex, resulting rot of the rootlets. This cycle can repeat itself "as long as conditions are favorable and susceptible tissue is available". Additionally, many *Phytopthora* species can produce chlamydospores and oospores that have thick wall. These spore forms can survive for long under unfavorable conditions. When conditions are favorable, however, they can germinate and form a new cycle. The pathogen requires a wound or natural growth crack for infection of suberized tree trunks and foot rot or gummosis occurs when zoospores or other propagules are splashed onto the trunk above the bud union (Widmer *et al.* 1998).

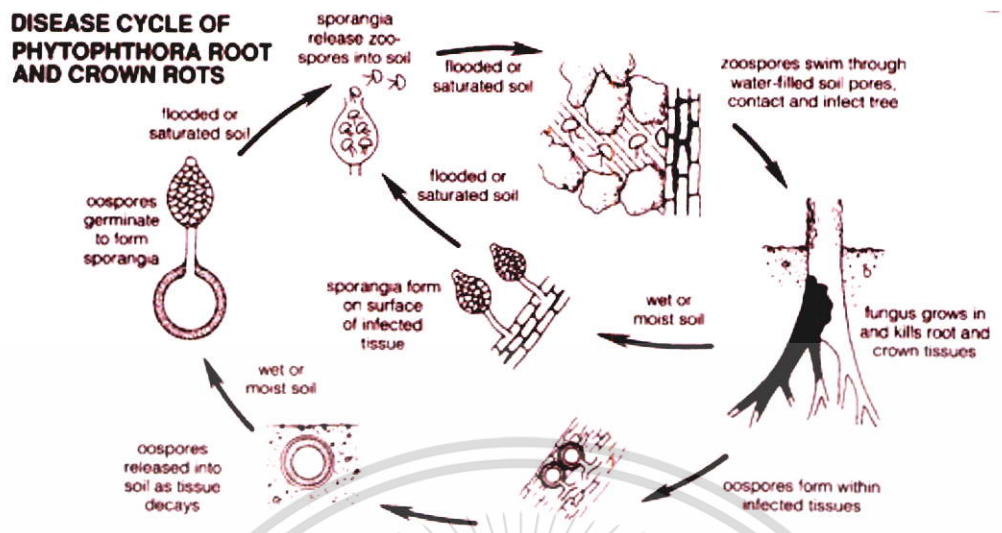


Figure 2.3. Life cycle of Phytophthora crown rots and root rots in citrus (Source: Graham and Timmer. 1992).

Ristaino and Gumpertz (2000) reviewed the five following modes as mechanisms of dispersal of *Phytophthora* species, which all occur in *Phytophthora* diseases of citrus.

- Dispersal from root to root in soil that involves root growth to inoculum, movement of inoculum to roots or root-to root contact
- Inoculum dispersal in surface water
- Splash dispersal from soil to aerial parts of plant
- Aerial dispersal from sporulating lesions on leaves, stems or fruit to other aerial parts of plant
- Dispersal by human or invertebrate activity including movement of soil, plants or propagules

The infected nursery plants are often the primary source of *Phytophthora* disease spread to citrus orchards (Graham and Timmer. 1992).

2.3.3. Economic impacts

Phytophthora diseases are the most serious of all citrus diseases, and annually decimate citrus production worldwide (Naqvi. 2004). The economic impact of *Phytophthora* diseases in citrus is difficult to estimate exactly because of the involvements of different *Phytophthora* species and other diseases. Moreover, the losses fluctuate according to seasonal and climatic conditions. The relationship between root rot and yield loss is proportionless, in addition (Graham and Feichtenberger. 2015). Nonetheless, it has estimated that *Phytophthora* spp. caused root rot in 8 – 20% of citrus orchards in California and Florida. Annually, from 3 – 6% of yields is lost due to the fibrous root rot, foot rot and gummosis in the United States (Graham and Menge. 1999; Graham and Feichtenberger. 2015). Under the wet climatic conditions often encountered in Thailand, infection with *Phytophthora* has become a major problem for the citrus industry, causing yield losses of approximately 6 – 12% and economic losses of at least 37 million USD/year. Overall, losses due to *Phytophthora* spp. are much more prevalent in some years in certain locations, because these diseases are particularly damaging under wet or flooded conditions (Drenth and Guest. 2004).

2.4. Isolation and identification of *Phytophthora* spp.

2.4.1. Detection and isolation of *Phytophthora* spp.

Although *Phytophthora* spp. are responsible for nearly 90 % of collar rots and 70% of all fine root diseases of woody plants, they are not often detected, leading to wrong diagnosis (Tsao. 1990). A woody tree can be infected by *Phytophthora* months to years before symptoms on foliage are visible when diseases are already shown in advance stage. A large tree can lose up to 50% of its feeder roots without loss of top growth. Therefore, early detection of *Phytophthora* diseases is difficult for not only growers but also plant pathologists. At the advance stage, the inoculum of

Phytophthora spp. is often decreased to a low and nearly detectable because of their limited saprophytic ability (Graham and Timmer. 2003). Moreover, most *Phytophthora* species grow slowly on culture media, thus are suppressed by fast growth organisms such as bacteria, *Pythium* spp. and fungi. Those make *Phytophthora* spp. difficult to isolate, especially from host tissues where *Pythium* spp. or the ubiquitous genus *Fusarium* often exist (Erwin and Ribiero. 1996). Not surprisingly, *Pythium* spp. and *Fusarium* spp., which are much easier to obtain from disease and soil samples, are frequently blamed for crown and root rots (Tsao. 1990).

Therefore, isolation of *Phytophthora* spp. requires different approaches and techniques as compare to those of true fungi.

2.4.1.1. Using of selective media

Unlike fungi and bacteria, *Phytophthora* spp. are insensitive to many fungicides and some groups of antibiotic. Thus, these chemicals inhibit associative organisms (such as fungi, other oomycetes and bacteria) but have little or no effect on target *Phytophthora*, are added to isolation media (Table 2.1). Dozen recipes for selective media have been developed using commercial prepared cornmeal agar (CMA) as basal medium (Erwin and Ribeiro. 1996). Of which, **P_{10}ARP** (CMA amended with pimaricin [10 µg/ml], ampicillin [250 µg/ml], rifampicin [10 µg/ml], and pentachloronitrobenzene [PCNB; 100 µg/ml]) and **PARPH** (P_{10}ARP medium amended with hymexazol 25 – 50 µg/ml) media are more commonly used for isolating most species of *Phytophthora* (Drenth and Sendall, 2001; Martin *et al.* 2012).

The presence of pimaricin in isolation media suppress most fungi, except for *Pythiaceae*, which consists of two genera *Pythium* and *Phytophthora*. Therefore, pentachloronitrobenzene (PCNB) and hymexazol are presented to suppress *Pythiaceae*. The pimaricin in all above media can be replaced by nystatin, which is a less effective but more readily and less expensive chemical (Drenth and Sendall. 2001). The presence of antibiotics in media will suppress bacteria. It found that the combination of

both rifampicin and ampicillin in media was more effective than vancomycin alone (Jeffers and Martin. 1986). Although the selective media are very useful for isolating *Phytophthora*, they do not "guarantee successful isolation". Quality of samples, in addition, influences greatly to the success of isolation *Phytophthora* (Tsao. 1983; Erwin and Ribiero. 1996; Martin *et al.* 2012).

Table 2.1. Common antibiotics and their properties used in selective media for isolating *Phytophthora* spp[1].

Chemical	**Activity**	**Target organism**	**Range used** (μg/ml)
Ampicillin	Antibacterial	Gram + ve bacteria	250 – 500
Benomyl	Antifungal	Most fungi except Zygomycetes and Oomycetes	10 – 25
Hymexazol	Antifungal	Most *Pythium* spp.	25 – 50
Nystatin (Mycostatin)	Antifungal	Most fungi except the Peronosporales	10 – 100
Penicillin G	Antibacterial	Gram +ve and Gram –ve cocci; Gram +ve bacilli	50 – 100
Pentachloronitrobenzene (PCNB)	Antifungal	Narrow antifungal spectrum	10 – 100
Pimaricin	Antifungal	Most fungi except the *Pythiaceae*	5 – 10
Polymixin B	Antibacterial	Gram –ve bacteria	20 – 50
Rifampicin	Antibacterial	Gram +ve and Gram –ve bacteria	10
Vancomycin	Antibacterial	Gram+ve and Gram-ve bacteria	100 – 200

[1]Source: Erwin and Ribeiro (1996) and Drenth and Sendall (2001)

2.4.1.2. Isolation from plant tissue

Using the selective media allows isolate *Phytophthora* spp. directly from infected plant tissues. The success of isolation will increase when newly infected samples are used, but will be greatly reduced when using old or dead samples (Tsao. 1983; Erwin and Ribiero. 1996). Because sterilization of sample surface can also kill

pathogen, this step may be unnecessary if samples are newly infected tissues, but it is required if necrotic tissues are used. For plants produce phenolic compounds, which can inhibit growth of *Phytophthora* spp., repeated washing tissue samples before placing on selective media is recommended (Streito *et al.* 2002; Martin *et al.* 2012).

2.4.1.3. Isolation from soil

A wide range of methods have been reported for the successful isolation of *Phytophthora* from soil and water samples, including soil plating and baiting methods.

Phytophthora spp. can be directly isolated from soil and water samples by using plating technique. The technique is typically utilized when *Phytophthora* inoculum in samples is high. Only small volume of soil or water samples is spread on plates containing selective agar media. After 2 - 4 days, *Phytophthora* each propagule in sample may develop into separate colony, thus the organisms can be isolated. This is common technique used for estimating population of *Phytophthora spp.* in soil and water samples by method of calculating the number of colonies developed on the agar plate (Timmer *et al.* 1988). Nonetheless, using of plating method is inefficient when *Phytophthora* inoculum in samples is low (Martin *et al.* 2012).

Soil baiting that uses *Phytophthora* attractive-materials as baits is the traditional but still very useful method for isolating *Phytophthora* spp. from soil. As compare to the direct plating, soil baiting has two main advantages. First, a larger volume of soil can be used, thus, it increases the chances of detecting species present at a low population density. Secondly, baiting is more suitable for detecting homothallic species that survive as dormant oospores, in soil (Erwin and Ribiero. 1996). Different techniques and plant materials as baits have been used for isolating *Phytophthora* spp. from soil (Table 2.2). However, recently, flotation of leaf tissue in soil-water suspension has been preferred technique in most recent studies rather than insertion soils in fruit reported in historical records (Erwin and Ribiero. 1996; Martin *et al.*

2012). Subsequently, isolation of *Phytophthora* from the infected baits can be made from healthy tissue surrounding lesions by using selective media (Ferguson and Jeffers. 1999; Drenth and Sendall. 2001). In addition, using un-wounded baits can reduce infection of *Pythium* spp. to the baits (Jeffers and Aldwinckle. 1987).

Table 2.2. Baiting techniques for isolating *Phytophthora* from soil[1].

Species	Bait material	Procedure
P. cinnamomi	Apple or pear	Make holes in fruit. Fill with wet soil and cover with plastic bag at 15-27°C for 5-10 days. Isolate from the edge of the rotted area around the hole. Suitable technique for many *Phytophthora* spp.
	Apple slices	Immerse slices in 200 ml water to which 25 g soil has been added, for 4-10 days
	Avocado fruit	Embed fruit partially in flooded soil; incubate at 20-27°C for 2-4 days.
	Avocado seedlings	Plant seedlings in wet soil; incubate at 21-27°C for 2-3 days.
	Avocado leaf pieces	Float leaf pieces on water added to soil for 4 days
P. citrophthora	Apple, lemon or orange fruit	Insert soil or citrus tissue into fruit as for *P. cinnamomi* above. Alternatively, place lemon or orange on the surface of soil for 4 or more days
	Lemon fruit	Immerse partially in 150 ml water to which 25 cc soil has been added. Incubate at 25°C for 6 days
P. nicotianae	Apple, lemon or orange fruit	Insert soil or citrus tissue into fruit as for *P. cinnamomi* above.
	Citrus leaf pieces	Float small leaf pieces on water 1-2 cm above 100 cc soil. Incubate at 22-28°C for 3-4 days.
	Tobacco leaves	Immerse the petiole end of leaf in water-soil mixture. Incubate at 25°C for 6 days
P. palmivora	Black pepper leaves	Immerse black pepper leaves or leaf discs partially in a water-soil mixture. Used for isolation from black pepper soils
	Cocoa pods	Insert soil beneath flaps of endocarp tissue of unripe cocoa pods. Used for isolation from cocoa soils
	Taro roots	Cut roots into 2.5 cm lengths, autoclave. Incubate in moistened soil (50 g/Petri dish) at 15°C for 7 days

[1]Source: Drenth and Sendall (2001)

Sampling is another important factor influences the success of *Phytophthora* isolation. Populations of *Phytophthora* spp. in soil vary according to season. Therefore, it is suggested that during or immediately after wet weather in rain season, when *Phytophthora* spp. are more active, is the best time to take soil sample for isolating. Sampling is often best from moist soils, under edge of plant canopy, 5 – 10 cm below soil surface. In addition, *Phytophthora* spp. in soil samples will lose their viability if are dried under high temperature (+ 40°C). Thus, after collection, the samples should be placed in plastic bag and kept at 10 – 15°C to prevent dry out (Drenth and Sendall. 2001; Martin *et al.* 2012).

2.4.2. Identification of *Phytophthora* spp.

2.4.2.1. Morphological approach

Many *Phytophthora* species can be easily identified which based on their morphological characteristics. Overlapping morphological features and minor-variable differences among *Phytophthora* spp., however, are making species difficult to identify accurately. Morphological identification of *Phytophthora* is based on the taxonomic keys of Waterhouse, which was later revised by Stamps *et al.* (1990). Useful characters that are used to classify species of *Phytophthora* include culture pattern; sporangium morphology (thickness of papilla; caducity; length-to-breadth ratio); morphology of sexual structures such as antheridia, oogonia and oospores; the presence or absence of chlamydospores and morphology of hyphae (Stamps *et al.* 1990; Erwin and Ribiero. 1996).

The thickness of papilla of sporangium is one of the most robust and useful characters for morphological identification of *Phytophthora*. Based on the thickness of papilla, *Phytophthora* are classified as papillate (papilla size $\geq$3.5 µm), semipapillate (papilla size <3.5 µm), and nonpapillate species with very slight apical thickening.

Currently, taxonomic classification (basing on analyzes of DNA sequences) divide described species (around 120 species) of genus *Phytophthora* into 10 clades (from 1 to 10) (Kroon *et al.* 2012). Interestingly, there are "natural arrangements" of the species by the type of papilla, with some exceptions, is observed in the groupings of a phylogenetic analysis (Martin *et al.* 2012). Papillate and semipapillate species place in clades 1 to 5. Clades 6, 7, 9, and subclade 8a contain nonpapillate species only. In subclades 8b and 8c are a mix of semipapillate and nonpapillate species. Clade 10 contains a mix of papillate and the nonpapillate species. Additionally, almost all nonpapillate species have persistent sporangia (non-caducous) except *P. pinifolia* and *P. foliorum,* which are partially caducous. Sporangial shape (ovoid, obovoid, pyriform, obpyriform, clavate, obclavate, reniform, irregular) and size (length-breadth ratios) are other important features for identification (Erwin and Ribiero. 1996).

For morphological identification of *Phytophthora*, it is useful to determine if a culture is homothallic or heterothallic, and whether the antheridium is amphigynous (around the oogonial stalk) or paragynous (next to the oogonial stalk). Homothallic species produce oogonia in single cultures, while heterothallic species produce them in the presence of a strain of the opposing compatibility type (A1 or A2).

The morphology of chlamydospores does not differ greatly between species. Thus, characteristics of this structure do not support much in species identification. Instead, the presence (for example, *P. palmivora*) or absence (for example, *P. heveae*) of chlamydospores is more useful for species identification (Erwin and Ribeiro. 1996; Drenth and Sendall. 2001).

2.4.2.2. DNA sequencing for identification of *Phytophthora* species

"DNA sequencing provided a big leap forward in knowledge on *Phytophthora* species" (Kroon *et al.* 2012). Sequencing analysis of specific loci is the most accurate molecular method for identification of isolates to a species level. If the sequences for

particular genes or DNA regions are identical or nearly identical, the isolates supposedly belong to the same species. Some specific loci from nuclear (ITS, large subunit rRNA, TigA gene fusion, translation elongation factor 1α) and mitochondrial (*cox1, nad1, cox2, nad9, rps10,* and *secY*) have been sequenced for identification purposes and phylogenetic resolution within *Phytophthora* (Grünwald *et al.* 2011; Martin *et al.* 2012). Of the loci, the non-coding ITS region consisting of ITS1, the 5.8S rDNA and ITS2, contain stretches of high homology that were used to design primers for polymerase chain reaction (PCR) amplification (Grünwald *et al.* 2011). For almost all *Phytophthora* species, the same primers can be used to amplify the region. The first extensive phylogenetic study of the genus *Phytophthora* based on ITS sequences was described by Cooke *et al.* (2000). This study based on analysis of ITS sequences of 234 isolates from 50 distinct *Phytophthora* species to divide the genus into 10 clades that currently used to group *Phytophthora* species and replaced the Waterhouse classification. Latterly, phylogenetic analysis of the genus was based on the combination of sequence data from nuclear loci and mitochondria (Kroon *et al.* 2004, 2012; Martin *et al.* 2014).

For molecular identification of certain isolates *Phytophthora* into species level, sequencing ITS regions usually is the first choice because it is easy to amplify and is diverse enough to distinguish among most species (Grünwald *et al.* 2011; Martin *et al.* 2012; Kroon *et al.* 2012). Not surprisingly, the ITS sequence data are the largest and more ready in databases than sequence data of other loci. When conducting BLAST analyses, it is important to use accurate dataset, which should be large and diverse, to ensure sequences reflect accurate species classification. There are some good databases that can used for BLAST analyses of Phytophthora sequences include the *Phytophthora* Database (http://www.Phytophthoradb.org) (Park *et al.* 2008), Phytophthora-ID (http://www.Phytophthora-ID.org) (Grünwald *et al.* 2011) and the National Center for Biotechnology Information (NCBI; http://www.ncbi/blast/).

For identification of a *Phytophthora* isolate to species level using DNA sequence, it is first suggested that sequencing the ITS regions. If sequences are 100% identical, then the *Phytophthora* sp. has been identified. If identity is <100% but ≥ 99%, a putative match was made, and it is suggested that follow-up identification of key morphological characters using microscopy be conducted. If identity is <99%, a new species might be detected and additional work will be required. In this case, sequencing of the *cox* spacer region and additional loci is suggested (Park *et al.* 2008; Grünwald *et al.* 2011).

2.5. Management of *Phytophthora* root rots in citrus

2.5.1. Using of resistant or tolerant rootstocks

Using tolerant and resistant rootstocks with desirable horticultural characteristics are the best solutions for controlling Phytophthora disease. Those are the best ways to reduce the costly applications of fungicides (Naqvi. 2004). Unfortunately, resistant rootstocks to *Phytophthora* spp. often have undesirable horticultural traits, are susceptible to other diseases or to abiotic stress such as PH, salinity, drought *etc*. The rootstocks with desirable horticultural characteristics, unfortunately, are susceptible to *Phytophthora* spp., virus and other diseases (Cacciola and San Lio. 2008; Graham and Feichtenberger. 2015). To gather the desirable traits of rootstocks, breeding through biotechnological means, therefore, has been carried out carried out (Grosser *et al.* 1994; Grosser *et al.* 2007; Dambier *et al.* 2011). The thorough screening results indicate that no citrus rootstocks are completely resistant to *Phytophthora* spp. However, there were wide variations in susceptibility of different rootstocks to different *Phytophthora* species (Naqvi. 2004; Graham and Feichtenberger. 2015). In general, while Cleopatra mandarin and sour orange group are tolerant to foot rot but are susceptible to root rot cause by *P. nicotianea*, trifoliate orange and its hybrids are tolerant or resistant. In contrast, Carrizo citrange and Swingle citrumelo, the hybrids of trifoliate orange, are susceptible to *P. palmivora*,

whereas Cleopatra mandarin and sour orange group are tolerant (Graham. 1990, 1995; Matheron *et al.* 1998; Graham and Feichtenberger. 2015). Additionally, pomelo (*C.maxima*) is considered susceptible to *Phytophthora* root rot (Naqvi. 2004).

2.5.2. Cultural practices

"Prevention is better than cure" should be followed strictly in *Phytophthora* managerial strategies. Appropriate cultural practices can immensely limit the spreads, multiplications, reproductions and infections of *Phytophthora* (Naqvi. 2004). Plants for new orchards should be selected from *Phytophthora* free nursery stock and with high budding. Because *Phytophthora* spp. abundant water to grow, disperse and infect plant, soils used for citrus orchard should be well drained and able to control water to avoid flooding and over watering. Thus, water management strategies can help immensely in management of *Phytophthora* diseases of Citrus (Naqvi. 2004). Additionally, it is better to apply nitrogen in nitrate form rather than in ammonium. The ammonium nitrogen is easy to metabolize to amino acids asparagine and glutamine, which attract zoospores and are ideal nutriments for Phytophthora spp.(Cacciola and San Lio. 2008).

2.5.3. Chemical control

Controls of *Phytophthora* ssp. still mainly rely on fungicides, despite the possibilities of their harmful consequences to human health and environment. Both systemic fungicides with more effectiveness and protestant fungicides (non-systemic) with lower costs are used to control phytophthora diseases in citrus.

Metalaxyl, a water-soluble phenylamide, is the most used systemic fungicide to control oomycetes including *Phytophthora* spp. (Bruin and Edgington, 1983; Erwin and Ribiero. 1996). The substance disrupts the ribosomal RNA synthesis, thus it disrupt the protein synthesis of Oomycetes (Gisi and Sierotzki. 2008). At low concentrations, metalaxyl strongly inhibit mycelial growth, formations of sporangia,

chlamycdospores and oospores of *Phytophthora* spp. (Farih *et al.* 1981). On other views, quick developments of resistance to metalaxyl of some species of *Phytophthora* spp. such as *P. infestans*, *P. citricola*, *P. nicotianae* and many other oomycetes, after the applications of these site-specific fungicides, have been reported (Ferrin and Kabashima. 1991; Csinos and Bertrand. 1994; Goodwin and McGrath. 1995; Gisi and Sierotzki. 2008). For instance, Timmer *et al.* (1998) found that 31 – 59% isolates of *P. nicotianae* from citrus nursery sites in Florida are resistant to metalaxyl. While the sensitive did not grow in the presence of 1 μg/ml of metalaxyl, these resistant isolates grew well at 100 μg/ml. Another systemic fungicide for control *Phytophthora* spp. in citrus is fosetyl-Al, an ethyl phosphonate fungicide. This substance works as a host defense inducer that triggers systemic acquired resistance (SAR) of plants (Bruin and Edgington. 1983; Erwin and Ribiero. 1996). Especially, fosetyl-Al still shows effective on isolates that resist to metalaxyl (Timmer *et al.* 1998). Both metalaxyl and fosetyl-Al can use in the ground to control *Phytophthora* root rots of citrus. Fosetyl-Al, however, is more effective when sprayed on foliar. In addition, using systemic fungicides in treatments against foot and root rots prevents infections of brown rots to fruit, in both pre- harvest and post-harvest phases (Cacciola and San Lio. 2008). Besides the uses of systemic fungicides, the non-systemic fungicides such as mancozeb, chlorothalonil and copper formulations still are used because of their lower cost (Naqvi. 2004; Gisi and Sierotzki. 2008).

Intensively chemical control, especially the use of site-specific fungicides, will result in resistance of *Phytophthora* spp. (Gisi and Cohen. 1996). The probability of resistant survivors will be smaller if the initial inoculum density is low. In addition, the populations and infections of *Phytophthora* spp. in soil vary widely according to season (Dirac *et al.* 2003; Alvarez *et al.* 2009). Therefore, estimates populations of *Phytophthora* spp. in soil before deciding applications of fungicides will reduce the resistance risk (Gisi and Sierotzki. 2008; Cacciola and San Lio. 2008; Graham and Feichtenberger. 2015). Currently, to avoid the developments of resistance, it suggests

that should not apply fungicides if density of *Phytophthora* is under 10 to 15 propagules per cm^3 of soil (Graham *et al.* 2014; Graham and Feichtenberger. 2015). However, it is also suggested that in young plant, fungicide treatment should be applied routinely for the first 2 years, because of the highly sensitive of young citrus plants to *Phytophthora* spp. (Cacciola and San Lio. 2008).

Additionally, to have maximum control of root rots, the applications of fungicides should be timed according to the physiological state of the plant and the population dynamics of pathogens. Normally, before plants begin to form new root generations is the best period to apply fungicides. At these periods of year, roots are sensitive to *Phytophthora* spp., thus need to be protected (Naqvi. 2004; Cacciola and San Lio. 2008). Citrus roots have distinct growth and dormant periods, which alternate with the periods of foliage growth (Lutz and Menge. 1986). The shoot flushes will precede the root flushes.

2.5.4. Biocontrol

To reduce the dependence of citrus production on noxious synthetic pesticides, the search for effective biological control agents (BCAs) against *Phytophthora* spp. has been widely carried out.

Fang and Tsao (1995a; 1995b) explored that *Pythium nunn* and *Penicillium funiculosum* inhibited mycelial growth, parasitized hyphae and spores of *P. cinnamomi*, *P. nicotianae*, and *P. citrophthora in vitro*. At 1,000 propagules per gram of soil, *Pythium nunn* and *Penicillium funiculosum* reduced root rot incidence of sweet orange seedlings caused by these pathogens. In addition, the *Penicillium funiculosum* increased significantly plant weight of the citrus seedlings, whereas *Pythium nunn* caused slight reduction of plant weight. Similarly, some isolates of *Trichoderma* spp. were reported to inhibit both mycelial growth of *P. parasitica in vitro* and reduced root rot incidence of citrus caused by the pathogen *in vivo* (Gade. 2012). *Myrothecium*

roridum also inhibited *in vitro* growth and *in vivo* infections of *Phytophthora* spp. to Citrus (Tuset *et al.* 1990). Co-inoculation of mycorrhizae *Acaulospora tuberculata* and *Glomus etunicatum* into roots of citrus seedlings increased root weight and phosphorus content in leaves, and reduced the infections of *P. nicotianae* (Watanarojanaporn *et al.* 2011). In addition, a hypovirulent isolate of *P. nicotianae* that naturally occurred on citrus roots could protect citrus rootstocks from infections of other virulence *P. nicotianae* and *P. palmivora*. However, the protection citrus root infections is not related to induction of systemic acquired (Colburn and Graham. 2007; Graham *et al.* 2012). Other fungi such as *Gliocladium penicillioides*, *Streptomyces* sp., *Fusarium solani* have also been reported to work as antagonists against Phytophthora root rot (Dandurand and Menge. 1992; Aryantha and Guest. 2006). Additionally, soil amendments such as composted organic matter have been evaluated for their potentials to act as biological control agents against Phytophthora root rot. Widmer *et al.* found that when a citrus soil was amended (20% vol/vol) with certain sources of composted municipal waste (CMW), the root rot incidence of susceptible citrus seedlings caused by *P. nicotianae* was reduced from 95% to as low as 5% (Widmer *et al.* 1998, 1999). Morales-Rodríguez (2014) reported that fresh tissues of *Brassica* spp. when treated as bio-fumigant could inhibit mycelial growth of *P. nicotianae*. Meanwhile brassica pallets showed effective control root rots of pepper caused by *P. nicotianae* in green house.

Maintaining adequate BCAs population in soil entry season, however, is major constrain of bio-control approach for root diseases. For instance, a bio-control agent developed from *Pseudomonas putida* 06909-rif/nal when was weakly applied for citrus orchards, population of *P. parasitica* in citrus rhizosphere was reduced by 84% after 1 year, but did not consistently reduced in the next years. However, the population of *Pseudomonas putida* was quickly declined when was applied once a years (Steddom *et al.* 2002).

2.5.5. *Chaetomium* spp. as the biological control agents

Chaetomium species are ubiquitous fungi most found in soils and death materials, with more than 350 species exist (Zhang *et al.* 2012). They decompose cellulose and other organic material. Some certain strains of these fungi can act as antagonists against plant pathogens.

Of which, *C. globosum* has received most concerns. Aggarwal *et al.* (2004) found that 06 potent isolates of *C. globosum* reduced 50 – 57% radial growth of *Drechslera sorokiniana* causing spot blotch of wheat, *in vitro*. At the meantime, crude extracts (ethyl-acetate extract) of their culture substrates reduced 65 – 83% colony growth of the pathogen *in vitro*, gave disease control rates of 70 – 84% *in vivo*. The authors found that interaction between two isolates of these *C. globosum* and the pathogen showed mycoparasitism, but the others showed antibiosis. Similarly, Shanthiyaa *et al.* (2013) evaluated biological potentials of 08 isolates of *C. globosum* to control *P. infestans* causing potato late blight in India. They found that the antagonists inhibited 45 – 72% colony growth of *P. infestans* in *vitro*. Moreover, the antagonists and compound named Chaetomin isolated from their culture filtrate reduced infections of the pathogen and increased the tuber yield of potato when treated for potato tubers.

Kanokmedhakul *et al.* (2002) found some compounds had antifungal, antibacterial activities from *C. globosum* N0802. This fungus and its crude extracts were reported to exhibit good control effects against *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt both *in vitro* and *in vivo*. Especially, the isolates could promote growth of tomato (Charoenporn *et al.* 2010).

Recently, Zhang *et al.* (2013) isolated chaetoglobosin A and chaetoglobosin C from an endophytic isolate of *C. globosum*. Of which, the chaetoglobosin A exhibited antifungal activity against *Setosphaeria turcica* causing Northern corn leaf blight both *in vitro* and *in vivo*. Additionally, certain isolate of *C. globosum* can produce different

compounds with different bioactivities such as phytotoxicity, cytotoxicity, mycotoxins (Li *et al.* 2014).

Moreover, there have been some reports on antagonisms of other *Chaetomium* spp. against different plant pathogens. *C. lucknowense* CL01, *C. elatum* ChE01 and their crude extracts exhibited abilities to control *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt (Sibounnavong *et al.* 2011). Crude extracts of *C. aureum*, *C. bostrychodes*, *C. cochliodes*, and *C. cupreum* exhibited antifungal activity against *Pythium aphanidermatum* causing root rots of pineapple (Pornsuriya *et al.* 2010). Nguyen *et al.* (2013) reported that *C. lucknowense* and *C. cupreum* inhibited mycelial growth and spore formation *in vitro* of *Pestalotia* sp., the causal agent of leaf spot in tea. In another study, Phung *et al.* (2014) reported that *C. lucknowense* and *C. cupreum* inhibited growth and spore formation of *Colletotrichum gloeosporioides* and *Pythium aphanidermatum* that associated with anthracnose and root rots of citrus.

In Thailand, *Chaetomiun* species have been screened for using as antagonist in since 1989. Potent strains of *C. cupreum* and *C. globosum* even developed as commercial product call Ketomium® that is widely used in Thailand and some countries in Southeast Asia. The product has shown good effects in controlling *Phytophthora* spp. infected tangerine (*Citrus reticulata*), black pepper, strawberry and durian in Thailand (Soytong *et al.* 2001).

Chaetomium spp. are well known as producers of hundreds secondary metabolites that have different bioactivities (Li *et al.* 2011; Zhang *et al.* 2012). Most the studies described those *Chaetomium* spp. suppressed plant pathogens through productions of substance(s), which have antifungal activities. In which, the antagonists did not parasitize pathogens; and no mycoparasitic phenomenon was observed or described. Moreover, Heller and Theiler-Hedtrich (1994) observed *C. globosum* grew over, coiled hyphae and degraded colonies of *P. parasitica* and *P. cinnamomi* in dual culture tests, suggesting mycoparasitic phenomenon. Nonetheless, to date, this is the

only evidence to suggest mycoparsitism as one of mechanisms of antagonist *Chaetomium* spp. use to control oomycetous pathogens. Thus, the understanding of interactions between the antagonists *Chaetomium* spp. and oomycetes including *Phytophthora* spp. is inadequate. Additionally, there is a significant limitation of research on the abilities of *Chaetomium* species and their metabolites to control the *Phytophthora* root rots in citrus.

CHAPTER 3

RESEARCH METHODOLOGY

3.1. Isolation, identification and pathogenicity test of *Phytophthora* spp. causing root rots of pomelo

3.1.1. Media for isolation and morphological study of *Phytophthora* spp.

The media were prepared and used for isolation and morphological study of *Phytophthora* spp. Potato dextrose agar (**PDA**: potato infusion from 200 g/L, 20 g/L dextrose, and 15 g/L agar), potato dextrose broth (**PDB**: potato infusion from 200 g/L, 20 g/L dextrose)V8 juice agar (**V8A**; 200 mL/L V8 juice [Cambell soup Co., Camden, NJ, USA], 3 g/L CaCO3, 20 g/L agar, 800 mL/L water) cornmeal agar (**CMA** [Hardy Diagnostics Co., Santa Maria, CA, USA]: 2 g/L corn meal infusion from solids, 15 g/L agar) and water agar (**WA**; 20 g/L, 1 L water) were autoclaved at 121°C before using to culture *Phytophthora* spp. Additionally, the selective medium **PARPH** was also prepared by adding pimaricin (10 µg/ml), ampicillin (250 µg/ml), rifampicin (10 µg/ml), pentachloronitrobenzene (PCNB; 100 µg/ml) and hymexazol (50 µg/ml) to cool molten CMA (50 - 55°C).

3.1.2. Isolation of *Phytophthora* spp.

Phytophthora species causing root rots of pomelo were isolated from soil and infected roots, which were taken from orchards in Chachoengsao province and Bangkok, Thailand. Tissue transplanting and baiting methods were used to isolate the pathogens.

3.1.2.1. Isolation of *Phytophthora* spp. from infected roots

Tissue transplanting method was used to isolate the pathogen. The infected root samples were washed under running water to remove soil and other debris. The

areas contained both healthy and diseased tissues on the roots were cut into small pieces (approximately 2 × 2 mm), and transferred to plates containing selective medium PARPH. The isolating plates were incubated in the dark, at room temperature (25 – 30°C) for 2 – 3 days. The mycelia growing from the root pieces were transferred to plates containing a thin layer of WA medium. The hyphal tips from growing colonies were transferred into PDA until get pure culture.

3.1.2.2. Isolation of *Phytophthora* spp. from soil

Baiting method was used for isolation of pathogens from soil. Soil samples (100 g) were placed in plastic cups, and were add 200 ml of sterile-distilled water at depth 5 – 7 cm. Healthy leaves of pomelo were cut into small pieces, and floated on water in the cups. After 3 – 4 days incubation in the dark the mycelia growing from the leaf pieces were transferred to plates containing a thin layer of WA medium. The hyphal tips from growing colonies were transferred into PDA until get pure culture.

All isolates were further identified by morphology and molecular phylogeny to confirm species.

3.1.3. Morphological identification

All obtained isolates were cultured on PDA, V8 agar, and CMA for morphological study. Growth rates of isolates on PDA medium were recorded. Sporangia were produced by floating mycelial discs which taken from margin of a 3-day-old culture on V8 agar, in 10 mL of sterilized distilled water. The discs were incubated under fluorescent light, at temperature of 25 – 28°C for 3 – 4 days (Erwin and Ribiero. 1996). The sporangia and other structures were observed and measured by a camera with associated software attached to an Olympus compound microscope (CH40; Olympus Optical Co. Ltd., Tokyo, Japan). At least 50 spores of each spore type were measured for each isolate then mean and standard deviation were reported.

The isolates were morphologically identified based on their morphological characteristics.

3.1.4. Molecular phylogeny identification of *Phytophthora* spp. based on DNA sequences

The isolates PHY02 and KA1, which represented for slow-growth and fast-growth isolates respectively, were chosen to identify into species by DNA sequencing.

3.1.4.1. DNA extraction

Mycelial mass of each isolate was collected from a purified colony grown in V8A, and ground with mortar and pestle in liquid nitrogen to fine powder. Genomic DNA of isolates was extracted by CTAB method with some modifications (Prabha *et al.* 2011). The DNA extraction procedure consisted of following steps:

(1) The frozen powder was transferred to a 2 ml centrifuge tube, and 500 µl of extraction buffer solution (200 mM Tris-HCl, pH 7.5; 25 mM EDTA and 250 mM NaCl and 0.5% SDS) was added. The reaction mixture was then vortexed for 5 sec and kept at room temperature for 30 min.

(2) The reaction mixture was centrifuged at 13,000 rpm for 1 min.

(3) The supernatant was transferred to a new centrifuge tube, and an equal volume of cold phenol chloroform was added.

(4) The reaction mixture was shortly vortexed and centrifuged again at 13,000 rpm for 2 min.

(5) The supernatant was again transferred to a new centrifuge tube, and re-extracted twice with 300 µl of chloroform and centrifuged as in step 3.

(6) The final supernatant was then transferred to a new centrifuge tube, and 300 µl of cold iso-propanol was added to it and gently mixed by inverting the tubes several times.

(7) The reaction mixture was incubated for 30 min at –20°C.

(8) The nucleic acids were then recovered by centrifugation at 13,000 rpm for 5 min.

(9) The supernatant was discarded, and pellet was washed with 70% cold ethanol, and then with 1 ml absolute ethanol.

(10) The tube was then dried for 15 min at 37°C.

(11) Finally, the pellets were re-suspended in 100 µl warm (55° C) TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA).

3.1.4.2. Polymerase chain reaction (PCR)

The internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S ribosomal DNA fragments (Fig. 3.1) of isolates PHY02 and KA1 were amplified by couple of primers ITS5/ ITS4 and ITS6/ ITS4, respectively, in polymerase chain reaction (PCR) with previously described conditions (Table 3. 2).

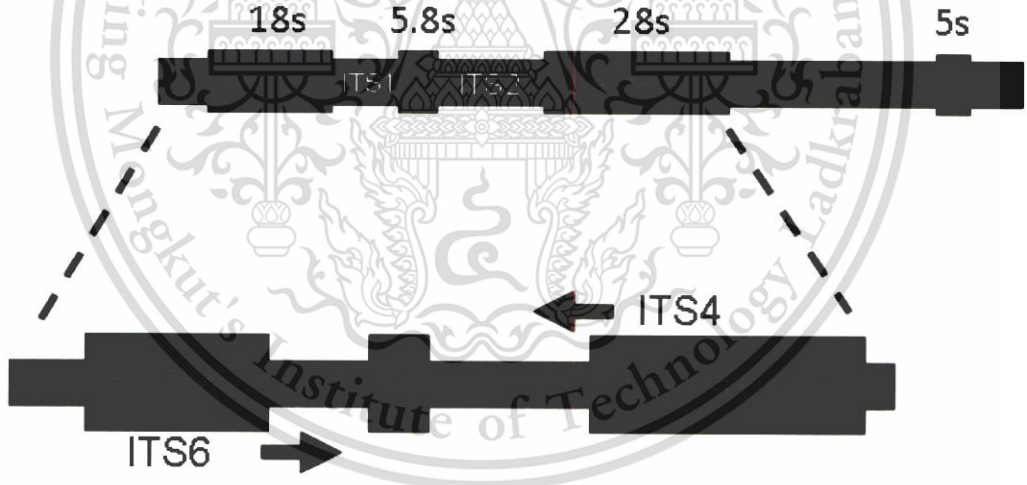


Figure 3.1. ITS regions on chromosomes of eukaryotes (Source: Grünwald *et al.* 2011).

Table 3.1. Primers, mix and program for PCR.

Primers used for PCR and sequencing		
Primers	Sequence (5′-3′)	References
ITS 6	GAA GGT GAA GTC GTA ACA AGG	Cooke *et al.* 2000
ITS 4	TCC TCC GCT TAT TGA TAT GC	White *et al.* 1990
ITS5	GGA AGTA AAA GTC GTA ACA AGG	
PCR mix (Grünwald *et al.* 2011)		
Components		Final concentration
DNA		3 ng/µl
dNTP (2.0mM)		200 µM
Primers (10uM stock)		0.4 µM (for each primer)
10 x Taq Buffer with 15mM MgCl2		1x
Taq 5u/µl		0.05 u/µl
dWater NA		NA
PCR Program (Grünwald *et al.* 2011)		
steps	cycles	conditions
1	1	95°C for 3min;
2		95°C for 1min;
3	35 (repeat steps 2-4)	55°C for 1min,
4		72°C for 1min;
5	1	72°C for 10min;
-	-	Store at -20C until used

3.1.4.3. Sequencing and phylologeny analysis

To identify *Phytophthora* isolates into species level, the sequencing of cloned fragments (PCR products) of isolates were performed at First Base Laboratory (Selangnor, Malaysia) using the same primers. The full-length determined ITS nucleotide sequences of isolates were used as queries for BLAST searches in GenBank

of National Center for Biotechnology Information (NCBI; http://www.ncbi/blast/) or Phytophthora Database (http://www.phytophthoradb.org). Subsequently, the sequences of isolates and related taxa (obtained from GenBank databases) were aligned and analyzed to construct a phylogenetic tree using software MEGA ver. 5.2 (Tamura *et al.* 2011).

3.1.5. Pathogenicity test

3.1.5.1. Preparation of inoculum

Sporangia of each isolate were produced by floating 30 – 40 mycelial discs, which taken from margins of a 3-day-old culture on V8 agar, in a Petri dish containing 10 mL of sterilized distilled water. The discs were incubated under fluorescent light, at temperature of 25 – 28°C for 3 – 4 days. The sporangia were separated from mycelia by low centrifugation, and were determined the concentration (Erwin and Ribiero. 1996). Potting medium contained sterilized clay soil, sand, and compost at a v/v/v ratio of 3 : 1 : 1 was prepared. The inoculum of each isolate was prepared as infested soil. Sporangia of *Phytophthora* sp. were adjusted with potting medium to get the infested soil with 5 sporangia per cubic centimeter of soil. The infested soil was incubated in the dark and moist condition at temperature of 25 – 28°C for 3 days before using to plant the pomelo seedlings.

3.1.5.2. Pathogenicity test

Pathogenicity of each *Phytophthora* isolate was proved by artificial inoculation into roots of pomelo seedlings var. Khaonampung in order to satisfy Koch's postulate.

Twelve-week-old pomelo seedlings were thoroughly washed to be free of potting mix, and planted in plastic pots (10 × 15 cm) containing the prepared-infested soil. Controls were prepared by planting the seedlings in same size pots, containing the sterilized potting medium. All pots including the controls were maintained in the green house at temperature of about 25-30°C and flooded with water for 24 hr each week.

After 6 wk, the plants were carefully removed from plastic tubes and washed free of potting mix. The root systems were then evaluated on following scales: 0 = all roots healthy; 1 = rotted roots apparent; 2 = obvious root rot, root system small; 3 = severe root rot, taproot necrotic, few new roots; 4 = no healthy root, stem girdled. The evaluation was independently made by two observers and the average rating presented. Additionally, root tips of each seedling were noted as rotted or healthy and the data expressed as root rot percentage using the following formula:

Root rot (%) = 100 x (Numbers of root rots/ Total roots).

The pathogen was re-isolated from infected root symptom, and morphological characteristics were compared with the inoculated isolate. The highest pathogenic isolates were selected for further experiments.

3.2. Biological control of *Phytophthora* spp.

3.2.1. Pathogenic and antagonistic isolates used for experiments

Two *Phytophthora* isolates denominated as PHY02 and KA1 were used as targets for biological control in this research. Meanwhile, *Chaetomium globosum* CG05, *Chaetomium lucknowenese* (CL01) and *Chaetomium cupreum* CC3003, which were proved as effective antagonists against different plant pathogens, were used as biological control agents. These strains were kindly provided by Dr. Soytong Kasem (KMITL).

