BIOLOGICAL CONTROL OF RICE BLAST CAUSED BY *Magnaporthe oryzae*

JIAOJIAO SONG

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENT FOR
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FACULTY OF AGRICULTURAL TECHNOLOGY
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Thesis	**การควบคุมโดยชีววิธีของข้าวใบไหมที่มีสาเหตุจากเชื้อรา *Magnaporthe oryzae***
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Student ID	**59604002**
Degree	**Doctor of Philosophy**
Program	**Agriculture**
Year	**2020**
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บทคัดย่อ

แยกเชื้อรา *Magnaporthe oryzae* (= *Pyricularia oryzae*) ได้หลายไอโซเลท (isolates) จากเมล็ดข้าวหลายสายพันธุ์ และจากใบไหม้ข้าวที่เป็นโรค และพบไอโซเลทที่มีความสามารถทำให้เกิดโรคไหม้รุนแรง ในข้าวสายพันธุ์ กข 57 และพันธุ์พิษณุโลก 2 จากการทดลองพบว่าเชื้อรา *Chaetomium elatum*, *Chaetomium lucknowense* and *Chaetomium brasiliense* และ *Chaetomium cochliodes* เป็นจุลินทรีย์ต่อต้านเชื้อรา *M. oryzae* เชื้อรา *Magnaporthe oryzae* และ *Chaetomium* spp. ทุกไอโซเลท ยืนยันการจัดจำแนกทางสัณฐานวิทยาและชีววิทยาระดับโมเลกุล จากการเลี้ยงเชื้อร่วม พบว่า *C. elatum*, *C. lucknowense* , *C. brasiliense* และ *C. cochliodes* สามารถควบคุมการเจริญเติบโตของเชื้อรา *M. oryzae*.

สารสกัดหยาบ จากเชื้อรา *C. elatum*, *C. lucknowense* *C. brasiliense* และ *C. cochliodes* มีประสิทธิภาพในการยับยั้งเชื้อรา *M. oryzae* สารสกัดหยาบด้วยเฮกเซน จาก *C. brasiliense* ยับยั้งสปอร์ได้สูงสุด มีค่า ED_{50} 35 ppm สารสกัดหยาบด้วยเอทธิลอะซิเตรท *C. lucknowense* ยับยั้งการสร้างสปอร์เชื้อสาเหตุโรค มีค่า ED_{50} 57 ppm และสารสกัดหยาบด้วยเอทธิลอะซิเตรท จาก *C. elatum* ยับยั้งการสร้างสปอร์เชื้อสาเหตุโรค มีค่า ED_{50} 106 ppm สารสกัด

หยาบจาก *C. cochliodes* (CCoH, CCoE and CCoM) ยับยั้งการสร้างสปอร์ *M. oryzae* ที่ 12 วัน มีค่า ED_{50} 85, 144 และ 374 ppm ตามลำดับ

นอกจากนี้ อนุภาคนาโนที่ผลิตจากสารออกฤทธ์ ของ *C. elatum*, *C. lucknowense* *C. brasiliense* และ *C. cochliodes* สามารถยับยั้งเชื้อรา *M. oryzae* สาเหตุโรคใบไหม้ข้าว นอกจากนี้ อนุภาคนาโน Nano-CLM (*C. lucknowense*) ยับยั้งการสร้างสปอร์ มีค่า ED_{50} 5.24 ppm อนุภาคนาโน Nano-CBH (*C. brasiliense*)) ยับยั้งการสร้างสปอร์ มีค่า ED_{50} 6.86 ppm อนุภาคนาโน Nano-CEE (*C. elatum*) ยับยั้งการสร้างสปอร์ มีค่า ED_{50} 7.89 ppm

ขนาดอนุภาคนาโน ของ nano-CCoH, nano-CCoE และ nano-CCoM จาก SEM วัดค่านาไฟเบอร์ ได้เฉลี่ย 567-611, 422-566 และ 415-472 nm ตามลำดับ อนุภาคนาโน จาก *C. cochliodes* (nano-CCoH, nano-CCoE and nano-CCoM) ที่ความเข้มข้น 3-15 ppm ยับยั้งการสร้างสปอร์เชื้อสาเหตุโรค *M. oryzae* ที่ 12 วัน มีค่า ED_{50} เท่ากับ 9, 16 และ 33 ppm ตามลำดับ

อนุภาคนาโนที่ผลิตจากสารออกฤทธิ์ *C. cochliodes* (CTh05) สามารถควบคุมโรคใบไหม้ข้าว ที่มีสาเหตุจาก *M. oryzae* isolate PO1 จากการทดลองพบว่าสามารถลดการเกิดโรคไหม้ได้ 38 % หลังจากฉีดพ่นอนุภาคนาโนที่ผลิตจากสารออกฤทธิ์รวม *C. cochliodes* (CTh05) ที่อัตรา 10 ppm ในเวลา 30 วัน ในขณะที่ การใช้สารเคมี Tricyclazole ลดการเกิดโรคได้ 29 % อย่างไรก็ตาม โรคไหม้ข้าวลดลง ถึง 60 % ใน 30 วัน หลังฉีดพ่นอนุภาคนาโน nano-CCoM ที่ 7 ppm รองลงมาคือ อนุภาคนาโน nanoCCoE