3.2.2. *In vitro* antagonism test

Each of the *Chaetomium* species was separately assessed its antagonism against two *Phytophthora* isolates using bi-culture techniques. A 5-mm-diameter mycelial disc from the active growing area of a 5-day-old colony of *Phytophthora* sp. was placed alone (as a control) or opposite a mycelial disc from one of the above antagonists on PDA plates (9-cm diameter). All plates were incubated at temperature of 28°C in the dark, and the colony diameters of the pathogen were measured after 30 days. The

inhibition of mycelial growth and spore production of the pathogen was then calculated as a percentage using the formula below:

$$\text{Inhibition (\%)} = 100 \times (1 - B/A) \qquad (1)$$

In which, A = colony diameter or spore number of the pathogen in control plates; B = colony diameter or spore number of the pathogens in bi-culture plates. The experiments were performed in completely randomized design with 4 replications. The collected data as colony diameter and spore number were subjected to analysis of variance (ANOVA) and treatment means were compared using Duncan's multiple range tests (DMRT) at P = 0.05 and P = 0.01.

3.2.3. *In vitro* effects of antagonistic crude extracts on *Phytophthora* spp.

3.2.3.1. Extraction of antagonistic crude extracts

The fungal antagonists *C. globosum* CG05, *C.lucknowense* CL01 and *C. cupreum* CC003 were separately cultured in potato dextrose broth (PDB), and incubated at room temperature (25 – 28°C) for 45 days. Fungal biomass of each antagonist was separately collected as fresh biomass, then was dried out at room temperature. Subsequently, the extraction of dried biomass of each antagonist was performed by the method described by Kanokmedhakul *et al.* (2006) as showing in Fig. 3.2.

Each dried biomass was ground and extracted with hexane (1:1 v/v) (in a 1000-ml bottle) by shaking-incubation for 72 hr at room temperature. Filtrate was then separated out the marc by filtering through filter paper (Whatman No.4). The hexane filtrate was evaporated through rotary vacuum evaporator to yield crude hexane extract. The marc from hexane extraction was further extracted with ethyl acetate and followed with methanol using the same procedure as hexane to yield crude ethyl acetate (EtOAc) and methanol extract (MeOH).

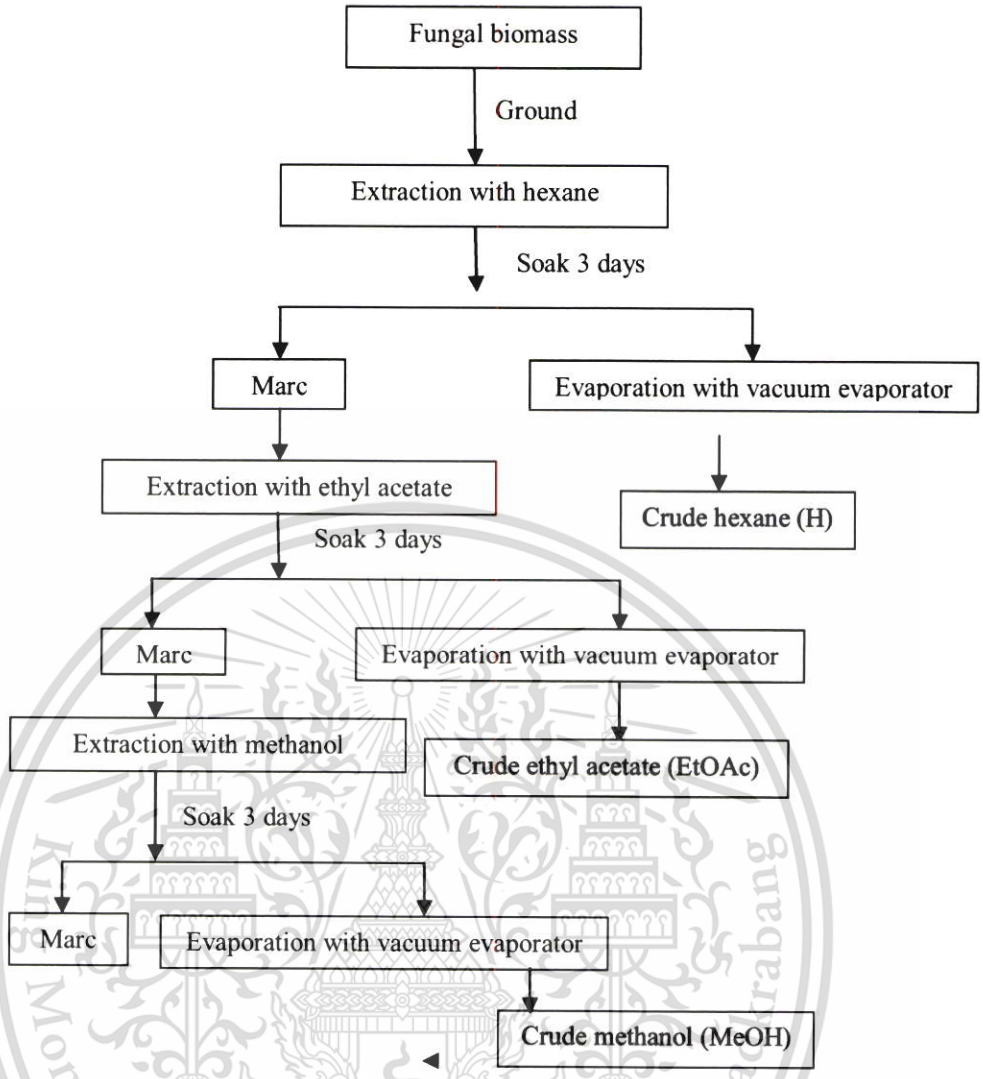


Figure 3.2. Extraction protocol of antagonistic crude extracts.

3.2.3.2. Bioactivity test of antagonistic crude extracts against *Phytophthora* spp.

The crude extracts of each antagonist were tested for their potential to inhibit the mycelial growth and/or spore formation of the *Phytophthora* sp. PHY02 and *Phytophthora* sp. KA1.

The experiments were designed as 2 factors factorial in completely randomized designs (CRD) with 4 replications. Factor A represented different crude extracts: A1 = crude hexane, A2 = crude EtOAc and A3 = crude MeOH. Factor B represented different concentrations of 0, 10, 50, 100, 500, and 1,000 µg/mL. A 5-mm-diameter

mycelial disc of *Phytophthora* sp. was placed on the center of PDA plates (5 cm in diameter) containing each crude extract. To obtain the desired concentrations, stock crude extract was weighed, dissolved in 2% dimethyl sulfoxide, mixed into molten PDA, and then autoclaved for 20 min at 121°C (15 psi). All plates were then incubated at temperature of 28°C in the dark, and colony diameters and spore number were measured calculated when the pathogen colonies developed fully on the control plates (about 5 days). Finally, the inhibition of mycelial growth of the pathogens was calculated as a percentage using the same formula (1) above. In which, A and B are the colony diameters (after subtracting the diameter of the inoculum disc) or spore production of the pathogens in the control and crude extract plates, respectively. In addition, effective dose (ED_{50}) values for inhibition of mycelial growth and spore formation were computed by probit analyses using SPSS Statistics ver. 19.0 software (IBM Co., USA). All experiments were repeated twice.

3.2.4. *In vivo* test of *Chaetomium* spp. and their crude extracts for control *Phytophthora* spp. in pot experiments

Spores and the most effective crude extract of each antagonist were used to control *Phytophthora* sp. PHY02 and *Phytophthora* sp. KA1 in separate experiments, under greenhouse conditions. Infested soil with 2.5 propagules of either *Phytophthora* sp. PHY02 or *Phytophthora* sp. KA1 per cubic centimeter of soil was prepared as described in pathogenicity test. Five-wk-old pomelo seedlings were planted in plastic tubes (10 x 15 cm) containing either infested soil or sterilized potting mix (non-inoculated control seedlings). Spores suspension at 10^4 spores/ml and the most effective crude extract each antagonist at concentration of 50 µg/mL were also prepared. The experiments were arranged in Randomized Complete Block Designed (RCBD) with 4 replications, treatments were performed as follows:

T_1 = Non-inoculated

T_2 = Inoculated (control)

T_3 = Crude MeOH of *C. globosum* CG05

T_4 = Crude MeOH (or EtAOc) of *C. lucknowense* CL01

T_5 = Crude MeOH of *C. cupreum* CC003

T_6 = Spore suspension of *C. globosum* CG05

T_7 = Spore suspension of *C. lucknowense* CL01

T_8 = Spore suspension of *C. cupreum* CC003

The treatments were biweekly applied by pouring 100 ml of the crude extract at concentration of 50 µg/mL or the spore suspension of tested *Chaetomium* spp. in each tube, for 8 weeks. The non-inoculated and inoculated plants were treated with water.

After 8 wk maintained in the green house, data were collected as plant weight and number root rots. The tested plants were washed off soil, and root tips in each seedling were visually evaluated as number of root rots. Percentage of root rots was calculated using following formula:

- Root rot (%) = 100 x (Numbers of root rots/ Total roots)
- Percentage of root rot reduction = 100 x (% Root rots of inoculated control seedlings – % Root rots of seedlings in treatments)/ % Root rot of inoculated control seedlings

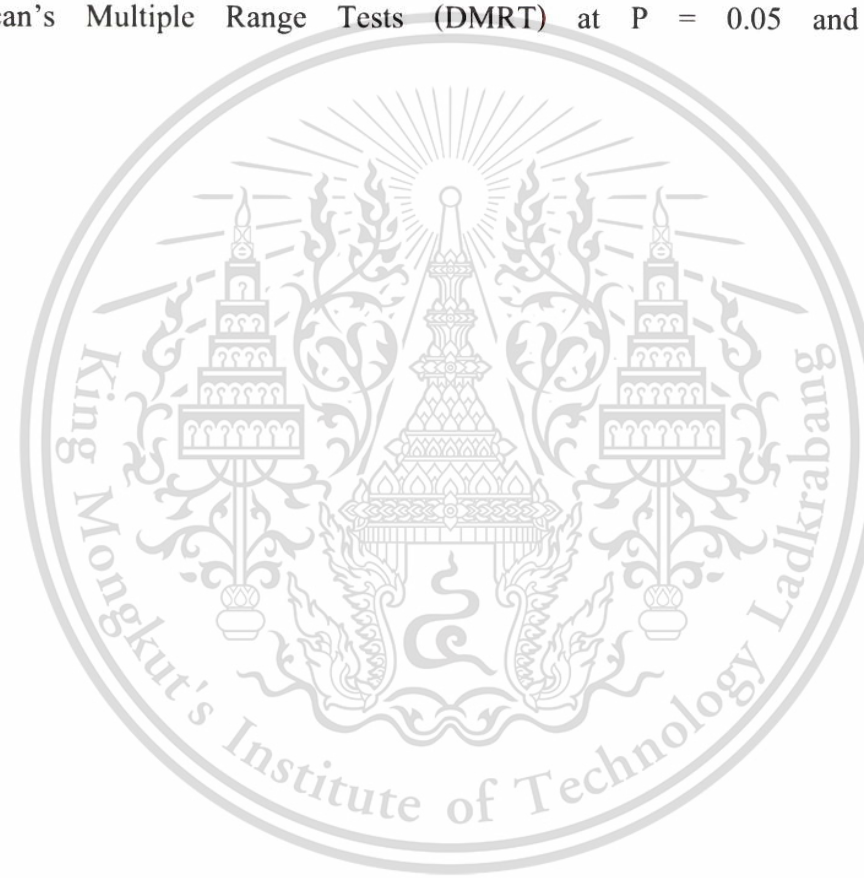
The whole plants were dried in hot air oven at 65°C for 12 hr, and weighed. Percentage of increase and decrease of dry plant weights were calculated by following formulas:

- Increase of dry plant weights (%) = 100 x (Dry plant weight of seedlings in treatments – Dry plant weight of control seedlings)/ Dry plant weight of inoculated control seedlings

- Decrease of dry plant weights (%) = 100 x (Dry plant weight of non-inoculated seedlings – Dry plant weight of seedlings in treatments)/ Dry plant weight of non-inoculated seedlings

3.3. Data analysis

All data were subjected to analysis of variance (ANOVA) using SPSS Statistics ver. 19.0 software (IBM Co., Armonk, NY, USA). Means were compared using Duncan's Multiple Range Tests (DMRT) at P = 0.05 and P = 0.01.



CHAPTER 4

RESULTS

4.1. Isolation, identification and pathogenicity of Phytophthora spp. causing root rot of pomelo

4.1.1. Morphological identification

Six isolates of *Phytophthora* spp. were isolated from soil and root samples from pomelo orchards. The isolates were morphologically identified into 02 groups.

The first group consisted of three isolates PHY01; PHY02; and PHY03, was isolated from samples collected in Chachoengsao province. All these isolates were similar morphological characteristics. They were slow growth organisms, with colony diameters after 7 days grown on PDA were less than 4 cm (Fig. 4.1). Colonies of these isolates show stellate pattern with aerial mycelia when grown on PDA. Hyphae are lumpy-branching with hyphal swellings. Sporangia produce readily and abundantly on agar surfaces of PDA and V8A after 3 – 5 days, occur in groups on sympodium or irregularly. Sporangia are papillate and caducous with short pedicels (mean 3.3 µ long). Sporangial shape vary from ellipsoid, ovoid, pyriform, obpyriform to near spherical, with a length to breadth ratio of 1.6 – 1.7 : 1 (Table 4. 1, Fig. 4.2, Fig. 4.3 and Fig. 4.4). Zoospores are directly released from sporangia when flooded in distilled water. Most of chlamydospores are globose in shape, produced abundantly from mycelia on agar surfaces of PDA and CMA. No sexual organ (oospores) was observed in cultures of these isolates, thus, they are a heterothallic species.

The identity in morphological characteristics of PHY01, PHY02 and PHY03 suggests they belong to the same species. These characteristics are consistent with descriptions of *Phytophthora palmivora* (Erwin and Ribiero. 1996).

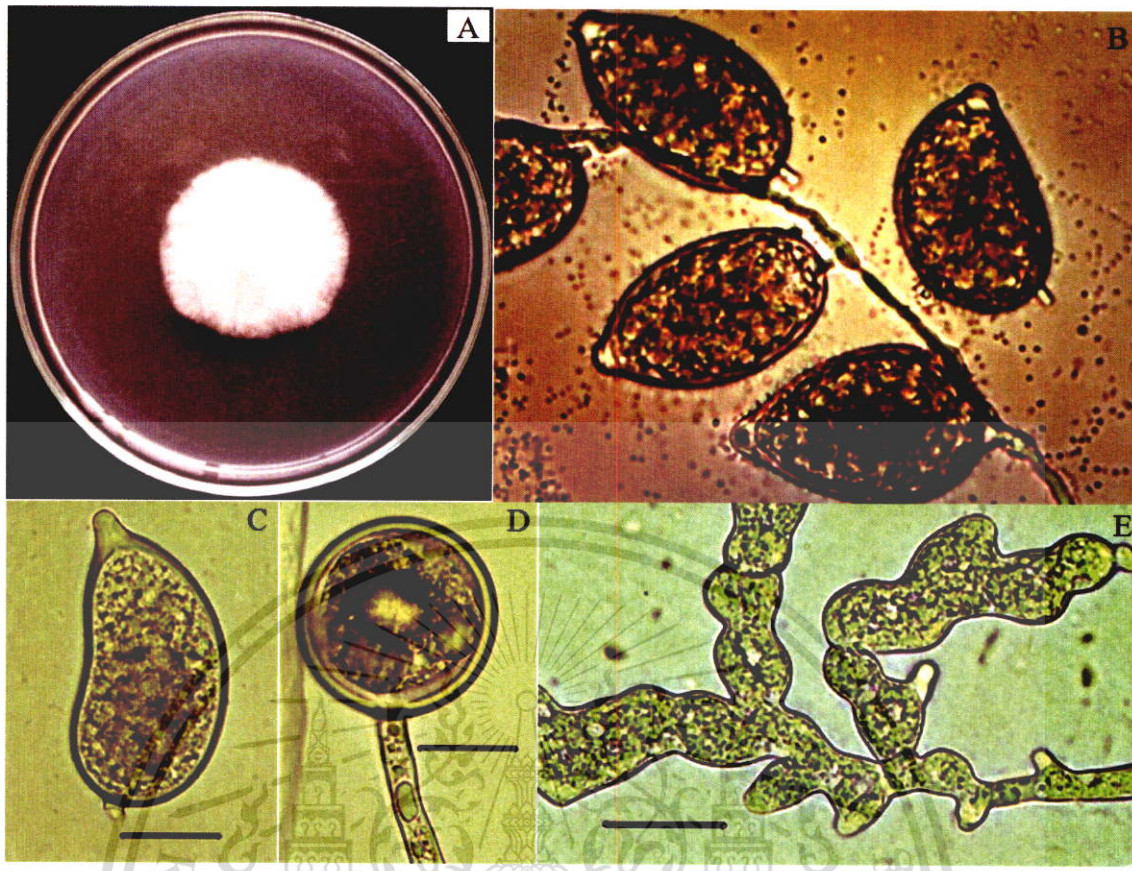


Figure 4.2. Morphological characteristics of isolate PHY01: A = 7-day-old culture on PDA, B = Sporangia on a sympodium, C = Detached sporangium, D = Chlamydospore, E = hyphae swelling (scale bars = 20 μm).

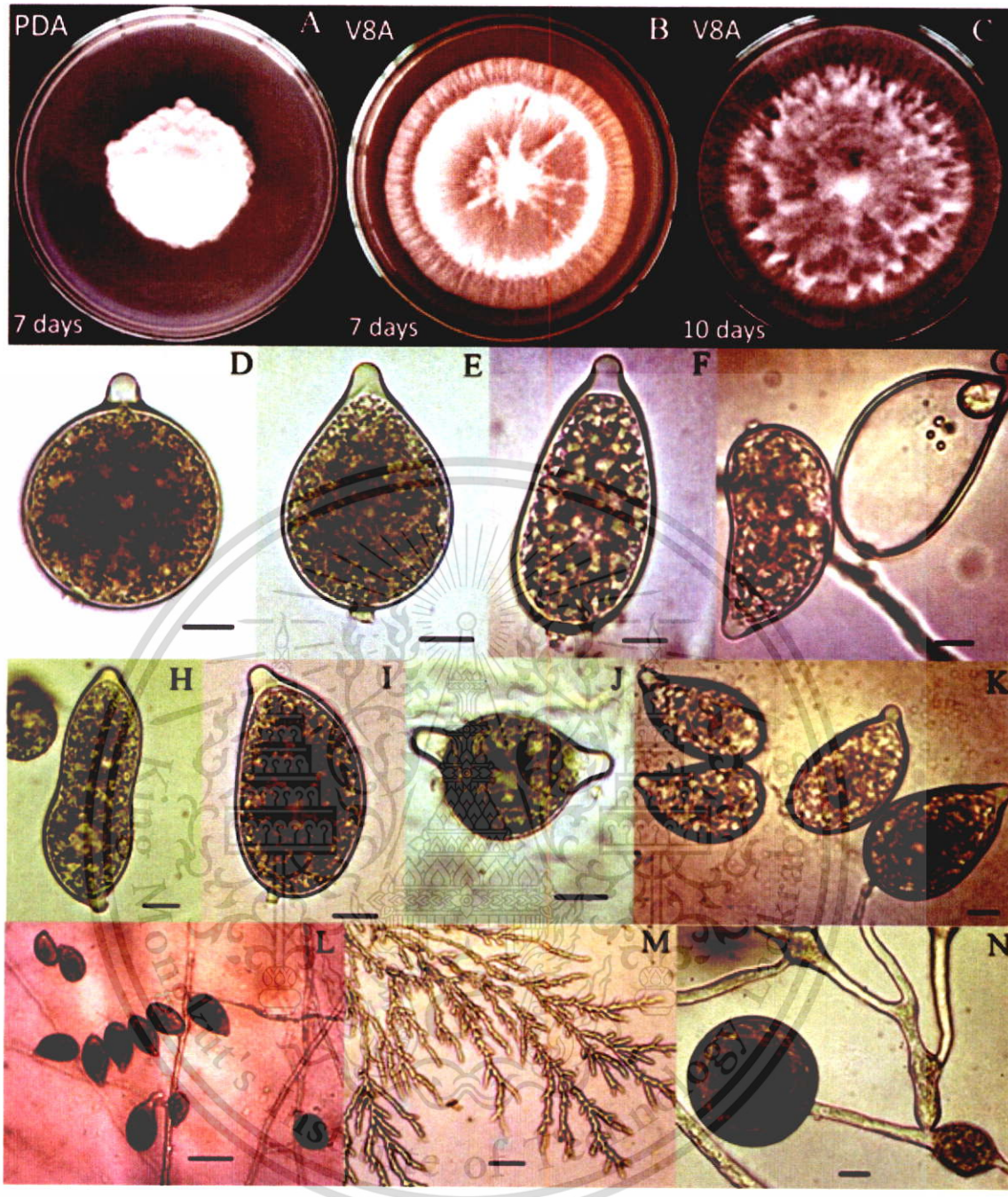


Figure 4. 3. Morphological characteristics of isolate PHY02: A – C = Colony types of PHY02 at 7 days grown on different media, D – J = Sporangia, K and L = Sporangia on sympodium, M = Lumpy-branching mycelia, N = Chlamydospore and swelling hyphae (scale bars: L and M = 50 μm, the others = 10 μm).

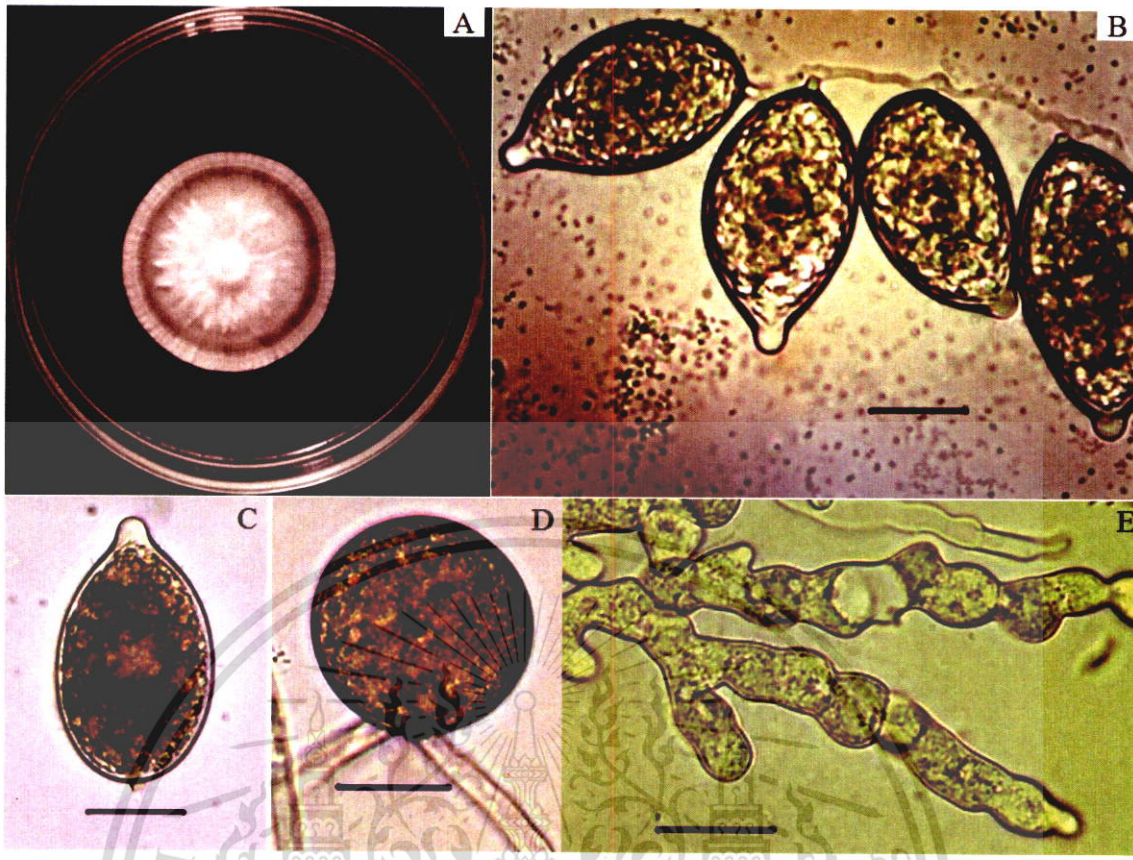


Figure 4.4. Morphological characteristics of isolate PHY03: A = 7-day-old culture on PDA, B = Sporangia on a sympodium, C = Detached sporangium, D = Chlamydospore, E = hyphae swelling (scale bars = 20 µm).

The second group consisted of three isolates KA1; KA2; and KA3, which was isolated from samples that collected in Bangkok. Unlike the first group, all these isolates of this group are fast-growth organisms, with colony diameters after 7 days grown on PDA ranging from 83 – 87 mm (Fig. 4.1). Colonies of these isolates show dense-aerial, arachnoid, and branched mycelia with hyphal swelling when grown on PDA and V8A (Fig. 4.5, Fig. 4.6 and Fig. 4.7). However, their mycelia are sparse when grown on CMA. No isolates of this group produce any spore type on agar surfaces of the used media. The isolates form sporangia only when flooded with distilled water. All the three isolates produce papillate, caducous sporangia with very short pedicels with mean length ranging from 3.0 – 3.2 µm (Table 4.1). The sporangial shape is predominantly subspherical and turbinate, with an average length-to-breadth ratio of

1.3:1. Chlamydospores of these isolates, which are globose in shape, form abundantly when followed the method of Tsao (1971). Additionally, no sexual organs (oospores) are observed in single culture of these isolates. Because of the identity in morphological characteristics of isolates KA1, KA2 and KA3, they probably belonged to one species. Most cultural and morphological characteristics of these isolates are consistent with descriptions of *Phytophthora nicotianae*.

The isolates PHY02 and KA1, which represented for slow-growth and fast-growth isolates respectively, were selected for molecular identification based on DNA sequencing.

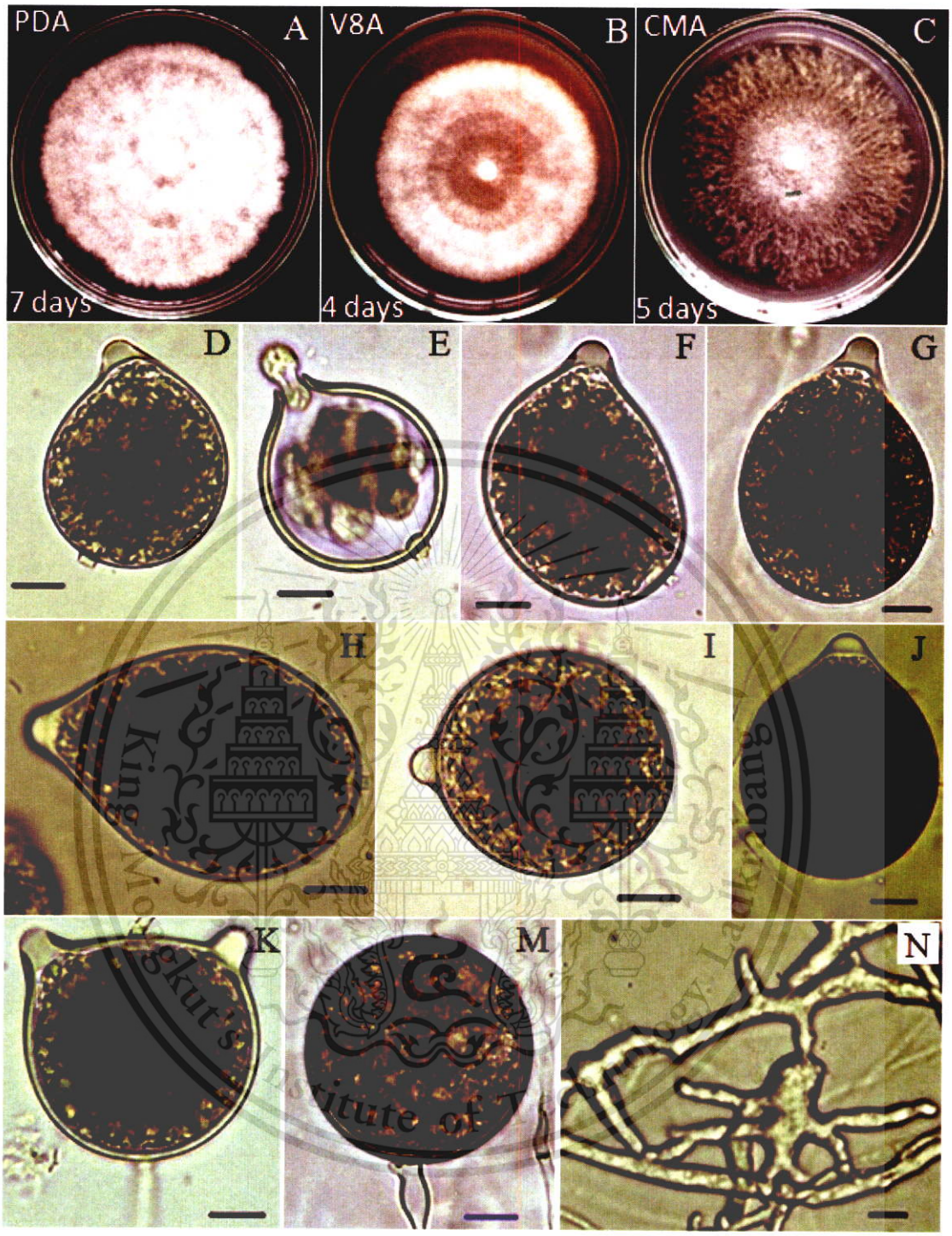


Figure 4.5. Morphological characteristics of isolate KA1: A – C = Cultures of KA1 on different media, D – K = Sporangia, M = Chlamydospore, N = Hyphae swelling (scale bars = 10 μm).

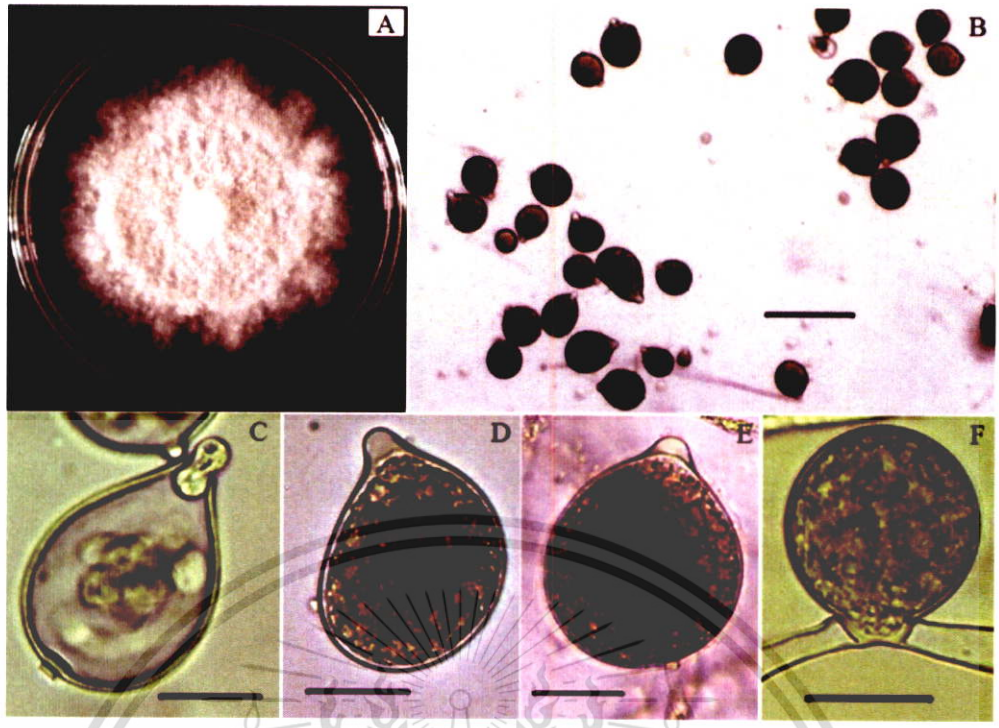


Figure 4.6. Morphological characteristics of isolate KA2: A = 7-day-old culture on PDA, B = Detached sporangia, C – E = Sporangia, F = Chlamydospore (scale bars = 20 µm).

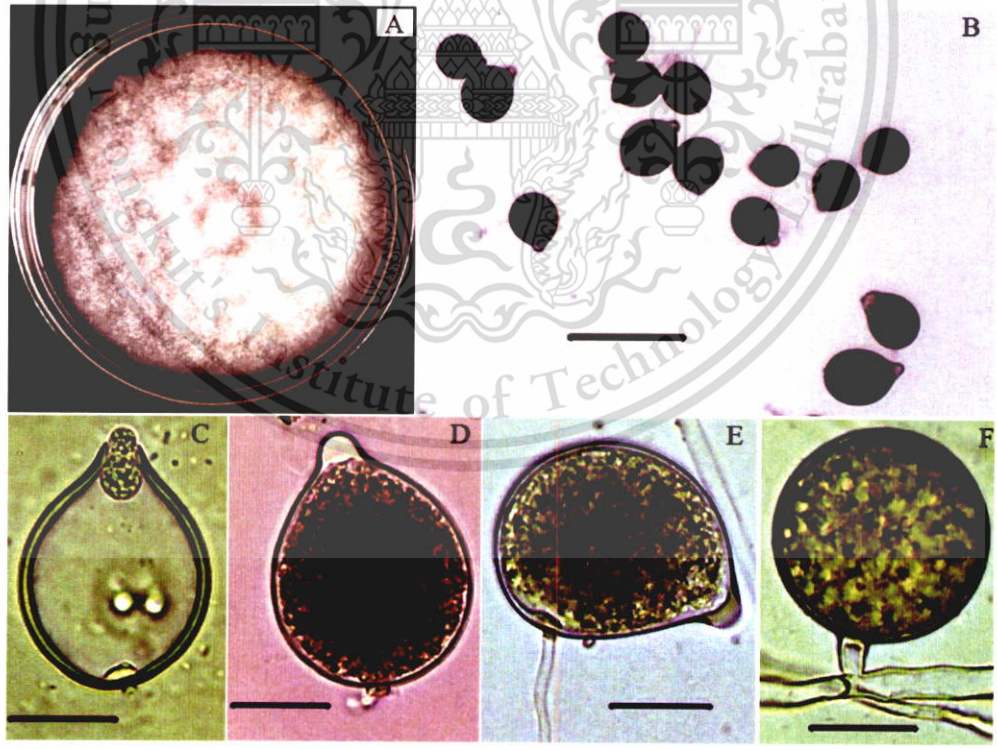


Figure 4.7. Morphological characteristics of isolate KA3: A = 7-day-old culture on PDA, B = Detached sporangia, C – E = Sporangia, F = Chlamydospore (scale bars = 20 µm).

4.1.2. Molecular phylogeny based on DNA sequences

Furthermore, molecular was investigated to confirm species. Primers ITS5 and ITS4 were used to amplify internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S ribosomal DNA fragments of isolates PHY02. Meanwhile, primers ITS6 and ITS4 were used for amplifying the same regions of isolates KA1. Both PCR products of isolates were about 900 kb. Nucleotide sequences of the ITS ribosomal DNA fragments of isolates PHY02 and KA1 were then determined and deposited in the GenBank under accession number KT175509 and KT175508, respectively. The DNA sequences of the two isolates were used as queries to search in GenBank (NCBI) using the BLAST function.

The BLAST analysis showed that the nucleotide sequences of PHY02 shared 99.75% identity with those of *Phytophthora palmivora* accession numbers PD00627, PD01515 and PD00491; and 99.87% identity with those of PD02505. Phylogenetic analysis confirmed the relationships between PHY02 and these related taxa (Fig. 4.8). The isolate PHY02 was identified as *Phytophthora palmivora*, based on its morphology and the molecular analysis.

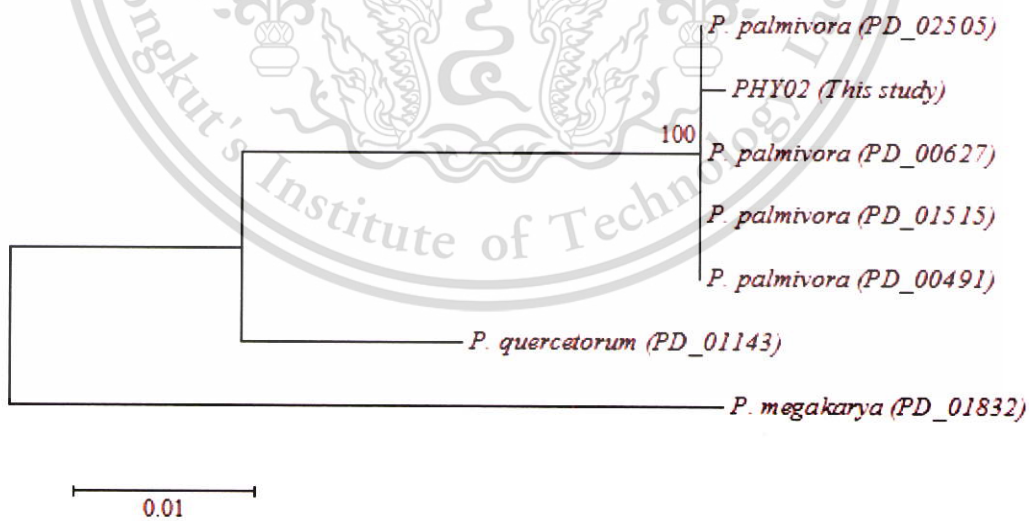


Figure 4.8. Phylogenetic tree showing relationship between *Phytophthora palmivora* PHY02 and related taxa inferred using a neighbor joining method based on internal transcribed spacer (ITS) rDNA sequences. Bootstrap value based on 1,000 replications is shown above the branch.

For the isolate KA1, the analysis showed that its nucleotide sequences shared 100% identity with those of *Phytophthora nicotianae* accession Nos. GU111681 and GU111670 from *Citrus* spp. in Taiwan; JF792541 and JF792530 from citrus soils in India; and many other isolates existing in the GenBank database. Phylogenetic analysis confirmed the relationships between KA1 and the related taxa (Fig.4.9). Thus, the pathogenic isolate KA1 was identified as *Phytophthora nicotianae*.

According to identification, which based on morphology and molecular phylogeny, it is confirmed the isolate PHY02 is *Phytophthora palmivora*, and related to isolates PHY01 and PHY02. The isolate KA1 is confirmed as *Phytophthora nicotianae*, which morphological closely related to isolates KA2 and KA3.

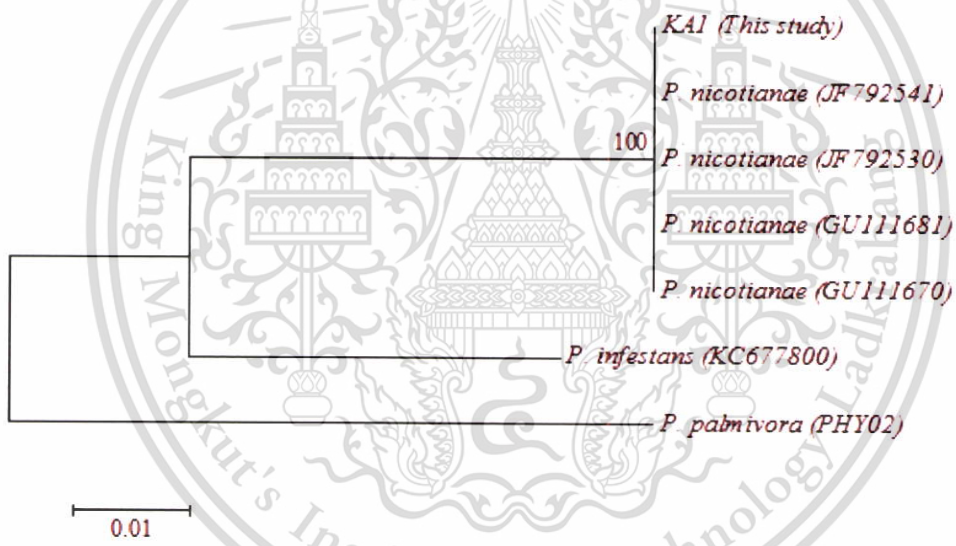


Figure 4.9. Phylogenetic tree showing relationship between *Phytophthora nicotianae* KA1 and related taxa base on the internal transcribed spacer ribosomal DNA sequences, using the neighbor-joining method with 5,000 bootstrap replicates. *P. infestans* (KC677800) (the species placed in the same clade 1 with *P. nicotianae*) was isolated from a potato in India, and *P. palmivora* PHY02 (This study; KT175509).

4.1.3. Pathogenicity test

All the six isolates were pathogenic to pomelo seedlings. At the inoculum level of 5 propagules per cubic centimeter of soil, the isolates PHY01, PHY02, PHY03, KA1, KA2 and KA3 exhibited the root rot rates of 45.7, 47.6, 45.1, 45.3, 44.7 and 43.6% on 12-wk-old pomelo seedlings, with the disease rating were 2.8, 2.9, 2.6, 2.8, 2.6 and 2.5, respectively (Table 4.2). However, there were no significant differences among the root rot percentages produced by isolates of *P. palmivora* (PHY01, PHY02 and PHY03). In the same way, root rot percentages produced by the isolates of *P. nicotianae* (KA1, KA2 and KA3) were not significantly different.

Table 4.2. Effect of different *Phytophthora* isolates on root rots of 12-week-old pomelo seedlings after six weeks of inoculation.

Isolates	Source	Root rot (%)	Disease severity
PHY01	Roots	45.7 ab[1]	2.8[2]
PHY02	Roots	47.6 a	2.9
PHY03	Soil	45.1 ab	2.6
KA1	Roots	45.3 ab	2.8
KA2	Soil	44.7 ab	2.6
KA3	Soil	43.6 b	2.5
Uninoculated seedlings		0.0 c	0.0

[1] Mean four replications, the same letter represents no significant difference among treatments base on the DMRT at $p = 0.05$

[2]Rate on a scale of 0 = all roots healthy to 4 = no healthy root, stem girdled.

All the inoculated seedlings produced very few new roots and leaves (Fig. 4.10). Root tips of infected roots were soft and discolored. Cortices of infected roots in inoculated seedlings were turned soft and sloughed, leaving only the white stele. These are typical symptoms of *Phytophthora* root rot in citrus and are similar to symptoms observed in the affected orchards in Chachoengsao and Bangkok. Meanwhile, the Un-

inoculated seedlings remained normally with abundant new roots and no symptom of rots.

The testing isolates were re-isolated from newly infected roots of inoculated seedlings. The results suggested that the obtained organisms were the causal agents of root rots in pomelo orchards in Chachoengsao and Bangkok.

The isolates PHY02 and KA1 were selected for further studies.

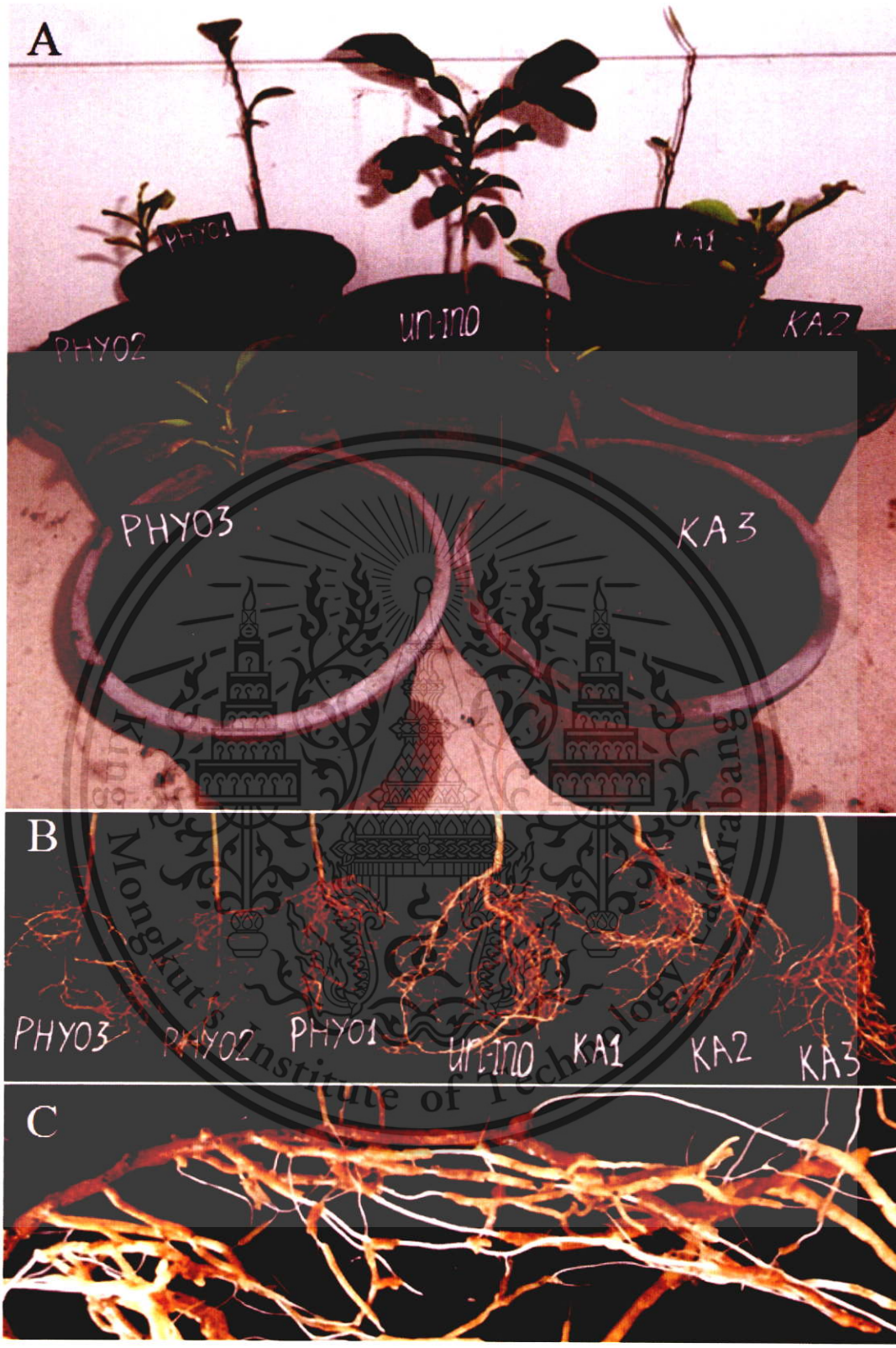


Figure 4.10. Effects of *Phytophthora* isolates on pomelo seedlings. A and B = Seedlings and their root systems after six weeks inoculation with different isolates; C = Closed symptoms of root rots in an inoculated seedlings.

4.2. Biological control of *Phytophthora* spp.

4.2.1. Antagonism of *Chaetomium* spp. to *Phytophthora* spp.

The antagonists were tested *in vitro* for their antagonism to *P. palmivora* PHY02 and *P. nicotianae* KA1 using bi-culture technique.

As shown in Table 4.3, in bi-culture plates at 30 days, the *C. globosum* CG05; *C. lucknowense* CL01; and *C. cupreum* CC3003 respectively reduced 61, 59, and 60% colony diameter, 99, 97.5 and 92% spore production of *P. palmivora* PHY02, when compared to the controls. Moreover, all tested *Chaetomium* spp. grew over, parasitized hyphae and degraded mycelia of *P. palmivora* PHY02, resulting in change of color from white to light yellow-brown and a part or entire colony death (Fig. 4.11).

In both the inhibition of mycelial growth and spore production, *C. cupreum* CC3003 was significantly less efficient than *C. globosum* CG05 and *C. lucknowense* CL01.

Table 4.3. Inhibitory effect of *Chaetomium* spp. on mycelial growth and spore production of *Phytophthora palmivora* PHY02 in bi-culture test.

Treatments	Colony diameter (cm)	Colony inhibition (%)	Number of spore ($x10^4$)	Spore inhibition (%)
CG05 vs *P. palmivora* PHY02	3.5 c[1]	61. 0 a	1.12 c	99.0 a
CL01 vs *P. palmivora* PHY02	3.7 c	59.2 a	2.8 c	97.5 a
CC3003 vs *P. palmivora* PHY02	4.5 b	49.7 b	8.5 b	92.2 b
P. palmivora PHY02 (control)	9.0 a	-	109.3 a	-

[1] Data collected at 30 days after incubation, mean of four replications. In one column, the same letter represents no significant difference among treatments base on the DMRT at $p = 0.05$.

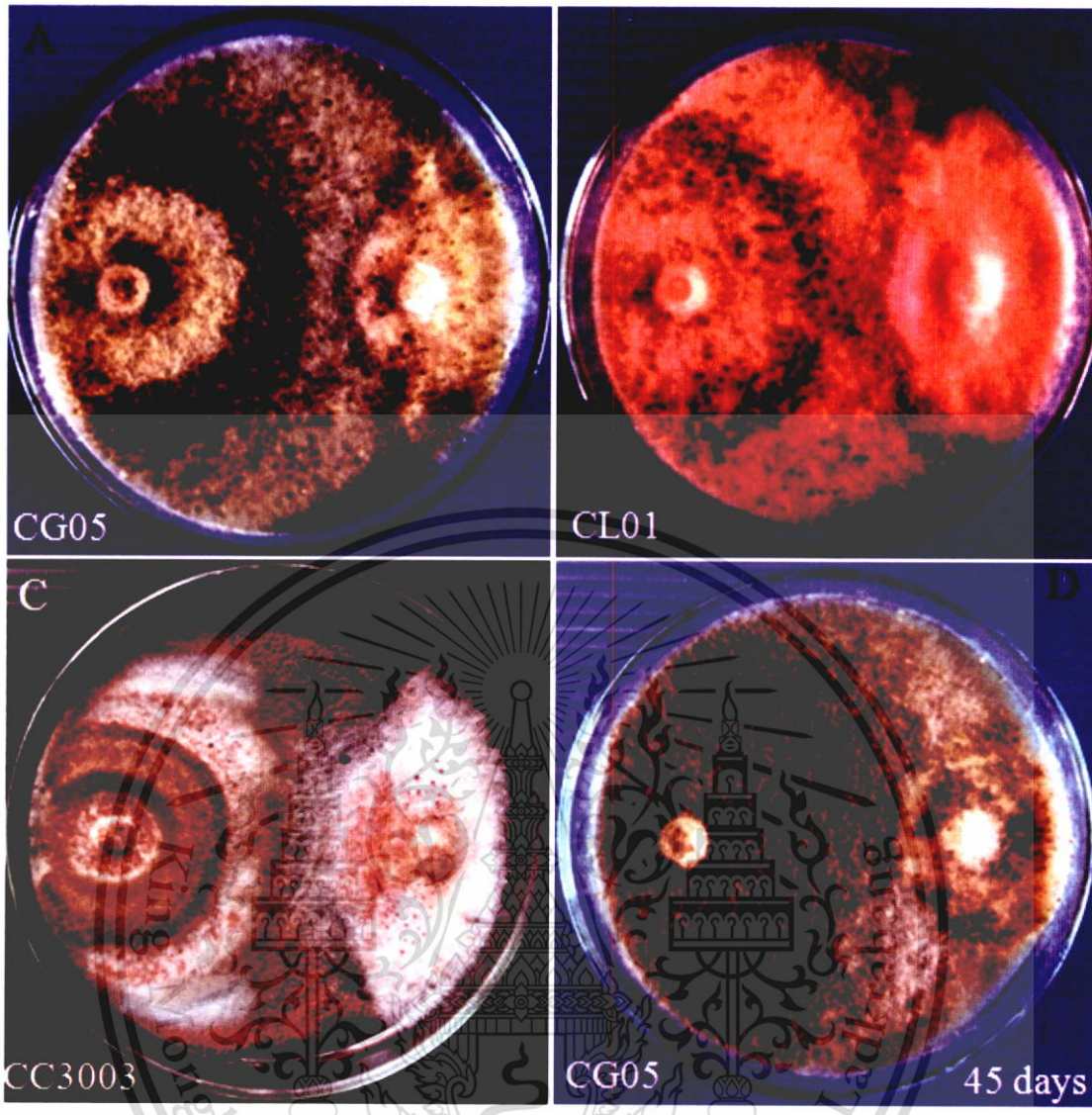


Figure 4.11. Growth of *Phytophthora palmivora* PHY02 in bi-culture tests against *Chaetomium* spp. (A, B and C: photos were taken at 30 days after incubation).

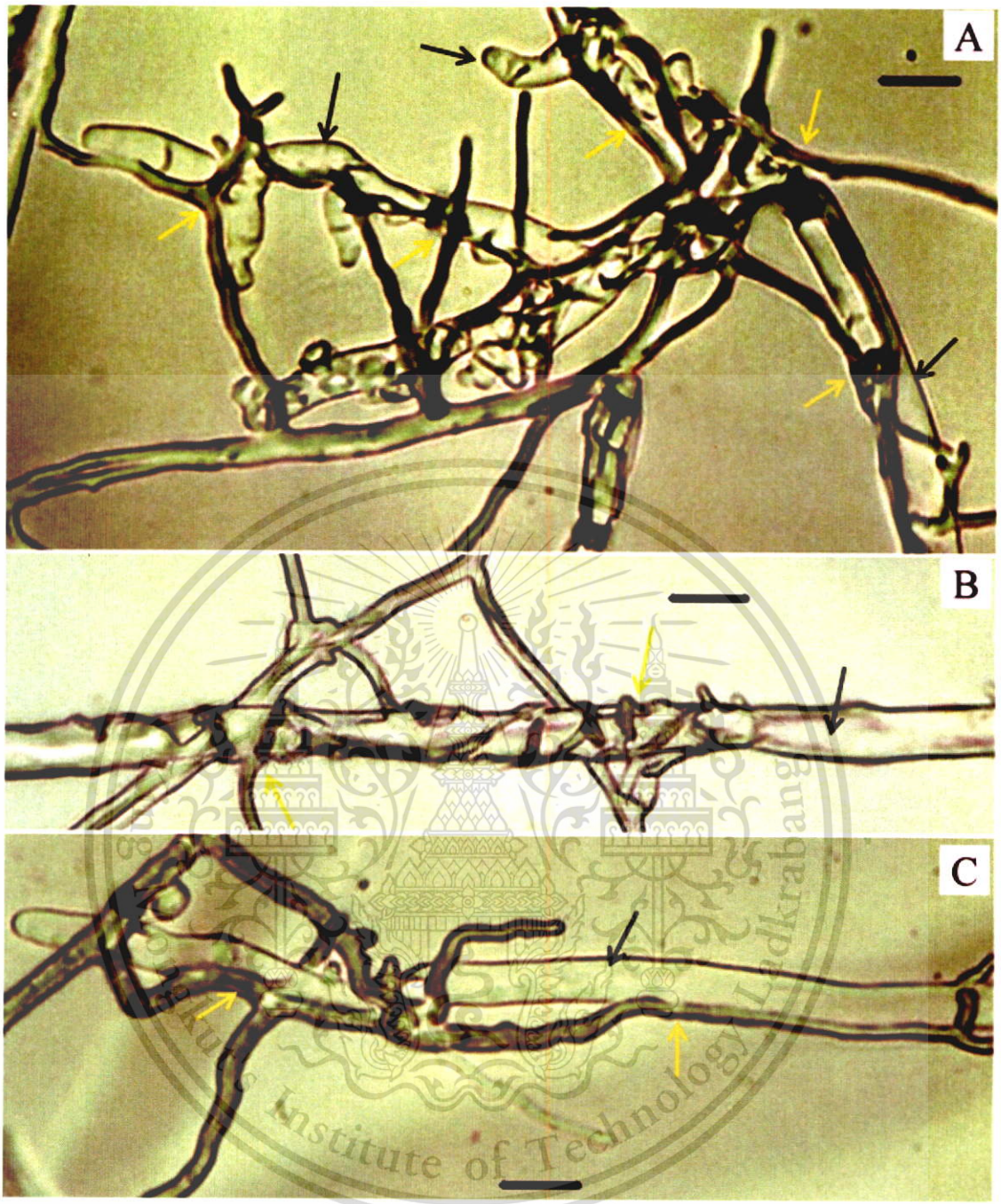


Figure 4.12. Mycopasitisms of *Chaetomium* spp. (yellow arrows) on hyphae of *Phytophthora palmivora* PHY02 (black arrows) in bi-culture plates: A = *Chaetomium globosum* CG05, B = *Chaetomium lucknowense* CL01, C = *Chaetomium cupreum* CC3003.

As shown in the Table 4.4, after 30 days incubation, growth of *P. nicotianae* KA1 was reduced 49.7%, 51.7%, and 56.4% by *C. globosum* CG05, *C. lucknowense* CL01 and *C. cupreum* CC3003, respectively, when compared to that of the control.

The *C. cupreum* CC3003 caused significantly higher growth inhibition of *P. nicotianae* KA1 than *C. globosum* CG05 and *C. lucknowense* CL01. Interestingly, substances released into the agar by *C. cupreum* CC3003 prevented colony growth of *P. nicotianae* KA1, resulting to show a clear zone of inhibition between the antagonist and pathogen. In contrast, colonies of *C. globosum* CG05 and *C. lucknowense* CL01 made contact with colonies of pathogen soon without the clear zone of inhibition (Fig. 4.13).

At 30 days after incubation, all tested antagonists grew over colonies of *P. nicotianae* KA1 in bi-culture plates. Hyphae of the antagonists were observed to penetrate or coil around hyphae of *P. nicotianae* KA1, resulting in the degradation and discoloration of pathogen colonies (Fig 4.14). *P. nicotianae* KA1both in bi-culture and control plates did not produce any spore types, the effect of testing antagonists on spore formation of the pathogen were not examined.

Table 4.4. Inhibitory effect of *Chaetomium* spp. on mycelial growth of *Phytophthora nicotianae* KA1 in bi-culture test.

Treatments	Colony diameter (cm)	Colony inhibition (%)
CG05 vs *P. nicotianae* KA1	4.5 a[1]	49.7 b
CL01 vs *P. nicotianae* KA1	4.4 a	51.7 b
CC3003 vs *P. nicotianae* KA1	3.9 b	56.4 a
P. nicotianae KA1	9.0	-

[1] Data collected at 30 days after incubation, mean of four replications. In one column, the same letter represents no significant difference among treatments base on the DMRT at $p = 0.05$.

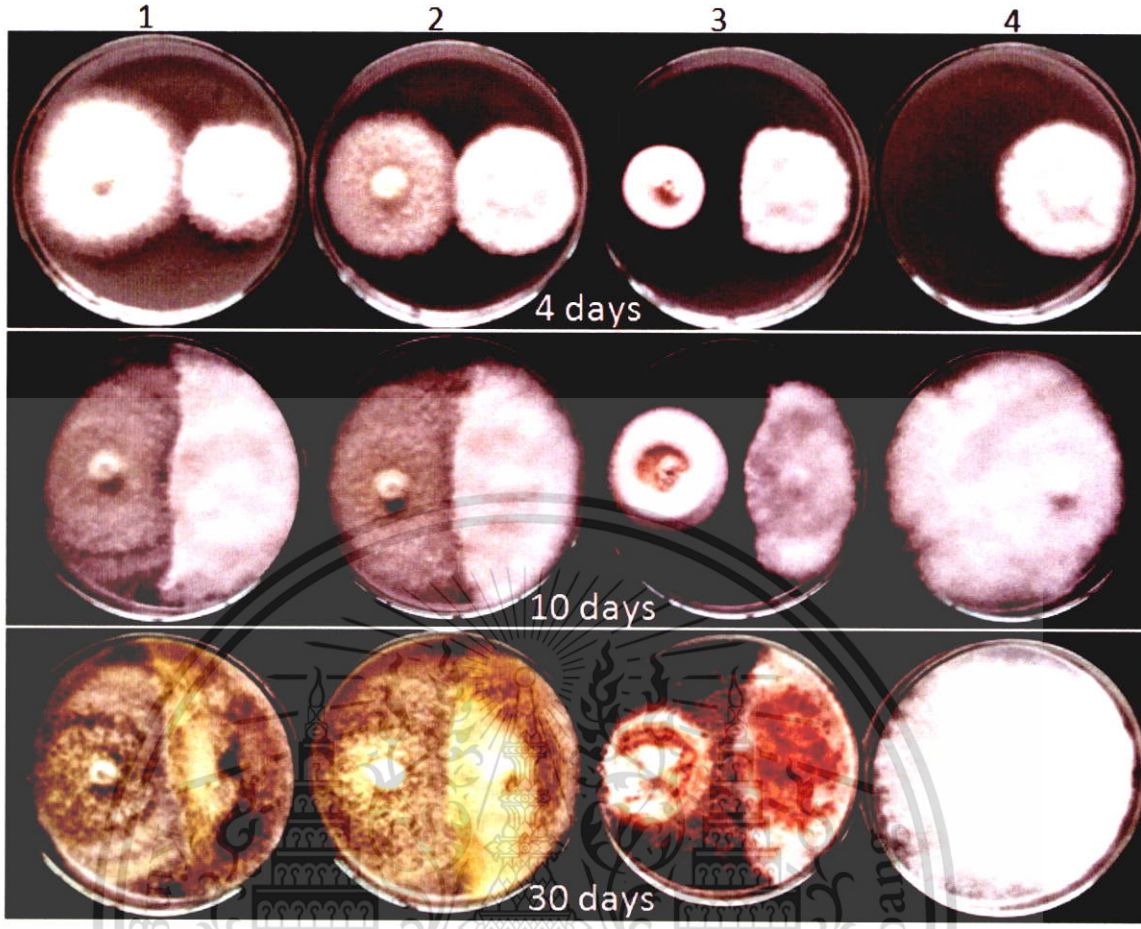


Figure 4.13. Growth of *Phytophthora nicotianae* KA1 in bi-culture test with *Chaetomium* spp. after 4, 10 and 30 days incubation: 1, 2, and 3 = *P. nicotianae* KA1 (placed on the right side of the plates) versus *Chaetomium globosum* CG05, *Chaetomium lucknowense* CL01, and *Chaetomium cupreum* CC3003, respectively; 4 = *Phytophthora nicotianae* KA1 alone.

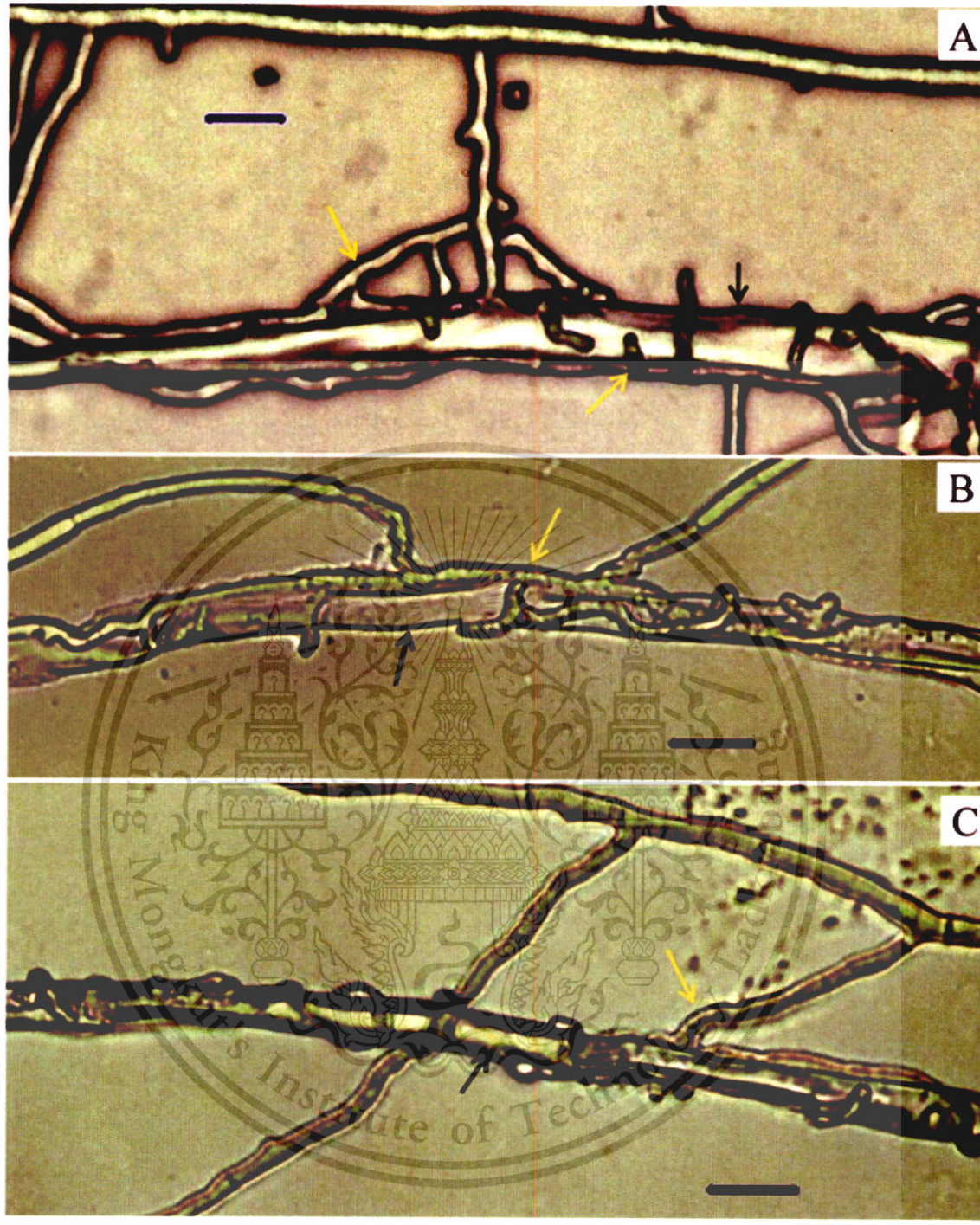


Figure 4.14. Mycopasitism of *Chaetomium* spp. (yellow arrows) on hyphae of *Phytophthora nicotianae* KA1 (black arrows) in bi-culture plates: A = *C. globosum* CG05, B = *C. lucknowense* CL01, C = *C. cupreum* CC3003

4.2.2. *In vitro* effects of antagonistic crude extracts on *Phytophthora* spp.

Crude extracts of *C. globosum* CG05, *C. lucknowense* CL01 and *C. cupreum* CC3003 were tested at different concentrations to evaluate their capacities to inhibit mycelial growth and spore formation of *P. palmivora* PHY02 and *P. nicotianae* KA1. The effective doses (ED_{50}) of each crude extract on mycelial growth and sporangia formation were also examined to determine their fungicidal spectrums.

4.2.2.1. Effects of antagonistic crude extracts on mycelial growth and spore formation of *P. palmivora* PHY02

All antagonistic crude extracts caused inhibitory effects on mycelial growth and spore formation of *P. palmivora* PHY02. However, their fungicidal spectrum was quite different. Thus, the pathogen considerably responded to different crude extracts.

As shown in Table 4.5, Table 4.6 and Fig. 4.15, effective dose values ED_{50} of crude Hexane, EtOAc and MeOH extracts of *C. globosum* CG05 on mycelial growth of *P. palmivora* PHY02 were 85.6, 33.4 and 26.5 µg/mL, respectively. Of which, crude MeOH extract was significantly more effective than others, gave an inhibitory rate of 100 %, at concentration 500 µg/mL. This compared with the inhibitory rates of 86% and 70% showed by crude EtAOc and Hexane extract, respectively, at the same concentration.

Among the crude extracts of *C. lucknowense* CL01, the crude EtOAc extract was most effective in suppressing mycelial growth of *P. palmivora* PHY02 with ED_{50} value was 77.0 µg/mL, and showed the inhibition of 91%, at concentration 500 µg/mL. While, ED_{50} values of crude hexane and MeOH extracts were 919.0 and 143.9µg/mL, and only caused 59 and 43 % growth inhibition of the tested pathogen, respectively, at concentration 500 µg/mL.

All crude extracts of *C. cupreum* CC3003 were less effective on mycelial growth of *P. palmivora* PHY02. No crude extracts of *C. cupreum* CC3003 could

reduce significantly the colony diameter of PHY02 at 10 µg/mL. The crude MeOH extract showed the ED_{50} of 596.8 µg/mL. While, both ED_{50} values of crude EtAOc and hexane extracts were more than 1,000 µg/mL. Additionally, the crude MeOH extract was more effective as compared to the others, caused 86% growth inhibition of the pathogen, at 1,000 µg/mL. This compared with 53 % and 29% caused by crude EtAOc and hexane, respectively.

Table 4.5. Colony diameter (cm) of mycelia growth of *Phytophthora palmivora* PHY02 in antagonistic crude extracts.

Crude extracts	Concentrations (µg/ml)					
	0	**10**	**50**	**100**	**500**	**1,000**
CG05 - Hexane	4.5 a[1]	3.0 b	2.2 c	2.0 f	1.3 h	0.9 i
CG05 - EtOAc	4.5 a	2.6 c	2.1 ef	1.5 g	0.6 j	0 k
CG05 - MeOH	4.5 a	2.4 d	1.6 g	1.3 h	0 k	0 k
CL01- Hexane	4.5 a	3.8 b	3.7 b	3.3 cd	2.6 f	1.6 h
CL01- EtOAc	4.5 a	3.2 d	2.8 e	1.9 g	0.4 j	0.3 j
CL01- MeOH	4.5 a	3.4 c	2.9 e	2.8 e	1.8 g	0.7 i
CC3003- Hexane	4.5 a	4.4 a	4.4 a	4.4 a	3.9 c	3.2 e
CC3003- EtOAc	4.5 a	4.5 a	4.2 b	3.9 a	3.2 e	2.1 g
CC3003- MeOH	4.5 a	4.5 a	4.0 c	3.4 d	2.8 f	0.7 h

[1]Mean of 4 replications. Means followed by the same letters in crude extracts of each antagonist were not significantly different by DMRT at $p = 0.05$.

Table 4.6. Inhibition percentage of mycelia growth of *Phytophthora palmivora* PHY02 by antagonistic crude extracts.

Crude extracts	Concentrations (µg/ml)					ED_{50} on mycelial growth (µg/mL)
	10	50	100	500	1,000	
CG05 - Hexane	33.9 j[1]	51.7 g	56.1 f	70.3 d	80.6 c	85.6
CG05 - EtOAc	41.7 i	54.4 fg	65.8 c	86.7 b	100.0 a	33.4
CG05 - MeOH	46.9 h	65.0 e	71.9 d	100.0 a	100.0 a	26.5
CL01- Hexane	16.1 j	18.3 j	27.2 i	43.1 e	65.6 c	919.0
CL01- EtOAc	30.0 h	37.5 fg	58.9 d	91.1 a	92.8 a	77.0
CL01- MeOH	24.7 i	35.0 g	37.8 f	59.2 d	84.4 b	143.9
CC3003- Hexane	0.6 h	1.9 h	2.2 h	13.3 f	28.9 d	>1,000
CC3003- EtOAc	1.1 h	7.8 g	13.9 f	28.3 d	52.8 b	>1,000
CC3003- MeOH	2.2 h	12.2 f	23.9 e	37.2 c	85.6 a	596.8

[1]Mean of 4 replications. Means followed by the same letters in crude extracts of each antagonist were not significantly different by DMRT at p = 0.05.

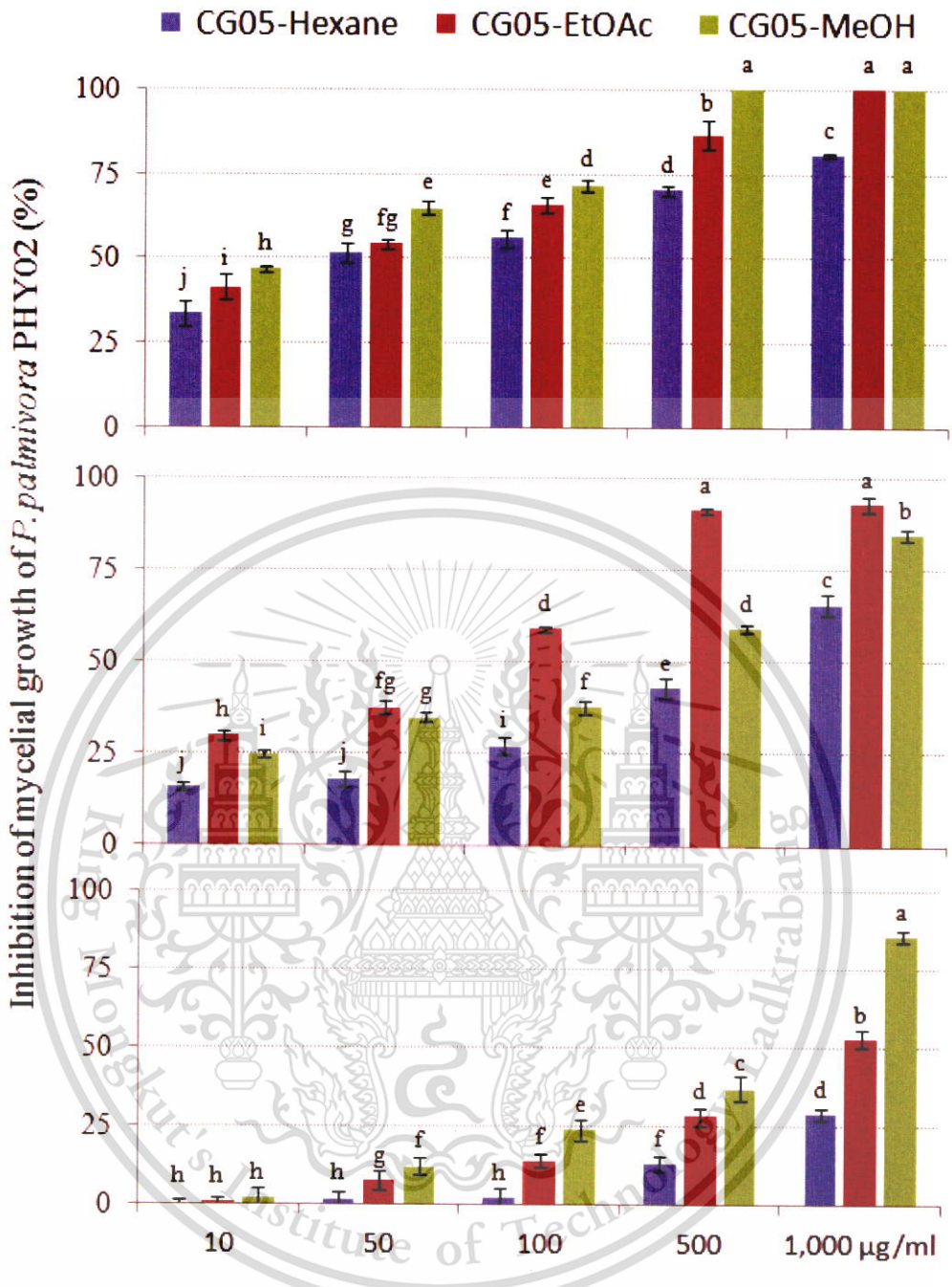


Figure 4.15. Inhibitory effect of antagonistic crude extracts on mycelial growth of *Phytophthora palmivora* PHY02. The columns represent the means of four replications. Bars represent standard ± deviation. In each chart, columns with the same letter are not significantly different based on DMRT at $p = 0.05$.

All of the tested crude extracts exhibited stronger inhibitory effect on spore formation than on mycelial growth of *P. palmivora* PHY02 (Table 4.7, Table 4.8 and Fig. 4.16). The spore formation of the pathogen was most sensitive to crude extracts of *C. globosum* CG05, with ED_{50} values of 5.1, 3.0, and 2.3µg/mL shown by the crude hexane, EtAOc and MeOH extracts, respectively. At the concentration of 100 µg/mL, these crude extracts reduced 91, 95, and 99% spore production of tested pathogen, respectively, as compared to the control. At the tested concentrations 10, 50 and 100 µg/mL, the crude MeOH extract was significantly better in suppressing spore formation of the pathogen, when compared to the others. However, at the higher concentrations (500 and 1,000 µg/mL), the spore inhibitory rates caused by these crude extracts were not significantly different, which all were more than 98%.

In contrast, crude extracts of *C. cupreum* CC3003 were less effective on spore formation of *P. palmivora* PHY02 with ED_{50} of 307.9, 145.7 and 93.6 µg/mL shown by the crude hexane, EtAOc and MeOH extracts, respectively. At concentration of 500 µg/mL, these crude extracts exhibited the inhibitory rates of 53, 74, and 79%, respectively. At all tested concentrations, the crude MeOH was significantly more effective than crude hexane and EtAOc extracts.

Spore formation of *P. palmivora* PHY02 was rather sensitive to the crude Hexane, EtAOc and MeOH extracts of *C. lucknowense* CL01, with ED_{50} values of 16.7, 3.5, and 4.0 µg/mL, respectively. Of which, the crude EtAOc extract suppressed spore formation of the pathogen better than crude hexane and MeOH extracts, at the concentrations 10, 100, and 500 µg/mL. However, at concentration of 1,000 µg/mL, inhibitory rate produced by the crude EtAOc were not significantly different from that produced by crude MeOH extract, both were more than 99 %.

All the crude hexane of the tested antagonists were less effective than the crude EtAOc and MeOH extracts in both inhibitions of mycelial growth and spore formation of *P. palmivora* PHY02. It is observed that although all the crude extracts exhibited

antifungal activities against mycelial growth and spore formation of *P. palmivora* PHY02, they did not cause degradations of hyphae of the pathogen.

Based on these results, the crude MeOH extracts of *C. globosum* CG05 and *C. cupreum* CC3003, and crude EtOAc extract of *C. lucknowense* CL01 were selected and used for *in vivo* control of *P. palmivora* PHY02 in pot experiment.

Table. 4.7. The number of spores of *Phytophthora palmivora* PHY02 ($x10^4$) when grown in antagonistic crude extracts.

Crude extracts	**Concentrations** (µg/ml)					
	0	**10**	**50**	**100**	**500**	**1,000**
CG05 - Hexane	50.2[1] a	18.3 b	10.3 d	4.8 e	1.1 fg	0.6 fg
CG05 - EtOAc	51.0 a	13.8 c	4.8 e	2.6 ef	0.3 fg	0.0 g
CG05 - MeOH	50.4 a	10.0 d	2.4 fg	0.6 fg	0.0 g	0.0 g
CL01- Hexane	50.1 a	29.6 b	17.9 c	12.2 e	8.9 fg	5.8 h
CL01- EtOAc	50.1 a	15.1 d	11.5 e	3.0 i	0.5 j	0.2 j
CL01- MeOH	50.0 a	17.6 c	9.2 f	7.5 g	3.1 i	0.6 j
CC3003- Hexane	51.2 a	48.4 b	47.0 bc	30.8 f	24. 2	12.8 i
CC3003- EtOAc	50.8 a	45.3 cd	44.5 d	25.9 g	13.4 i	6.7 k
CC3003- MeOH	50.6 a	41.1 e	39.6 e	23.7 h	10.6 j	1.8 l

[1]Mean of 4 replications. Means followed by the letters in crude extracts of each antagonist were not significantly different by DMRT at $p = 0.05$.

Table 4.8. Inhibition percentage of spore production of *Phytophthora palmivora* PHY02 by antagonistic crude extracts.

Crude extracts	Concentrations (µg/ml)					ED_{50} on spore formation (µg/mL)
	10	50	100	500	1,000	
CG05 - Hexane	63.6 c	79.4 e	90.5 d	97.7 abc	98.7 abc	5.1
CG05 - EtOAc	73.0 f	90.6 d	94.8 c	99.4 ab	100.0 a	3.0
CG05 - MeOH	80.1 e	95.3 bc	98.8 ab	100.0 a	100.0 a	2.3
CL01- Hexane	41.0 i	64.3 h	75.8 f	82.3 de	88.4 c	16.7
CL01- EtOAc	69.7 g	81.5 e	94.0 b	99.0 a	99.5 a	3.5
CL01- MeOH	64.9 6 h	77.1 f	84.9 d	93.8 b	98.9 a	4.0
CC3003- Hexane	5.5 i	8.3 hi	39.8 f	52.8 e	75.1 cd	307.9
CC3003- EtOAc	10.8 h	12.3 h	49.0 e	73.5 d	86.7 b	145.7
CC3003- MeOH	18.6 g	21.6 g	53.1 e	79.0 c	96.5 a	93.6

[1]Mean of 4 replications. Means followed by the same letters in crude extracts of each antagonist were not significantly different by DMRT at $p = 0.05$.

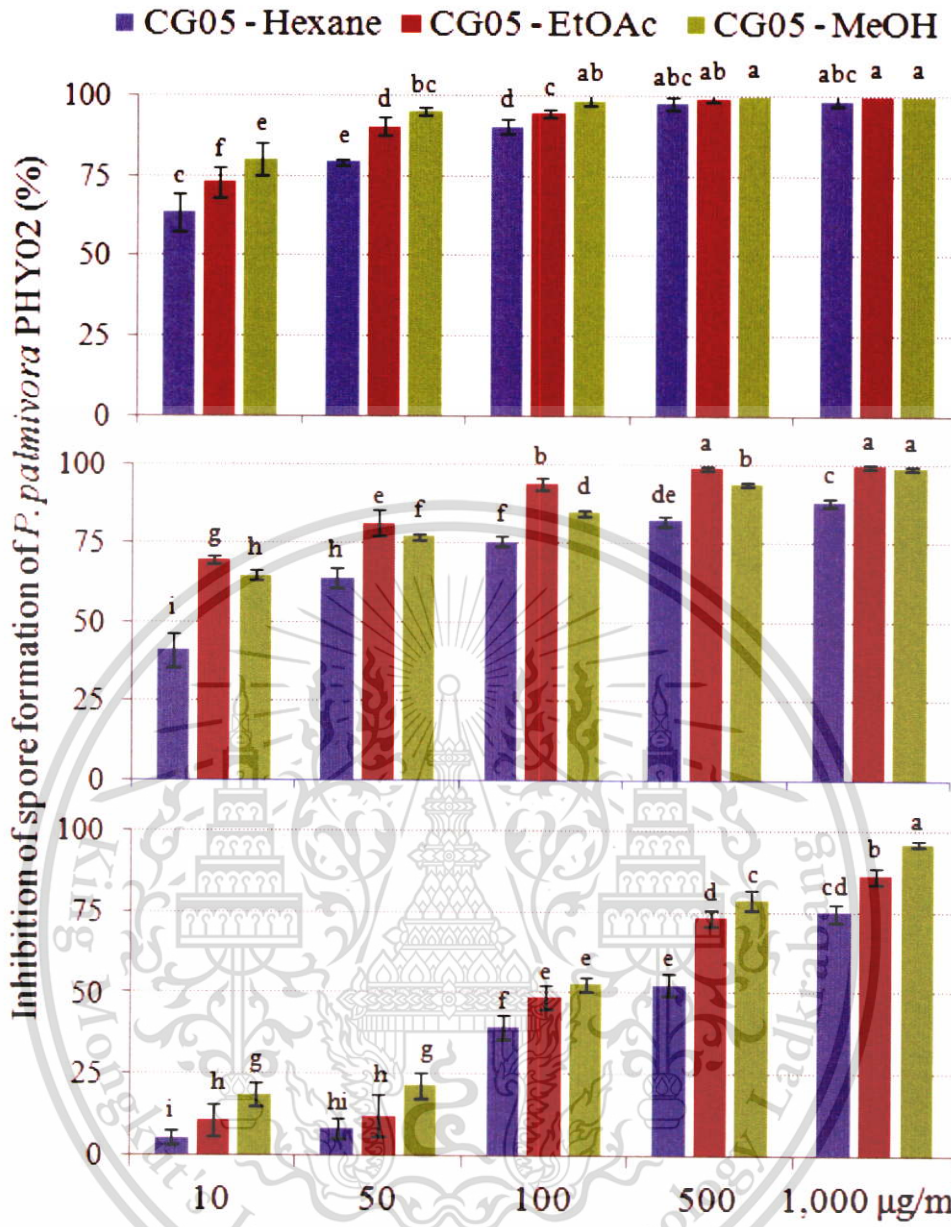


Figure 4.16. Inhibitory effects of crude extracts of the tested *Chaetomium* spp. on spore formation of *Phytophthora palmivora* PHY02. The columns represent the means of 04 replications. Bars represent standard ± deviation. In each chart, columns with the same letter are not significantly different based on DMRT at $p = 0.05$.

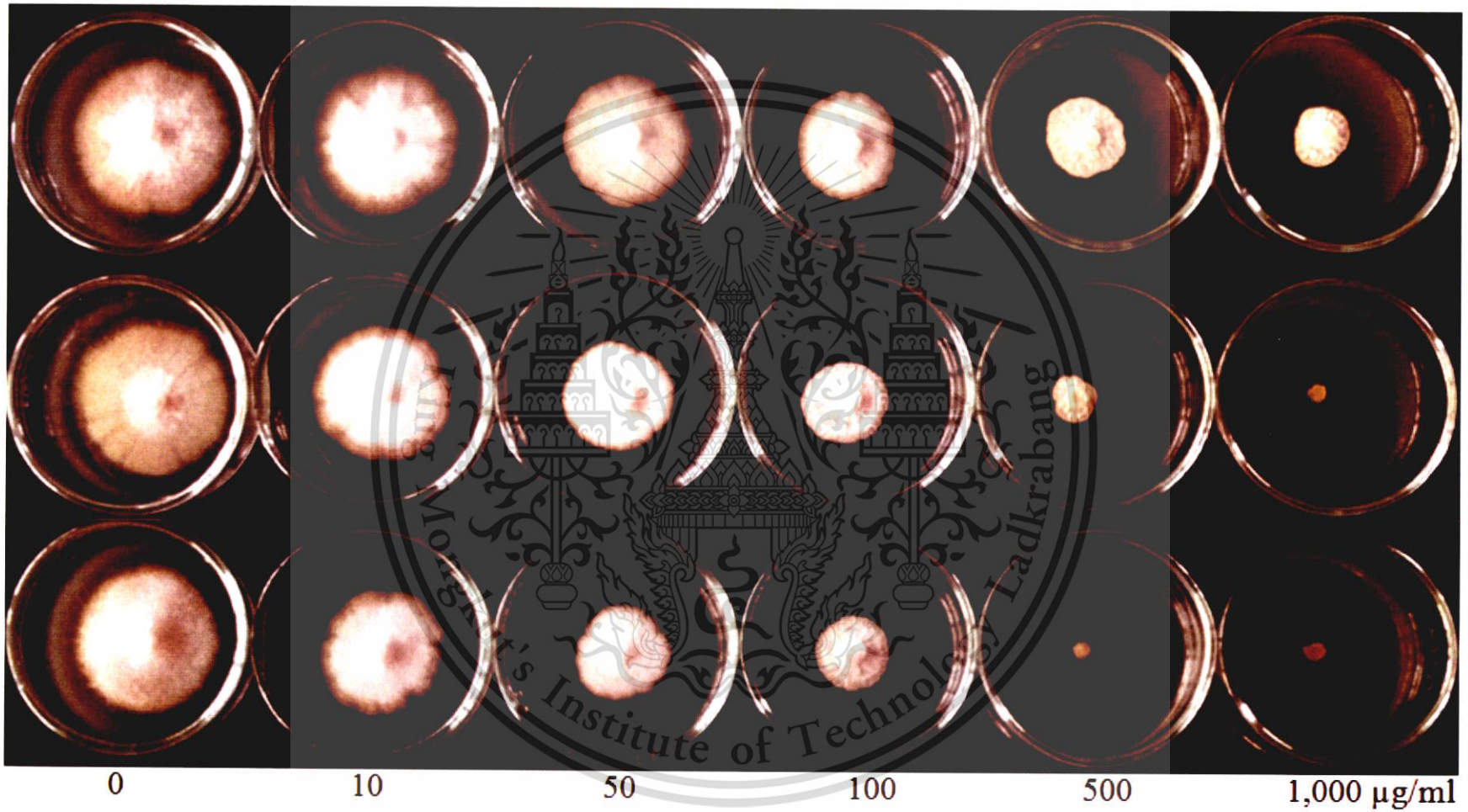


Figure 4.17. Growth of *Phytophthora palmivora* PHY02 at different concentrations of different crude extracts of *Chaetomium globosum* CG05 (upper line: crude Hexane extract; middle line: crude EtAOc extract; and bottom line: crude MeOH extract).