นอกจากนี้ จากการฉีดพ่นใบข้าวด้วย อนุภาคนาโน nano-CBH ที่ผลิตจาก *C. brasiliense* พบว่าต้นข้าวสามารถผลิตสารต่อต้านการเกิดโรคไหม้ในข้าว คือ Sakuranertin และ Oryzalexin B มีค่า R_f 0.08 และ 0.28 ตามลำดับ ซึ่งเป็นการวิจัยค้นพบครั้งแรกในการใช้อนุภาคนาโนที่ผลิตจากสารออกฤทธิ์จุลินทรีย์ เป็น elicitors ชักนำให้ข้าวสร้างภูมิคุ้มกันโรค โดยผลิตสารต่อต้านการเกิดโรค (phytoalexin) จึงนับว่าเป็นงานวิจัยค้นพบใหม่ในการใช้อนุภาคนาโนที่ผลิตจากสารออกฤทธิ์จุลินทรีย์ *Chaetomium* spp ชักนำให้ข้าวสร้างภูมิคุ้มกันโรคไหม้

Thesis **Biological control of rice blast caused by *Magnaporthe oryzae***

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Degree **Doctor of Philosophy**

Program **Agriculture**

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ABSTRACT

The isolates of *Magnaporthe oryzae* (= *Pyricularia oryzae*) were isolated from rice seeds and diseased leaves. It was proved to be vilurence isolates to cause rice blast in rice var. RD 57 and PSL 2 by pathogenicity test. *Chaetomium elatum*, *Chaetomium lucknowense, Chaetomium brasiliense* and *Chaetomium cochliodes* were antagonized to *M. oryzae*. All isolates of *Magnaporthe oryzae* and antagonistic *Chaetomium* spp. were confirmed identification by morphology characterization and molecular phylogeny. Biculture tests demonstrated that all the antagonistic *C. elatum*, *C. lucknowense* and *C. brasiliense* and *C. cochliodes* could suppress the growth of *M. oryzae*.

Then, the crude extract metabolites from *C. elatum*, *C. lucknowense* and *C. brasiliense* and *C. cochliodes* were effectively inhibited *M. oryzae.* Hexane extract of *C. brasiliense* gave the highest spore inhibition with ED_{50} of 35 ppm. EtOAC extract of *C. lucknowense* inhibited spore production at 57 ppm and EtOAC extract of *C. elatum* inhibited spore production at 106 ppm. The crude metabolites of *C. cochliodes* (CCoH, CCoE and CCoM) expressed antifungal activity against the *M. oryzae*, resulting in inhibited spore production in 12 days with effective dose (ED_{50}) values of 85, 144 and 374 ppm, respectively.

Furthermore, the nanoparticles loaded with crude metabolites of each antagonistic *C. elatum*, *C. lucknowense, C. brasiliense* and *C. cochliodes* were proved to be antifungal activity against *M. oryzae in vitro*. Nano-CLM (*C. lucknowense*) inhibited sprore production at 5.24 ppm, Nano-CBH (*C. brasiliense*) inhibited spore production at 6.86 ppm and Nano-CEE (*C. elatum*) inhibited spore production at 7.89 ppm. The nanoparticles sizes of nano-CCoH, nano-CCoE and nano-CCoM were ranged between 567-611, 422-566 and 415-472 nm., respectively. Nanoparticles derived from *C. cochliodes* (nano-CCoH, nano-CCoE and nano-CCoM) at concentrations of 3-15 ppm significantly inhibited *M. oryzae* in 12 days with ED_{50} values of 9, 16 and 33 ppm, respectively.

Nanoparticles containing active compounds derived from *C. cochliodes* (CTh05) was proved to control rice blast disease caused by *M. oryzae* isolate PO1. *In vivo* experiments revealed a reduction of 38 % in blast disease after application of nanoparticles constructed from crude extract mixtures from *C. cochliodes* at 10 ppm for 30 days. Meanwhile, the chemical fungicide Tricyclazole resulted in reduction of blast disease by 29 %. Rice blast disease was decreased in 30 days after applying nano-CCoM at the concentration of 7 ppm which resulted 60 % disease reduction, and followed by nanoCCoE and nanoCCoH which disease reduction of 58 and 50 % respectively, and tricyclazole resulted in a 56 % reduction in rice blast.

Moreover, the rice leaves treated with nano-CBH from *C. brasiliense* produced Sakuranertin and Oryzalexin B as seen on the thin layer chromatography where the R_f value was 0.08 assuming to be Sakuranertin, and R_f values of 0.28 supposing to be Oryzalexin B. It is firstly found that these nanoparticles act as elicitors to induce immunity in rice plants through the production of phytoalexin.

The research findings are found to be a new discovery of nanoparticles construed from natural product fungal metabolites from *Chaetomium* spp. that used to control rice blast disease and induced phytoalexin production against blast disease.

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Jiaojiao Song

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CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

Rice (*Oryza sativa*) is accepted to be the most important crops worldwide. It deserves as a major of energy source over half of the world population, and the major food in India, China and Asian countries, which 92% of the world rice growing areas (Gnanamanickam, 2009). In 16 December 2002, the United Nation has declared the year of 2004 to be the International Year of Rice. The declaration was supported by 40 countries. Rice becomes the one of the major cereal grains such as maize, wheat and rice) to feed the increased population. It is consumed by 3.5 billion people around the world which mostly in Asia. The increasing rice production have achieved to help the hunger in the world (Gnanamanickam, 2009).

The fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) causing blast disease is the first recorded diseases of rice which known as rice fever disease in China in early 1637. It is recorded as *Imochibyo* in Japan in 1704. The rice production has expanded throughout Asia, Africa and Latin America in last few centuries. The blast disease is found over 85 countries worldwide. *M. oryzae* infects all parts of the rice causing losses of hundreds of millions tons annually. The yield losses have encountered to shortages in many developing countries in the last recent years. Then, the control strategy of blast disease must concern for global food security and social well-being (Wang, 2009).