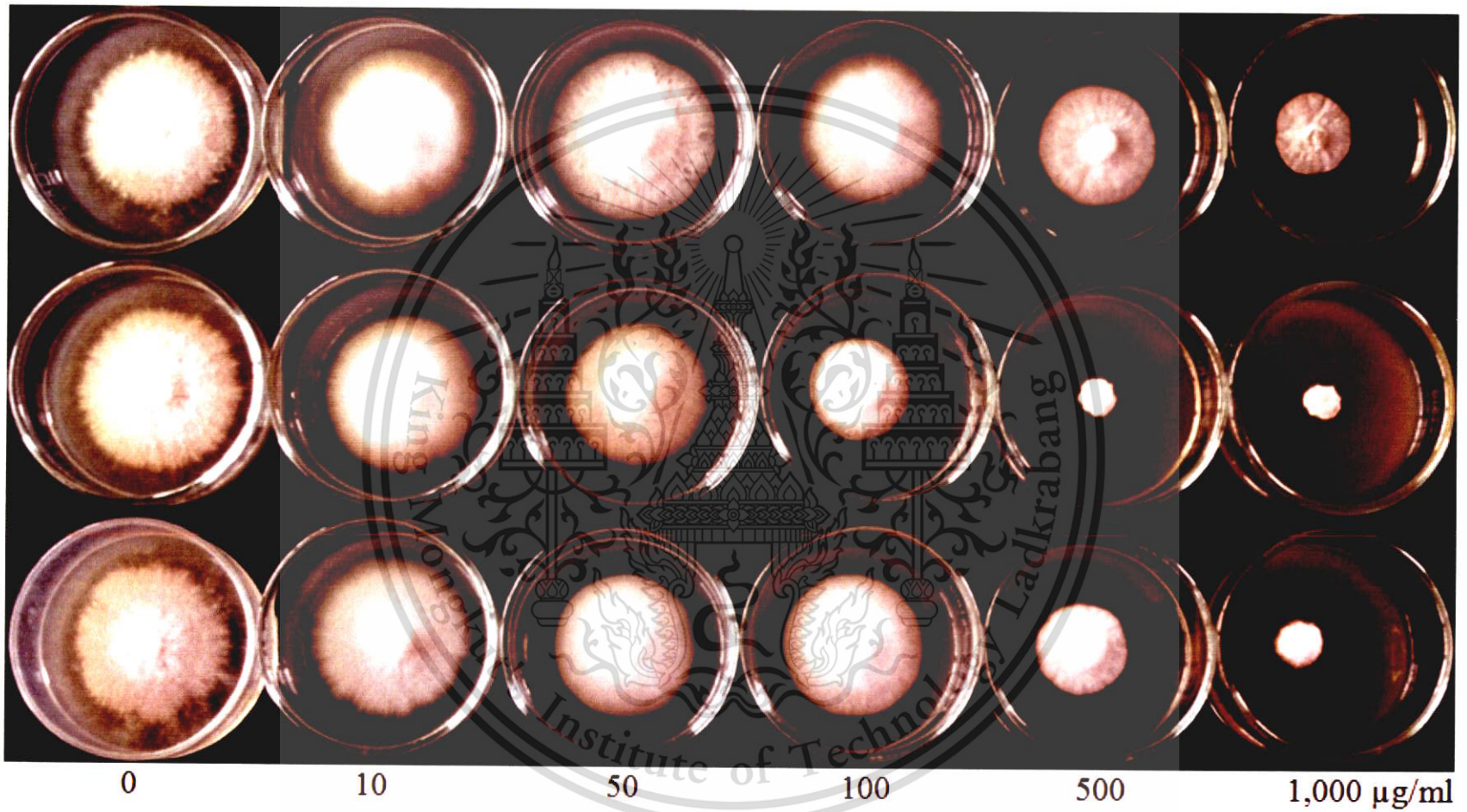


Figure 4.18. Growth of *Phytophthora palmivora* PHY02 at different concentrations of different crude extracts of *Chaetomium lucknowense* CL01 (upper line: crude Hexane extract; middle line: crude EtAOc extract; and bottom line: crude MeOH extract).

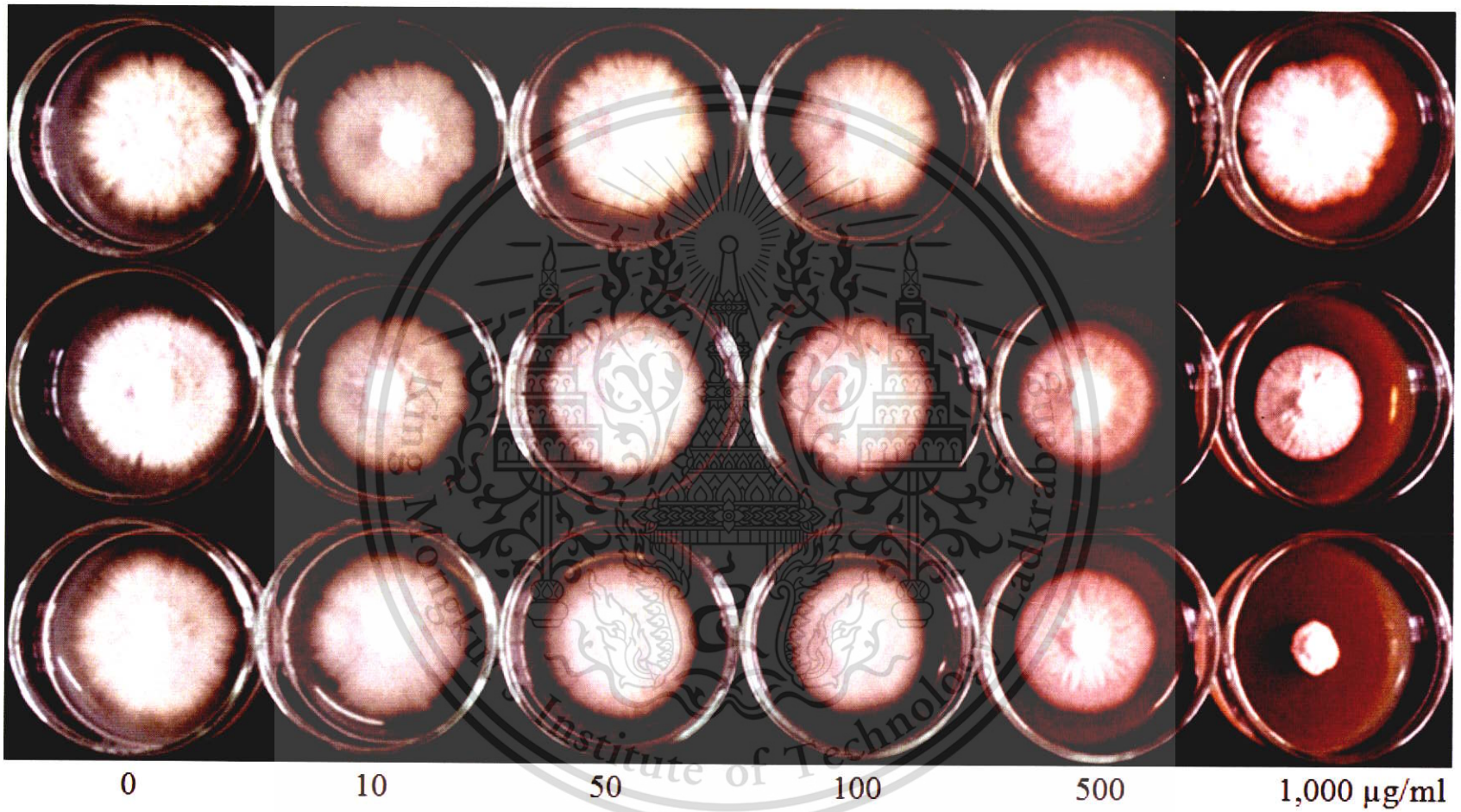


Figure 4.19. Growth of *Phytophthora palmivora* PHY02 at different concentrations of different crude extracts of *Chaetomium cupreum* CC3003 (upper line: crude Hexane extract; middle line: crude EtAOc extract; and bottom line: crude MeOH extract)

4.2.2.2. Effects of antagonistic crude extracts on the mycelial growth of *Phytophthora nicotianae* KA1

The antagonistic crude extracts suppressed mycelial growth of *P. nicotianae* KA1 far better from they showed on *P. palmivora* PHY02, with much lower effective dose ED_{50} values were recorded.

The crude hexane, EtAOc and MeOH extracts of *C. globosum* CG05 respectively exhibited the low effective dose ED_{50} values of 67.2, 4.6 and 4.6 µg/mL on mycelial growth of *P. nicotianae* KA1 (Table 4.10). The growth inhibitions caused by crude MeOH and EtOAc extracts were not significantly different at all the tested concentrations. However, these inhibitory rates were significantly greater than that caused by the crude hexane extract, at concentrations 10, 50, 100 and 500 µg/mL. At concentration of 100 µg/mL, crude MeOH and EtOAc extracts exhibited the inhibitory rates of 93.3 and 92.8%, comparing with 35.6% produced by crude hexane extract. Moreover, in the presence of MeOH or EtOAc extract at concentrations 100, 500, and 1,000 µg/mL, the mycelial growth of *P. nicotianae* KA1 was inhibited nearly 100% (colony diameter did not increase significantly).

Similarly, both crude MeOH and EtOAc extracts of *C. lucknowense* CL01exhibited strong antifungal activity against mycelial growth of *P. nicotianae* KA1, with effective dose ED_{50} values of 2.9 and 2.6 µg/mL, respectively. The inhibitory rates produced by the two crude extracts were not significantly different at all tested concentrations. At concentration of 50 µg/mL, these crude extracts inhibited 94.2 and 95.2% mycelial growth of *P. nicotianae* KA1, respectively. In contrast, crude hexane extract was less effective, with an effective dose ED_{50} value of 101.4 µg/mL, showing the inhibitory rates of 15.3%, at concentration of 50 µg/mL. Furthermore, colony diameter of the pathogen did not increase significantly at concentrations 100, 500 and 1,000 µg/mL of crude MeOH or EtOAc extracts of *C. lucknowense* CL01, with growth inhibitions were more than 99%.

Among the crude extracts of *C. cupreum* CC3003, the crude MeOH extract exhibited strongest antifungal activity against mycelial growth of *P. nicotianae* KA1, with ED_{50} value of 4.5 µg/mL. This compared with 20.8 and 41.2 µg/mL, which shown by crude hexane and EtOAc extracts, respectively. At concentration of 50 µg/mL, the crude MeOH extract produced the inhibitory rates of 90.6%. This compared with 40.6% and 67.2% produced by crude hexane and EtOAc extracts, respectively. At concentration of 1,000 µg/mL, however, all three crude extracts completely inhibited colony growth of *P. nicotianae* KA1.

Because of their strong inhibitory effects against the tested pathogen, the crude MeOH extracts of antagonists were selected and used for *in vivo* control *P. nicotianae* KA1.

Table 4.9. Colony diameter (cm) of mycelia growth of *Phytophthora nicotianae* KA1 in antagonistic crude extracts.

Crude extracts	Concentrations (µg/ml)					
	0	**10**	**50**	**100**	**500**	**1,000**
CG05 - Hexane	4.5 a[1]	3.6 b	2.9 b	1.5 d	0.4 f	0.1 g
CG05 - EtOAc	4.5 a	1.3 c	0.3 f	0.1 g	0.0 g	0.0 g
CG05 - MeOH	4.5 a	1.3 c	0.4 f	0.1 g	0.0 g	0.0 g
CL01- Hexane	4.5 a	4.2 b	3.8 c	2.8 d	1.0 e	0.3 g
CL01- EtOAc	4.5 a	0.9 ef	0.3 g	0.0 h	0.0 h	0.0 h
CL01- MeOH	4.5 a	0.7 f	0.2 g	0.0 h	0.0 h	0.0 h
CC3003- Hexane	4.5 a	3.0 c	1.5 e	0.6 f	0.0 i	0.0 i
CC3003- EtOAc	4.5 a	3.3 b	2.7 d	1.4 e	0.2 h	0.0 i
CC3003- MeOH	4.5 a	1.4 e	0.4 g	0.0 i	0.0 i	0.0 i

[1]Mean of 4 replications. Means followed by the letters in crude extracts of each antagonist were not significantly different by DMRT at $p = 0.05$.

Table 4.10. Inhibition percentage of mycelial growth of *Phytophthora nicotianae* KA1 by antagonistic crude extracts.

Crude extracts	Concentrations (µg/mL)					ED_{50} on mycelial growth (µg/mL)
	10	50	100	500	1,000	
CG05 - Hexane	21.1 f[1]	35.6 e	66.4 d	90.8 b	97.2 a	67.2
CG05 - EtOAc	71.1 c	93.3 b	98.3 a	100.0 a	100.0 a	4.6
CG05 - MeOH	70.6 c	92.8 b	97.8 a	100.0 a	100.0 a	4.6
CL01- Hexane	6.4 g	15.3 f	38.1 e	78.3 d	94.4 b	101.4
CL01- EtOAc	81.1 cd	94.2 b	99.2 a	100.0 a	100.0 a	2.9
CL01- MeOH	83.6 c	95.2 b	99.4 a	100.0 a	100.0 a	2.6
CC3003- Hexane	33.9 e	67.2 d	86.1 c	99.2 a	100.0 a	20.8
CC3003- EtOAc	26.1 f	40.6 e	70.0 d	95.3 b	100.0 a	41.2
CC3003- MeOH	68.9 d	90.6 c	98.9 a	100.0 a	100.0 a	4.5

[1]Mean of 4 replications. Means followed by the letters in crude extracts of each antagonist were not significantly different by DMRT at $p = 0.05$.

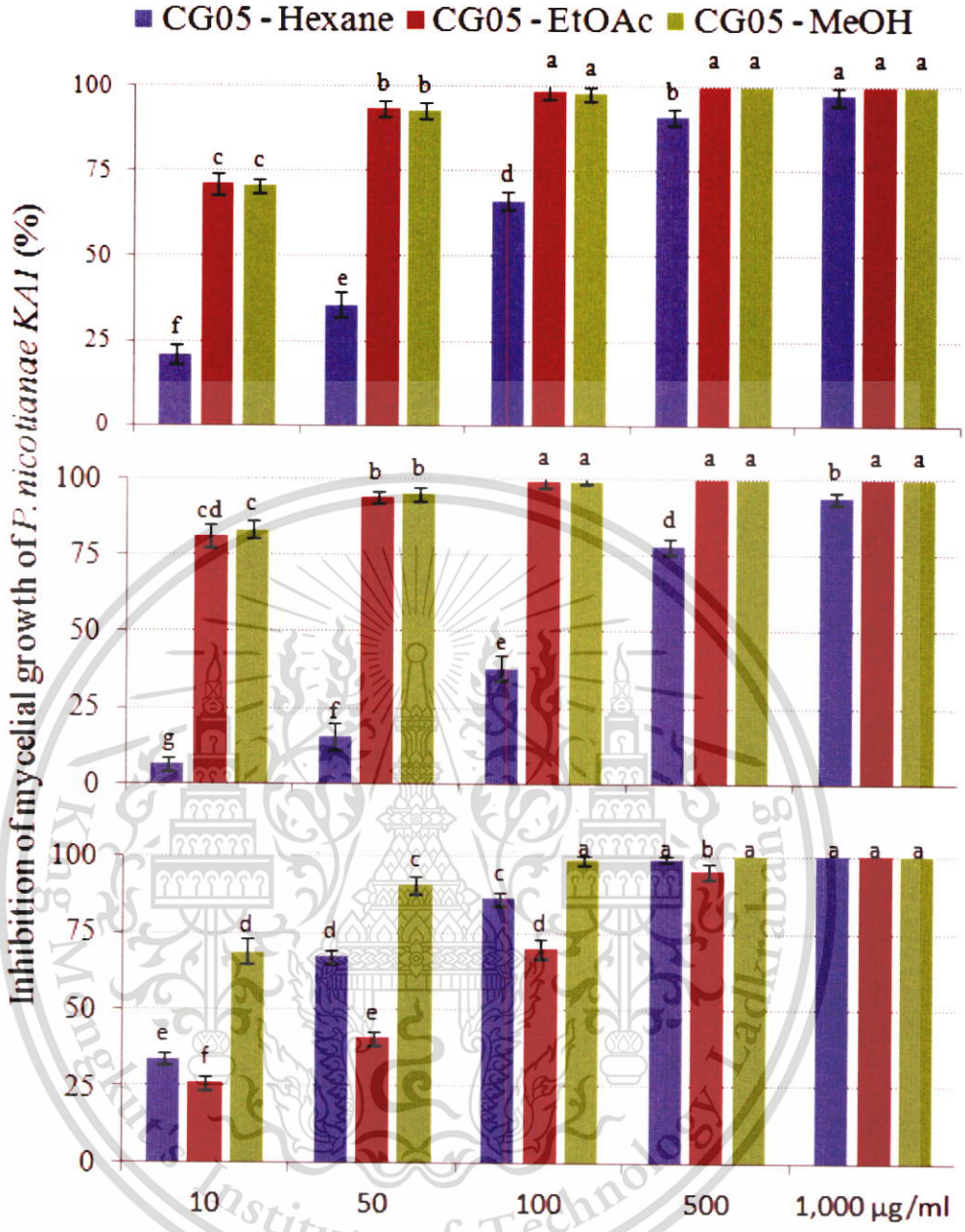


Figure 4.20. Inhibitory effects of crude extracts of the tested *Chaetomium* spp. on mycelial growth of *Phytophthora nicotianae* KA1. The columns represent the means of 04 replicates. Bars represent standard ± deviation. In each chart, columns with the same letter are not significantly different based on DMRT at $p = 0.05$.

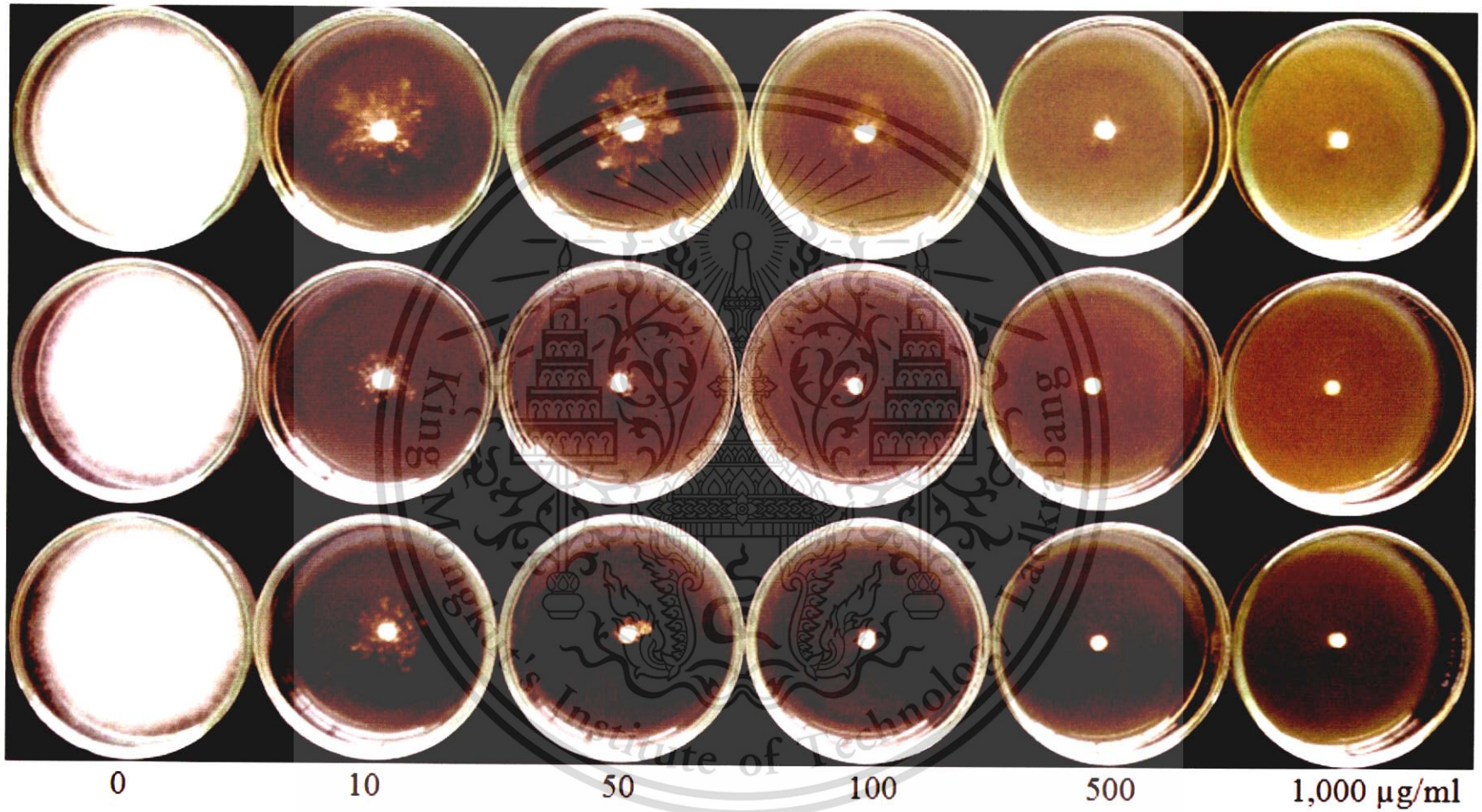


Figure 4.21. Growth of *Phytophthora nicotianae* KA1 at different concentrations of different crude extracts of *Chaetomium globosum* CG05 (upper line: crude Hexane extract; middle line: crude EtAOc extract; and bottom line: crude MeOH extract).

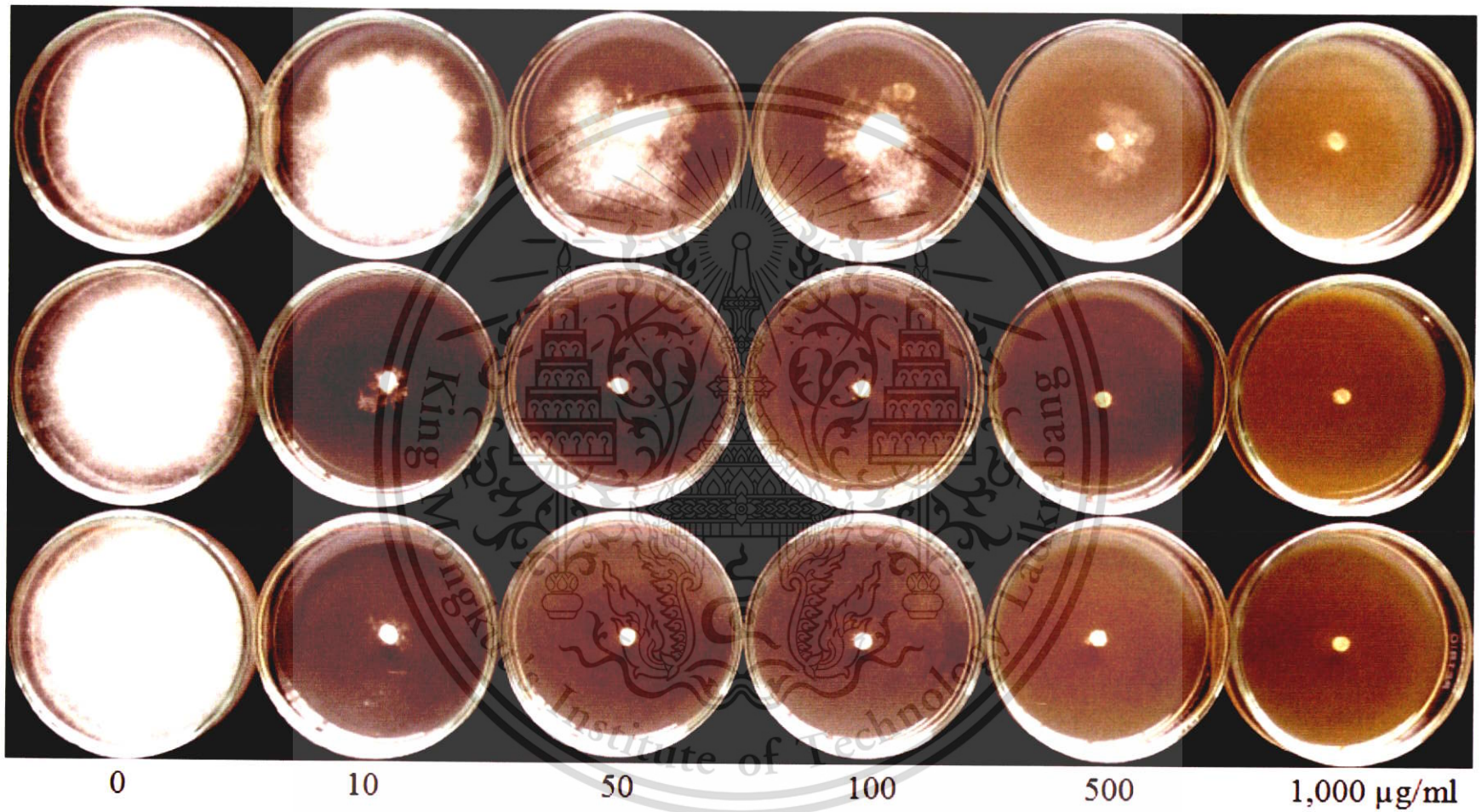


Figure 4.22. Growth of *Phytophthora nicotianae* KA1 at different concentrations of different crude extracts of *Chaetomium lucknowense* CL01 (upper line: crude Hexane extract; middle line: crude EtAOc extract; and bottom line: crude MeOH extract).

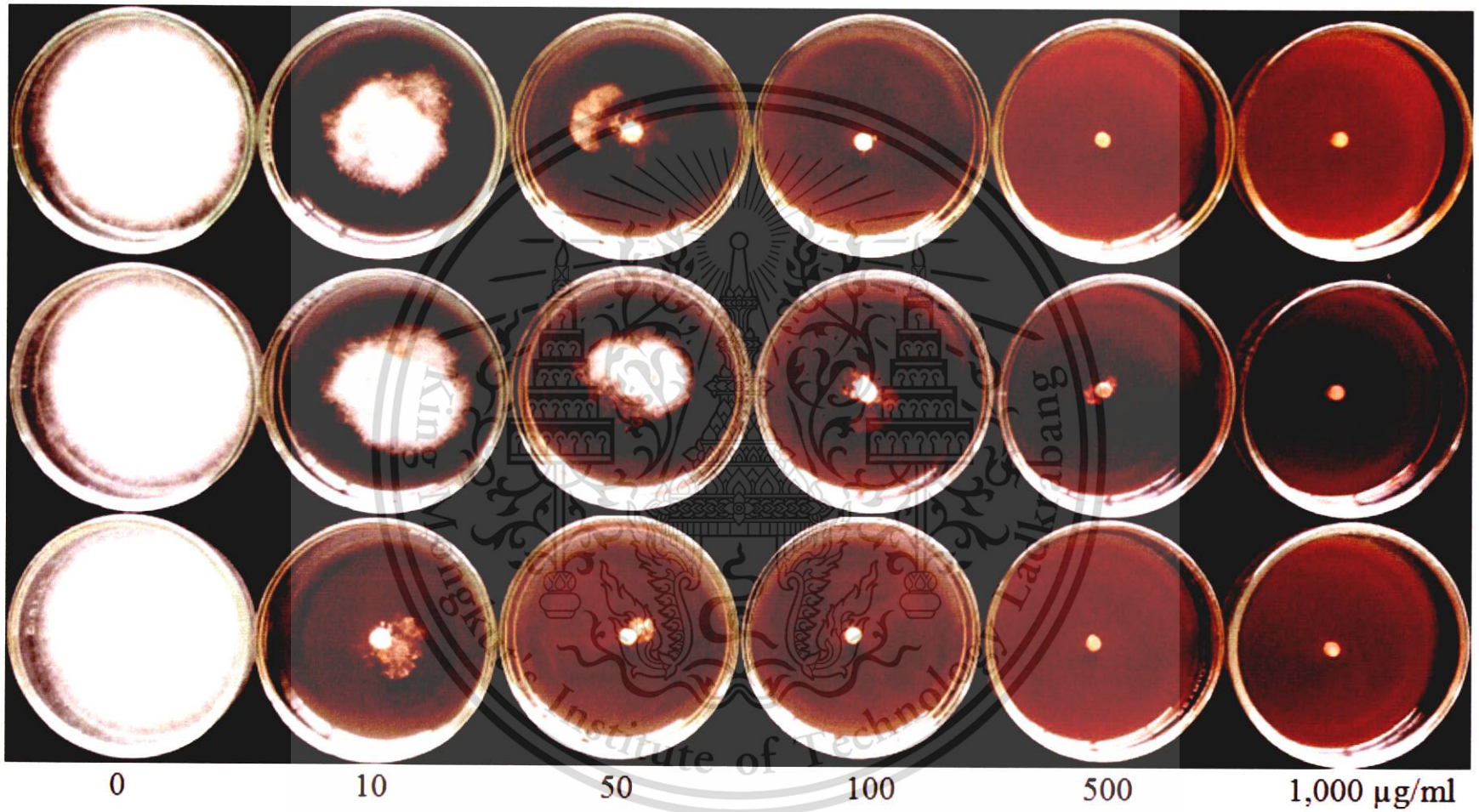


Figure 4.23. Growth of *Phytophthora nicotianae* KA1 at different concentrations of different crude extracts of *Chaetomium cupreum* CC3003 (upper line: crude Hexane extract; middle line: crude EtAOc extract; and bottom line: crude MeOH extract).

4.2.3. *In vivo* effects of *Chaetomium* spp. and their crude extracts on *Phytophthora* spp. causing root rots of pomelo

Applications of spores and crude extracts of the antagonists showed highly significant effects on root rot rates and plant weights of pomelo seedlings inoculated with 2.5 propagules of either *P. palmivora* PHY02 or *P. nicotianae* KA1 per cubic centimeter of soil.

As shown in 4.5, in the absence of *Chaetomium* spp. and their crude extracts, seedlings inoculated with *P. palmivora* PHY02 (control) produced root rot rates of 52.8%. This compared with no root rot in the non-inoculated seedlings. Furthermore, dry plant weight of the control seedlings was decreased by 58.3 % compared to that of non-inoculated seedlings. Meanwhile, the seedlings that were treated with spores of *C. globosum* CG05 (S-CG05), *C. lucknowense* CL01(S-CL01) and *C. cupreum* CC3003 (S-CC3003) respectively exhibited root rot rates of 18, 20 and 21%, which were decreased by 66, 62 and 61%, compared to that shown on the inoculated control seedlings. Seedlings that were treated with crude MeOH extract of *C. globosum* CG05(M-CG05), crude EtAOc of *C. lucknowense* CL01 (E-CL01) and crude MeOH of *C. cupreum* CC3003 (M-CC3003) respectively showed root rot rates of 19, 28 and 33, which decreased by 63, 47 and 38%, compared to that produce by the inoculated control seedlings. Following treatments with spores and their crude extracts of tested antagonists (S-CL01, S-CG05, S-CC3003, M-CG05, E-CL01and M-CC3003), dry plant weight of seedlings increased by 85, 83, 51, 91, 44 and 27 %, respectively, as compared to that of inoculated control seedlings. In comparisons with the non-inoculated seedlings, however, dry plant weights of seedlings in these treatments were also decreased by 23, 24, 37, 21, 40 and 47%.

Table 4.11. Effects of *Chaetomium* spp. and their crude extracts on root rots and plant weights of pomelo seedlings inoculated with *Phytophthora palmivora* PHY02.

Treatments	Root rot		Plant weight		
	Root rot (%)[6]	Decrease compared to the control seedlings (%)	Dry plant weight (mg)	Increase compared to the inoculated control seedlings (%)	Decrease compared to non-inoculated seedlings (%)
Non-inoculated[1]	0.0 e[6]	100	971 a	139.8	-
PHY02[2](inoculated control)	52.8 a	-	405 e	-	58.3
Me[3]-CG05	19.7 d	62.6	773 b	90.7	20.5
E[4]-CL01	28.2 c	46.5	585 c	44.4	39.8
Me-CC3003	32.9 b	37.7	513 d	26.5	47.2
S[5]-CG05	18.0 d	65.9	750 b	85.2	22.8
S-CL01	19.9 d	62.3	740 b	82.7	23.8
S-CC3003	20.7 d	60.8	613 c	51.2	36.9

[1]Non-inoculated plants treated with water; [2]Inoculated plants treated with water; [3]Inoculated plants treated with crude MeOH extracts of antagonists; [4]Inoculated plants treated with crude EtAOc of *C. lucknowense* CL01; [5]Inoculated Plants inoculated with *P. palmivora* PHY02 and spores of antagonists; For each parameter, values indicated by the same letter are not significantly different according to DMRT at $p = 0.05$.

Seedlings treated with crude MeOH extract of *C. globosum* CG05 had significantly lower root rot rate and higher dry plant weight, as compared with the seedlings treated with crude EtAOc of *C. lucknowense* CL01and crude MeOH of *C. cupreum* CC3003. In contrast, there was no significant difference among root rot rates produced by seedlings treated with spores of different antagonists. However, the dry plant weight of seedlings treated with spores of *C. cupreum* CC3003 was significantly less than seedlings treated with spores of *C. globosum* CG05 and *C. lucknowense* CL01. The root rot rates were not significantly different between seedlings treated with

crude MeOH extract and those treated with spores of *C. globosum*. Seedlings treated with spores of *C. lucknowense* CL01and *C. cupreum* CC3003 even showed significantly lower root rot rates and higher dry plant weight than those treated with crude extracts.

As shown in the Table 4.12, in the absence of *Chaetomium* spp. and their crude methanol extracts, seedlings inoculated with *P. nicotianae* KA1 (controls) exhibited a root rot rate of 49.8%, and dry plant weight was reduced by 51.6% compared with that of non-inoculated seedlings. In contrast, the seedlings that treated with spores and their crude methanol extracts of tested antagonists (S-CL01, S-CG05, S-CC3003, M-CG05, M-CL01and M-CC3003) respectively, produced root rot rates of 15, 17, 14, 16, 14 and 15%, which were 71, 66, 71, 69, 73 and 69 % lower than those observed in the inoculated control seedlings. Moreover, dry plant weights of seedlings in these treatments were respectively reduced by only 13, 17, 11, 16, 11 and 17% compared with that in non-inoculated plants, and increased by 79, 72, 83, 74, 85, and 73 % compared with that of the inoculated control seedlings. Little differences in root rot percentage and plant weight of seedlings were observed among the 6 different treatments, according to DMRT (P= 0.05). These parameters were not significantly different whether seedlings were treated with spores or with crude MeOH of *C. globosum* CG05 and *C. cupreum* CC3003. However, seedlings that treated with spores of *C. lucknowense* CL01 had lower dry plant weight and root rot percentage than seedlings treated with its crude MeOH extracts.

Additionally, in general, the *Chaetomium* spp. and their crude extracts controlled *P. nicotianae* KA1 better than *P. palmivora* PHY02.

Table 4.12. Effects of *Chaetomium* spp. and their crude extracts on root rot and plant weight of pomelo seedlings inoculated with *Phytophthora nicotianae* KA1.

Treatments	Root rot		Plant weight		
	Root rot (%)	Decrease compared to the control seedling (%)	Dry plant weight (mg)	Increase compared to the inoculated control seedling (%)	Decrease compared to non-inoculated seedling (%)
Non-inoculated[1]	0.0 d[5]	100	998 a	106.7	-
KA1[2] (inoculated control)	49.8 a	-	483 d	-	51.6
Me[3]-CG05	15.6 bc	69.1	840 bc	74.1	15.8
Me-CL01	13.7 c	72.6	893 b	85.0	10.5
Me-CC3003	15.4 bc	68.6	833 bc	72.5	16.5
S[4]-CG05	14.7 c	70.6	865 bc	79.3	13.3
S-CL01	17.1 b	65.6	828 c	71.5	17.0
S-CC3003	14.2 c	71.4	885 bc	83.4	11.3

[1]Non-inoculated plants treated with water; [2]Inoculated plants treated with water; [3]Inoculated plants treated with crude MeOH extracts of antagonists; [4]Inoculated Plants inoculated with *P. nicotianae* KA1 and spores of antagonists; [5]Mean of 4 replications; For each parameter, values indicated by the same letter are not significantly different according to Duncan's multiple range test at $p = 0.05$.

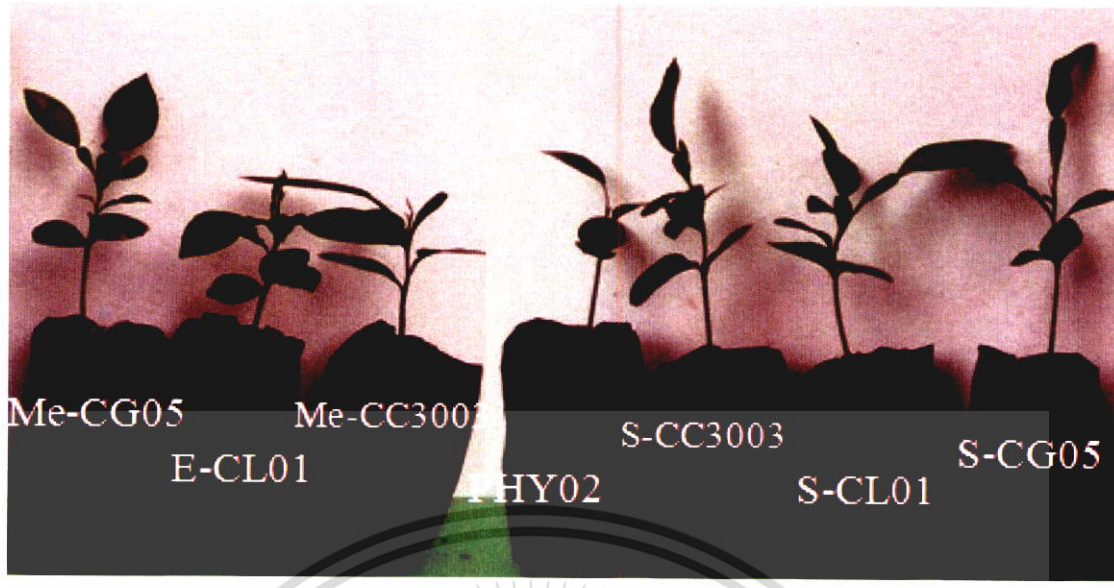


Figure 4.24. Pomelo seedlings inoculated with *Phytophthora palmivora* PHY02 and treated with *Chaetomium* spp. and their crude methanol extracts

Figure 4.25. Pomelo seedlings inoculated with *Phytophthora nicotianae* KA1 and treated with *Chaetomium* spp. and their crude methanol extracts.

CHAPTER 5

DISCUSSION

In this research, six isolates of *Phytophthora* spp., dividing into two different groups, were isolated from soil and infected roots of pomelo var. Khaonampung. The first group contained slow-growth isolates that had papilate-caducous sporangia with short pedicels, and occurring in groups of 5 – 15 on one sympodium. These are typical characters of *P. palmivora* according to Erwin and Ribeiro (1996), which described the occurrences of groups (up to 20) conspicuous papillate sporangia, with short pedicels on a sympodium as the distinguished characteristics of this species. More importantly, the analysis of their ITS sequences confirmed these isolates were *P. palmivora*. The appearance of only *P. palmivora* in samples from the orchards affected by root rots in Chachoengsao province and its high virulence for roots of pomelo in pathogenicity test suggested that this species is the causal agent. This finding is in correspondent with previous findings. Zitko *et al.* (1991) demonstrated that *P. palmivora* is often more aggressive and damages even larger root than *P. parasitica* on citrus. Serious root rots of citrus caused by *P. palmivora* have been recorded in America, India (Zitko and Timmer. 1992; Naqvi. 2004). Additionally, *P. palmivora* is known as a prominent plant pathogen with a wide host range, which infects various important crops such as black pepper, durian, citrus, rubber *etc*, in Southeast Asia (Drenth and Guest. 2004).

All morphological characteristics of isolates from the second group were similar to those of *P. nicotianae* (syn. *P. parasitica*), according to the descriptions of Erwin and Ribeiro (1996), except the production of caducous sporangia. Despite the unusual characteristics of the sporangia, the ITS sequences of this isolate were identical to those of many isolates of *P. nicotianae* found in GenBank. Moreover, the phylogenetic analysis strongly confirmed the relationships between the isolate KA1and the related taxa. Caducity of sporangia is an important and useful character for

morphological study and identification of *Phytophthora* species. According to the extensive reviews by Erwin and Ribeiro (1996), *P. nicotianae* does not produce caducous sporangia, but produces persistence sporangia on the long stalks. However, Cacciola *et al.* (1994) found that isolates identified as *P. nicotianae* obtained from affected *Forsythia* plants had caducous sporangia with a very short pedicels (less than 5 μm). From lavender (*Lavandula angustifolia* Mill.), Álvarez *et al.* (2007) also obtained 05 isolates, which identified as *P. nicotianae*, had caducous sporangia with short pedicels (2.1 – 3.8 μm). These descriptions are consistent with the observation for isolates of *P. nicotianae* obtained from pomelo, in this study.

The appearance and high virulence of isolate of *P. nicotianae* in pomelo, as shown in this study, supported the conclusion that *P. nicotianae* is the main causal agent of root rot in all types of citrus worldwide (Erwin and Ribiero. 1996; Naqvi. 2004). The species is well known to distribute widely and cause root rots of citrus plants in citrus-growing areas in America. It is estimated that 20 – 80% citrus orchards in Florida are infested by *P. nicotianae* (Graham and Timmer. 2008). Recently, *P. nicotianae* is reported to be the most predominant pathogens of citrus root rots, foot rots and gummosis in Egypt (Ahmed *et al.* 2012), South Africa (Meitz-Hopkins. 2014). In Thailand and other countries in Southeast Asia, *P. nicotianae* is responsible for root rots of various citrus types and many other crops such as papaya, tobacco, durian, black pepper, pineapple *etc.* (Drenth and Guest. 2004). Therefore, it is unsurprised when such virulence pathogen infects and causes root rots of pomelo, which is widely grown in Thailand. Additionally, pomelo (*Citrus maxima*) is considered susceptible to *Phythopthora* species (Naqvi. 2004).

The antagonistic activity of bio-control microorganisms is often demonstrated by the inhibition of growth, infection or reproduction of pathogen (Alabouvette *et al.* 2006). *Chaetomium* spp. in this study, both as living organisms and as crude extracts showed great inhibitory effects on developments of *Phytophthora* spp. *in vitro* and *in vivo*.

Representing as antagonists, *Chaetomium* spp. inhibited 50 – 61% colony growth; 92 – 99% spore production of *P. palmivora* PHY02; and inhibited 50 – 56% colony growth of *P. nicotianae* KA1, in bi-culture tests. The abilities of *Chaetomium* spp. to suppress sporulation and mycelial growth of fungal pathogens are well documented. Many studies showed that *Chaetomium* spp. *in vitro* inhibited colony growth of different plant pathogens such as *Drechslera sorokiniana* (Aggarwal *et al.* 2003), *P. infestans* (Shanthiyaa *et al.* 2013), *Pestalotia* sp. (Nguyen *et al.* 2013), *Colletotrichum gloeosporioides* and *Pythium aphanidermatum* (Phung *et al.* 2014). In which, parasitism was not detected, but the *Chaetomium* spp. inhibited colony growth of the pathogens by production of antibiotics, suggesting the antibiosis mechanism. In this study, however, all tested antagonists grew over, parasitized hyphae and strongly degraded colonies of *P. palmivora* PHY02 and *P. nicotianae* KA1, in bi-culture plates. These phenomena are typical of mycoparasitism (Alabouvette *et al.* 2006; Narayanasamy, 2013). Similarly, hyphae of *P. cinnamomi* and *P. nicotianae* can be parasitized and lysed by *C. globosum* (Heller and Theiler-Hedtrich. 1994). Mycoparasitism relies on various lytic enzymes for degradation of the cell wall of the host, causing death of the target organism, which results in a decrease in inoculum density, whereas antibiosis is a type of antagonism resulting from the production of secondary metabolites toxic to other microorganisms (Sun *et al.* 2006, Alabouvette *et al.* 2006; Narayanasamy. 2013). The secondary metabolites may include antibiotics, bacteriocines, volatile compounds with an antifungal activity, and enzymes such as lytic enzymes (Alabouvette *et al.* 2006). *Chaetomium* species are well known as producer of lytic enzymes and hundreds of other secondary metabolites, which may be involved in their antagonistic activity (Zhang *et al.* 2012). Degrading enzymes such as chitinase and beta-1,3-glucanase have been shown to be secreted by *Chaetomium* spp. in culture substrates and under mycoparasitism conditions (Sun *et al.* 2006; Liu *et al.* 2008). On the other hand, all the antagonists used in this study were known to produce antibiotics with antifungal activities against different plant pathogens (Soytong *et al.*

2001; Kanokmedhakul *et al.* 2006; Sibounnavong *et al.* 2011). Therefore, the inhibitions of growth and spore formation of *P. palmivora* PHY02 and *P. nicotianae* KA1 might be involved the consequences of both antibiosis and parasitism interaction between the tested *Chaetomium* spp. and the pathogens. Indeed, the interaction between *C. cupreum* CC3003 and *P. nicotianae* KA1 found in this study showed typical characteristics of both antibiosis and mycoparasitism. At first *C. cupreum* CC3003 prevented growth, then it parasitized hyphae and degraded colony of *P. nicotianae* KA1. These interactions were different from those between *C. cupreum* CC3003 and *P. palmivora* PHY02, in which, the growth of the pathogen was not prevented further. The interaction between antagonists and pathogens is varied. One antagonist could respond differently to different fungal pathogens, even through different isolates of one species (Alabouvette *et al.* 2006; Narayanasamy. 2013). Both *C. globosum* and *C. cupreum* have been reported to control *P. nicotianae* root rots (Soytong *et al.* 2001). However, this is reported for the first time of *C. lucknowense* effectively against *Phytophthora* spp.

All crude extracts of *Chaetomium* spp. exhibited antifungal activities on mycelial growth (ED_{50}: 26.5 – 2,495 µg/mL) and spore formation (ED_{50}: 2.3 – 307.9 µg/mL) of *P. palmivora* PHY02. The crude extracts showed strongly antifungal activities on mycelial growth of *P. nicotianae* KA1with ED_{50} values of 2.6 – 101.4 µg/mL. *Chaetomium* species are well known as producers of hundreds metabolites with different bioactivities that play important roles in their biological control activities (Sun *et al.* 2006; Zhang *et al.* 2012). From culture filtrate of a certain isolate of *C. globosum*, Park *et al.* (2005) isolated chaetoviridins A and B. Of which, chaetoviridins A exhibited more antifungal activities against *Pythium ultimum*, *P. capsici*, *P. infestans* and other plant pathogens with IC_{50} valued of 1.23 – 33 µg/mL. Chaetoglobosins A and C, which were found from another isolate of *C. globosum*, exhibited antifungal activity against *Setosphaeria turcica*, causing northern corn leaf blight (Zhang *et al.* 2013). Qin *et al.* (2009) found that Chaetomugilin D produced by *C. globosum* showed

antifungal activity against *Mucor miehei*. Additionally, *C. globosum* CG05 and *C. lucknowense* CL01used in this study are known to produce chaetoglobosins C, which is frequently indicated to account for the antifungal activities against plant pathogens (Soytong *et al.* 2001; Charoenporn *et al.* 2010; Sibounnavong *et al.* 2011). Kanokmedhakul *et al.* (2006) isolated six different compounds from *C. cupreum* CC3003. Of which, four compounds (rubrorotiorin, rotiorinols A and C, and rotiorin) expressed strongly antifungal activities against mycelial growth of fungus *Candida albicans* with IC_{50} values of 0.6, 10.5, 16.7, and 24.3 μg/ml, respectively.

These findings suggested that multiple metabolites might contribute to the antifungal activity of a single crude extract. Thus, the inhibitory effects on *P. palmivora* PHY02 and *P. nicotianae* KA1 observed in this study may have resulted from the combined antifungal activities of different metabolites within the crude extracts. One *Chaetomium* species may produce many metabolites with different bioactivities and molecular weight, this lead to differences in antifungal activity of its crude extracts. In addition, different species probably produce different metabolites (Kanokmedhakul *et al.* 2006). Different species or even different isolates within a species can differently react to a metabolite (Narayanasamy. 2013). These explained for the great variation in response of the tested pathogens to different crude extracts, which observed in this study. The *P. nicotianae* KA1 expressed more sensitive to crude extracts than *P. palmivora* PHY02. Crude Hexane extracts showed less effective in both inhibition of mycelial growth and spore formation of *P. palmivora* PHY02 compared to crude MeOH and EtAOc. However, crude Hexane of *C. cupreum* CC3003 inhibited mycelial growth of *P. nicotianae* KA1better than crude EtAOc. Similarly, Kumar *et al.* (2013) reported that crude Hexane of *C. globosum* exhibited less antifungal activity against *Sclerotinia sclerotiorum* than crude MeOH and EtAOc. Sporangium formation is the most sensitive stage in life cycle of *Phytophthora* (Erwin and Ribiero. 1996). This study demonstrated that spore formation of *P. palmivora* PHY02 was much more sensitive than mycelial growth to all the antagonistic crude

extracts. The high inhibitory effects of crude extracts from *Chaetomium* spp. on spore formation of *Fusarium oxysporum* and *P. parasitisca* have been noted by previous authors (Charoenporn *et al.* 2010; Sibounnavong *et al.* 2011). Unlike in the bi-culture test, despite being suppressed, mycelia of *P. palmivora* PHY02 and *P. nicotianae* KA1were not degraded when grown in crude extracts. Similarly, in previous studies, crude extracts and even the pure compounds such as rubrorotiorin, chaetoviridins A, chaetoglobosins C *etc* also did not cause degradation of mycelia of tested pathogens (Park *et al.* 2005; Kanokmedhakul *et al.* 2002;2006; Charoenporn *et al.* 2010; Sibounnavong *et al.* 2011). Therefore, it is suggested that lytic enzymes might not involve in antifungal activities of crude extracts against the pathogens. Antifungal activities of crude extracts did not account for all bio-control activities of the tested *Chaetomium* spp. against *P. palmivora* PHY02 and *P. nicotianae* KA1, in this study. Besides the known metabolites, degrading enzymes should be considered as effective factors involved in bio-control activities of the tested *Chaetomium* spp. against the pathogens.

This study demonstrated that *Chaetomium* spp. and their crude extracts reduced 38 – 66% root rot, and increased dry plant weight by 27 – 91%, compared to those of the control seedlings that inoculated with *P. palmivora* PHY02. In cases of seedlings inoculated with *P. nicotianae* KA1and treated with *Chaetomium* spp. and their crude MeOH extract, root rot rates were reduced by 66 – 73% and dry plant weight increased by 72 – 85%. Similarly, the introduction of antagonists into soil for controlling *Phytophthora* root rot in citrus had some success under greenhouse conditions in prior studies. Fang and Tsao (1995a) reported that when *Pythium nunn* and *Phytophthora* spp. were together inoculated into roots of sweet orange (*Citrus sinensis*), the population of *Phytophthora* spp. in soil and root rot incidence on plants was significantly reduced. However, the effective control only achieved when very high concentration (1,000 propagules/ cm^3 of soil) of *Pythium nunn* was applied. At that concentration, their data showed that number of roots and weight of plants also

reduced. In another similar study with *Penicillium funiculosum* as bio-control agent, Fang and Tsao (1995b) also reported the same effects of the antagonist on citrus plant inoculated with *Phytophthora* spp. Moreover, a bio-product named Ketomium® (Strong crop Co. Ltd., Samatprakaru Province, Thailand), developed from 22 strains of *C. globosum* and *C. cupreum* has showed great ability to control *Phytophthora* sp. causing citrus root rot in the field (Soytong *et al.* 2001). In addition, the spores and crude MeOH of *C. globosum* and *C. lucknowense* were reported to be effective in controlling *Fusarium* tomato wilt (Charoenporn *et al.* 2010; Sibounnavong *et al.* 2011).

All tested antagonists showed abilities to control pathogens *in vitro*, and reduced 60 – 71% root rots of plants *in vivo*. The control mechanisms *in vivo* probably are still both mycoparsitism and antibiosis. The *Chaetomium* spp. might also parasitize the pathogen in the soil. Moreover, antifungal compounds including enzymes and antibiotics produced by the antagonists may accumulate in soil, resulting in a suppressive environment harmful to zoosporangia, zoospores, oospores, chlamydospores and mycelium of the pathogen" (Erwin and Ribiero. 1996). All crude extracts exhibited much more antifungal activities against *P. nicotianae* KA1than against *P. palmivora* PHY02 *in vitro*. Therefore, not surprisingly, in this study, crude extracts controlled *P. nicotianae* KA1 *in vivo* better than *P. palmivora* PHY02. In this study, in plants inoculated with *P. nicotianae* and treated with *Chaetomium* spp. and their crude extracts, the plant weight was less than the non-inoculated seedlings. That was similar with observation of Fang and Tsao (1995a) who found that *Pythium nunn* while significantly reduced the root rot rate caused by *P. parasitica* in citrus, also reduced plant weight.

Interestingly, in most cases during the *in vivo* experiments, spores of tested antagonists gave the same or even better positive effects in controlling *P. nicotianae* KA1and *P. palmivora* PHY02 than their crude extracts. Thus, using spores of these antagonists to control *Phytophthora* root rot in pomelo may provide a simple and

economical approach, and may be easier to use in large-scale applications than crude extracts. The effectiveness of the tested *Chaetomium* strains and their crude extracts in the control of *P. nicotianae* in this study provided a convincing reason to promote the applications of these strains to control *Phytophthora* root rot in citrus plants. Moreover, these biological control agents showed high potentials to alternative harmful synthesis fungicides.

CHAPTER 6

CONCLUSION

P. palmivora and *P. nicotianae* were the causal agents of pomelo root rot in Thailand. Six isolates of *Phytophthora* spp., which isolated from infected roots and soils of pomelo, again were highly virulent for roots of the plants in the pathogenicity tests. They were divided into 02 groups, slow-growth and fast-growth isolates, each group contained 03 isolates that had identically morphological characteristics. The analyses of ITS ribosomal DNA sequences and phylogeny of isolate PHY02, the representative of the slow-growth isolates, confirmed it was *P. palmivora*. Meanwhile those analyses of isolate KA1, the representative of the fast-growth isolates, proved it was *P. nicotianae*.

Both antibiosis and mycoparasitism mechanisms probably involved in antagonistic activities of *C. globosum* CG05, *C. lucknowense* CL01 and *C. cupreum* CC3003 against *P. palmivora* PHY02 and *P. nicotianae* KA1. All the antagonists grew over and parasitized hyphae, resulting in inhibition of mycelial growth, spore formation and colony degradations of *P. palmivora* and *P. nicotianae* KA1, during the bi-culture tests. These were typical phenomena of mycoparasitism mechanisms. On the other hand, all crude extracts of antagonists exhibited antifungal activities against the mycelial growth and spore formation of the pathogens, explaining for antibiosis mechanism.

The crude extracts of the tested *Chaetomium* spp. exhibited varyingly antifungal activities against *P. palmivora* and *P. nicotianae*, *in vitro*. In general, the crude hexane extracts of antagonists exhibited less antifungal activities against mycelial growth and spore formation than the crude ethyl acetate and methanol extracts. Crude extracts of *C. lucknowense* CL01 were more effective than those of *C. cupreum* CC3003, but were less effective than those of *C. globosum* CG05 in

controlling the mycelial growth and spore formation of *P. palmivora* PHY02. In addition, the antagonistic crude extracts suppressed the spore formation of *P. palmivora* PHY02 better than they did with its mycelial growth. Moreover, the antagonistic crude extracts inhibited mycelial growth of *P. nicotianae* far better than they did with that of *P. palmivora*.

The antifungal activities of crude extracts of *C. globosum* CG05, *C. lucknowense* CL01 and *C. cupreum* CC3003 did not reflect all the bio-control activities of these antagonists against *P. palmivora* PHY02 and *P. nicotianae* KA1. Unlike in bi-culture tests, in spite of causing great reductions of colony growth, the crude extracts did not cause degradations of mycelia of the pathogen, which often results from activities of lytic enzymes. Therefore, lytic enzymes might involve in bio-control activities of the tested *Chaetomium* spp. against the *Phytophthora* spp. in this study.

During the *in vivo* trials, spores and crude extracts of the tested *Chaetomium* spp. greatly reduced root rot rates and increased plant weights of pomelo seedlings inoculated with *P. palmivora* PHY02 or *P. nicotianae* KA1. Furthermore, the *Chaetomium* spp. and their crude extracts protected the seedlings from infections of *P. nicotianae* KA1 better than those from *P. palmivora* PHY02.

In most cases during the *in vivo* trials, the spores of tested antagonists gave the same or even better positive effects in controlling *P. nicotianae* KA1and *P. palmivora* PHY02 than their crude extracts. Thus, using spores of these antagonists to control *Phytophthora* pomelo root rots may provide a simple and economical approach, and may be easier to use in large-scale applications than crude extracts as fungicides.

C. lucknowense is reported as the new effective antagonist of *Phytophthora* spp. causing root rot of citrus. The *C. lucknowense* CL01 not only showed antagonism to *P. palmivora* PHY02 and *P. nicotianae* KA1 *in vitro*, the fungus also protected plants from infections of the pathogens under green house conditions.

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AUTHOR BIOGRAPHY

Name: Mr. PHUNG MANH HUNG

Date of Birth: 1st Oct 1981

Resident address: Phuho commune, Phutho town, Phutho province, Vietnam

Email address: manhhungnomafsi@gmail.com

EDUCATION:

University	Degree	Year
Vietnam National University of Agriculture (Hanoi, Vietnam)	B.Sc.	2004
Vietnam National University of Agriculture (Hanoi, Vietnam)	M.Sc.	2010
Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand	Ph.D.	2016

CONFERENCES:

Phung Manh Hung, W. Pongnak and Soytong, K. 2013. Isolation plant pathogens from pomelo and their pathogenicity test. In: Proceeding of the 2rd International Conference of Integrated Sustainable Agricultural Technology, Nov 28-29, KMITL, Bangkok, Thailand.

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PUBLICATIONS FOR Ph.D. PROGRAM:

Phung Manh Hung, W. Pongnak and Soytong, K. 2014. Biological control of Pomelo diseases using *Chaetomium* spp. **Journal of Agricultural Technology** 10(4):833-844. (*TC index journal*).

Phung Manh Hung, Pongnak Wattanachai, Soytong Kasem and Supattra Poeaim. 2015. Biological Control of *Phytophthora palmivora* Causing Root Rot of Pomelo Using *Chaetomium* spp. **Mycobiology**. 43(1): 63-70. (*ISI and Scopus index journal*).

Phung Manh Hung, Pongnak Wattanachai, Soytong Kasem and Supattra Poeaim. 2015. Efficacy of Chaetomium Species as Biological Control Agents against Phytophthora nicotianae Root Rot in Citrus. **Mycobiology**. 43(3): 288-296. (*ISI and Scopus index journal*).

APPENDIX

Mycobiology

Research Article

Biological Control of *Phytophthora palmivora* Causing Root Rot of Pomelo Using *Chaetomium* spp.

Phung Manh Hung[1,2,*], Pongnak Wattanachai[1], Soytong Kasem[1] and Supattra Poeaim[3]

[1]*Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand*
[2]*Northern Mountainous Agriculture and Forestry Science Institute (NOMAFSI), Phutho 293840, Vietnam*
[3]*Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand*

Abstract *Phytophthora* diseases have become a major impediment in the citrus production in Thailand. In this study, an isolate of *Phytophthora* denominated as PHY02 was proven to be causal pathogen of root rot of Pomelo (*Citrus maxima*) in Thailand. The isolate PHY02 was morphologically characterized and identified as *Phytophthora palmivora* based on molecular analysis of an internal transcribed spacer rDNA sequence. This work also presents *in vitro* evaluations of the capacities of *Chaetomium* spp. to control the *P. palmivora* PHY02. As antagonists, *Chaetomium globosum* CG05, *Chaetomium cupreum* CC3003, *Chaetomium lucknowense* CL01 inhibited 50~61% mycelial growth, degraded mycelia and reduced 92~99% sporangial production of *P. palmivora* PHY02 in bi-culture test after 30 days. Fungal metabolites from *Chaetomium* spp. were tested against PHY02. Results showed that, methanol extract of *C. globosum* CG05 expressed strongest inhibitory effects on mycelial growth and sporangium formation of *P. palmivora* PHY02 with effective dose ED50 values of 26.5 μg/mL and 2.3 μg/mL, respectively. It is interesting that *C. lucknowense* is reported for the first time as an effective antagonist against a species of *Phytophthora*.

Keywords Biological control, *Chaetomium*, *Phytophthora palmivora*, Pomelo

Citrus are important fruit crops, being widely and commercially grown in Southeast Asia. However, under prevailing wet climatic conditions, *Phytophthora* is a major impediment in the citrus production and has caused annual loss of about 6~12% of yield in Thailand alone [1].

Phytophthora palmivora (Butl.) is a ubiquitous and prominent plant pathogen with a wide host range, which infects various important crops in Southeast Asia [1, 2]. On citrus, this fungal-like organism has been reported to infect almost every part of the plant at any stage of its growth [3]. The spread and pathogencity were reported to be faster and more aggressive to roots of citrus than *P. parasitica* and *P. citrophthora* [4, 5]. Since *Phytophthora* and other oomycetous organisms have different biochemical pathways compared to the true fungi, they are insensitive to many fungicides [6]. In addition, the resistance of *Phytophthora* species to an important group of fungicides such as phenylamides (metalaxyl and related compounds) has become a serious problem in their chemical control [2]. In order to develop eco-friendly management of *Phytophthora* diseases and to reduce the costly applications and harm of fungicides, screening of bio-control agents against citrus *Phytophthora* has become a vital research aspect and is being carried out all over the world [3].

Chaetomium Kunze is a large genus of saprophytic ascomycetes with more than 350 species [7]. Relying on lytic enzymes, they decompose cellulose and other organic materials [8, 9]. Some *Chaetomium* species are reported to act as antagonists against various plant pathogens. Even commercial bio-product has also been developed from potent strains of *Chaetomium* spp. [10]. Furthermore, over 200 metabolites with a wide range of bioactivities have been found from the genus *Chaetomium* and many of which exhibited antifungal activity against plant pathogens [7]. However, there is a significant limitation of research on the abilities of *Chaetomium* species and their metabolites to control the *Phytophthora*.

Recently, we have isolated a single species of *Phytophthora* (denominated as PHY02) from roots of pomelo (*Citrus*

Mycobiology 2015 March, **43**(1): 63-70
http://dx.doi.org/10.5941/MYCO.2015.43.1.63
pISSN 1229-8093 • eISSN 2092-9323

***Corresponding author**
E-mail: manhhungnomafsi@gmail.com

Received November 6, 2014
Revised January 19, 2015
Accepted January 27, 2015

maxima) in orchards having serious root rot problems in Chang chen Sao province, Thailand. This study reports characteristics and identification of this isolate as well its *in vitro* bio-control using *Chaetomium* species as antagonists and their metabolites.

MATERIALS AND METHODS

Characteristics and identification. The *Phytophthora* (PHY02) was cultured on potato dextrose agar (PDA; potato infusion from 200 g/L, 20 g/L dextrose, and 15 g/L agar), cornmeal agar (CMA [Hardy Diagnostics Co., Santa Maria, CA, USA]: 2 g/L corn meal infusion from solids, 15 g/L agar) and V8 juice agar (V8A; 200 mL/L V8 juice [Cambell soup Co., Camden, NJ, USA], 3 g/L $CaCO_3$, 20 g/L agar, 800 mL/L water) for morphological study. Sporangia and other structures of PHY02 were observed under a light microscope (Olympus CH40; Olympus Optical Co. Ltd., Tokyo, Japan) then taken photos and measured by a camera (Moticam 2000; Motic Incorporation Ltd., Hong Kong, China) using enclosed software MoticPlus 2.0.

To identify the pathogen at molecular level, universal primers ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG) and ITS 4 (5′-TCCTCCGCTTATTGATATGC) were used to amplify an internal transcribed spacer (ITS) rDNA region of isolate PHY02, with previously described PCR conditions [11], then was directly sequenced with the same primers at First BASE Laboratories Sdn Bhd (Selangnor, Malaysia). The full-length ITS rDNA nucleotide sequence of PHY02 was used as a query to search the GenBank DNA database of Phytophthora Database (http://www. phytophthoradb. org/blast/) using BLAST tool. Reference ITS rDNA sequences of related taxa were also downloaded from the Phytophthora Database. Subsequently, the sequences of PHY02 and related taxa were aligned and performed phylogenetic analysis using MEGA ver. 5.2. Finally, phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replications [12].

Pathogenicity test. Pathogenicity was proved by artificial inoculation PHY02 into roots of pomelo seedlings (*Citrus maxima*) var. Khao nam Pueng (the same affected variety where PHY02 was obtained).

Root inoculation was done using the infested soil method of Zitko *et al.* [4]. Six-month-old pomelo seedlings were thoroughly washed to be free of potting mix and then planted in plastic tubes (10 × 15 cm) containing sterilized clay soil-sand (1 : 1) with 5 chlamydospores of PHY02 per cubic centimeter. Controls were prepared by planting the seedlings in same size tubes, but containing sterilized clay soil-sand (1 : 1) only. All pots including the controls were maintained in the green house at temperature of about 25~30°C and flooded with water for 24 hr each week. After 6 wk, the plants were carefully removed from plastic tubes, the soil was washed out and rating was done based on degree of the root rot symptoms. The pathogen then was re-isolated from newly infected roots symptom and morphology characteristics were compared with the isolate PHY02.

Fungal isolates. *C. globosum* strain CG05, *C. cupreum* strain CC3003, and *C. lucknowense* strain CL01 (from collection of Dr. Soytong K., KMITL, Thailand) were used as antagonists and produced antagonistic substances. The isolate PHY02 was represented as the target control in this study.

Bi-culture test. A mycelial disc of PHY02 (5 mm diameter) was placed singly (as controls) or oppositely to a mycelial disc of each above antagonist on 9-cm-diameter Petri dishes, which contained PDA. After incubation at 25°C for 30 days, data were collected as colony diameter and number of sporangia produced by PHY02 in both bi-culture and control plates. Numbers of sporangia were counted by using haemacytometer. Data were computed in a form of inhibition percentage of mycelial growth and sporangial production of the pathogen by using the formula below:

$$\% \text{ Inhibition} = 100 \times (A - B)/A$$

, where A = colony diameter or numbers of sporangia of PHY02 in control plates; B = colony diameter or numbers of sporangia of PHY02 in bi-culture plates.

Finally, variance and the treatment means were analyzed and compared by using Duncan's multiple range tests at 0.05.

***In vitro* test of crude extracts from *Chaetiomium* spp. to inhibit *P. palmivora* PHY02.** *C. globosum* CG05, *C. lucknowense* CL01, and *C. cupreum* CC3003 were separately cultured in potato dextrose broth (500 plates per antagonist) at room temperature in 45 days. Fungal biomass of each antagonist was separately collected as fresh biomass, then was dried out at room temperature. Subsequently, the extraction of dried biomass from each antagonist was performed by the method described by Kanokmedhakul *et al.* [13]. Each dried biomass was ground and extracted with hexane (1 : 1 v/v) and incubated by shaking for 72 hr at room temperature. The filtrate was separated out the marc by filtration through filter paper (Whatman No. 4). The hexane filtrate was performed through rotary vacuum evaporator to yield crude hexane extract. The marc from hexane extraction was further extracted with ethyl acetate and followed with methanol using the same procedure as hexane to yield crude ethyl acetate and methanol extract.

The three different crude extracts of each antagonist were conducted in a factorial experiment to test for inhibition of mycelial growth and sporangium formation of *P. palmivora* PHY02 by using poisonous food method. A mycelial disc (5 mm in diameter) of PHY02 placed on PDA plates (5 cm in diameter), which contained different concentrations of each crude extract (0, 10, 50, 100, 500, and 1,000 µg/mL). To obtain the desired crude extract concentrations, stock crude extract was weighted then dissolved in 2% dimethyl

sulfoxide and added to molten PDA before autoclaving at 121°C (15 psi) for 20 min. After incubating at 25°C for 10 days, colony diameter and numbers of sporangia production of the pathogen were collected and then expressed as inhibition percentage using the same formula above. Effective dose ED_{50} values on mycelial growth and sporangial production were calculated by probit analysis using the software SPSS Statistics ver. 19.0 (IBM Co., Armonk, NY, USA).

RESULTS

Pathogenicity of *Phytophthora* PHY02. After 6 wk of inoculation with chlamydospores of PHY02 on roots, the pomelo seedlings had an average of 47.6% root tips rotted and produced very few new roots (data not shown). Cortex of feeder roots turned soft and was sloughed to leave only steles, which were similar to symptoms of root rot in the affected orchards in Chang chen Sao, Thailand (Fig. 1). Meanwhile, non-inoculated seedlings produced abundantly new roots and no symptom of rot was observed. A single

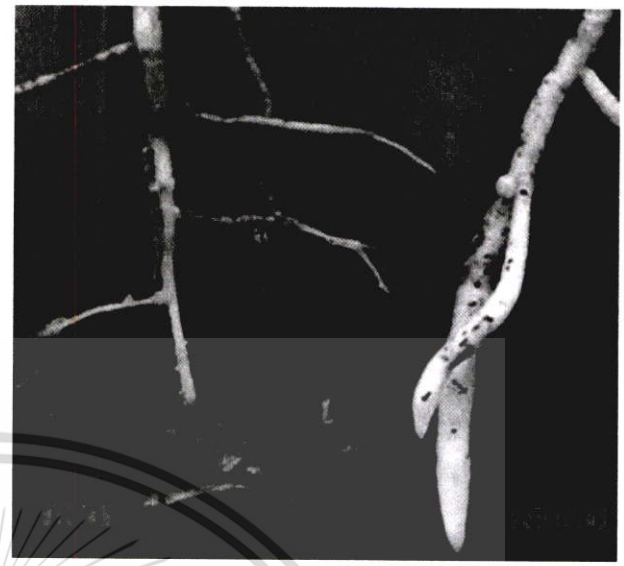

Fig. 1. Symptoms caused by *Phytophthora palmivora* PHY02 on root of pomelo in pathogenicty test (INO, inoculated; N-INO, non-inoculated).

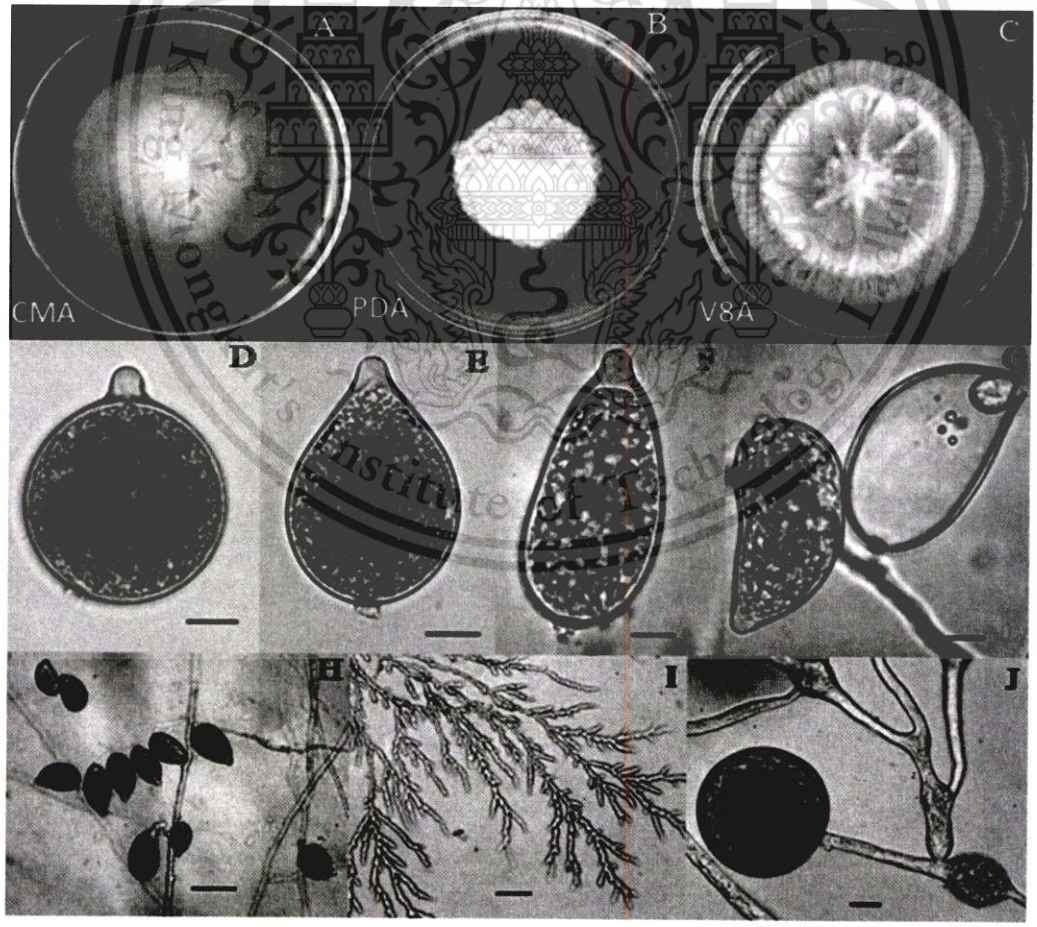

Fig. 2. Morphological characteristics of *Phytophthora palmivora* PHY02. A~C, Colony types of PHY02 at 7 days grown on different media; D~F, Different shapes of zoosporangia; G, Zoospore release directly from sporangium; H, Occurrence of sporangia on sympodium; I, Lumpy-branching mycelia; J, Chlamydospore and swelling hyphae (scale bars: D~G, J = 10 μm, H, I = 50 μm). CMA, cornmeal agar; PDA, potato dextrose agar; V8A, V8 juice agar.

species of *Phytophthora* was re-isolated from disease symptoms on newly infected roots, which all had identical morphology characteristics with the isolated PHY02. As a result, PHY02 was proven to be the causal pathogen of the pomelo root rot in Chang chen Sao, Thailand.

Characteristics and identification of *Phytophthora* PHY02. Colonies of PHY02 showed stellate pattern, with aerial mycelia on V8A and PDA whereas nearly no aerial mycelium on CMA (Fig. 2). The isolate PHY02 produced lumpy-branching hyphae with hyphal swellings. Zoospores were directly released from sporangia when flooded in distilled water. Sporangia produced abundantly when grown on PDA and V8A after 3~5 days, occurred in groups on sympodium or irregularly, were papillate and caducous with short pedicels up to 6 μm long (mean 3.3). Sporangial shape varied from ellipsoid, ovoid, pyriform, obpyriform to near spherical, and mean of sizes were 53.8 × 33.3 μm with a length to breadth ratio of 1.2~2.2 (mean 1.6) (Table 1). Most of chlamydospores were globose in shape, produced abundantly from mycelia when incubated in dark. No sexual organ was observed in cultures of this isolate since it was a heterothallic species.

The sequence with 951 bases of the ITS rDNA PCR product of PHY02 was determined and used as a query to search the Genbank DNA database of the Phytophthora Database using the BLAST search. It was found that there was 99.75% (809/811), 99.87% (782/783) to identity with *Phytophthora palmivora* accession number PD_00627 and PD_02505, respectively. The phylogenetic tree, which illustrated relationships between PHY02 and related taxa was constructed as shown in Fig. 3. The isolate PHY02 was identified as *Phytophthora palmivora* (Butl.), based on its morphology and the molecular analysis.

Table 1. Characteristics of *Phytophthora palmivora* PHY02 from *citrus maxima*

Structure	Mean	Max	Min
Sporangia[a]			
Length (μm)	53.8	76.7	34.2
Breadth (μm)	33.3	45.6	23.6
L/B (μm)	1.6	2.2	1.2
Pedicel length (μm)	3.2	5.6	0.9
Papilla length (μm)	6.4	9.8	3.1
Chlamydospore[b] diameter (μm)	36.9	54.7	25.3

[a]Data collected from 200 separate sporangia.
[b]Data collected from 150 separate chlamydospores.

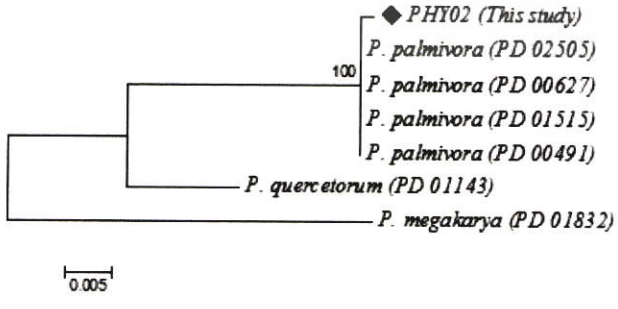

Fig. 3. Phylogenetic relationship between *Phytophthora palmivora* PHY02 and related taxa inferred using a neighbor-joining method with internal transcribed spacer (ITS) rDNA sequences. Bootstrap value based on 1,000 replications is shown above the branch.

Table 2. Inhibition of mycelial growth and sporagial production of *Phytophthora palmivora* PHY02 in bi-culture test with *Chaetomium* spp.

Treatments	Colony inhibition (%)	Sporangium inhibition (%)
CG05 vs. PHY02	61.0 ± 1.0 a	99.0 ± 0.4 a
CL01 vs. PHY02	59.2 ± 1.2 a	97.5 ± 0.2 a
CC3003 vs. PHY02	49.7 ± 1.2 b	92.2 ± 0.9 b

Average of four replications, the same letter represents no significant difference between treatments, based on the Duncan's multiple range test at $p = 0.05$.

Bi-culture test. As shown in Table 2, the tested antagonists led to 50~61% growth inhibition and reduced 92~99% sporangial production of *P. palmivora* PHY02 in bi-culture plates at 30 days, when compared to the controls. Moreover, CG05 and CL01 were seen to grow rapidly over PHY02 colony after 10~15 days. In all bi-culture plates, both antagonists degraded mycelia of the pathogen, resulting in change of color from white to light yellow-brown and a part or entire colony death (Fig. 4). Differently, *C. cupreum* CC3003 was a slow growing fungus, as it grew over gradually and resulting in degradation of colony of PHY02 in some cases. Mycelia of all the tested antagonists were observed to penetrate mycelia of PHY02 in some cases. In both inhibitions of mycelial growth and sporangial production, CC3003 was significantly less efficient than CG05 and CL01.

***In vitro* tests of crude extracts from *Chaetomium* spp. to inhibit *P. palmivora* PHY02.** Total nine crude extracts from CG05, CL01, and CC3003 were tested at different concentrations to evaluate their capacities to inhibit mycelial growth and sporangium formation of *P. palmivora* (PHY02). The effective doses (ED_{50}) of each crude extract on mycelial growth and sporangial production were also examined to determine their fungicidal spectrum.

As shown in Table 3, the three crude extract of CG05 exhibited more antifungal activities against mycelial growth of PHY02 as compared to crude extracts of CL01 and CC3003. Particularly, the tested pathogen did not grow at all in the presence of higher 500 μg/mL methanol extract and 1,000 μg/mL ethyl acetate extract of CG05. At tested concentrations from 10~500 μg/mL, the methanol extract was more effective than the others (Figs. 4 and 5). Conversely, all crude extracts of CC3003 were less effective on mycelial growth of PHY02 with higher ED_{50} values (596.8~2,495 μg/

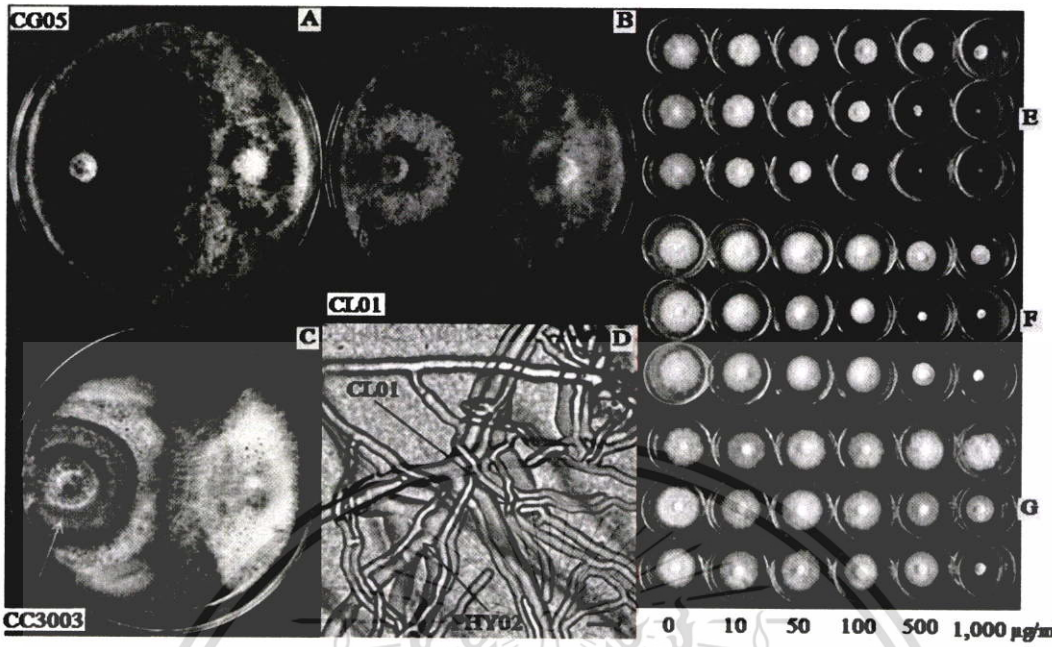

Fig. 4. Growth of *Phytophthora palmivora* PHY02 in bi-culture tests (at 30 days) and in crude extracts (at 10 days) of *Chaetomium* spp. A~C, PHY02 and *Chaetomium* spp. in bi-culture plates; D, Interaction between mycelia of CL01 and PHY02 in bi-culture test (scale bar = 10 μm); E~G, Growth of PHY02 at different concentrations of crude extracts of CG05, CL01, and CC3003, respectively (upper row: hexane extracts; middle row: ethyl acetate extracts; lower row: methanol extracts).

Table 3. Effective dose values (ED_{50}) of different crude extracts of *Chaetomium* spp. on mycelial growth and sporangial production of *Phytophthora palmivora* PHY02

Crude extracts		ED_{50} (μg/mL)	
		On mycelial growth	On sporangial production
CG05	Hexane	85.6	5.1
	EtOAc	33.4	3.0
	MeOH	26.5	2.3
CL01	Hexane	919.0	16.7
	EtOAc	77.0	3,5
	MeOH	143.9	4.0
CC3003	Hexane	> 2,495	307.9
	EtOAc	> 1,730	145.7
	MeOH	596.8	93.6

Hexane, hexane extract; EtOAc, ethyl acetate extract; MeOH, methanol extract.

mL). The methanol extract of CC3003 was more effective than the hexane and ethyl acetate at concentrations from 100~1,000 μg/mL. However, none of them showed significant inhibition of mycelial growth of PHY02 at 10 μg/mL. Whereas, mycelial growth of PHY02 was significantly inhibited by all crude extracts of CL01 at 10 μg/mL. Of which, the ethyl acetate extract was more significantly effective than the others at concentrations from 100~1,000 μg/mL, with ED_{50} value of 77.03 μg/mL and exhibited 83.5% growth inhibition of PHY02 at 1,000 μg/mL. Meanwhile, ED_{50} values of the methanol, hexane extract were 143.9 and 919.1 μg/mL, respectively. Unlike in bi-culture test, mycelia of PHY02 were not degraded by crude extracts of the antagonistic fungi.

All the tested crude extracts exhibited stronger inhibitory effects on sporangium formation than on mycelial growth of PHY02 with much lower ED_{50} values. The sporangium formation of PHY02 was most sensitive to crude extracts of CG05 with ED_{50} values of as 5.1, 3.0, and 2.3 μg/mL for the hexane, ethyl acetate and methanol extract, respectively.

Meanwhile, all three crude extracts of CC3003 were least effective on sporangial production of PHY02 with higher ED_{50} values. Of which, the methanol extract was more significantly effective than the others with ED_{50} value of 93.6 μg/mL, and gave an inhibitory rate of 96.5% at 1,000 μg/mL. The hexane and ethyl acetate extract gave the highest ED_{50} values among nine tested crudes, which were 307.9 and 145 μg/mL, respectively.

To reduce 50% sporangial production of PHY02, the hexane, ethyl acetate and methanol extract of CL01 required concentrations of 16.7, 3.5, and 4.0 μg/mL, respectively. However, none of them could reduce 90% sporangial production of PHY02 at 50 μg/mL. At concentrations from 10~500 μg/mL, the ethyl acetate extract of CL01 was more significantly effective than the others, showing an inhibitory rate of 94.0% at 100 μg/mL. While at concentration of 500 μg/mL, the hexane and methanol extract reduced 82.3% and 93.8% sporangial production of PHY02, respectively.