Rice blast becomes the mainly disease due to wide distribution and high incidence in favorable conditions. Valent (2004) stated that the rice blast disease becomes the world’s epidemic disease and needed to get more information. The blast is distributed in 85 countries wherever rice is cultivated. It is a seriously damaging disease in highland condition, especially faced the drought and soil stress to the rice production leading to severe infection to a variety of rice cultivars. The yield loss is happened due to blast infection can be reached as high as 50% when the disease occurs in epidemic areas. The damaged rice crop is often influenced by environmental condition. Rice blast disease can be found as biological terrorism for rice growing plants due to high devastation in rice production (Gnanamanickam, 2009).

Rice blast diseases are normally controlled by resistant varieties, cultivation management in fields and application of synthetic fungicides (Zeigler *et al.,* 1994). But the chemical fungicides application to control of the pathogen may possible harmful to human health and agroecosystem. The application of noxious synthetic fungicides in agriculture production has needed to decrease and search for new biological control agents against blast disease to safe human consumption and our environment. Biological control of plant disease is defined by applying microorganisms to control plant pathogen and given a high potential alternative method instead of synthetic chemical fungicides. It is the most cost effective and safety to surrounding environment (Sáenz-de-Cabezón, 2010; Narayanasamy, 2013).

Biological control of plant disease has become an effective control to many diseases. The biocontrol agents eg *Chaetomium*, *Penicillium, Emericella* and *Trichoderma* are reported to be effective to control many plant diseases. *Chaetomium* spp are reported that the formulated bioproducts as a broad spectrum mycofungicides can be actively control several plant diseases (Soytong and Soytong, 1997). *Emericella nidulans* was recorded to control *Colletotrichum gloeosporioides* (anthracnose of Vanilla) by Talubnak and Soytong (2010). *Chaetomium* species are unique fungi found in fertile soils, animal dungs and death organic materials which recorded to be found over 350 species (Zhang *et al,* 2012). Some species of *Chaetomium* species acts as decomposing fungi that produces enzyme cellulase to degrade cellolose in the organic materials. Some isolates are reported as antagonists against plant pathogens. Hung *et al.* (2015a) recorded that *Chaetomium* spp. gave a good control *Phytophora palmivora* PHY02 (root rot of Pomelo), *Chaetomium globosum*, *Chaetomium cupreum*, *Chaetomium lucknowense* expressed antifungal supression the growth of *P. palmivora* PHY02.

Nanotechnology has been increasingly interested in many reserch areas especially in agriculture to study food industries and plant protection (Li, *et al.*, 2011). Moreover, the nanotechnology in agriculture are searching by many researchers to find out nanomaterials that is being explored for farming along with nano-delivery systems are also approaching to the new era of industrial revolution in agricultural development (Soutter, 2012). Precision farming would possible be helped the agriculturists in crop production to minimum farm inputs for inorganic chemicals of pesticides and fertilizers. The micro-sensors and other automatic monitoring devices can be provided to crop or animal production to improve farming systems, and finally increasing in yield and income. It is a great potential for nanoscience and technology to be nanotechnological application to get a state-of-the-art solutions in various challenges which is encounterted by agriculture (Ditta, 2012). Nanoparticles can be served as 'magic bullets' which is containing pesticide, chemicals or genes to the targeted plants. It can easy to penetrate the protectative chemicals or metabolites into cells, cuticles and tissues and slowly release of the active substances in plants. The most popular of nanoparticles or nanomaterials are being used for plant protection that like nanospheres, nanocapsules, nanopowder and nanogels (Bruno Perlatti *et al.*, 2013).

1.2 Objectives

The management of rice disease is aimed to protect disease epidemic that usually done using plant resistant varieties and various chemical fungicides. The application of chemical fungicides have been proved to be toxic side effect to human being, non-target organisms and environment changes**.** These chemical fungicides are expensive leading for high production cost. The resistant cultivars are used in large scales but in long term, the resistant genes are usually broken down and could not resist the the rice blast. However, the research findings during the past two decades have been found out the other potential control for rice blast disease. It is a biological control method of rice blast diseases using antagonistic microorganism (Zahirul *et al.*, 2004). Biocontrol technology is a special significance of eco-friendly environment and cost effective control which can be applied to integrate with other disease control strategies for protection with sustained rice yields.

The objectives were performed as follows:

1) To isolate and identify seed-borne fungi from rice.
2) To study morphological and molecular phylogenetics of *Magnaporthe oryzae* causing rice blast and antagonistic fungi *Chaetomium* spp.
3) To perform *in vitro* testing antagonistic fungi and their abilities to control *M. oryzae*.
4) To perform *in vitro* bioassay test using nano-particles of antagonistic fungi against *M. oryzae*.
5) To perform *in vivo* test nano-products of antagonistic fungi to suppress the rice blast caused by *M. oryzae.*

1.3 Scope and location of research work

This study covered from collection diseased samples, isolation and identification of *Magnaporthe oryzae* causing rice blast in Thailand. Biological control of rice blast was investigated by using antagonistic fungi *Chaetomium* spp. Antgonistic metabolites as crude extract and nanoparticles were tested *in vitro* and their microbial elicitors were tested *in vivo*.

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 and antagonistic DNA(s) were extracted and amplified at Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL).

CHAPTER 2

REVIEW LITERATURE

2.1 General of rice

It is recognized that rice production has started in many countries over 6500 years ago. The first rice cultivation was recorded in China (Hemu Du region) in 5000 B.C. as in Thailand around 4500 B.C. Lately, it has found rice cultivation in Cambodia, Vietnam and southern India etc. It derived species of *Japonica* and *Indica* and expanded to other countries eg Indonesia, Japan, Korea, Myanmar, Philippines, Pakistan, Sri Lanka. *Japonica* rice is a well-irrigated rice cultivated in temperate zone. It is a medium or short grains (round grain) and prefered a rainfed lowland of warm tropical zones. *Indica* rice is a well-irrigated rice cultivated in warm tropical zone. It is long, thin and flat grains. Morover, rice (*Oryza sativa*) was introduced to farmers in Middle East and Mediterranean Europe around 800 B.C. Thereafter, the middle of the 15th century, rice had spreaded to France and Italy. In 1694, rice is introduced toi South Carolina, U.S.A. that was probably originated from Madagascar. The Spanish brought it to South America at the beginning of the 18th century (Source: UNCTAD. org) (Gnanamanickam, 2009).