All the hexane extracts of tested antagonists were less effective than the ethyl acetate and methanol in both inhibitions of mycelia growth and sporangium formation

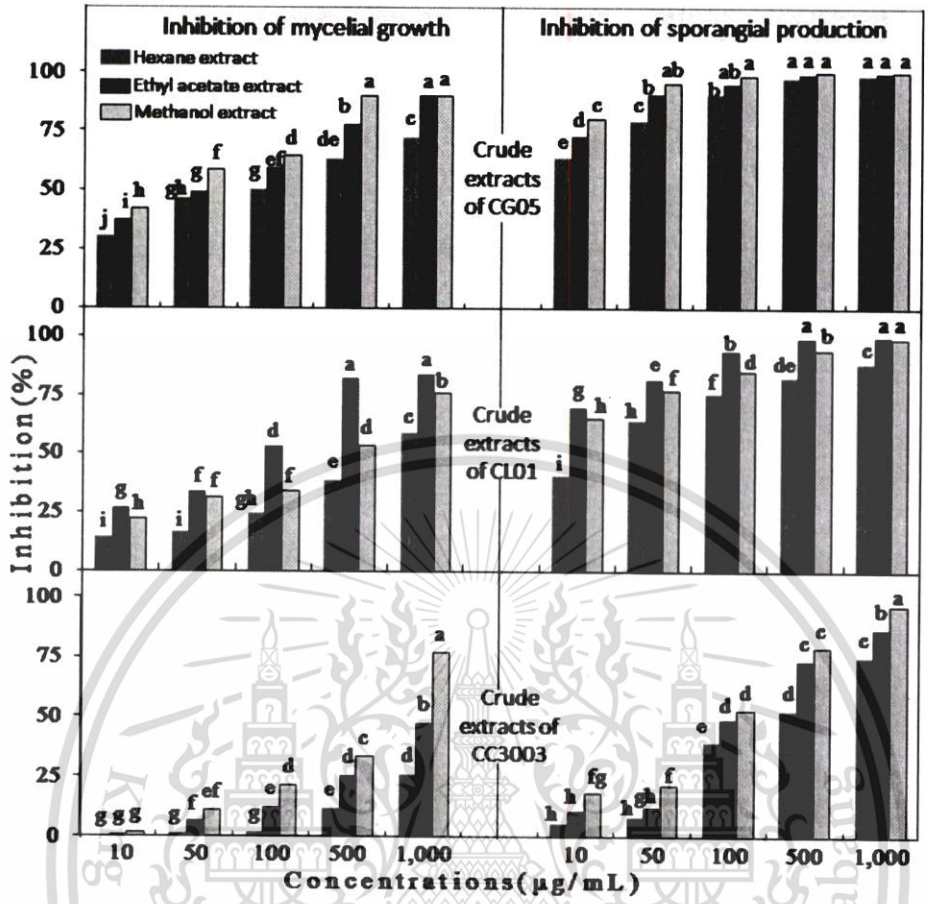

Fig. 5. Inhibitory effects of crude extracts of *Chaetomium* spp. on mycelial growth (the charts on the left site) and sporangial production (the charts on the right site) of *Phytophthora palmivora* PHY02 at different concentrations. The same letter above columns in each chart represents no significant difference between treatments, based on the Duncan's multiple range test at $p = 0.01$.

of *P. palmivora* PHY02.

DISCUSSION

We have designated the species isolated from root rot symptoms of pomelo (*Citrus maxima*) in Thailand as *Phytophthora palmivora* (Butl.). The highly pathogenic of *P. palmivora* PHY02 to roots of pomelo here is supported by previous studies which demonstrated that this species is more aggressive and damages even larger root than *P. parasitica* on citrus [4, 5]. Serious root rot disease of citrus caused by *P. palmivora* has been recorded in India, America [3, 14].

The antagonistic activity of bio-control microorganisms is often demonstrated by the inhibition of growth, infection or reproduction of pathogen [15]. The tested strains of *Chaetomium* spp. as antagonists here not only inhibited colony growth and degraded mycelia but also reduced 92~99% sporangial production of *P. palmivora* PHY02. The degradation of mycelia of tested pathogen probably resulted from the lytic enzymes which are commonly secreted by *Chaetomium* species [8, 9]. On other hand, every tested antagonist in this study was demonstrated to produce antibiotics exhibiting antifungal activities against different plant pathogens [10, 13, 16]. It refers that the inhibitions of *P. palmivora* here were resulted from both antibiotics and lytic enzymes secreted by the antagonists. *C. globosum* and *C. cupreum* have ever been reported to control *Phytophthora* spp. in dual culture test [17, 18]. *C. lucknowense* was concluded to control *Fusarium oxysporum* causing tomato wilt [16]. However, to best our knowledge, this is the first report of *C. lucknowense* as an effective antagonist against a species of *Phytophthora*.

Chaetomium species are known as producers of many different bioactive metabolites which play important roles in their biological control activities [7, 9]. One *Chaetomium* species may produce many metabolites with different bioactivities and molecular weight, this lead to differences in antifungal activity of its crude extracts [16, 19]. That explains why there were variations in responses of *P. palmivora* PHY02 to different crude extracts in this study. The less efficiency of hexane extracts of *Chaetomium* spp. in comparison with the ethyl acetate and methanol against fungal pathogens were also confirmed by previous authors

[16, 20].

C. globosum CG05 has been known to produce many bioactive compounds such chaetoglobosin (A, G, C, V, etc.) and chaetoviridins (A, B) [10]. Of which, the chaetoglobosin C is usually referred as antifungal principle of this strain and was reported to inhibit colony growth, sporangium formation of *P. parasitisca* and other fungal pathogens [10, 19]. However, the chaetoviridins A produced from *C. globosum* F0142 also has strong antifungal activities against *P. infestans*, *P. capsici* [21]. Thus, it suggests that chaetoglobosin C and also other bioactive compounds involved in crude extracts of *C. globosum* CG05 caused the high inhibitions of *P. palmivora* PHY02 in this study. *C. cupreum* CC3003 has been known to produce compound rubrorotiorin which inhibited growth of *Candida albicans* at low concentration (EC_{50} = 0.6 µg/mL) [13]. However, crude extracts of *C. cupreum* CC3003 here showed least inhibitory effects on *P. palmivora* with high ED_{50} values. Similarly, crude extracts of *C. cupreum* have been known to be less effective against *Pythium aphanidermatum* [20]. Both the crude extracts and chaetoglobosin C produced by *C. lucknowense* CL01 have been reported to inhibit colony growth and spore formation of *Fusarium oxysporum* causing tomato wilt [16]. However, this is the first time the inhibitory effects of metabolites from *C. lucknowense* on a species of *Phytophthora* are reported.

Sporangium formation is the most sensitive stage in life cycle of *Phytophthora* species [2]. This study demonstrated that sporangium formation of *P. palmivora* PHY02 was much more sensitive than mycelial growth to all tested crude extracts. The high inhibitory effects of crude extracts from *Chaetomium* spp. on spore formation of *Fusarium oxysporum* and *P. parasitisca* have been noted by previous authors [16, 19].

It is clear that only metabolites which were not decomposed through the extraction and autoclaving of the crude extracts resulted in the inhibitions of *P. palmivora* PHY02. All enzymes and many antibiotics will be decomposed because of heat and pressure when autoclaving [2]. That why mycelia of the pathogen were not degraded when growing in crude extracts. Therefore, the antifungal activities of crude extracts here probably did not account for all bio-control activities of the tested antagonists against *P. palmivora* PHY02. Beside the known metabolites, degrading enzymes should be considered as effective factors involved in bio-control activities of the tested antagonists against the pathogen. The roles and secretion of lytic enzymes of *Chaetomium* species under antagonism conditions with *Phytophthora* need to be investigated in the future. The effective crude extracts may possible develop to be microbial elicitors to induce immunity in citrus plants against *P. palmivora*.

ACKNOWLEDGEMENTS

This work was acknowledged under grant supports of the King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand.

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Mycobiology

Research Article

Efficacy of *Chaetomium* Species as Biological Control Agents against *Phytophthora nicotianae* Root Rot in Citrus

Phung Manh Hung[1,2,*], Pongnak Wattanachai[1], Soytong Kasem[1] and Supattra Poeaim[3]

[1]*Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand*
[2]*Northern Mountainous Agriculture and Forestry Science Institute (NOMAFSI), Phutho 293840, Vietnam*
[3]*Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand*

Abstract Thailand is one of the largest citrus producers in Southeast Asia. Pathogenic infection by *Phytophthora*, however, has become one of major impediments to production. This study identified a pathogenic oomycete isolated from rotted roots of pomelo (*Citrus maxima*) in Thailand as *Phytophthora nicotianae* by the internal transcribed spacer ribosomal DNA sequence analysis. Then, we examined the *in vitro* and *in vivo* effects of *Chaetomium globosum*, *Chaetomium lucknowense*, *Chaetomium cupreum* and their crude extracts as biological control agents in controlling this *P. nicotianae* strain. Represent as antagonists in bi-culture test, the tested *Chaetomium* species inhibited mycelial growth by 50~56% and parasitized the hyphae, resulting in degradation of *P. nicotianae* mycelia after 30 days. The crude extracts of these *Chaetomium* species exhibited antifungal activities against mycelial growth of *P. nicotianae*, with effective doses of 2.6~101.4 μg/mL. Under greenhouse conditions, application of spores and methanol extracts of these *Chaetomium* species to pomelo seedlings inoculated with *P. nicotianae* reduced root rot by 66~71% and increased plant weight by 72~85% compared to that in the control. The method of application of antagonistic spores to control the disease was simple and economical, and it may thus be applicable for large-scale, highly effective biological control of this pathogen.

Keywords Biological control, *Chaetomium*, Citrus root rot, *Phytophthora nicotianae*

Phytophthora nicotianae Breda de Haan (syn. *P. parasitica* Dastur) is known as a typical root pathogen that can infect hundreds of plant genera. This fungus-like organism infects and causes root rot in many different citrus types worldwide [1, 2]. According to the Food and Agricultural Organization of the United Nations Statistics Division (FAOSTAT), in 2013, approximately 0.1 million ha were dedicated to citrus cultivation in Thailand, resulting in the production of 1.2 million tons of fruit, which was slightly less than that in Indonesia in Southeast Asia [3]. With the prevalence of wet climatic conditions in Thailand, however, infection with *Phytophthora* has become a major problem for the citrus industry, causing yield losses of approximately 6~12% and economic losses of at least 37 million USD/yr [4].

Current practices for controlling *Phytophthora* diseases are largely based on cultivation management in fields and application of synthetic fungicides [1, 5]. Intensive use of chemical pesticides for the control of plant pathogens may lead to be harmful accumulation of toxins in the environment and adverse effects on human health. Moreover, the resistances of *Phytophhtora* species to fungicides are the topical questions in controlling the pathogens [1, 2]. In particular, many fungicides targeting chitin and sterol synthesis are ineffective against *Phytophthora* species because of the differences in their cell wall composition compared to that of true fungi [1, 5].

To reduce the dependence of agriculture production on noxious synthetic pesticides, the search for effective biological control agents (BCAs) against plant pathogens has been carried out worldwide. *Chaetomium* species are ubiquitous fungi of which more than 350 species exist. Some of these fungi can act as antagonists against various plant pathogens through the production of lytic enzymes and metabolites [6-8]. In our efforts to control *Phytophthora* root rot in *Citrus* plants, some potent strains of *Chaetomium* species

Mycobiology 2015 September, 43(3): 288-296
http://dx.doi.org/10.5941/MYCO.2015.43.3.288
pISSN 1229-8093 • eISSN 2092-9323

*Corresponding author
E-mail: manhhungnomafsi@gmail.com

Received June 7, 2015
Revised June 10, 2015
Accepted July 21, 2015

have been used as BCAs, and we have recently succeed in controlling *P. palmivora in vitro* using *Chaetomium* [9]. However, the applications of these BCAs for the control of *Phytophthora* species in citrus production are limited because our inadequate understanding of the mechanisms through which *Chaetomium* species control *Phytophthora* species.

In our recent investigation, we isolated a *Phytophthora* sp. (denominated as KA1) from rotted roots of a pomelo orchard near Bangkok, Thailand affected by root rot. Therefore, in this study, we characterized this isolate and examined the capacity of *Chaetomium* species and their crude extracts for biological control of this strain.

MATERIALS AND METHODS

Characterization and identification of *Phytophthora* sp. KA1. For morphological studies, the following media were used to culture *Phytophthora* sp. KA1: V8 juice agar (200 mL/L V8 juice [Campbell Soup Co., Camden, NJ, USA], 3 g/L $CaCO_3$, 20 g/L agar, and 800 mL/L water) and potato dextrose agar (PDA; HiMedia Laboratories Pvt. Ltd., Mumbai, India). A camera with associated software attached to an Olympus light microscope (CH40; Olympus Optical Co. Ltd., Tokyo, Japan) was used for observation and measurement of sporangia and other structures of *Phytophthora* sp. KA1.

Sporangia of the studied isolate were produced by floating some mycelial discs (obtained from margins of a 3-day-old culture on V8 agar) in 10 mL of double distilled water. The discs were then incubated under fluorescent light, at temperature of 25~28°C for 3~4 days. To determine the caducity of sporangia, the floating mycelial discs (bearing sporangia) were raised in a drop of distilled water several times, and the length of pedicels was measured under a light microscope. Sporangia caducity was determined based on the uniformity of the pedicel length [1]. *Phytophthora* sp. KA1 was identified into species level based on sequence analysis of the internal transcribed spacer (ITS) ribosomal DNA. Primers ITS6 (5′-GAA GGT GAA GTC GTA ACA AGG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used to amplify ITS1, ITS2 and the 5.8S ribosomal DNA fragments by polymerase chain reaction under previously described conditions [10]. The sequencing of the cloned fragments then was performed at First Base Laboratory (Selangnor, Malaysia), using the same primers. The full-length determined ITS nucleotide sequences of *Phytophthora* sp. KA1 then were used as queries for BLAST searches in GenBank (http://www.ncbi/blast/). Subsequently, the sequences of *Phytophthora* sp. KA1 and related taxa (obtained from GenBank database) were aligned and analyzed to construct a phylogenetic tree using software MEGA ver. 5.2 [11].

Fungal isolates and *in vitro* antagonism tests. *Phytophthora* sp. KA1 was the target for biological control in this study. Meanwhile, *C. globosum* (CG05), *C. lucknowense* (CL01), and *C. cupreum* (CC3003), which have all been proven to control *P. palmivora*-induced root rot in *Citrus* sp. [9], were used as antagonists and to produce antagonistic crude extracts.

Each of the *Chaetomium* species was tested for antagonism against *Phytophthora* sp. KA1 using bi-culture techniques [9]. A 5-mm-diameter mycelial disc from the active growing area of a 5-day-old colony of *Phytophthora* sp. KA1 was placed alone (as a control) or opposite a mycelial disc from one of the above antagonists on PDA plates (9 cm diameter). All plates were then maintained at temperature of 28°C in the dark, and the colony diameters of the pathogen were measured after 10 and 30 days. The inhibition of mycelial growth of the pathogen was then calculated as a percentage using the formula below:

$$\text{Inhibition (\%)} = 100 \times (1 - B/A) \quad (1)$$

, where A and B are the colony diameters of *Phytophthora* sp. KA1 in the control and bi-culture plates, respectively. The experiment was repeated twice, and performed in a completely randomized design with 4 replicates.

***In vitro* effects of antagonistic crude extracts on the growth of *Phytophthora* sp. KA1.** Crude extracts of the tested antagonists were produced by method of Kanokmedhakul *et al.* [12]. Each fungal antagonist was cultivated in potato dextrose broth (200 g/L potato infusion and 20 g/L dextrose) at 25~28°C for 45 days. Fungal biomass then was collected, air-dried, ground, and extracted sequentially with hexane, ethyl acetate (EtOAc), and methanol (MeOH) to produce crude hexane, crude EtOAc, and crude MeOH extracts, respectively. The crude extracts were obtained by evaporating the extracted-solvents in a vacuum.

These 3 crude extracts from each antagonist were tested at different concentrations (10, 50, 100, 500, and 1,000 µg/mL; untreated samples [0 µg/mL] were used as controls) for their potential to inhibit the growth of *Phytophthora* sp. KA1. A 5-mm-diameter mycelial disc of *Phytophthora* sp. KA1 was placed on the center of PDA plates (5 cm in diameter) containing the crude extracts. To obtain the desired concentrations, stock crude extract was weighed, dissolved in 2% dimethyl sulfoxide, mixed into molten PDA, and then autoclaved for 20 min at 121°C (15 psi). All plates were then incubated at temperature of 28°C in the dark, and colony diameters were measured when the pathogen colonies developed fully on the control plates (about 5 days). Finally, the inhibition of mycelial growth of the tested pathogen was calculated as a percentage using the same formula Eq. 1 above. In which, A and B are the colony diameters of *Phytophthora* sp. KA1 (after subtracting the diameter of the inoculum disc) in the control and crude extract plates, respectively. Effective dose (ED_{50}) values for inhibition of mycelial growth were also computed by probit analyses using SPSS Statistics ver. 19.0 software (IBM Co., Armonk, NY, USA). All experiments were repeated twice,

and performed in completely randomized designs with 4 replicates.

Preparation of inocula. For morphological studies and inoculum production, chlamydospores of *Phytophthora* sp. KA1 were produced as described by Tsao [13]. Mycelia of *Phytophthora* sp. KA1 were first grown at 28°C for 1 wk in 250-mL bottles containing 25 mL of cleared V8 broth. Subsequently, 100 mL of sterile-distilled water was added to each bottle, and then the bottles were maintained at temperature of 18°C in the dark for 3 wk. For use as the inoculum, chlamydospores were separated from the mycelium by repeated blending and low-speed centrifugation.

The pathogen inoculum was prepared by mixing chlamydospores of *Phytophthora* sp. KA1 into sterilized fine sand before dark-moist incubation at 28°C. After 3 days, the propagule density of the inoculum was then determined as described by Timmer *et al.* [14] using selective agar medium pimaricin-ampicillin-rifampicin-pentachloronitrobenzene-hymexazol (PARPH). Infested soil was then prepared by mixing the inoculum with potting media (sterilized clay soil, sand, and compost at a v/v/v ratio of 3 : 1 : 1) to give concentrations of 5 or 2.5 propagules of *Phytophthora* sp. KA1 per cubic centimeter of soil; these samples were used in the pathogenicity and greenhouse tests, respectively.

To provide BCAs for the greenhouse test, each antagonist was separately cultured on PDA plates for 30 days, and spores were then collected by scraping of the agar surface of the plates and ground before addition to 300 mL of potato dextrose broth in a 500-mL bottle for semi-germination. The bottle was incubated at 30°C for 8 hr, and then propagule density was determined under a microscope using a haemocytometer. The crude MeOH extract of each antagonist was weighed and then dissolved in 2% dimethyl sulfoxide before being added to sterilized distilled water to give a 50 μg/mL solution.

Pathogenicity and greenhouse tests. For the first experiment, pathogenicity was examined by artificial inoculation of *Phytophthora* sp. KA1 into roots of pomelo seedlings. Seedlings (3-mon-old) were washed the roots under running water to remove potting mix, then were transferred into either the infested soil or the sterilized potting media only (as controls) in plastic tubes (10 × 15 cm). Each treatment was performed with 5 seedlings and repeated once.

For the second experiment, spores and crude MeOH extracts of each antagonist were used as BCAs to control *Phytophthora* sp. KA1 under greenhouse conditions. For crude treatments, 5-wk-old pomelo seedlings were planted in plastic tubes containing infested soil. The prepared solutions of crude MeOH were used to treat the seedlings via bi-weekly watering of 100 mL per tube. For each spore treatment, the infested soil was mixed with the prepared spores of a single antagonist at a concentration of 100 propagules per cubic centimeter of soil, and these soils were then used for planting the seedlings. In addition, seedlings were also planted in infested soil as controls and in sterilized potting media as references. Whenever the crude treatments were applied, the plants in the other groups were also watered with the same volume of fresh water. Each treatment was performed with 4 plants (with 1 plant considered as 1 replication). The experiment was performed in a completely randomized block design, under greenhouse conditions (temperature of 28~33°C), and repeated twice.

All plants in the experiments were weekly flooded with fresh water for 24 hr, and the root rots were rated after 6 wk. The potting mix was washed away, then up to 100 root tips on each seedling were visually rated as healthy or rotted, and the root data were expressed as the percent root rot. Plants from the second experiment were then dried at 65°C for 12 hr before being weighed. The selective agar medium PARPH was used throughout the experiments to isolate the pathogen from the infected roots.

Data analysis. All data were subjected to analysis of variance (ANOVA) using SPSS Statistics ver. 19.0 software (IBM Co.). Because there was no significant differences between the experiments based on preliminary analysis of variance, data from the repeated experiments were combined. Means were compared using least significant differences with analysis of Duncan's multiple range test (DMRT).

RESULTS

Pathogenicity of *Phytophthora* sp. KA1. At the inoculum level of 5 chlamydospores of *Phytophthora* sp. KA1 per cubic centimeter of soil, an average of 45.3% of root tips were rotted, with very few new roots in pomelo seedlings. In contrast, the non-inoculated seedlings had no root rot and produced an abundance of new roots. Importantly, the tested organism was re-isolated from newly infected roots of the inoculated plants. This result indicates that *Phytophthora* sp. KA1 was the causal agent of the pomelo root rot observed in Thailand.

Morphological characteristics and identification of *Phytophthora* sp. KA1. Colonies of *Phytophthora* sp. KA1 grown on PDA showed aerial, arachnoid, and branched mycelia with hyphal swelling. Sporangia were produced abundantly when the pathogen was flooded with the distilled water, but no sporangia formed on the tested agar media. The isolate produced papillate, caducous sporangia with very short pedicels (mean length: 3.1 μm) (Fig. 1). The sporangial shape was predominantly subspherical and turbinate, with an average length-to-breadth ratio of 1.3 : 1 (Table 1). Chlamydospores formed abundantly in a globose shape when the method of Tsao [13] was applied; however, chlamydospores did not form on the agar media. No sexual organs were observed in single cultures of this isolate.

Nucleotide sequences of the ITS ribosomal DNA fragments of *Phytophthora* sp. KA1 were determined and deposited in

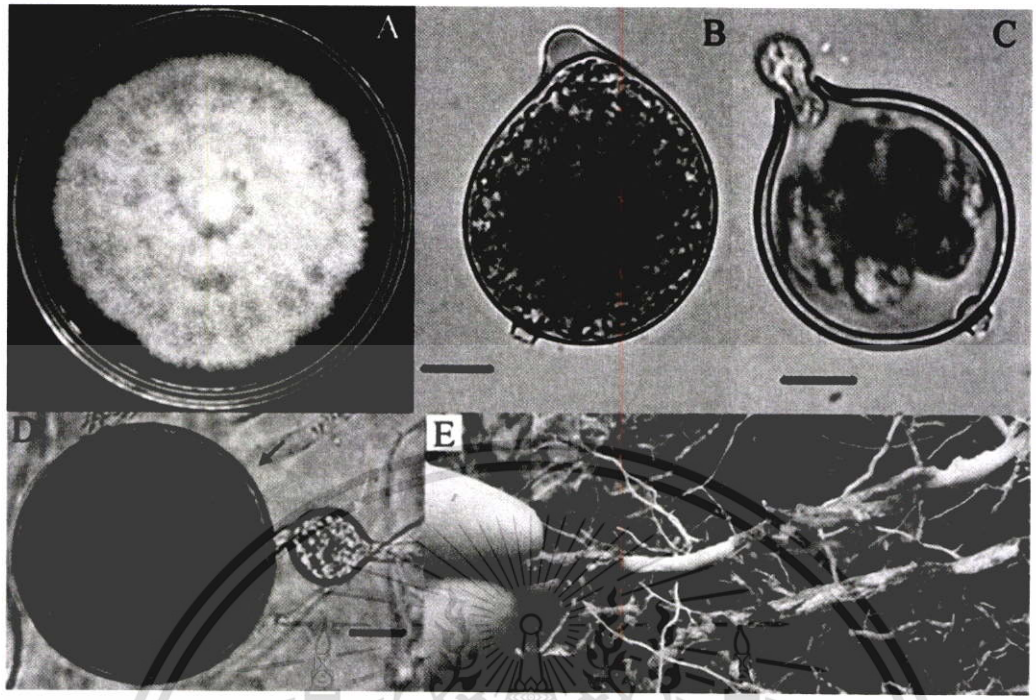

Fig. 1. Morphological characteristics of *Phytophthora nicotianae* KA1. A, Seven-day-old culture on potato dextrose agar; B, C, Caducous sporangium with a short pedicel; D, Chlamydospore (black arrow) and hypha swelling (yellow arrow) (scale bars: B~D = 10 μm); E, Root rot symptoms in a pomelo plant near Bangkok, Thailand.

Table 1. Characteristics of *Phytophthora nicotianae* KA1 from pomelo

Structure	Mean of size
Sporangia[a]	
Length (μm)	49 ± 8.6[b]
Breadth (μm)	37 ± 4.7
Length/Breadth ratio	1.3 ± 0.2
Papilla length (μm)	4.8 ± 1.0
Pedicel[c] length (μm)	3.1 ± 1.0
Chlamydospore[d] diameter (μm)	37 ± 4.6

[a]Data collected from 150 separate sporangia.
[b]Mean ± standard deviation.
[c]Data collected from 85 separate detached sporangia.
[d]Data collected from 85 separate chlamydospores.

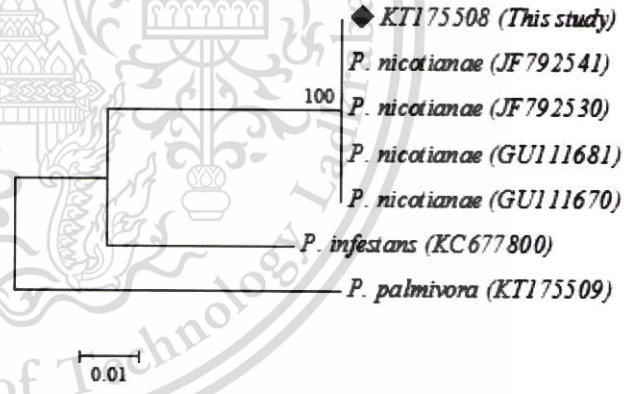

Fig. 2. Phylogenetic tree showing relationship between *Phytophthora nicotianae* KA1 and related taxa base on the internal transcribed spacer ribosomal DNA sequences, using the neighbor-joining method with 5,000 bootstrap replicates. *P. infestans* (KC677800) (the species placed in the same clade 1 with *P. nicotianae*) was isolated from a potato in India, and *P. palmivora* (KT175509) was isolated from a pomelo in Thailand [9].

the GenBank under accession No. KT175508. The DNA sequences of *Phytophthora* sp. KA1 were used as queries to search GenBank using the BLAST function. This analysis demonstrated that the nucleotide sequences of *Phytophthora* sp. KA1 shared 100% identity with those of *P. nicotianae* (accession Nos. GU111681 and GU111670 from *Citrus* spp. in Taiwan; JF792541 and JF792530 from citrus soils in India; and many other isolates existing in the GenBank database). Phylogenetic analysis confirmed the relationships between *Phytophthora* sp. KA1 and the related taxa (Fig. 2).

Antagonism of *Chaetomium* species to *P. nicotianae* KA1. The 3 potent strains CG05, CL01, and CC3003 of *Chaetomium* spp. were tested *in vitro* for antagonism to *P. nicotianae* KA1 using bi-culture techniques. Ten days after inoculation with these fungi in bi-culture plates, *P. nicotianae* KA1 growth was inhibited to 50.6%, 52.5%, and 59.7% of that of the control, respectively, with further reductions to 49.7%; 51.7%, and 56.4% of that of the control after 30 days. The growth of *P. nicotianae* KA1 was prevented by substances that were released into the agar medium by CC3003 colonies, resulting in a clear zone of

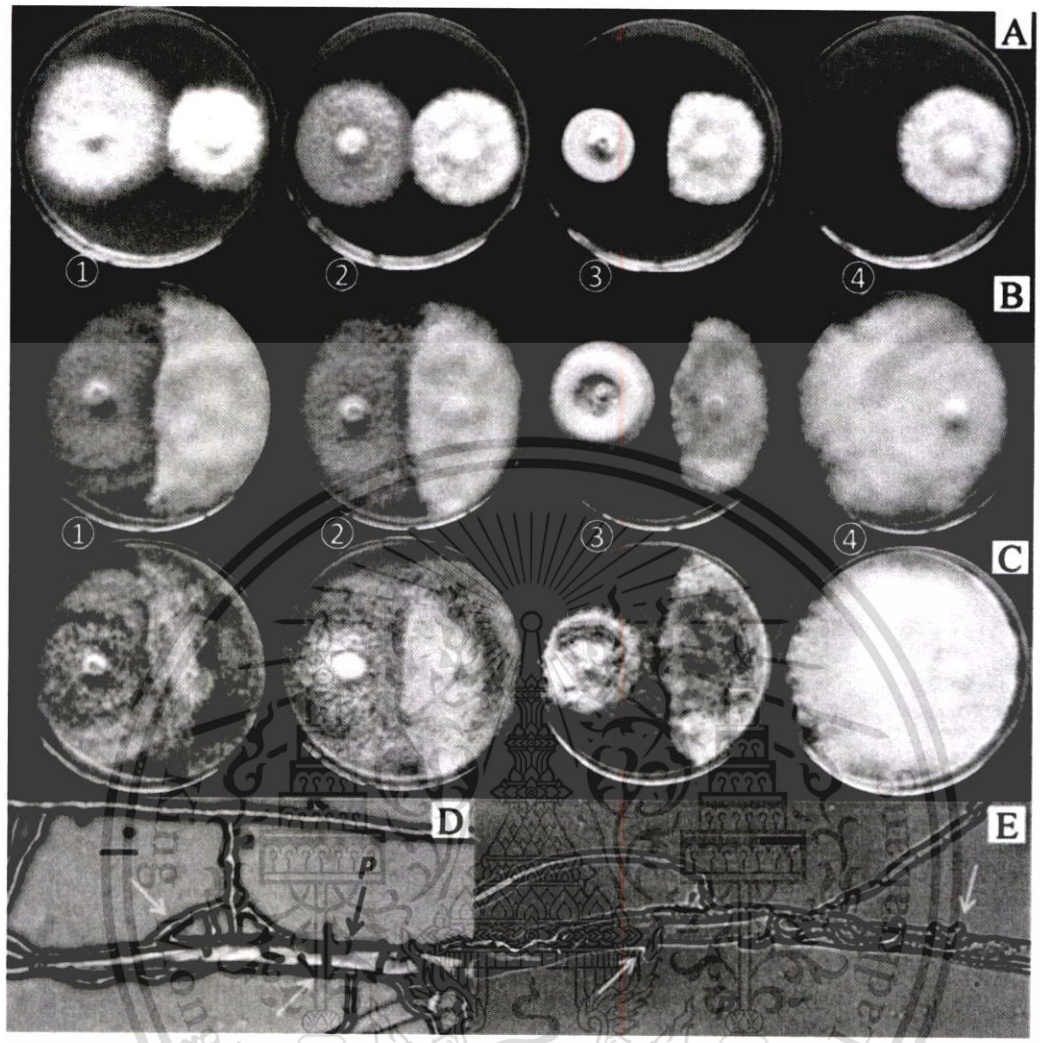

Fig. 3. Growth of *Phytophthora nicotianae* KA1 in bi-culture tests at 4 (A), 10 (B), and 30 days (C) after inoculation (1, 2, and 3: *P. nicotianae* KA1 [placed on the right side of the plates] inoculated with CG05, CL01, and CC3003, respectively; 4: *P. nicotianae* KA1 alone); D, E, *Chaetomium* hyphae (yellow arrows) coiled around and grew inside hypha of *P. nicotianae* KA1 (P) (scale bars: D, E = 10 μm).

inhibition between the antagonist and pathogen before contact was made. In contrast, CG05 and CL01 colonies made contact with pathogen colonies soon after inoculation without the clear zone of inhibition (Fig. 3A and 3B).

At 30 days after inoculation, all of the tested antagonists grew over colonies of *P. nicotianae* KA1 in bi-culture plates (Fig. 3C). Hyphae of the antagonists were shown to penetrate or coil around hyphae of *P. nicotianae* KA1 (Fig. 3D and 3E), resulting in the degradation and discoloration of pathogenic colonies (from white to light yellowish-brown). According to DMRT, at both points of measurement (10 and 30 days), CC3003 caused significantly higher growth inhibition of *P. nicotianae* KA1 than CG05 and CL01 ($p = 0.05$).

Effects of antagonistic crude extracts on the growth of *P. nicotianae* KA1. Nine antagonistic crude extracts were tested at different concentrations for growth inhibition of *P. nicotianae* KA1. As shown in Table 2 and Fig. 4, mycelial growth of the tested pathogen was highly sensitive to the crude MeOH extracts of all 3 antagonists and to the crude EtOAc extracts of CG05 and CL01, with very low ED_{50} values (2.6~4.6 μg/mL). In particular, in the presence of these crude extracts at a concentration of 100 μg/mL, the growth of *P. nicotianae* KA1 was inhibited completely (the colony diameter did not increase significantly). In contrast, the mycelial growth of *P. nicotianae* KA1 was moderately sensitive to other tested crude extracts (ED_{50} values ranging from 20.8 to 101.4 μg/mL).

According to DMRT, the effects of crude MeOH and EtOAc extracts of CG05 on mycelial growth of *P. nicotianae* KA1 were not significantly different at all tested concentrations. However, when the tested concentrations were from 10 to 500 μg/mL, greater inhibition of mycelial growth was observed with these extracts than with the crude hexane extract. Similarly, whereas the crude MeOH

Table 2. Inhibition of *Phytophthora nicotianae* KA1 growth at different concentrations of antagonistic crude extracts

Crude extract		Inhibition of *P. nicotianae* KA1 growth (%)					Effective dose (ED_{50}, μg/mL)
		Concentration (μg/mL)					
		10	50	100	500	1,000	
CG05	Hexane	21 ± 2.9 f[a]	36 ± 3.6 e	66 ± 2.6 d	91 ± 2.3 b	97 ± 2.8 a	67.2
	EtOAc	71 ± 3.1 c	93 ± 2.4 b	98 ± 2.1 a	100 ± 0 a	100 ± 0 a	4.6
	MeOH	71 ± 2.1 c	93 ± 2.3 b	98 ± 2.0 a	100 ± 0 a	100 ± 0 a	4.6
CL01	Hexane	6 ± 2.3 g	15 ± 4.3 f	38 ± 4.0 e	78 ± 2.6 d	94 ± 2.0 b	101.4
	EtOAc	81 ± 3.8 cd	94 ± 2.1 b	99 ± 1.7 a	100 ± 0 a	100 ± 0 a	2.9
	MeOH	84 ± 2.6 c	95 ± 2.3 b	99 ± 1.0 a	100 ± 0 a	100 ± 0 a	2.6
CC3003	Hexane	34 ± 2.1 e	67 ± 2.1 d	86 ± 2.1 c	99 ± 1.1 a	100 ± 0 a	20.8
	EtOAc	26 ± 2.1 f	41 ± 2.1 e	70 ± 2.9 d	95 ± 2.3 b	100 ± 0 a	41.2
	MeOH	69 ± 4.1 d	91 ± 2.8 c	99 ± 1.6 a	100 ± 0 a	100 ± 0 a	4.5

[a]Mean standard deviation of 4 replicates. For crude extracts of each antagonist, values indicated by the same letter are not significantly different according to Duncan's multiple range test at $p = 0.05$.

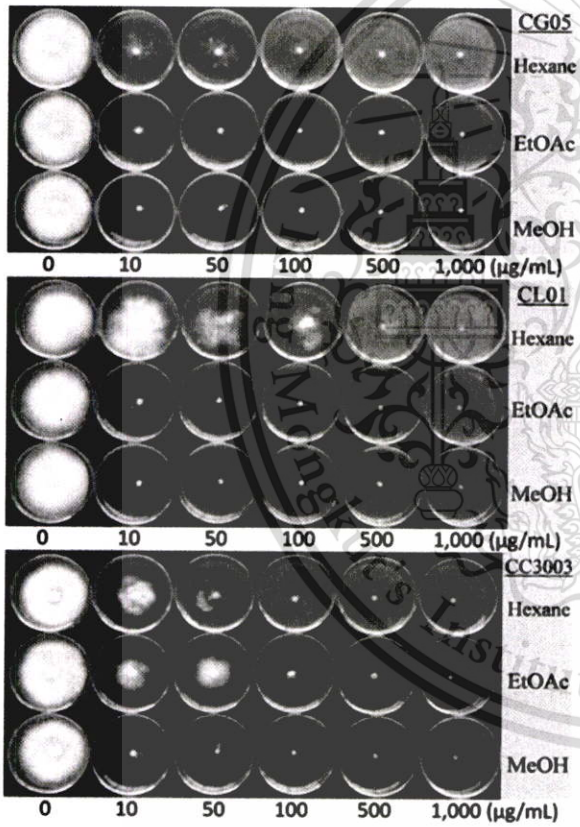

Fig. 4. Growth of *Phytophthora nicotianae* KA1 in the presence of different concentrations of antagonistic crude extracts.

and EtOAc extracts of CL01 did not cause significantly different inhibition at all tested concentrations, the growth inhibition caused by both of these extracts was higher than that caused by the crude hexane extract. In contrast, among the CC3003 extracts, the crude EtOAc extract of CC3003 had the lowest inhibitory effect on mycelial growth of *P. nicotianae* KA1 when the tested concentrations were from 10 to 500 μg/mL.

Because the MeOH extracts exhibited strong inhibitory effects against the tested pathogen, we chose these extracts as BCAs to control the pathogen in greenhouse experiments.

Table 3. Effects of biological control agents on root rot and plant weight of pomelo seedlings inoculated with *Phytophthora nicotianae* KA1

Treatment	Root rot (%)	Dry plant weight (mg)
Non-inoculated	0 ± 0 d[d]	998 ± 46 a
KA1[a] (control)	49.8 ± 1.8 a	483 ± 34 d
Me[b]-CG05	15.6 ± 1.4 bc	840 ± 47 bc
Me-CL01	13.7 ± 1.1 c	893 ± 29 b
Me-CC3003	15.4 ± 1.7 bc	833 ± 41 bc
S[c]-CG05	14.7 ± 1.6 c	865 ± 37 bc
S-CL01	17.1 ± 1.2 b	828 ± 45 c
S-CC3003	14.2 ± 1.7 c	885 ± 37 bc

[a]Inoculated plants were treated with water.
[b]Inoculated plants were treated with crude MeOH extracts of antagonists.
[c]Plants were inoculated with both chlamydospores of *P. nicotianae* KA1 and spores of antagonists.
[d]Mean standard deviation of 4 replicates. For each parameter, values indicated by the same letter are not significantly different according to Duncan's multiple range test at $p = 0.05$.

Efficacy of BCAs from *Chaetomium* antagonists against *P. nicotianae* KA1 infection under greenhouse conditions. Applications of spores and MeOH extracts of the antagonists showed highly significant effects on root rot rates and plant weights of pomelo seedlings inoculated with *P. nicotianae* KA1. As shown in the Table 3, in the absence of BCAs, inoculated seedlings (controls) exhibited a root rot rate of 49.8%. Following inoculation with *P. nicotianae* KA1, plant weight was reduced by 51.6% compared with that of non-inoculated seedlings (998 mg in non-inoculated seedlings versus 483 mg in inoculated seedlings). However, in the presence of BCAs, the plant weight of seedlings was reduced by only 11~17% compared with that in non-inoculated plants and increased by 72~85% compared with the inoculated controls. Plants treated with BCAs

Fig. 5. Pomelo plants at 6 weeks after application of different biological control agents.

exhibited root rot rates of 13.7~17%, which was 66~71% lower than that observed in inoculated controls. Non-inoculated plants did not exhibit root tip rot (Fig. 5). The pathogen *P. nicotianae* KA1 was detected in infected roots in all treatments except for non-inoculated controls.

Among the 6 treatments involving application of BCAs, little difference in root rot percentage and plant weight was observed according to DMRT. For BCAs from CG05 and CC3003, no significant difference in root rot percentage and plant weight was observed when comparing crude MeOH extracts and spores. However, for CL01, seedlings treated with crude MeOH extracts had slightly higher plant weight and lower root rot percentage than seedlings treated with spores.

DISCUSSION

In this study, we identified a pathogenic oomycetous isolate obtained from rotted roots of pomelo as *P. nicotianae*. The high virulence of this pathogen in pomelo, as shown in this study, supported the conclusion that "*P. nicotianae* is the main causal agent of root rot in all types of citrus worldwide" [1, 2]. We also clearly demonstrated that the tested *Chaetomium* species and their crude extracts strongly inhibited the growth of *P. nicotianae* KA1 *in vitro* and caused a dramatic reduction in the rate of root rot in plants infected with *P. nicotianae* KA1 under greenhouse conditions. Thus, our data provide important insights into the use of these BCAs in the control of *P. nicotianae* root rot of citrus plants.

All morphological characteristics of *P. nicotianae* KA1 were similar to those of *P. nicotianae* Breda de Haan, which has been described in detail in previous studies. According to extensive reviews by Erwin and Ribeiro [1], *P. nicotianae* is a non-caducous sporangia species. However, Cacciola *et al.* [15] found that isolates identified as *P. nicotianae* obtained from affected *Forsythia* plants had caducous sporangia with a very short pedicels (less than 5 μm). From lavender (*Lavandula angustifolia* Mill.), Álvarez *et al.* [16] also obtained 5 isolates identified as *P. nicotianae* that had caducous sporangia with short pedicels (2.1~3.8 μm). Their descriptions are consistent with our observations for *P. nicotianae* KA1 isolated from pomelo. Despite the unusual characteristics of the sporangia, the ITS sequences of this isolate were identical to those of many other isolates of *P. nicotianae* found in GenBank.

The inhibition of *P. nicotianae* KA1 growth at both 10 and 30 days in the bi-culture test demonstrated that the BCAs applied in this study could maintain inhibition of the pathogen over time. The coiling, penetration, and degradation of *P. nicotianae* KA1 mycelia in response to treatment with all tested *Chaetomium* species are typical of mycoparasitism. Similarly, all of the *Chaetomium* species used in this study have been reported to parasitize hyphae and to grow over and degrade *P. palmivora* colonies [9]. Hyphae of *P. cinnamomi* and *P. nicotianae* can be parasitized and lysed by *C. globosum* [17]. However, other studies also indicated antibiosis as the mechanism of the action of *C. globosum* and *C. cupreum* against *Colletotrichum gloeosporioides*, *Pyricularia oryzae*, *Rhizoctonia solani*, and

Curvularia lunata [8] and of *C. lucknowense* against *Fusarium* spp. [18, 19]. In these studies, parasitism was not detected. Interestingly, parasitism of *Chaetomium* spp. has only been reported on species within genus *Phytophthora*. The interactions between *C. cupreum* CC3003 and *P. nicotianae* KA1 found in this study showed characteristics typical of both of these mechanisms.

Mycoparasitism relies on various lytic enzymes for degradation of the cell wall of the host, causing "death of the target organism", which "results in a decrease in inoculum density", whereas antibiosis is a type of "antagonism resulting from the production of secondary metabolites toxic to other microorganisms" [7, 20]. Indeed, *Chaetomium* species are known to produce lytic enzymes and many other secondary metabolites, which may be involved in their antagonistic activity [6]. Degrading enzymes such as chitinase and beta-1,3-glucanase often are secreted by *Chaetomium* spp. in culture substrates and under mycoparasitism conditions [7, 21]. Additionally, various other compounds with antifungal activity against plant pathogens, such as chaetoviridins A and B [22] and chaetoglobosins A and C [23] have been isolated from *C. globosum*. The *C. lucknowense* CL01 has also known to produce chaetoglobosin C, which exhibited antifungal activity against both mycelial growth and formation of conidia in *Fusarium oxysporum* f. sp. *lycopersici* that is the causal agent of tomato wilt [19]. From crude EtOAc and MeOH extracts of *C. cupreum* CC3003, Kanokmedhakul *et al.* [12] isolated 3 compounds (rotiorinols A and C and rubrorotiorin), having strong antifungal activities against mycelial growth of fungus *Candida albicans* [12]. These findings suggested that multiple metabolites may contribute to the antifungal activity of a single crude extract. The growth inhibition of *P. nicotianae* KA1 observed in this study may have resulted from the combined antifungal activities of various metabolites within the crude extracts.

It is particularly notable that the mycelial growth of *P. nicotianae* KA1 in this study responded more sensitively to the crude extracts of tested antagonists (ED_{50} values of 2.6~101.4 μg/mL) than the mycelial growth of *P. palmivora* did (ED_{50} values of 26.5~2,495 μg/mL), which was reported in our previous study [9].