Oryza sativa is one of the major cereal grains that supports the growing world population. The population was double increased between the years of 1961 and 1999 from 3.07 to 6.05 billion. The production of these three major cereals including rice was increased 2.5–3.0 times. IR8 variety was developed as a hybrid between an Indonesian variety “Peta” and a Chinese variety “Dee Geo Woo Gen” (IRRI, 2006). IR8 rice variety became a novel rice management to increase yield for the hungers to one of food self-sufficiency in Asia. But it is not easy to overcome the goal achievement. Rice has to sustain for 3.5 billion people around the world, mostly in Asia. These achievements was tried to increase rice production to help in hungers in the world (Gnanamanickam, 2009).

2.2 Major diseases of rice

The pathogenic microorganisms are included the fungi, bacteria, virus, nematode and mycoplasma-like organisms that cause diseases to rice. The rice fungal diseases are blast caused by *Magnaporthe oryzae*(= *Pyricularia oryzae***)**, brown spot caused by *Bipolaris oryzae*, narrow brown leaf spot caused by *Sphaerulina oryzina*, stem rot caused by *Magnaporthe salvinii*, sheath blight caused by *Rhizoctonia solani*, sheath spot caused by *Rhizoctonia oryzae*, sheath rot caused by *Sarocladium oryzae*, Black kernel caused by *Curvularia* spp. The bacterial diseases of rice are bacterial blight caused by *Xanthomonas campestris* pv. *oryzae*, bacterial streak caused by *Xanthomonas campestris* pv. *oryzicola*, bacterial sheath rot caused by *Pseudomonas syringae* pv. *syringae*. The viral diseases of rice are dwarf caused by *Nephootettix cincticeps*, *N. nigropictus* and *Recilia dorsalis*, and rice tungro virus. These diseases are recorded to be seriously infected in rice production. (Zahirul *et al.*, 2004; Ou, 1985).

2.3 General of rice blast

2.3.1 History and distribution

The rice blast disease was recorded in China, the blight of rice seedlings is the first rice disease found which is stated by Song Ying-Shin who described in “Utilization of Natural Resources” in 1637. The disease was found to believe that caused by heat**,** then absorbed to the grain during drying in sunshine, the rice grains was being stored before cooled *off*. Thereafter, the seed*s w*ere sown in the field*s* in the presence of a warm, moist southeast wind, then the disease developed. The heat or fever was described like the causal agent of disease. It like the original basis for rice blast “rice fever disease”, it was a name used in Chinese and Japanese literatures (Ou, 1985).

Goto (1955) stated that the rice blast occurrence in Ichikawa Prefecture of Japan in 1704**.** The disease was recorded in Japan by S. Miayannaga in 1788, by Kojima in 1793 and Konishi in 1809. In Italy, the disease named *brusone* was reported by Astolfi (1828), Brugnatelli (1838), and Gera (1846) etc. The records of *brusone* were vagued and some authors had identified the disease as blast. It may be interacted with other diseases (Padwick, 1950). Metcalf (1906, 1907) stated that rice blast is caused the damage in South Carolina as early in 1876, and it was most seriously of eight rice diseases in the USA in 1907. He was expected to be the one who firstly called the disease "blast". Padmannabhan (1965) stated that the disease was first recorded in India in 1913 and recorded to be devastating epidemic occurred in 1919 in the Tanjore delta area of Tamil Nadu. The causal organism, *Pyricularia oryzae*, was named by Cavara in 1918, Italy and was described in Japan by Shirai in 1896 (Ou, 1985).

2.3.2 Casual organism

Rice blast is the most important and destructive rice disease that caused by *Pyricularia oryzae* (Imperfect of *Magnaporthe oryzae* Couch) which also known as *Pyricularia grisea* (Cooke) Sacc. (Couch and Kohn, 2002; Urayama *et al.*, 2010; Hosseini-Moghaddam and Soltani, 2013). The disease incidence and severity of rice blast are varied year by year depended on location and environmental conditions (Zhang *et al.*, 2009; Sukanya *et al.*, 2011; Bhat *et al.*, 2013).

However, *P. oryzae* Cavara is reported to be high pathogenic variation to host plant and specificity to each variety (Leung *et al.*, 1988). The level of pathogenic variation of *P. oryzae* isolates differs with rice varieties (Bonman *et al.,* 1987). Therefore, the studies on reaction of differential rice genotypes and molecular characterization are important in understanding pathogenic variation of *P. oryzae.* Rice blast was classified in many pathotypes depended on the infection pattern of differential rice genotypes (Kang and Lee, 2000).

2.3.3 Symptoms

Symptoms of blast disease occurs above ground parts of the rice plant. It is observed at early growing stages up to maturity. Symptoms appear on the leaves, nodes and panicles (Ghazanfar *et al.*, 2009; Urayama *et al.*, 2010). It has been reported to cause grain yield losses of up to 100 % (Prabhu *et al.*, 2009; Filippi *et al.*, 2011; Vanaraj *et al.*, 2013).

The blast pathogen produces lesions as spots on leaves, nodes, panicles and grains. The middle of spots is normally grey or whitish, and the margin is brown or reddish-brown in color. The shape and colour of spot lesions are varied which depending on environmental conditions, growing spots and susceptibility of the rice cultivar. The spots starts as small, water-soaked, whitish, greyish or bluish dots, then quickly enlarged under moist conditions on susceptible cultivars, and sometimes remain greyish. The blast symptoms are predominant in given area depend on conditions (Ou, 1987).

There are many spots which occur on leaves and are cell death soon. Rice seedlings at tiller stage are usually completely killed in the field. If the nodes are infected, then sheath rots, turned back in color and dried and all parts above the infected node died. The panicle may be infected, and produced brown lesions. The panicle base are usually infected leading to “rotten neck” or “neck rot”, and infected panicles often fall. Panicle branches and glumes are also infected (Ou, 1987). Kuilman (1940) stated that severe attack of blast causing stunting of the plant.

2.3.4 Disease cycle

The rice blast fungus starts its infection cycle when a three-celled conidium lands on the rice leaf surface. The spore attaches to the hydrophobic cuticle and germinates, producing a narrow germ tube, which subsequently flattens and hooks at its tip before differentiating into an appressorium. The single-celled appressorium matures and the three-celled conidium collapses and dies in a programmed process that requires autophagy. The appressorium becomes melanized and develops substantial turgor. This translates into physical force and a narrow penetration peg forms at the base, puncturing the cuticle and allowing entry into the rice epidermis. Plant tissue invasion occurs by means of bulbous, invasive hyphae that invaginate the rice plasma membrane and invade epidermal cells. Cell-to-cell movement can initially occur by plasmodesmata. Disease lesions occur between 72 and 96 hours after infection and sporulation occurs under humid conditions; aerial conidiophores with sympodially arrayed spores are carried to new host plants by dewdrop splash (Richard and Nicholas, 2009).

Spores produced by *Magnaporthe oryzae* (anamorph: *Pyricularia oryzae*) as the primary inoculum on the overwintering tissues produce the initial infections on young seedlings when the spores that are deposited on leaves, germinate and invade leaf tissues. Disease severity is often correlated with the amount of infested material. Lesions on the young seedlings appear within a few days after infection. These secondary lesions produce more spores and these spores are readily wind disseminated to nearby healthy leaf tissues. The secondary cycles can be repeated many times during the growing season, with the potential for very high amounts of disease within the crop. The number of cycles and the number of spores that are produced on each individual lesion can be influenced by many factors, including the temperature, rainfall, the depth of the water in the paddy, the amount of nitrogen used to fertilize the rice, and the level of genetic resistance in the cultivar that is infected. Generally, the leaf phase of the disease is most severe when daily temperatures are moderate, and when rice is over-fertilized, or if it is being grown in flood waters below the recommended depths. Under these conditions conducive to disease, very high incidences of disease have been recorded on susceptible cultivars. Spores produced near the end of the growing season may infect the collar of the flag leaf producing symptoms called collar rot. They may also infect the neck when it emerges from the infected collar upon which the head will be supported to produce a condition called rotten neck or neck blast. Yield losses are significantly correlated with the extent of rotten neck. Research has shown a 0.5% loss in yield for every 1% of rotten neck. Infections of the neck are generally considered the most deleterious phase of the disease because infection at this location can reduce seed set on the entire panicle. In addition, the fungus can infect the panicle, the branches on the panicle, and the peduncles upon which the seeds are carried on these branches. Finally, new information shows that the seed also can be infected. Infections of the neck, panicle and branches of the panicle are usually relatively nodescript grayish discolorations of the tissues (TeBeest *et al.,* 2007).

2.4 Management of rice blast

2.4.1 Varietal resistance

The blast resistant cultivars have been investigated to be the most effective strategy to protect rice crops against blast disease. The field tests had done and observes in many countries for varietal reaction to blast resistance. In United States, the blast resistant cultivar Katy (Moldenhauet *et al.*, 1990) was released for seeds and grower use in 1989, but it lacked the desirable agronomic characteriatics of Newbonnet. Also, Newbonnet sustained little damage when grown in open field under a proper moisture regime throughout the season. As a result, growers were reluctant to switch to Katy and other less productive cultivars for economic reasons, and they often did not fully appreciate the risks involved (Lee, 1994). The blast resistant variety become the most effective method to economize and control rice blast in the fields of resource-poor farmers in West Africa (Séré, 2007). Lately, *Magnaporthe oryzae* the causal agent can only overcome the blast resistance for a few years after the wide cultivation of improved resistant vareity cultivars. The races of blast pathogen are genetically changed according to the genotypes of resistance genes in the rice cultivar by breakdown of blast resistance genes. The relationship of virulence genes in the blast pathogen and resistance genes in the rice cultivar have been panciled in the gene-for-gene theory (Flor, 1971; Silué, 1992).

The races of *Magnaporthe oryzae* are established in Japan (Kiyosawa, 1967) to screen varietal resistance for blast and classified the cultivars on specific races or resistance genes. Many other cultivars were also classified, many resistant genes were found and the rice cultivars were reclassified. These research findings are clearly understood which resistance gene in each cultivar, but not to identify the overall resistance of cultivars.

There are many resistant rice cultivars had been developed against rice blast. But, as in disease-resistant cultivars of other cereals, their usefulness was short-lived, as their resistance was lost or "broke down" due to develop the new races or pathotypes of the blast pathogen (Ou, 1987).

2.4.2 Cultivation management

Effective alternative control measures to be used in the absence of acceptable cultivar resistance generally are not available. Blast severity may be reduced somewhat by planting in fields where local environment or cultural factors do not promote shading or inhibit wind circulation, avoiding excessive use of nitrogenous, and maintaining proper floodwater levels throughout the growing season. These strategies, however, often do not prevent substantial losses or are difficult for individual growers to implement (Lee, 1994).