Our results above indicate that *Chaetomium* species and their crude extracts could provide sufficient control of *P. nicotianae* root rot in the pomelo, under greenhouse conditions. The introduction of *Pythium nunn* [24] and *Penicillium funiculosum* [25] into soil for controlling *Phytophthora* root rot in citrus has been successful under greenhouse conditions in prior studies. Moreover, a bio-product named Ketomium® (Strong Crop Co. Ltd., Samatprakaru Province, Thailand), developed from 22 strains of *C. globosum* and *C. cupreum* has showed ability to control *Phytophthora* sp. causing citrus root rot in the field [8]. In our study, in plants inoculated with *P. nicotianae* and treated with the BCAs, the plant weight was less than that of the non-inoculated seedlings. Thus, although these antagonists and their MeOH extracts did provide protection against pathogen infection, they did not promote the growth of pomelo seedlings. Similarly, Fang and Tsao [24] found that *Pythium nunn* reduced significantly the root rot rate caused by *P. parasitica* in citrus, but did not favor plant growth. The pathogen was still detected after the BCAs were applied, suggesting that the antagonists and their crude MeOH extracts could not remove *P. nicotianae* KA1 from the soil, despite their strong inhibition of pathogen growth *in vitro*. Thus, these crude extracts are fungistatic rather than fungicidal. The ability of the tested crude extracts to control different plant pathogens *in vitro* has been well documented, but has never been reported under *in vivo* conditions.

Approximately 300 g of dry biomass of *Chaetomium* species will yield approximately 20 g of crude MeOH [12]. In contrast, a single PDA plate harboring 4-wk-old *C. globosum* yields hundreds of millions of spores [26]. Thus, mixing of spores from these antagonists with potting media to control root rot in pomelo plants caused by *P. nicotianae* may provide a simple and economical approach, and may be easier to use in large-scale applications than crude extracts. The effectiveness of the tested *Chaetomium* strains and their crude extracts in the control of *P. nicotianae* in this study provides a convincing reason to promote the application of these strains to control *Phytophthora* root rot in citrus plants.

ACKNOWLEDGEMENTS

This work is supported by King Mongkut's Institute of Technology Ladkrabang Research Fund (Grant number: KREF125602).

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Journal of Agricultural Technology 2014 Vol. 10(4): 833-844
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Biological control of Pomelo diseases using *Chaetomium* spp

Hung Phung Manh[1*], Pongnak Wattanachai[2] and Soytong Kasem[1]

[1]Department of Plant Production Technology, Department of Soil Science, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand

Hung Phung Manh, Pongnak Wattanachai and Soytong Kasem (2014) Biological control of Pomelo diseases using *Chaetomium* spp. Journal of Agricultural Technology 10(4):833-844.

Threeisolates of plant pathogenic fungi were isolated from anthracnose and root rot of pomelovarKhao Nam Pueng. The isolates were morphologically identified as *Colletotrichum gloeosporioides*CL01 causing anthracnose, two isolates were identified as *Pythium intermedium* PY.S01 and *Pythiumaph anidermatum* (PY.S02) which causing root rot of Pomelo. All isolates were proved for pathogenicity on PomelovarKhao Nam Pueng. *Chaetomium cupreum, Chaetomium globosum* and *Chaetomium lucknowense* as effective antagonists were significantly proved to inhibit *C. gloeosporioides* CL01 and *P.aphanidermatum* PY.S02 in bi-culture antagonistic test. *Ch. cupreum, Ch.globosum and Ch.lucknowenese* inhibited the colony growth and conidial production of the *C. gloeosporioides* CL01. The colony growth of *C. gloeosporioides* was significantly inhibited by *Ch. cupreum, Ch. globosum* and *Ch. lucknowenese* which were 30.69, 37.78 and 34.86 per cent respectively, when compared with the control. Moreover, *Ch.cupreum, Ch. globosum* and *Ch.lucknowenese* were completely grown over the colony of *P. aphanidermatum* PY.S02 in bi-culture plates at 30 days. *Ch. Globosum* and *Ch. Lucknowense* were significantly inhibited sporangia, oospores and chlamydospore production of *P. aphanidermatum* PY.S02 at 89.01 and 86.41% respectively which significantly higher than *Ch. Cupreum* (53.89%). Moreover, *Ch. lucknowense* is reported for the first time to inhibit *C. gloeosporioides* causing anthracnose of Pomelo. Futher investigation would study on their control mechanism through fungal metabolites aganist these pathogens and would also test *in vivo*.

Keywords: *Chaetomium cupreum, Chaetomiumg lobosum,Chaetomium lucknowenese*, pomelo diseases

Introduction

Pomelo (*Citrus maxima*L) is considered as one of the most important fruit in Southeast Asia where it originated (TFNet, 2013). Along with grapefruit, pomelo is an important fruit crop grown commercially in many countries around the world. According to FAOSTAT, in 2011, total area of world production for pomelo and grapefruit is estimated at 276,222 ha and production

* Corresponding author: Hung Phung Manh; e-mail: manhhungnomafsi@gmail.com

at 7.7 million tons (FAOSTAT, 2011). Citrus in general and pomelo in particular is susceptible to a number of pathogens causing incalculable losses to the crop (Naqvi, 2004). Beyond good agronomic and cultural practices, growers often rely heavily on chemical fungicide application for control diseases (Agrios, 2005; Baker, 1987). The overuse chemical fungicides are happening in many crops including pomelo, even some banned fungicides that are still used by farmers (Thaipinta, A., Hudak, P.F., 2000). Finally, their products are not safe for consumers and have been refused or difficult to access to some biggest market such as Japan, EU (CAP, 2008). Therefore, alternative control has been being studied and needed to search more safety disease control. The objective of this study, therefore, was to evaluate *Chaetomium cupreum*, *Chaetomium globosum* and *Chaetomium lucknowense* as effective antagonists to inhibit some plant pathogenic of Pomelo.

Materails and methods

Sample collection and isolation

The anthracnose symptom on leaves and root rot disease were collected in Chachengsao province, Thailand and brought to laboratory. The pathogens were isolated by transplanting tissue method for anthracnose and baiting technique for root rot disease as modified methods from Burgess *et al.* (2008) and Drenth & Sendall (2001). All isolates were cultured in potato dextrose agar (PDA) in Petri dishes (9cm diameter) and incubated in the room temperature approximately (27–30^{0}C). Pure cultures were morphologically studied under binocular compound microscope. The characteristics were obsereved and recorded hyphae characteristics, shape and size of spore and other structures that needed for morphological characters, measured and taken photo under compound microscope.

Pathogenicity test

The three isolates were tested for pathogenicity to pomelo leaves varKhao Nam Peung using Koch's postulates to confirm pathogenic isolates. The isolates were sub-cultured on PDA dishes for 7 – 10 days at room temperature. The pomelo leaves were plucked, cleaned by sterile water before made wound. A 0.5 cm diameter sterilize cork borer was used to cut agar plugs from the active growing of sub-culture dishes in each isolate and were separately inoculated on the wounded leaves. The inoculated leaves were placed in Petri dishes which contained moist sterilized tissue paper and incubated at room temperature. After 4 – 5 days, the diameter of symptoms was recorded for

evaluation virulence of each isolate. The experiment was done using Completely Randomized Design (CRD) with four replications.

Bi-culture antagonistic test

Chaetomium cupreum, Chaetomium globosum and *Chaetomium lucknowenese* as antagonistic fungi are provided by Assoc Prof Dr Kasem Soytong, KMITL, Thailand which tested to inhibit plant pathogens using bi-culture test. The experiment was arranged in CRD with 4 replications. The antagonistic fungi and pathogens were separately cultured on PDA at room temperature (30–32ºC) for seven days. A 0.5 cm diameter agar plug from actively growing edge of the pathogen was placed oppositely to an agar plug of the antagonist in 9 cm diameter Petri dish containing PDA media. At the same time, a single plug of an antagonistic fungus or of the pathogen was placed on one side of other plates as the controls. The plates were incubated at room temperature for 30 days.Data were collected including colony diameter (cm) and the number of spore production by the pathogen. The number of spore production was counted by using haemacytometer. Percentage inhibition of mycelial growth or spore production of pathogen was calculated according to the following formula: %inhibition = 100 x (colony diameter or number of spore production of pathogen in control plate – colony diameter or number of spore production of pathogen in bi-culture plate)/ colony diameter or spore production of pathogen in control plate. Colony diameter and number of spore were statistically computed analysis of variance, the treatment means were compared using Duncan's Multiple Range Test (DMRT) at P = 0.05 and 0.01.

Results and discusssions

Isolation of pathogens

Three isolates were found which one isolates from leave anthacnose and two isolates from root rotof pomelo. Of which, one isolate was identified as *Colletotrichum gloeosporiodes* CL01. Two isolates were identified as *Pythium intermedium* PY.S01 and *Pythiumaph anidermatum* PY.S02. The species description wererecorded as follows:

***Colletotrichumgloeosporioides*C.L01**

Colonieson PDA with well developed aerial mycelia, 6 – 8 cm after 7 days, cottony, white to smoke – gray, with small black or peach – colored dots corresponding to the fungal sporulation. Conidia slimy, formed singly,

cylindrical, 8 –17 × 4 – 6μm on conidiophore, apex obtuse, aseptate, guttulate, hyaline, smooth, formed septum before germination (Fig.1). The present species is morphologically closed to *C. gloeosporioides*as epitypified by Cannon *et al.* (2008).

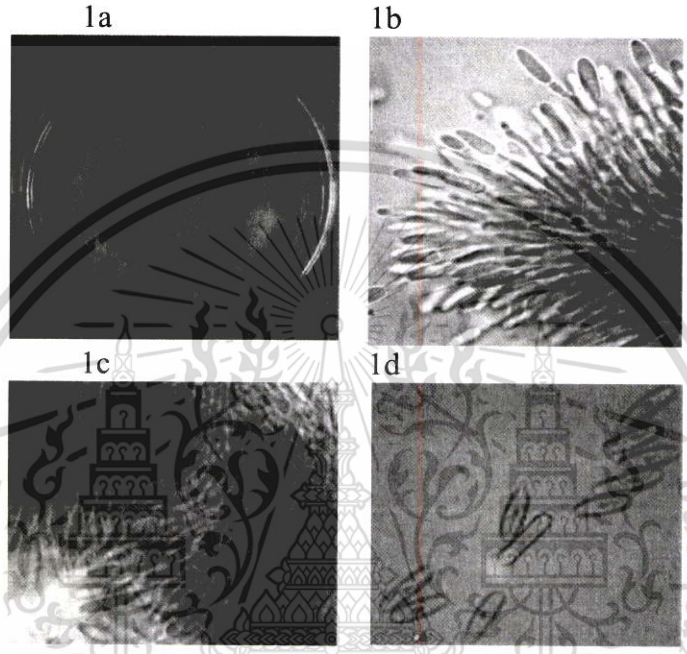


Fig. 1. *Colletotrichum gloeosporioides*from pomelo: A. Colony on PDA after inoculation 7 days. B and C. Dense fascicle conidiophores bearing conidia. D. Conidia showing guttulation

***Pythiumintermedium* PY.S01**

Colonies grew well with much aerial mycelia, reached to 9 cm diameter in less than 3 days on PDA medium. Hyphaeare non-septate, swelling mostly spherical, intercalary or terminal, 18 – 20μm in diameter, branching, tangled knots were formed (Fig 2). The morphology of this isolate is closed to *Pythiumintermedium,* which described in previous studies (K.H. Domsh and W. Gams, 1993).

***Pythiumaphanidermatum* PY.S02**

Colonies grew very fast with much aerial mycelia, covered full PDA plate (9 cm diameter) in 48h. Oogoniaand oospores formed readily in PDA, which confirmed it is a homothallic species. The shape of oogonium was mostly

terminal, spherical, 24 – 27µm in diameter (Fig 3). The present isolate is morphologically identified as *Pythiumaph anidermatum* that was described byWaterhouse (1967, 1968). The occurrence and o btainment easily of *Pythium* spp from soil sample confirmed earlier studies that the organisms is one of the most common soil borne and wide distribution (K.H. Domsh and W. Gams, 1993).

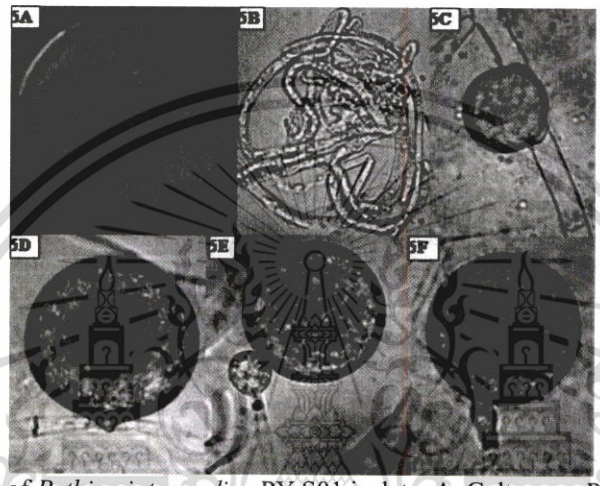


Fig. 2. Morphology of *Pythiumintermedium*PY.S01 isolate. A. Culture on PDA after 3 days. B. Tangled knots of hyphea. C – F. Hyphea swelling spherical in shape

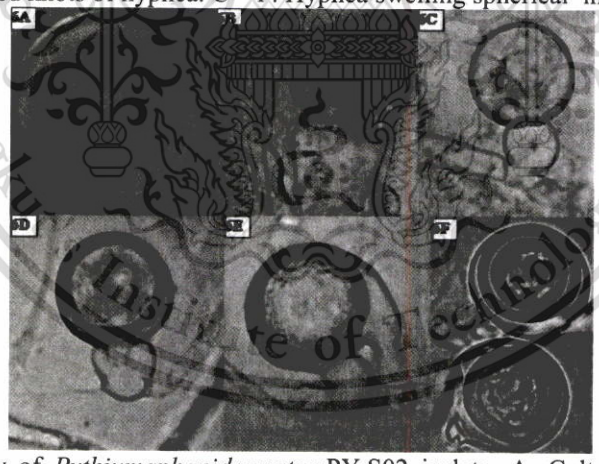


Fig. 3. Morphology of *Pythiumaphanidermatum*PY.S02 isolate. A. Culture in PDA after 3 days. B. Inflated zoosporangium. C. Young oogonium. D. Terminal oogonium with one antheridium. E and F. Terminal oogonium with two antheridia and formimg oospores.

Pathogenicity test

All isolates were proved to be pathogenic to Pomelo var Khao Nam Pueng. The leaves were inoculated with *Pythium aphanidermatum* PY.S02 and *P. intermedium* PY.S01 showing symptoms within 16 – 24 hours. *Pythium* spp are very common and important pathogens cause of seed rot, seedling damping-off, and root rot of all types of plants (Agiros, 2005) including citrus, and also of soft rots of fleshy fruits in contact with the soil (Naqvi, 2004). In many instances, poor germination of seeds or poor emergence of seedlings is the result of damping-off infections in the pre-emergence stage. However, older plants are seldom killed when infected with the damping-off pathogen, but they develop root and stem lesions and root rots, their growth may be retarded considerably, and their yields may be reduced drastically (Agiros, 2005).

Colletotrichum gloeosporioides showed symptoms within 36 hours, whereas there was no symptom on uninoculated control (Fig 4). The lesion sizes were measured at 4 days after inoculation that significantly (at $P<0.01$) differed those three species (Table 1). All symptoms were re-isolated the pathogens from the lesion of inoculated leaves. The morphology of re-isolates appeared to be the same to the isolates that obtained from collected samples. *C.gloeosporioides* have been recorded causing anthracnose on some serious disease in citrus both pre-harvest and post harvest such as leaf blight, anthracnose (Timmer, *et al.*, 2004). The conidia of *C. gloeosporioides*(Penz) Sacc are produced on dead twigs of the mother plant and dispersed by rainsplashes to developing fruits. These conidia germinate on fruit surface and remainquiescent till maturity of the fruit. Ethylene treatment and / or natural colour breakdownof fruit makes it susceptible for invasion of infection hyphae from the appressoria (Brown, 1977, 1978). The lesions developed on the fruit surface remain firm brown tobrownish black and in long term storage, the affected rind eventually develops soft rot (Timmer, *et al.*, 2004).

Table 1. Pathogenicity tests of *Pythium aphanidermatum, P. intermedium* and *Colletotrichum gloeosporioides* on detached leaves of Pomelo for 4 days

Isolates	Lesion size (cm)
*C.gloeosporioides*C.L01	2.1 b
P.intermedium PY.S01	4.6 a
*P.aphanidermatum*PY.S02	4.6 a
CV%	7.96

[1]Mean of four replacations. Mean followed by a common letter are not significantly different by DMRT at P =0.01.

The symptoms showed quickly and clearly in the inoculated leaves which demonstrated these isolates of the *C.gloeosporioides, P.aphanidermatum* and *P.intermedium*were virulence for PomelovarKhao Nam Pueng. It is confirmed previous comments that these pathogens are seriously attacked citrus trees in general including Pomelo (Naqvi, 2004; Agrios, 2005).

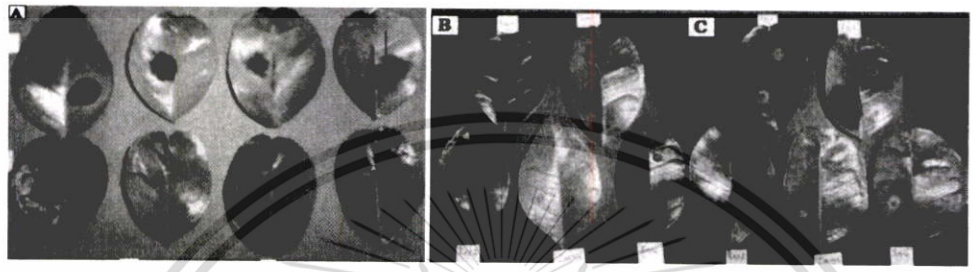


Fig. 4. Pathogenicity test for 4 days after inoculation. A=*C.gloeosporioides* C.L01; B=*P.intermedium*PY.S01 *C* =*P.aphanidermatum* PY.S02.

Bi-culture antagonistic test

Ch. cupreum, *Ch.globosum* and *Ch.lucknowense* were proved for ability to inhibit *C. gloeosporioides* causing anthracnose of Pomeloin bi-culture test. Results showed that *Ch. cupreum, Ch. globosum* and *Ch.lucknowense* inhibited both colony growth and conidia production of the tested pathogen. The colony growth of *C. gloeosporioides*was inhibited by *Ch. cupreum, Ch. globosum* and *Ch. lucknowense*as 30.69, 37.78 and 34.86 %, respectively, when compared with the controls (Fig. 5; Table 2). Whereas, the conidia production of *C. gloeosporioides* was inhibited by *Ch. globosum* of 70.10 % followed by *Ch. lucknowense* (60.54%) and *Ch. Cuperum* (51.71%).

The crudes extract from *Ch. cupreum* and *Ch. globosum* were reported to suppress both colony growth and conidia production of *C. gloeosporioides* caused anthracnose of *Citrus maxima* (Nuanjamrat, N., 2004) and *Citrus reticulate* (S. Kanokmedhakul, *et al.*, 2007) in vitro test. However, the studies did not evaluate abilities of *Chaetomium* spp as the antagonistic organisms to control the *C. gloeosporioides*. Other research fiding, Noiaium and Soytong (1997) repoted that *Ch.globosum* could inhibit the mycelial growth and spore production of *C.gloeosporioides* caused anthracnose of Mango as 62.38 and 76.20%, respectively, in bi-culture test. *Ch.cupreum* gave the potential to inhibit the mycelial and spore production of the fungal pathogen as 52.02 and 53.17 per cent. In this study, the inhibition of mycelial growth and spore production of *C. gloeosporioides* due to *Ch. globosum* and *Ch. cupreum* which both are higher than our result. The reasons probadly are different strain of *C.*

gloeosporioides, one is from Pomelo (*citrus maxima*) and one is from Mango (*Magniferaindica*). Morevover, *Ch. lucknowense* is reported for the first time to inhibit *C. gloeosporioides* causing anthracnose of Pomelo.

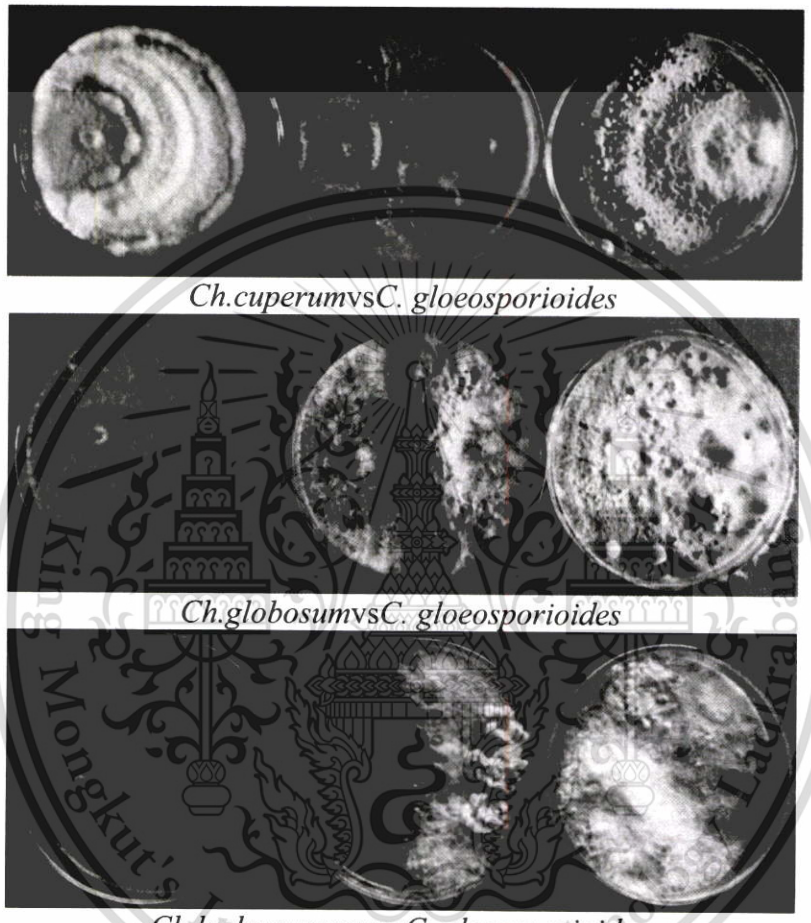
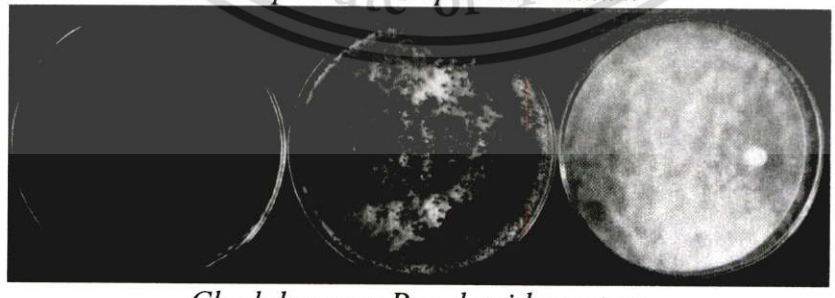


Fig. 6. *Chaetomium*spp against *C. gloeosporioides*in bi-culture test at 30 days

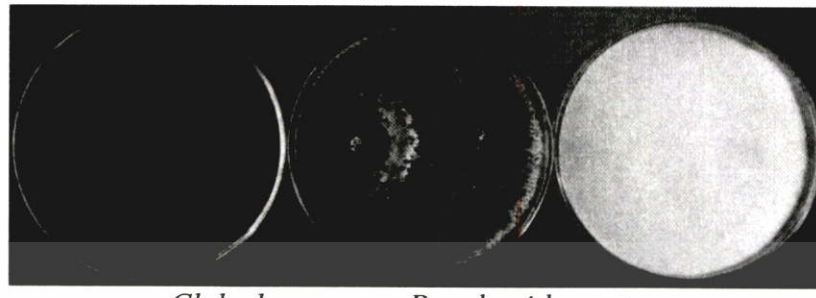
Ch. cupreum, Ch. globosum and *Ch.lucknowense* were completely inhibited and grew over *P.aphanidermatum* PY.S02 in bi-culture plates. However, *Ch. globosum* and *Ch.lucknowense* grew over the pathogen colony at 30 days (Fig. 7, Table 3). With this, *Ch.globosum* inhibited oospore production of 89.01 % followed by *Ch.lucknowense* (86.41 %) and *Ch. cuperum* (53.89 %) when compared with the controls. Beside reduction of oospore formation, it is relized that the lysis of mycelia of *P. aphanidermatum* in bi-clture plates with *Ch. globosum* and *Ch.lucknowense* implies mechanism of control. That is probadly resulted from effection of the atangonists, because *Ch. globosum* has

been reported to be a strong cellulose decomposer (Umikalsom *et al.*, 1998). *Ch. cupreum, Ch. globosum* in this study that are the same isolates reported by Kanokmedhakul *et al.* (2006) who stated that *Ch.cupreum* produced three new azaphilones named rotiorinols A-C (1-3), two new stereoisomers, (-)-rotiorin (4) and epi-isochromophilone II (5), and a known compound, rubrorotiorin (6), were isolated from *Ch.cupreum* CC3003. Compounds 1, 3, 4, and 6 exhibited antifungal activity against *Candida albicans* with IC_{50} values of 10.5, 16.7, 24.3, and 0.6ug/mL, respectively. *Ch.globosum* produces chaetomanone which also active against *Mycobacterium tuberculosis* (Kanokmedhakul *et al.,* 2001). Meanwhile, Soytong *et al.* (2001) also reported that those compounds could inhibit plant pathogens, *C. gloeosporioides* and *P. aphanidematum* as well. Moreover, Soytong *et al.* (2013) stated that the bioactive compounds Chaetoglobosin C of *Ch. lucknowense* and chaetomanone A produced from *Chglobosum* can be used as microbial elicitors to elicit phytoalexin, tomatineintomato seedlings var. Sida inoculated with *Fusarium oxysporum* f sp *lycopersici*. The inhibition oospore production of *P. aphanidermatum* caused root rot of pineappleby crude extract from *Ch. cupreum* was repored by Pornsuriya, *et al.* (2010). Nuanjamrat (2004) also reported that crude extract from *Ch. globosum* and *Ch. cupreum* could inhibit both sporangia and oospore production of *Pythium*sp caused root rot of pomelo, but this study did not identified into species.

*Ch.cuperum*vs*P. aphanidermatum*



*Ch.globosum*vs*P. aphanidermatum*



*Ch.lucknowense*vs*P. aphanidermatum*

Fig. 7.*Chaetomium* spp against *P. aphanidermatum*PY.S02 in bi-culture test at 30 days.

Table 2. Bi-culture test between *Chaetomium* spp and *Pythium aphanidermatum* PY.S02 for colony and conidia inhibition at 30 days

Treatments	Colony diameter of pathogens (cm)	% inhibition of colony	Number of conidia (x 10^6)	% inhibition of conidia
*Ch.cuperum*vs C.L01	6.24 b[1]	30.70 b	42.43 a	51.71 c
*Ch.globosum*vs C.L01	5.60 c	37.78 a	26.69 c	70.11 a
Ch.lucknowenese vs C.L01	5.86 c	34.86 a	34.50 b	60.54 b
Control	9.00 a	-		-
CV%	2.10	4.00 %	6.51	6.23

[1]Mean of four replacations. Means followed by a common letter are not significantly differed by DMRT at P =0.01.

Table 3. Bi-culture test between *Chaetomium* spp and *Pythium aphanidermatum* PY.S02 for oosporse inhibition at 30 days

Treatments	Number of oospores (x 10^4)	% inhibition of oospores
Control 1	32.22 a[1]	
*Ch.cuperum*vs PY.S03	14.45 b	53.89 b
Control 2	30.55 a	
*Ch.globosum*vs PY.S03	3.35 c	89.01 a
Control 3	32.87 a	
*Ch. lucknowenese*vs PY.S03	4.35 c	86.41 a
CV%	10.36	9.68

[1]Mean of four replacations. Means followed by a common letter are not significantly differed by DMRT at P =0.01.

Chaetomium species has been reported to produce numerous types of compounds such as benzoquinone derivatives, tetra-*S*-methyl derivativesand chaetoglobosinanalogs, most of them are mycotoxins (Soytong, 1991). For example, Chaetoglobosin C was isolated from *Ch. globosum* and *Ch.lucknowense* are reported to suppress many plant pathogens from different crops such as *Colletotrichum dematium, C. gloeosporioides, Fusarium oxysporum, Phytophthora parasitica, P. palmivora, P. cactorum* (Soytong, 2001).

Acknowledgements

The authors wish to acknowledge the support of Faculty of Agricultural Technology, KMITL, Bangkok, Thailand for funding this research.

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(Received 28 April 2014; accepted 30 June 2014)

ICIST 2013, KMITL, Bangkok, Thailand
November 28-29, 2013

Isolation plant pathogens from pomelo and their pathogenicity test

Hung, Phung Manh W. Pongnak and Soytong, K.

Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang(KMITL), Bangkok, Thailand

Pomelo is an important fruit crop grown commercially in many countries, especially, in the Southeast Asia. Growing in the tropical and temperate zone with a high rate of rainfall and humidity, pomelo is attacked by various pathogens and causing some prevalence disease such as anthracnose, brown rot, root rot, gummosis and damming off. In this study, the prevalence diseases and rooted soil samples from infeted pomelo orchards were collected in Vietnam and Thailand to isolate and tested for pathogenicity. Nine isolates were found which 4 isolates from leave diseases and 5 isolates from soil samples planted to Pomelo were obtained. In which, 6 isolates were morphological identified as *Colletotrichum* spp. (C.L01) causing anthracnose, 2 isolates identified as *Pythium* spp.(PY.S01; PY.S02) and 3 isolates identified as *Phytopthora* spp. (PH.S01; PH.S02 and PH.S03) which both causing root rot disease. All isolates were proved for pathogenicity to Pomelo. With this, six isolates appeared to be aggressive or virulence isolates to infect Pomelo var. Khao Nam Pueng.

Introduction

Pomelo (*Citrus maxima*.L) is considered one of the best and most important fruit of counties in Southeast Asia where it originated (TFNet, 2013). Along with grapefruit, pomelo is an important fruit crop grown commercially in many countries around the world. According to FAOSTAT, in 2011, total area of world production for pomelo and grapefruit is estimated at 276,222 ha and production at 7.7 million tons (FAOSTAT, 2011).

Citrus in general and pomelo in particular is susceptible to a number of pathogens causing incalculable losses to the crop. Occurrence of a particular pathogen, its ability to cause disease, survival and subsequent spread to cross threshold level in order to damage the crop are governed by agro-climatic conditions, varietal susceptibility, soil type etc. Disease problems in Citrus, if not attended properly, may become a limiting factor for its successful

cultivation. A number of fungal, viral and few bacterial pathogens right from nursery level to bearing stage attack citrus plants. Fortunately, due to the geographical isolation, different climatic and edaphic factors, cultivation of wide range of Citrus cultivars, only a few diseases in any Citrus growing area cause significant damage and require due attention for their effective management (S.A.M.H. Naqvi ,2004).

Growing in the tropical and temperate zone with a high rate of rainfall and humidity, pomelo is attacked by various pathogens and causing some prevalence disease such as anthracnose, brown rot, root rot, and gummosis damming off (Timmer *et al.*, 2004). The objective of this study, therefore, was to isolate and measure virulence of pathogens causing disease prevalence of pomelo in Viet Nam and Thailand. The preliminary objective of this research stusy was to isolate plant pathogenic fungi from Pomelo and test there ability to infect to Pomelo as to screen for aggressive or virulent isolates for further study on biological control of plant pathogens.

Methodology

Collection and isolation

The diseases prevalence on leaves and root which affected pomelo orchards in Vietnam and Thailand were collected. The pathogens were isolated by transplanting tissue method for leaf disease and baiting techniquesfor root disease (Lester W. Burgess, *et al.*, 2008; Drenth and B. Sendall, 2001). All isolates of pathogens were cultured in potato dextrose agar (PDA) in Petri dishes (9cm diameter) and incubated in the room temperature approximately (27 – 30^0C). Pure cultures were morphological studied under binocular compound microscope. The characteristics were obsereved and recorded hyphae characteristics, shape and size of spore and other structures that needed for morphological characters, measured and taken photo under compound microscope.

Pathogenicity test

All isolates were tested for pathogenicity to pomelo leaves var Khao nam Peung using Koch's postulates to confirm pathogenic isolates. The isolates were sub-cultured on PDA dishes for 7 – 10 days at room temperature. The pomelo leaves were plucked, cleaned by sterile water before made wound. A 0.5 cm diameter sterilize cork borer was used to cut agar plugs from the active growing of sub-culture dishes in each isolate and were separately inoculated on the wounded leaves. The inoculated leaves were placed in Petri dishes

contained moist sterilize tissue paper and incubated at room temperature. After 4 – 5 days, the diameter of symptoms was recorded for evaluation virulence of each isolate. The experiment was done using Completely Randomized Design (CRD) wuth four replications.

Result and Discusssion

Isolation of pathogens from pomelo

Nine isolates were found which 4 isolates from leave diseases and 5 isolates from rooted soil samples of pomelo. Of which 6 isolates were identified as *Colletotrichum* spp. (C.L01) causing anthracnose disease, 2 isolates identified as *Pythium* spp.(PY.S01; PY.S02) and 3 isolates identified as *Phytopthora* spp. (PH.S01; PH.S02 and PH.S03) which both causing root rot disease of Pomelo as seen in Table 1.

Table 1. The isolates obtained after isolation from the samples

Pathogens	**Isolates**	**Number of isolate**
From leaf samples		***04***
Colletotrichum .spp	C.L01	01
Glomerella cingulata	G.L02	01
Alternaria spp.	A.L03	01
Xanthomonas campestris p.v. citri	X.L04	01
From the soil samples		***05***
Pythium spp	PY.S01	01
	PY.S02	01
Phytophthora .spp	PH.S01	01
	PH.S02	01
	PH.S03	01

***Colletotrichum* spp.** C.L01

Symptom: anthracnose disease attacks leaves and fruits at any growth stage. The symptoms are most visible on leaves. At first, *Colletotrichum* .spp generally appears on leaves as small and irregular yellow, brown or dark brown spots. The spots can expand and merge to cover the whole affected area. The colour of the infected part darkens. Infected fruit has small, water soaked, sunken, circular spots that may increase in size (Fig 1).

Colonies: on PDA with well developed aerial mycelium, 6 – 8 cm after 7 days, cottony, white to smoke – gray, with small black or peach – colored dots corresponding to the fungal sporulation. *Conidia* slimy, formed singly, cylindrical, 8 –17 × 4 – 6μm, apex obtuse, aseptate, guttulate, hyaline, smooth, forming septum before germination. The present species is morphologically very closed to *C. gloeosporioides* as epitypified by Cannon *et al.* (2008).

Glomerella cingulata G.L02 is a perfect state of *Colletotrichum* sp (Argios, 2005) which was obtained from the leaf samples (Fig 2).

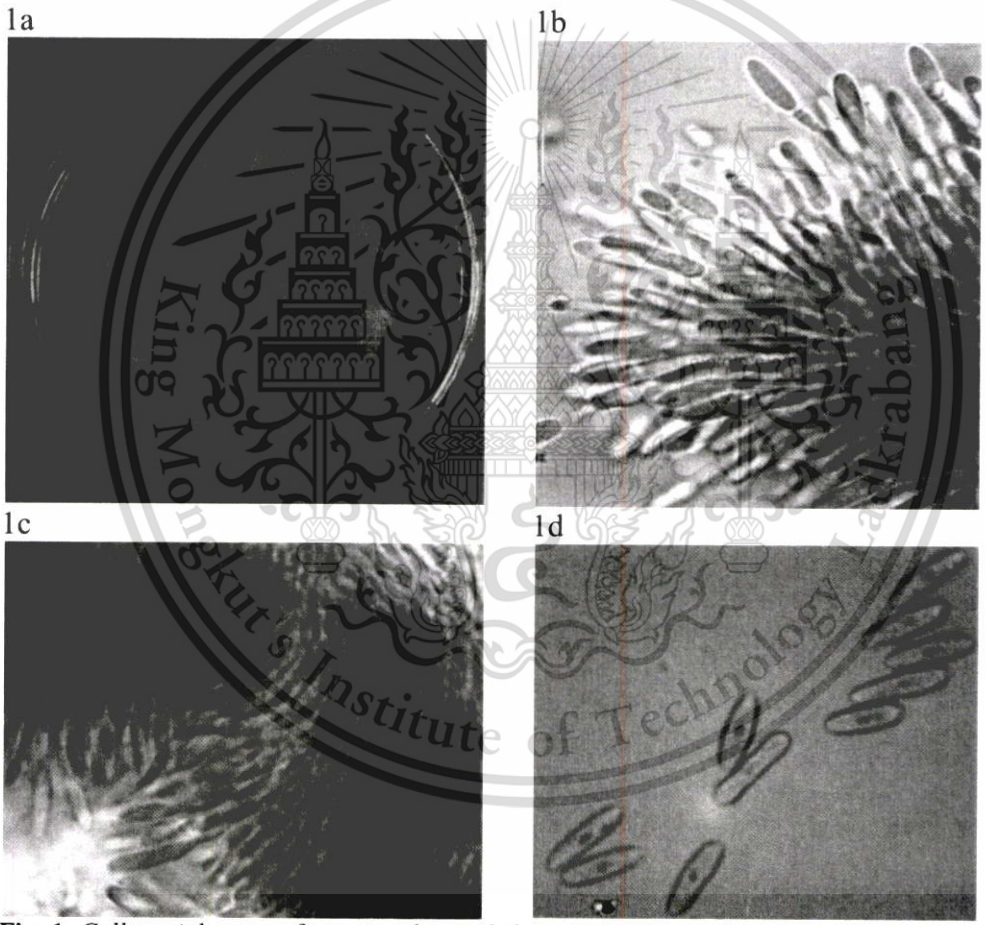


Fig. 1. *Colletotrichum s*pp from pomelo: A. Colony on PDA after inoculation 7 days. B and C. Dense fascicle conidiophores bearing conidia. D. Conidia showing guttulation.

***Alternaria* spp.** A.L03

Colonies: on PDA fast growing (9cm in 7days) aerial mycelium well developed, cottony, sporulation, smoke – gray to gray – olivaceous. *Conidia* single or catenulate, pyriform to ovoid in shape with a thickened, darkened scar, 4 – 6 transversal septa, 2 – 4 longitudinal septa, smooth or slightly roughened (Fig 3).

***Xanthomonas campestris* p.v. citri** X.L04 is the pathogen cause citrus canker was isolated easily from the affected leaves of pomelo (Fig 4).

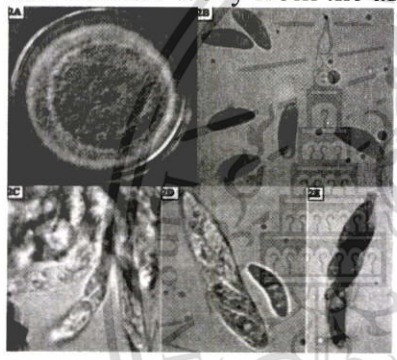


Fig. 2. *Glomerella cingulata* from pomelo leaf. A. The colony on PDA. B. Conidia. C – E. Ascus with conidia inside

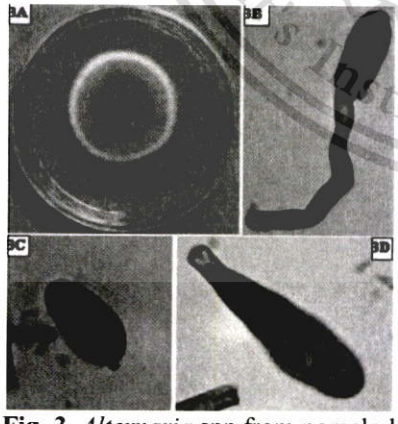


Fig. 3. *Alternaria*.spp from pomelo leaf. A. Colony on PDA. B – C. Conidia.

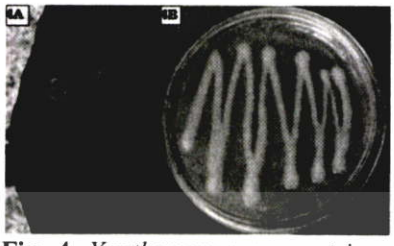


Fig. 4. *Xanthomonas campestris* p.v. *citri* from leaves of pomelo. A. Symptoms on leaf. B. Culture on potato dextrose peptone agar (PDPA)

Two isolates of *Pythium* spp were obtained when isolation from the rooted soil samples used baiting technique, PY.S01 and PY.S02 isolates.

Pythium. **spp (PY.S01)**

Colonies with well developed aerial mycelium, 9 cm diameter in less than 3 days on PDA. *Hyphea* swelling mostly spherical, intercalary or terminal, 18 – 20μm in diameter, branching, tangled knots were formed (fig 5). The *sporangium* and *zoospores* did not produce in agar or water, *oogonium* did not produce even inoculated on carrot agar, that confirmed this isolates was heterothallic. The morphology of this isolate is very closed to *Pythium intrmedium,* that was described in previous studies (K.H. Domsh and W. Gams, 1993).

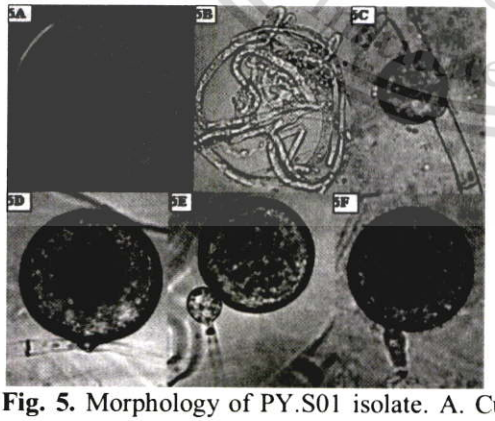


Fig. 5. Morphology of PY.S01 isolate. A. Culture on PDA after 3 days. B. Tangled knots of hyphea. C – F. Hyphea swelling spherical in shape.

***Pythium* spp PY.S02**

Colonies: grew very fast, covered full a 9 cm diameter Petri dish in 48h. Zoospores produced readily when transferred to water. Oogonium formed readily in single culture on both PDA, confirmed it is homothallic species. The shape of oogonium were mostly terminal, spherical, 24 – 27μm in diameter (Fig 6). The present isolate is morphologically very closed to *Pythium aphanidematum* that was described by Waterhouse.The occurrence and o btainment easily of *Pythium* spp. from soil sample confirmed earlier studies that the organism is one of the most common soil born and wide distribution (K.H. Domsh and W. Gams, 1993).

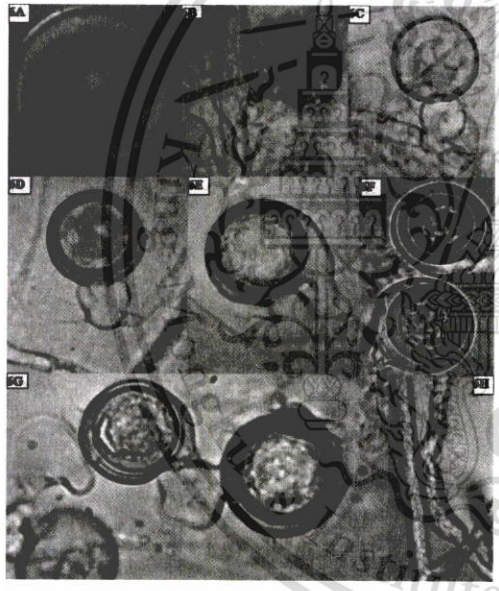
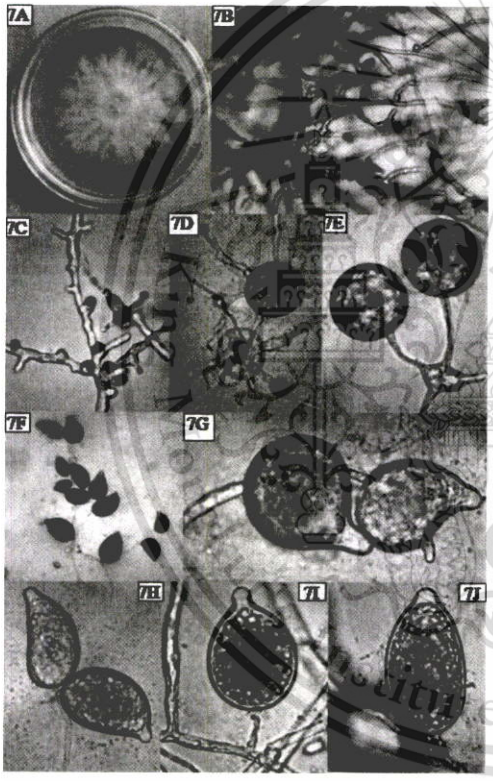


Fig. 6. Morphology of PY.S02 isolate. A. Culture in PDA after 3 days. B. Inflated zoosporangium. C. Young oogonia D. Terminal oogonium with one antheridium. E and F. Terminal oogonium with two antheridium. G. Intercalary oogonium. H. Oospores. I. Hyphae swelling.