In Japan, timing of planting has been demonstrated to be an important factor in blast development, early plantings usually having less disease than later plantings. This is explained by the fact that in early plantings under low temperature at the tiller stage and too high at the heading stage for vigorous disease development (Hashioka, 1950a, 1950b; Kuribayashi and Ichikawa, 1952). In India, Chandramohan and Palaniswamy (1963) reported on the relation between time of planting and incidence of blast and noted that severe blast was correlated with low temperature, high humidity and heavy dews.

Seedlings raised in upland nurseries are more susceptible to blast even after they are transplanted. This is explained by the lower silicon content of the epidermal cells (Ou, 1987). Otani (1949, 1950,1952) also showed that these seedlings contained more soluble nitrogen, amino acids and amines, which favoured disease development. Upland seedlings had higher physiological activities, producing more roots and absorbing more nitrogen than seedlings grown in wet nurseries (Yamada and Ota, 1956).

2.4.3 Chemical control

Since the use of commercial fungicides became routine in the early part of 19th century, many different products have been used to control rice blast disease, caused by *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*).

Chemicals have been used extensively in Japan for controlling blast disease, and virtually all rice fields have been treated with chemicals including Copper compounds, Mercuric compounds and Antibiotics (Ou, 1987). Extensive studies on antibiotics had been made in China, and Qingfengmycin (Shanghai Institute of Plant Physiology, 1975) and other have been used for blast control.

But, like other microorganism, *P. oryzae* develops strains resistant to chemicals. Yoshii *et al.* (1958) reported its resistance to phenylmercuric acetate (PMA) by repeated culture on media containing PMA. The seriousness of the problem was not realized until the effectiveness of kasugamycin was found to decrease in the field in Yamagata Prefecture, Japan, beginning in 1971, due to the frequent use of the antibiotic (Miura *et al.*, 1975). The population of resistant strains decreased in the field after the use of kasugamycin ceased (Ito and Yamaguchi, 1979).

2.4.4 Biological control

Initial screening of a range of microorganisms for the suppression of *Pyricularia oryzae* growth in vitro indicated that many species were promising candidates for use as biological control agents for blast disease (Sy *et al.*, 1983a). Eight species microorganisms were selected from the 120 microorganisms that were initially tested in vitro and in vivo based on their sound level of efficiency (Sy *et al.* 1983a,b; Sy, 1987). The highest rates of disease incidence reduction varied from 77.5% to 91.6% following folia treatment by *Trichothecium roseum*, *Chaetomium globosum*, *Micromonospora* sp. and *Trichoderma harzianum* (CP0-80). Similarly, leaf blast severity inhibition rates varied from 90.3% to 97.4% with biotreatments from *Trichoderma* sp. (str-IKP-6), *T. roseum, C. globosum, Micromonospora* sp. (PS6-2) and *T. harzianum.* When the same concentrations were used, *C. globosum* was the most effective of all the 120 microorganisms tested (Sy *et al.*, 1994).

A diverse group of biocontrol agents such as bacteria, fungi, virus exist in nature. Among them bacterial antagonist are considered ideal candidates because of their rapid growth, ease of handling and aggressive colonizing character. Bacterial antagonists, *Pseudomonas* and *Bacillus* in particular, are good candidates for biological control. As many as 23 bacterial antagonists belonging to the genera *Bacillus*, *Pseudomonas*, *Serratia*, and *Erwinia* have been found to inhibit mycelial growth of *R. solani*, while a few of them also inhibit growth of other fungal pathogens like *Sclerotium oryzae* (stem rot), *B. oryzae* (brown spot), *P. grisea* (blast), *Sarocladium oryzae* (sheath rot) and *Fusarium fuijkuroi* (bakanae). Laboratory studies also revealed that a large number of bacterial strains possess the ability to protect rice plants from diseases such as blast, sheath blight, sheath rot, and stem rot. Pseudomonas fluorescens was reported can inhibit the growth of *P. grisea* (*P. oryzae*) caused rice blast (Zahirul *et al.*, 2004; Vasudevan *et al.,* 2002).

Important fungal antagonists for rice major disease include *Trichoderma* spp., *Penicillium*, *Myrothecium verrucaria*, *Chaetomium globosum* and *Laerisaria arvalis*. (Zahirul *et al.*, 2004). The bio-efficacy of plant growth-promoting microorganisms: *Pseudomonas aeruginosa* (UPMP1), *Corynebacterium agropyri* (UPMP7), *Enterobacter gergoviae* (UPMP9) and *Bacillus amyloliquefaciens* (UPMS3), *Trichoderma harzianum* (UPMT1) and *Trichoderma virens* (UPMT2) in induction of defense-related enzymes against *Pyricularia oryzae* was evaluated in rice cultivated under aerobic conditions by Ng. *et al.* (2015). Ng. *et al.* (2015) reported that under dual culture inoculation of all PGPMs significantly reduced rice leaf blast severity at day eight after *P. oryzae* inoculation. The reduction in rice leaf blast disease severity was associated to the increase of peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activities in rice seedlings when pre-inoculated with PGPMs. The highest leaf blast disease reduction (59.17 %) occurred with rice seedlings pre-inoculated with *C. agropyri* (UPMP9), followed by *P. aeruginosa* (UPMP1) (40.65 %), *T. harzianum* (UPMT1) (42.23 %), *T. virens* (UPMT2) (20.85 %), *E. gergoviae* (UPMP9) (17.84 %) and *B. amyloliquefaciens* (UPMS3). The high efficiency of PGPM in leaf blast disease suppression was associated with significant increase in total microbial activity (FDA hydrolysis) in rhizosphera soil (4.80–7.86 μg/g/0.5 h) compared to the control (2.25 μg/g/0.5 h). Thus, the application of PGPM is a potential alternative approach in rice leaf blast disease management of aerobic rice.

Jacqueline *et al.* (2015) identified the class of the secondary metabolites produced by the mycorrhizal fungus *Waitea circinata* obtained from a Cerrado orchid and to assess the in vitro and in vivo antagonism of the extracts against *M. oryzae* (anamorph *Pyricularia oryzae*). Three mycorrhizal extracts (crude, mycelial and lyophilized) were obtained and tested. In the *in vitro* antagonism assay, *W. circinata* produced the largest inhibition zone. The crude extract reduced the mycelial growth of the pathogen by 75 %, inhibited the formation of appressoria by 100 % and reduced the AUDPC by 25 % compared to the control. The results suggested that *W. circinata* extract has the potential for rice blast control.

2.5 Phytoalexins in rice

Phytoalexins are inducible secondary metabolites possessing antimicrobial activity toward phytopathogens (Ahuja *et al.*, 2012; Großkinsky *et al.*, 2012). The rice phytoalexins that have been identified fall into two major groups: the first group includes 15 diterpenoid-type phytoalexins: momilactones A and B (Cartwright *et al.,* 1981), oryzalexin A–F (Akatsuka *et al.,* 1983; Akatsuka *et al.*, 1985; Kato *et al.,* 1993; Kato *et al.,* 1994; Kono *et al.*, 1984; Kono *et al.,* 1985; Sekido *et al.*, 1986), oryzalexin S (Kodama *et al.*, 1992a), phytocassanes A–E (Koga *et al.*, 1995; Koga *et al.,* 1997; Yajima and Mori, 2000), which are collectively known as labdane-related diterpenoids, and *ent*-10-oxodepressin, amacrocyclic diterpenoid (Inoue *et al.,* 2013). The second group includes a single phytoalexin, the flavonoidtype phytoalexin sakuranetin (Kodama *et al.*, 1992b). Among these, momilactones, phytocassanes, *ent*-10- oxodepressin and sakuranetin are considered biologically important phytoalexins because they show high antimicrobial activity in in vitro assays, demonstrating potent inhibitory activity on germ tube elongation of *M. oryzae*, with an ED_{50} value below 1–10 $\text{mg}\cdot\text{l}^{-1}$ (Cartwright *et al.,* 1977; Inoue *et al.,* 2013).

Sakuranetin is considered one of the most biologically important phytoalexins in terms of its high antimicrobial activity and high accumulation in rice leaves infected by *Magnaporthe oryzae*, a major phytopathogenic fungi (Kodama *et al.*, 2012). Momilactone A, a diterpenenoid-type phytoalexin, displayed an inhibitory effect on the growth of rice blast fungus at the site of infection in rice leaves. In contrast, no direct evidence has shown that sakuranetin contributes to fungal growth prevention and spore germination at the infection site in rice. Addressing whether sakuranetin also plays a role in the inhibition of fungal growth at the site of infection will be important (Hasegawa *et al.*, 2010; Shimizu *et al.*, 2012).

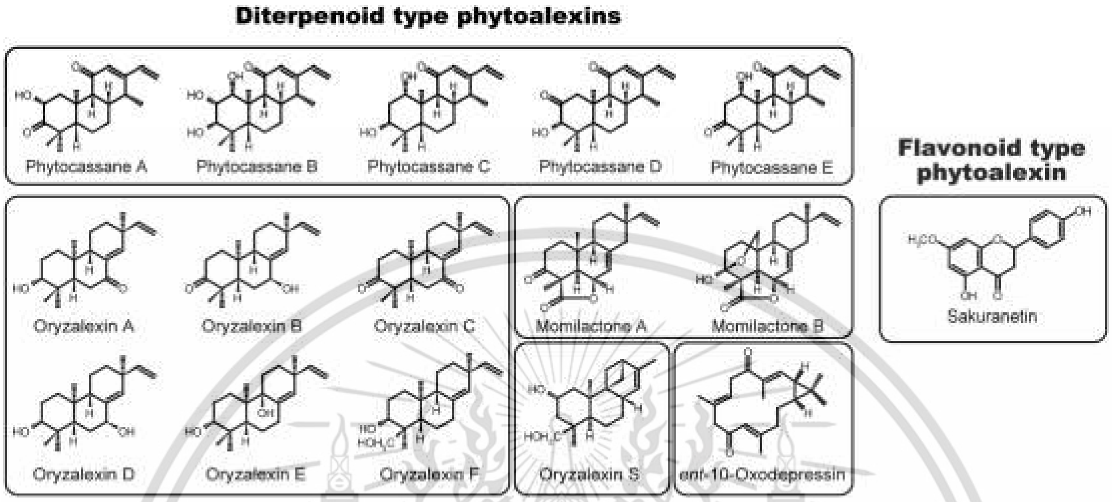


Figure 2.1. Chemical structures of rice phytoalexins (Koji *et al.*, 2004)

2.6 *Chaetomium* spp. as antagonistic fungus against plant pathogens

Chaetomium is a fungus belonging to Ascomycota of the family Chaetomiaceae which established by Kunze in 1817 (Arx *et al.*, 1986). It is one of the largest genera of saprobic ascomycetes with more than 300 species worldwide (Arx *et al.*, 1986; Soytong and Quimio, 1989). *Chaetomium* species are potential degraders of cellulosic and other organic material and can be antagonistic against various soil microorganisms. Screening of *Chaetomium* species and strains isolated from soils for use as potential biological control agents commenced in Thailand in 1989 (Soytong *et al.*, 2001).

Reports indicate that strains of *C. cupreum* and *C. globosum* are able to suppress plant pathogens such as *Curvularia lunata*, *Pyricularia oryzae* and *Rhizoctonia oryzae* in vitro (Soytong, 1989, 1992a). Viable spores of *Chaetomium* spp. were able to reduce tomato wilt caused by *Fusarium oxysporum* f.sp. lycopersici in greenhouse and field trials (Soytong, 1990, 1992b) and also prevent basal stem rot of corn caused by *Sclerotium rolfsii* (Soytong, 1991). These effective strain" of

Chaetomium have been formulated into bio-pellets and biopowders for the biological control of plant diseases and have been patented and registered under the name Ketomium® (Soytong *et al.*, 2001).