In this study, 03 isolates of *Phytopthora.*spp (PH.S01, PH.S02 and PH.S03 were obtained when isolation and used pomelo leaves as baits. The morphology of the 3 isolates were almost the same which shown in Fig 7.

The colonies grew very slowly on PDA medium, less then 5cm diameter in 7 days, but grew faster on agar carrot. The characteristic of colonies on agar carrot also were difference from PDA with more aerial mycelia. *Mycelium* was tufted, with no pattern, spreading, and arachnoid aerial. *Hyphae* was much swelling in all isolates. *Sporangial shapes* ranged from ellipsoid, ovoid, pyriform, obpyriform, to spherical with a prominent papilla, mostly terminal, sometime intercalary and non-caducous. Sporangia size averaged at 37 x 26µm and length-breadth ratio at 1.42 : 1. *Sporangiophores* were irregular, sympodium or sympodium branched. *Chlamydospores* produced abundantly in all isolates intercalary or terminally and no sexual structures in single cultures.



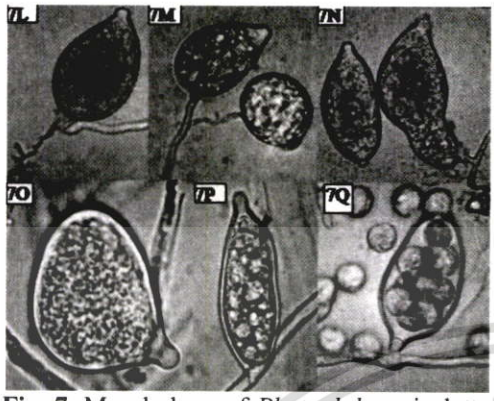


Fig. 7. Morphology of *Phytophthora* isolates. A. Culture in PDA after 10 days. B. Tufted and branching mycelium. C. Swelling hyphae. D and E. Chlamydospores. F. Sporangiophores. G. Intercalary sporangia H – M. common shape of sporangium of isolates with prominent papillas. N – P. Irregular sporangium. Q. Sporangium releasing oospore.

The occurrence of *Phytophthora* species many citrus fields both Vietnam and Thailand citrus fields confirmed earlier studies that these pathogens are present in almost all orchards (Ann PJ *et al*., 2004). The morphology of these present isolates close to *Phytophthora nicotianae* (sym. *Ph. Paratica*) described by Erwin and Ribeiro (1996). However, the morphological differences among some species are few and variable, making it difficult to classify the species accurately (Drenth and B. Sendall, 2001). Thus, need more studies to identify these isolates at species level.

Pathogenicity test

Colletotrichum gloeosporioides have been recorded causing some serious disease in citrus both pre-harvest and post harvest such as leaf blight, anthracnose (Timmer, *et al*., 2004). Whereas, *Pythium* spp. and *Phytophthora* ssp. with wide host range are considered the most serious soil bone for all of plant (Naqvi, 2004; Agrios 2005). *Alternaria* spp. also cause some disease of citrus such as fruit drop or leaf spot but not really dangerous for pomelo. Bacterial canker (*Xanthomonas campestris* pv. *citri*) is major disease on citruses, but not a serious on pomelo (TFNet, 2013).

For those reasons, the 6 isolates of C.L01 of *Colletotrichum* spp., PY.S01 and PY.S02 of *Pythium* spp., PH.S01, PH.S02 and PH.S03 of *Phytopthora* spp. were proved for pathogenic on Pomelo var Khao nam Pueng.

All 6 tested isolates were proved to be pathogenic which infected to the leaves of pomelo var Khao Nam Pueng. The leaves were inoculated by PY.S01 and PY.S02 of *Pythium* spp. stared to show symptoms, 16 – 24 hours after inoculation, the isolates of *Colletotrichum* spp. and *Phytopthora* spp. showed symptoms at 36 hours after inoculation. Whereas, there was no symptoms on uninoculated control leaves (Fig 8). The lesion sizes were measured 4 days (96 hours) after inoculation that were significantly (at $p<0.01$) different between *Colletotrichum* spp. and *Pythyum* spp.and *Phytophthora* spp. isolates. However, there were not significantly difference between 2 isolates of *Pythium* spp. PY.S01 and PY.S02. In a similar way, there were not significantly difference between 3 isolates of *Phytophthora* spp. PH.S01, PH.S02 and PH.S03 (Table 2). All isolates were re-isolated from the lesion of inoculated leaves. The morphorlogy of re-isolates appeared to be the same to the isolates that obtained from collected samples.

Table 1. Diameter of lesions induced on pomelo leaves 4 days after inoculation

Isolate	**Pathogenicity Test Lesion size** (cm)
Colletotrichum spp.	
C.L01	2.1
Pythium spp.	
PY.S01	4.6
PY.S02	4.6
Phytopthora spp.	
PH.S01	3.38
PH.S02	3.26
PH.S03	2,95
CV%	***7.96***
$LSD_{0.01}$	***0.56***

The symptoms showed quickly and clearly in the inoculated leaves which demonstrated these isolates of the *Colletotrichum* spp., *Pythyum* spp. and *Phytophthora* spp. very virulence for pomelo var Khao Nam Pueng in particular and for other pomelo varieties in general, because, all samples were collected from affected orchards with difference varieties. That confirmed previous studies that these organism are the top dangerous for citrus, in which has pomelo (Naqvi, 2004; Agrios 2005).

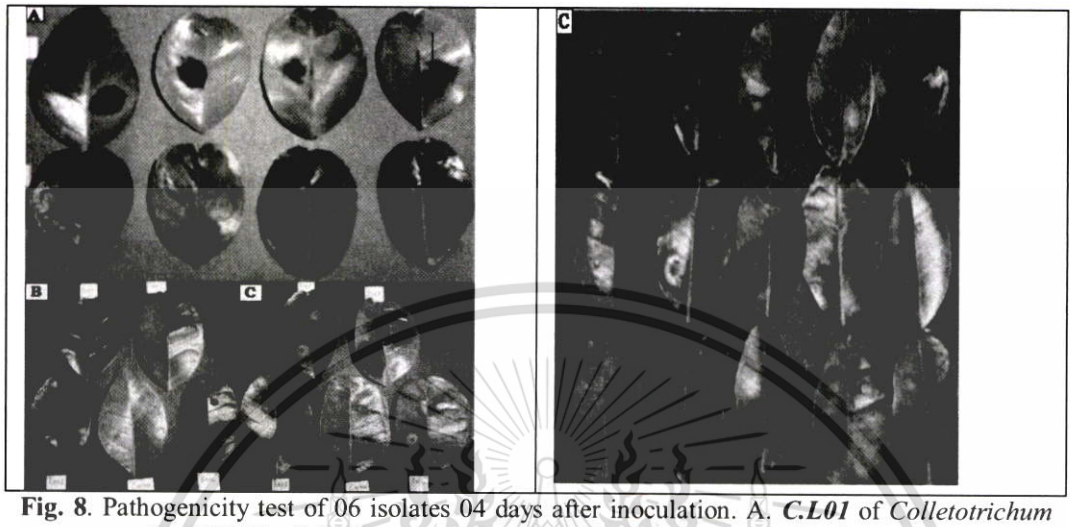


Fig. 8. Pathogenicity test of 06 isolates 04 days after inoculation. A. ***C.L01*** of *Colletotrichum* spp. B. ***PY.S01*** of *Pythium* spp. C. ***PY.S02*** of *Pythium* spp. D. from top to bottom ***PH.S01***, ***PH.S02*** and ***PH.S03*** of *Phytopthora* spp.

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Journal of Agricultural Technology 2015 Vol. 11(8): 1897-1910
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Identifications of *Phytophthora* spp. causing citrus root rots in Thailand

Phung Manh Hung[1, 2,*], Pongnak Wattanachai[1], Soytong Kasem[1] and Supattra Poeaim[3]

[1]Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand
[2]Northern Mountainous Agriculture and Forestry Science Institute (NOMAFSI), Phutho 293840, Vietnam
[3]Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand

Phung M.H., Pongnak W., Soytong K. and Poeaim S. (2015). Identifications of *Phytophthora* spp. causing citrus root rots in Thailand. Journal of Agricultural Technology 11(8):1897-1910.

Thailand is one of the largest citrus producers in Southeast Asia. Pathogenic infection by *Phytophthora*, however, has become one of major impediments to production. In this research, 3 slow-growth and 3 fast-growth isolates of *Phytophthora* spp. were obtained from samples in Chancheng Sao province and Bangkok, Thailand, respectively. Based on morphological characteristics and ITS ribosomal DNA sequence analysis, the slow-growth isolates were identified as *Phytophthora palmivora*. Meanwhile, the fast-growth isolates were identified as *Phytophthora nicotianae*. All obtained isolates showed high virulence for pomelo seedlings in pathogenicity test. The appearances and virulence of these *Phytophthora* spp. suggested they were causal agents of pomelo root rots in Chancheng Sao and Bangkok in Thailand.

Keywords: *Phytophthora*, citrus root rots

Introduction

Thailand currently, is one of the largest citrus producers in Southeast Asia, with the harvested area in 2013 is estimated at 0.1 million ha, resulting in production of 1.2 million tons of fruit (FAOSTAT 2013). With the prevalence of wet climatic conditions in Thailand, however, infection with *Phytophthora* has become a major problem for the citrus industry, causing yield losses of approximately 6~12% and economic losses of at least 37 million USD/yr (Drenth and Guest 2004).

***Coressponding Author:** Phung Manh Hung, **Email:** manhhungnomafsi@gmail.com

Although *Phytophthora* spp. are responsible for nearly 90 % of collar rots and 70% of all fine root diseases of woody plants, they often are not detected, leading to wrong diagnoses (Tsao 1990). Disease symptoms that caused by *Phytophthora* species often are confused with damages from other pathogens and abiotic agents. Therefore, the heavy losses of citrus and other crops due to *Phytophthora* are often the results of delays in recognition of the organisms as causal agents of the diseases under investigation. Isolation and identification of *Phytophthora* species are the only accurate method of early detection the pathogens, although that are always difficult (Erwin and Ribiero 1996).

This paper presents the isolation and identifications of *Phytophthora* spp. causing root rots pomelo (*Citrus maxima*) in Thailand.

Materials and Methods

Isolations of Phytophthora spp.

Phytophthora spp. were isolated from newly infected roots that taken from pomelo orchards having serious root rot problems, in Thailand. Transplanting methods being described below and selective medium were used to isolate the pathogens (Drenth and Sendall. 2001).

Newly infected roots were washed under running water to remove soil and other debris. The areas contained both healthy and diseased tissue on the roots then were aseptically cut into small pieces (approximately 2 × 2 mm). The root pieces then were transferred to selective medium PARPH. After incubating in the dark, at room temperature (25 – 30°C) for 2-3 days, materials from the margin of colonies that developed from the root pieces were transferred to plates containing a thin layer of WA medium. Pure cultures using for further studies then were obtained from hyphal tips developed from the colonies grown in the WA medium.

Pathogenicity test

Pathogenicity of each *Phytophthora* isolate was proved by artificial inoculation into roots of pomelo seedlings in order to satisfy Koch's postulate.

Twelve-week-old pomelo seedlings were thoroughly washed to be free of potting mix and then planted in plastic pots (10 × 15 cm) containing infested soil (with 5 propagules of *Phytophthora* sp. per cubic centimeter). Controls were prepared by planting the seedlings in same size pots, containing the sterilized potting medium.

All pots including the controls were maintained in the green house at temperature of about 25-30°C and flooded with water for 24 hr each week.

After 6 wk, the plants were carefully removed from plastic tubes and washed free of potting mix. The root systems were then evaluated on following scales: 0 = all roots healthy; 1 = rotted roots apparent; 2 = obvious root rot, root system small; 3 = severe root rot, taproot necrotic, few new roots; 4 = no healthy root, stem girdled. Evaluations were independently made by two observers and the average rating presented. Additionally, up to 100 root tips of each seedling were rated as rotted or healthy and the data expressed as root rot percentage. The pathogens then were re-isolated from newly infected root symptoms and morphological characteristics were compared with the inoculated isolate.

Morphological characterization

All obtained *Phytophthora* isolates were cultured on PDA, V8 agar, and CMA for morphological study. Growth rates of isolates on PDA medium were recorded. Sporangia of isolates were produced by floating some mycelial discs (obtained from margins of a 3-day-old culture on V8 agar) in 10 mL of double distilled water. The discs were then incubated under fluorescent light, at temperature of 25-28°C for 3-4 days. To determine the caducity of sporangia, the floating mycelial discs (bearing sporangia) were raised in a drop of distilled water several times, and the length of pedicels was measured under a light microscope. Sporangia caducity was determined based on the uniformity of the pedicel length (Erwin and Ribeiro. 1996). The sporangia and other structures were observed and measured by a camera with associated software attached to an Olympus light microscope (CH40; Olympus Optical Co. Ltd., Tokyo, Japan). At least 50 spores of each spore type were measured for each isolate then mean and standard deviation were reported.

Identification of pathogens based on DNA sequences

DNA extraction and polymerase chain reaction (PCR)

Mycelium of isolates was collected from purified colonies grown in PDA, and separately ground with mortar and pestle in liquid nitrogen to fine powders. DNA of isolates then was extracted by CTAB method with some modifications (Prabha *et al*., 2013).

Either couple of universal primers ITS6/ ITS4 or ITS5/ ITS4 was used to amplify internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S ribosomal DNA fragments by polymerase chain reaction (PCR) under previously described conditions (White *et al.* 1990; Cooke *et al.* 2000).

Sequencing and phylologeny analysis

To identify *Phytophthora* isolates into species level, the sequencing of cloned fragments (PCR products) of isolates then were performed at First Base Laboratory (Selangnor, Malaysia), using the same primers. The full-length determined ITS nucleotide sequences of isolates then were used as queries for BLAST searches in GenBank of National Center for Biotechnology Information (NCBI; http://www.ncbi/blast/) or Phytophthora Database (http://www.phytophthoradb.org). Subsequently, the sequences of isolates and related taxa (obtained from GenBank databases) were aligned and analyzed to construct a phylogenetic tree using software MEGA ver. 5.2 (Tamura *et al.* 2011).

Results

Morphological characteristics and pathogenicity of Phytophthora spp. from pomelo

Total 06 isolates of *Phytophthora* spp., dividing into 02 groups, were isolated from soil and root samples that collected from affected-pomelo orchards.

The first group, which comprised three isolates PHY01; PHY02; and PHY03, was isolated from samples collected in Chang chen Sao province. All these isolates have similar morphological characteristics. They were slow growth organisms, with colony diameters after 7 days grown on PDA were less than 4 cm (Fig. 1). Colonies of these isolates showed stellate pattern with aerial mycelia when grown on PDA. Hyphae are lumpy-branching with hyphal swellings. Sporangia produced readily and abundantly on agar surfaces of PDA and V8A after 3-5 days, occurred in groups on sympodium or irregularly. Sporangia were papillate and caducous with short pedicels (mean 3.3 μ long). Sporangial shape varied from ellipsoid, ovoid, pyriform, obpyriform to near spherical, with a length to breadth ratio of 1.6 – 1.7 : 1. Zoospores were directly released from sporangia when flooded in distilled water. Most of chlamydospores were globose in shape, produced abundantly from mycelia on agar surfaces of PDA and CMA. No sexual organ was observed in cultures of these isolates, thus, they were a heterothallic species. Morphological characteristics of isolate PHY02, the typical isolate of this group, are showing in Fig. 2. The identity in morphological characteristics of PHY01, PHY02 and PHY03 suggested they were same species.

The second group, which consisted of three isolates KA1; KA2; and KA3, was isolated from samples that collected near Bangkok. Unlike the first group, all these isolates of this group were faster-growth organisms, with

colony diameters after 7 days grown on PDA were from 83 – 87 mm. Colonies of these isolates showed dense-aerial, arachnoid, and branched mycelia with hyphal swelling when grown on PDA and V8A. However, their mycelia were spare when grown on CMA. No isolates of this group produced any spore type on agar surfaces of tested media. The isolates formed sporangia only when were flooded with distilled water. All the three isolates produced papillate, caducous sporangia with very short pedicels (mean length: 3.1 µm). The sporangial shape was predominantly subspherical and turbinate, with an average length-to-breadth ratio of 1.3:1. Chlamydospores of these isolates, which are globose in shape, formed abundantly when the method of Tsao (1971) was applied. Additionally, no sexual organs were observed in single cultures of these isolates. Morphological characteristics of KA1, the typical isolate of this group, are showing in Fig. 3. Because the isolates KA1, KA2 and KA3 had identical morphological characteristics, they probably belonged to one species.

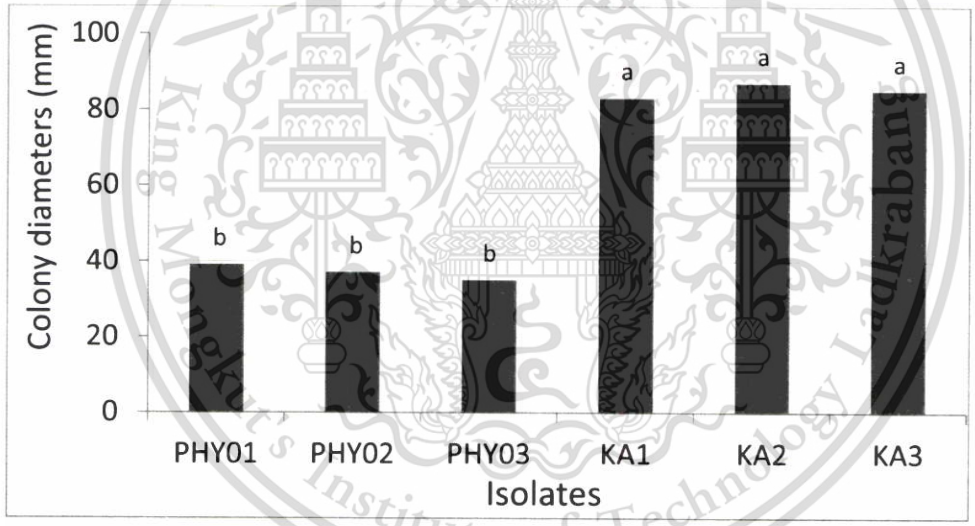

Fig. 1. Colony diameter of isolates of *Phytophthora* after 7 days grown on PDA (the same letter are not significantly different among isolates base on DMRT at $p = 0.05$)

Pathogenicity of isolates

As shown in table 4.2, all the six isolates were pathogenic to pomelo seedlings. Pomelo seedlings (12-week-old), which inoculated with 5 propagules of the isolates per cubic centimeter of soil, exhibited the root rot rates of 43.6 –

47.6%, with disease rating ranging from 2.5 – 2.9. These compared with no root rot and disease rating of 0.0 in the un-inoculated seedlings.

According to DMRT, there were no significant differences among the root rot percentages produced by isolates except for the slight difference between isolates PHY02 and KA3. All the inoculated seedlings produced very few new roots and leaves. Root tips of infected roots were soft and discolored. Root cortices of inoculated seedlings were turned soft and sloughed, leaving only the white stele (Fig. 4). These are typical symptoms of *Phytophthora* root rot in citrus, which was similar to symptoms observed in the affected orchards in Chang chen Sao and near Bangkok. In addition, all the isolates were re-isolated from newly infected roots of inoculated seedlings. Meanwhile, the Un-inoculated seedlings remain normally with abundant new roots and no symptom of rots. The results suggested that the obtained organisms were the causal agents of root rots in pomelo orchards in Chang chen Sao and near Bangkok.

Based on the pathogenicity and morphological characteristics of isolates in each group, we chose the isolates PHY02 and KA1 for further studies.

Table. 1. Morphological characteristics of *Phytophthora* isolates obtained from pomelo soil and roots in Thailand

Structures	Mean of size					
	PHY01	PHY02	PHY03	KA1	KA2	KA3
Sporangia[1]						
Length (μm)	54 ± 10.1[2]	54 ± 9.8	56 ± 9.5	49 ± 8.6	52 ± 7.7	49 ± 8.2
Breadth (μm)	34 ± 3.7	33 ± 4.4	34 ± 4.5	37 ± 4.7	39 ± 4.3	37 ± 4.6
Length/Breadth ratio	1.6 ± 0.3	1.6 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2
Papilla length (μm)	6.6 ± 1.6	6.4 ± 1.6	6.3 ± 1.6	4.8 ± 1.0	4.9 ± 1.0	5.0 ± 1.0
Pedicel length[3] (μm)	3.1 ± 1.1	3.2 ± 1.1	3.2 ± 1.1	3.1 ± 1.0	3.2 ± 1.0	3.0 ± 1.0
Chlamydospore[4] *diameter* (μm)	37 ± 6.4	37 ± 6.0	37 ± 6.6	37 ± 4.6	37 ± 6.4	36 ± 6.2

[1]Data collected from at least 100 separate sporangia; [2]Mean ± standard deviation; [3]Data collected from 85 separate detached sporangia; [4]Data collected from 50 separate chlamydospores

Table 2. Effect of different *Phytophthora* isolates on root rots of 12-week-old pomelo seedlings after six weeks of inoculation

Isolate	Source	Root rot (%)	Disease severity
PHY01	Roots	45.7 ab[1]	2.8[2]
PHY02	Roots	47.6 a	2.9
PHY03	Soils	45.1 ab	2.6
KA1	Roots	45.3 ab	2.8
KA2	Soil s	44.7 ab	2.6
KA3	Soil s	43.6 b	2.5
Uninoculated seelings		0.0 c	0.0

[1] Mean four replicates, the same letter represents no significant difference among treatments base on the Duncan's multiple range test at $p = 0.05$
[2]Rate on a scale of 0 = all roots healthy to 4 = no healthy root, stem girdled.

Identification and phylogeny of Phytophthora spp. caused root rots of pomelo in Thailand

Primers ITS5 and ITS4 were used to amplify internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S ribosomal DNA fragments of isolates PHY02. Meanwhile, primers ITS6 and ITS4 were use for amplifying the same regions of isolates KA1. Both PCR products of isolates were about 900 kb. Nucleotide sequences of the ITS ribosomal DNA fragments of isolates PHY02 and KA1 were then determined and deposited in the GenBank under accession number KT175509 and KT175508, respectively. The DNA sequences of the two isolates were used as queries to search GenBank (NCBI) using the BLAST function.

The BLAST analysis showed that the nucleotide sequences of PHY02 shared 99.75% (809/811) with those of *Phytophthora palmivora* accession numbers PD00627, PD01515 and PD00491; and 99.87% (782/783) identity with those of PD02505. Phylogenetic analysis confirmed the relationships between PHY02 and these related taxa (Fig. 5). The isolate PHY02 was identified as *Phytophthora palmivora* (Butl.), based on its morphology and the molecular analysis.

For the isolate KA1, the analysis showed that its nucleotide sequences shared 100% identity with those of *Phytophthora nicotianae* accession Nos. GU111681 and GU111670 from *Citrus* spp. in Taiwan; JF792541 and JF792530 from citrus soils in India; and many other isolates existing in the GenBank database. Phylogenetic analysis confirmed the relationships between KA1 and the related taxa (Fig. 5). Thus the pathogenic isolate KA1 was identified as *Phytophthora nicotianae* (Breda de Haan).

Discussions

Phytophthora spp. are one of the major pathogens of many horticultural crops causing incalculable losses. The pathogens are decimating citrus industry worldwide (Naqvi, 2004). However, due to the difficulties in their isolations, early and accurate diagnosis of *Phytophthora* diseases in plant often is very difficult not only for grower but also for plant pathologists (Erwin and Ribeiro, 1996). In this research, six isolates of *Phytophthora* spp., dividing into two different groups, were isolated from samples collected in the affected pomelo orchards.

The first group contained slow-growth isolates that had papilate-caducous sporangia with short pedicels, and occurring in groups of 5 – 15 on one sympodium. These are typical characters of *P. palmivora* (Butl.) according to Erwin and Ribeiro (1996), which described the occurrences of groups (up to 20) conspicuous papillate sporangia with short pedicels on a sympodium as the distinguished characteristics of this species. More importantly, the analysis of their ITS sequences confirmed these isolates were *P. palmivora* (Butl.). The appearance of only *P. palmivora* in the samples from affected orchards in Chang chen Sao and its high virulence for roots of pomelo suggest that this species is the causal agent of the disease appearing there. This finding is in correspondent with others of previous authors. Zitko *et al.* (1991) demonstrated that *P. palmivora* often is more aggressive and damages even larger root than *P. parasitica* on citrus. Serious root rot disease of citrus caused by *P. palmivora* has been recorded in America, India (Zitko and Timmer, 1994; Naqvi, 2004). Additionally, *P. palmivora* are known as a prominent plant pathogen with a wide host range, which infects various important crops such as black pepper, durian, citrus, rubber...*etc*, in Southeast Asia (Drenth and Guest, 2004).

All morphological characteristics of isolates from the second group were similar to those of *P. nicotianae* Breda de Haan (syn. *P. parasitica*) (Erwin and Ribeiro, 1996), except the production of caducous sporangia. Despite the unusual characteristics of the sporangia, the ITS sequences of this isolate were identical to those of many isolates of *P. nicotianae* found in

GenBank. The Phylogenetic analysis also strongly confirmed the relationships between the isolate KA1and the related taxa. Caducity of sporangia is an important and useful character for morphological study and identification of *Phytophthora* species. According to the extensive reviews by Erwin and Ribeiro (1996), *P. nicotianae* does not produce caducous sporangia, but produces persistence sporangia on the long stalks. However, Cacciola *et al.* (1994) found that isolates identified as *P. nicotianae* obtained from affected *Forsythia* plants had caducous sporangia with a very short pedicels (less than 5 µm). From lavender (*Lavandula angustifolia* Mill.), Álvarez *et al.* (2007) also obtained 05 isolates identified as *P. nicotianae* that had caducous sporangia with short pedicels (2.1 – 3.8 µm). Their descriptions are consistent with our observations for isolates of *P. nicotianae* obtained from pomelo. The appearance and high virulence of isolates of *P. nicotianae* in pomelo, as shown in this study, supported the conclusion that "*P. nicotianae* is the main causal agent of root rot in all types of citrus worldwide" (Erwin and Ribeiro,1996; Naqvi, 2004). The species is well known to distribute widely and cause root rots of citrus plants in citrus-growing areas America. It is estimated that 20 – 80% citrus orchards in Florida are infested by *P. nicotianae* (Graham and Timmer, 2008). Recently, *P. nicotianae* is report to be the most predominant pathogens of citrus root rots, foot rots and gummosis in Egypt (Ahmed *et al.*, 2012), South Africa (Meitz-Hopkins, 2014). In Thailand and other countries in Southeast Asia, *P. nicotianae* not only infects various citrus types, causing root rots and foot rots, but also cause root rots of many other crops such as papaya, tobacco, durian, black pepper, pineapple ... *etc* (Drenth and Guest, 2004). Therefore, it is unsurprised when the virulence pathogen infects and causes root rots of pomelo, which is widely grown in Thailand and considered to be susceptible to *Phythopthora* (Naqvi, 2004).

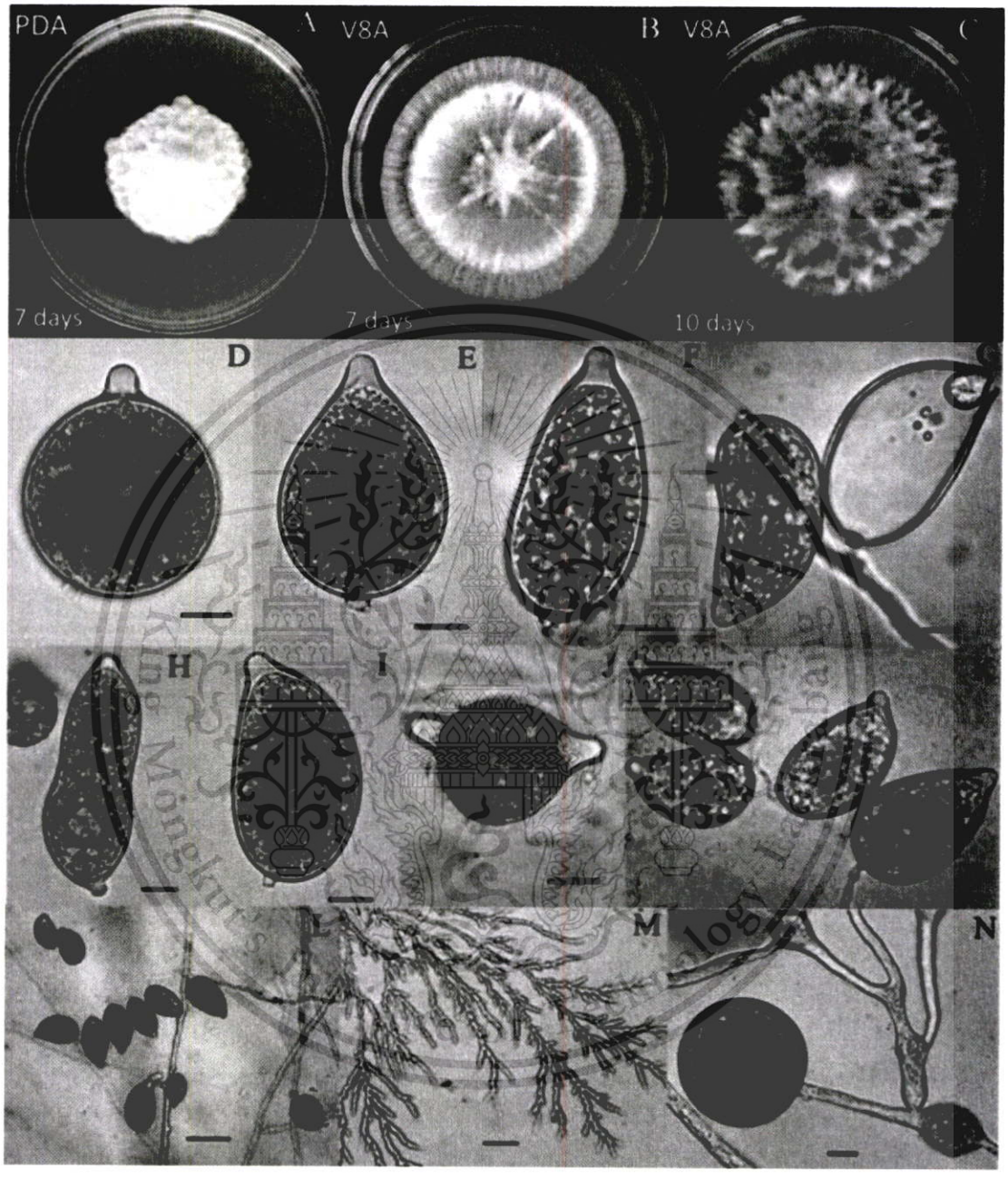

Fig. 2. Morphological characteristics of isolate PHY02. A-C, Colony types of PHY02 at 7 days grown on different media; D-J, Zoosporangia; K and L, Occurrence of sporangia on sympodium; M, Lumpy-branching mycelia; J, Chlamydospore and swelling hyphae (scale bars: D-G, N = 10 μm, L, M = 50 μm).

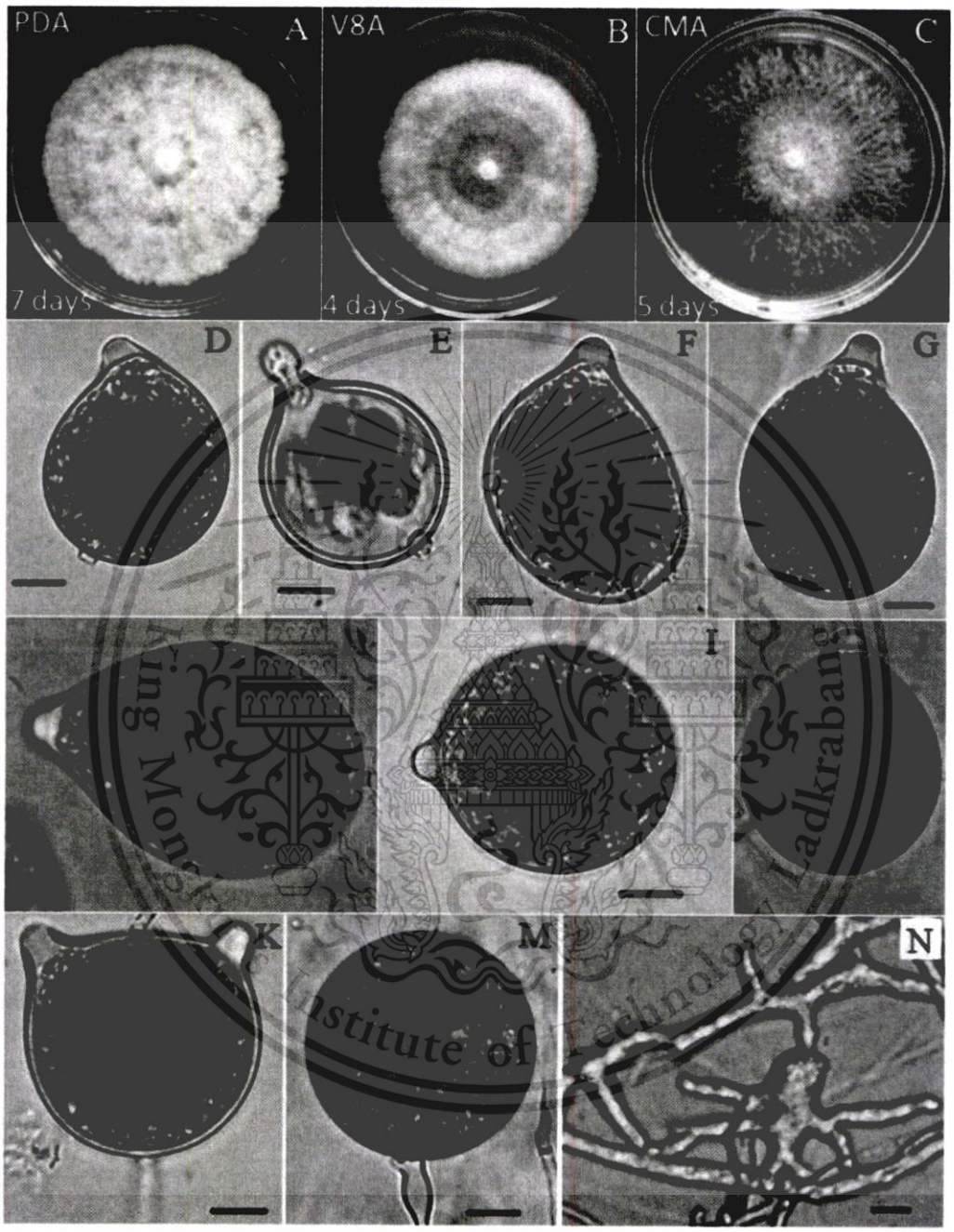

Fig. 3 a. Morphological characteristics of *Phytophthora* sp. KA1. A – C, cultures of KA1 on different media; D – K, Sporangia ; M, Chlamydospore; N, Hyphae swelling (scale bars: D – M = 10 μm; N = 50 μm).

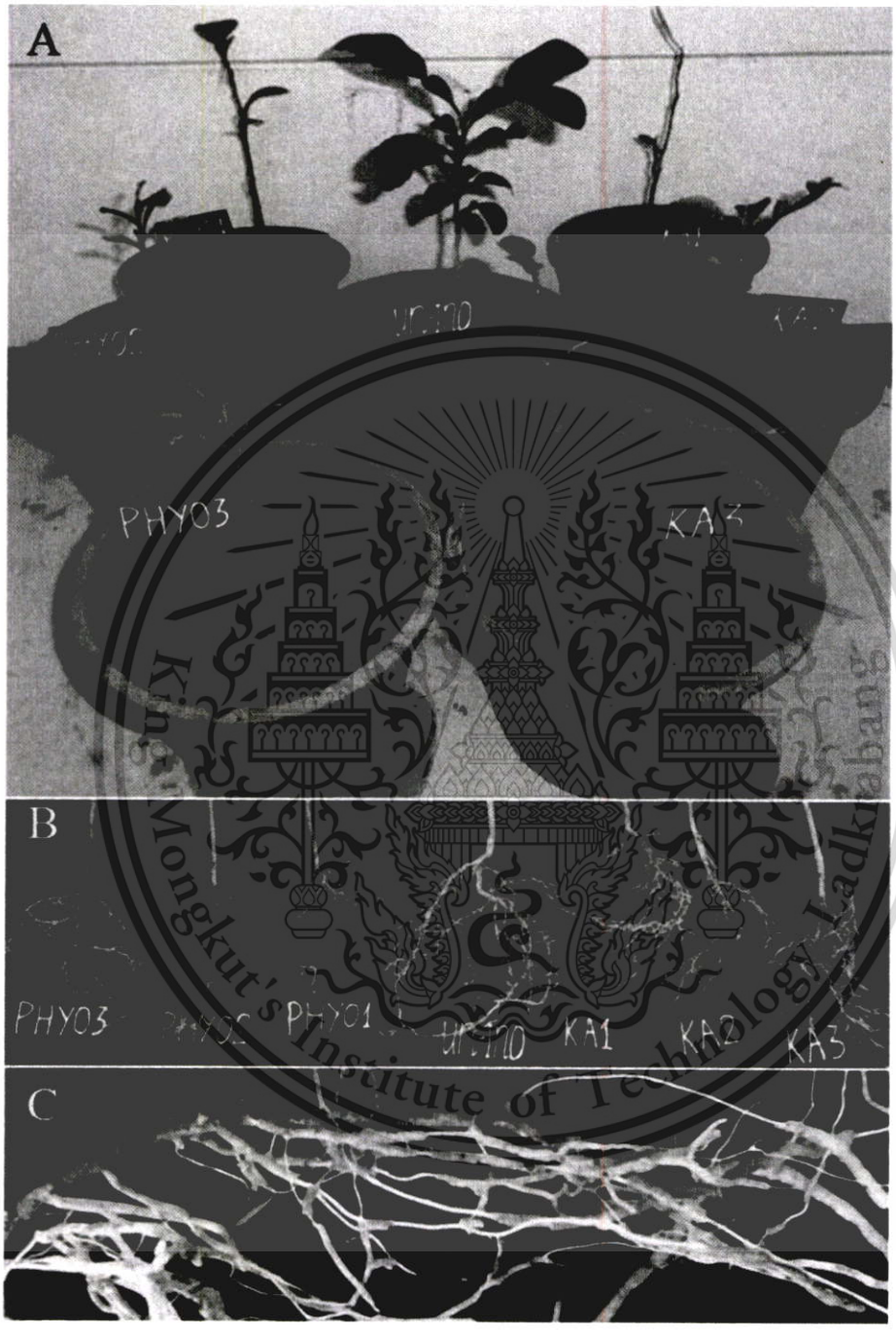

Fig. 4. A and B – Seedlings and their root systems after six weeks inoculation with different *Phytophthora* isolates ; C – closed symptoms of root rots in an inoculated seedling.

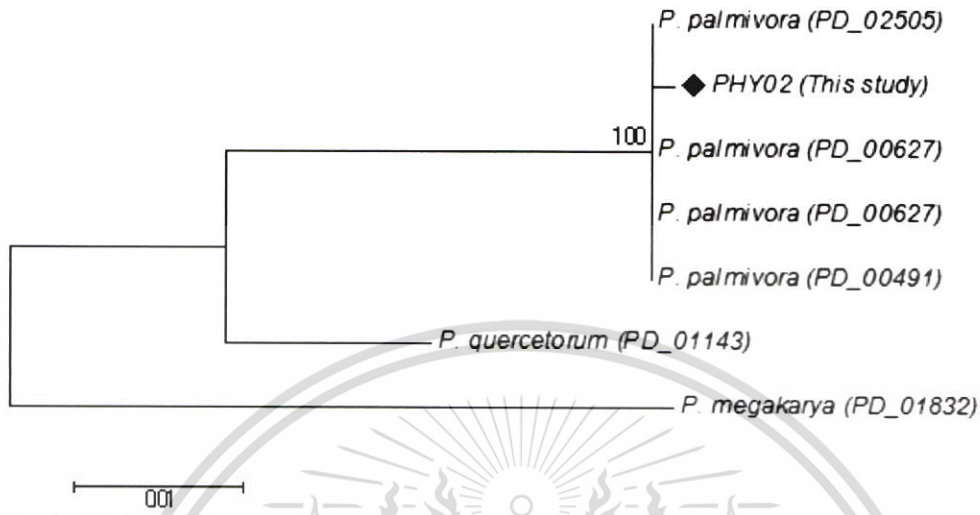

Fig. 4. Phylogenetic relationship between *Phytophthora palmivora* PHY02 and related taxa inferred using a neighbor joining method with internal transcribed spacer (ITS) rDNA sequences. Bootstrap value based on 1,000 replications is shown above the branch.

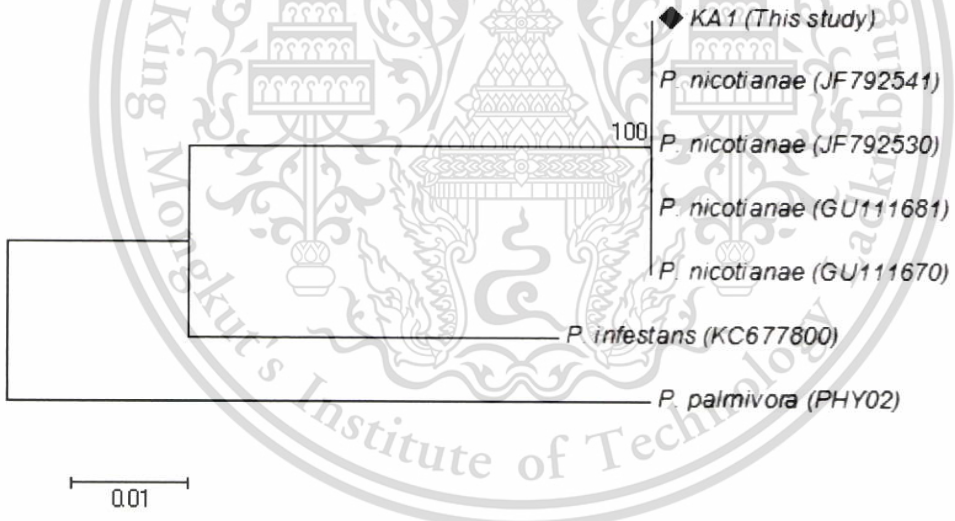

Fig.5. Phylogenetic tree showing relationship between *Phytophthora nicotianae* KA1 and related taxa base on the internal transcribed spacer ribosomal DNA sequences, using the neighbor-joining method with 5,000 bootstrap replicates. *P. infestans* (KC677800) (the species placed in the same clade 1 with *P. nicotianae*) was isolated from a potato in India, and *P. palmivora* PHY02 (KT1755

Acknowledgements

This work is supported by King Mongkut's Institute of Technology Ladkrabang Research Fund (Grant number: KREF125602).

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