Soytong *et al*. (2005) application antagonistic fungi to control anthracnose disease of grape caused by *Colletotrichum gloeosporioides*. Assays using crude extracts from *Chaetomium cupreum* CC, *C. globosum* CG, *Trichoderma harzianum* PC01, *T. hamatum* PC02, *Penicillium chrysogenum* KMITL44. In addition the isolated antibiotic substances Rotiorinol, Chaetoglobosin-C and Trichotoxin A50 from *C. cuprem* CC, *C. globosum* CG, *T. harzianum* PC01, *T. hamatum* PC02, respectively were carried out to test bioactivity. All extracts and compounds inhibited the growth of *C. gloeosporioides* strain WMF01, with average ED_{50} values between 1 to 50 ppm. Applications of bioproducts of *Chaetomium*, *Penicillium* and *Trichoderma*, and a mixture of those bioproducts in a powder formulation and a chemical control were conducted in the field to control anthracnose disease of 5-varieties of grape. All bioproducts significantly reduced the disease incidence on leaves, twigs and fruits of grape in all varieties as compared to the chemical control.

C. brasilense and *C. cupreum* antagonized *F. oxysporum* f.sp. lycopersici NKSC02 race 2 causing wilt of tomato var sida and cherry varieties (Sibounnavong *et al.*, 2012). The metabolite assay test proved the antifungal activity of *C. brasilense* and *C. cupreum* against the sporulation of *F. oxysporum* f. sp. *lycopersici* race 2. Control mechanism of *Chaetomium* spp are concerned for inhibition of the tested pathogen. Crude extracts from *C. brasilense* and *C. cupreum* were proved to control the tested pathogen. All metabolites of crude extracts from *C. brasilense* and *C. cupreum* were significantly inhibited the sporulation of *F. oxysporum* f. sp. lycopersici race 2.

Hung *et al.* (2015b) stated that the effects of *C. globosum*, *C. lucknowense*, *C. cupreum* and their metabolites to *Phytophora nicotianae* causing rot of Pomelo. As results, *Chaetomium* spp suppressed the mycelial growth by 50~56% and antagonized the pathogen hyphae, then degraded *P. nicotianae* mycelia within 30 days. The crude metabolite extracts of *Chaetomium* spp showed antifungal activities suppressed the growth of *P. nicotianae*, with ED_{50} was 2.6~101.4 µg/mL. In greenhouse condition, application of ascospores and metabolite extracted with methanol from *Chaetomium* spp to pomelo seedlings inoculated with *P. nicotianae* that reduce root rot of pomelo by 66~71% and increased in plant weight by 72~85% compared to the control.

Chaetomium spp. are recorded to produce many metabolites that expressed different bioactivities (Li *et al.,* 2011; Zhang *et al.,* 2012) against human and plant pathogens. Most the studies described those *Chaetomium* spp. suppressed plant pathogens through productions of substance(s), which have antifungal activities. Sy *et al*. (1994) assess the efficiency spectrum of *Chaetomium globosum* towards three combinations of rice cultivars and *Pyricularia oryzae* isolates, it was found that *C. globosum* (N 76-1) provides full protection of these rice cultivars against the associated strain of *P. oryzae* with 100% inhibition of the development of leaf blast.

2.7 Nanotechnology

The nanometer defines as a metric unit of length which one billion of a meter or 10^{-9} m. The ‘nano’ is used to describe objects, systems, and phenomena with many characteristics from nanometer-scale structures. The nanotechnology defines as the building, re-structuring the materials to be molecular level. Nano is derived from the root word of *nanometer*. A nanometer (nm) defines as one-billion of a meter. The nano scales are shown for example as the width of human hair is 80,000 nm and the smallest things to see with the naked human eye are 10,000 nm across. The nano scales are the physics and chemistry basic rules (Brahic and Shanahan, 2005). The technological example is concerned the carbon nanotube that discovered in 1991, which is only a few nanometers in diameter but can conduct electricity better than copper, and 100 times stronger than steel that only one sixth of its weight (Perea-de-Lugue, 2009).

Nanotechnological application become a new strategy for agricultural development to be achieved for manufacturing the nano-pesticides, nano-fertilizer, and nano elicitor in disease elimination in poultry, plant production, food processing, and evn agricultural waste. The nanosensors to precise agricultural practices and used in livestock and fisheries. Nanotechnology become the potential to revolutionize agriculture and food systems in the areas of nano-fertilizers, pesticide career, microfluidics, nucleic acid bioengineering, nanobioprocessing, bioanalytical nanosensors, bioselective surfaces, environmental processing, pathogen detection, plant/animal production, biosecurity, molecular biology, protection of the environment to reduce and converse the agricultural materials to valuable products, design and development of new nanocatalysts to convert vegetable oils into biobased fuels and biodegradable industrial solvents, control ecological life support system (Monserate, 2013) and nano elicitors for animal and plant immunity.

Nanotechnology is a broad advanced agricultural development such as reproductive bioscience and biotechnology, conversion of agricultural and food wastes to bioenergy and other byproducts through enzymatic nanobioprocessing, disease protection and application to plants using various nanocides or nanopesticides (Carmen *et al.,* 2003) Nanoscale devices with novel properties to support the agricultural systems “smart”. There are able to respond in different situations by themselves, and an appropriate remedial action. These smart systems deliver chemicals to control and target the manner as similar to the proposed using nanodrug in human being (Roco *et al.,* 2003; Lu *et al.*, 2008).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Isolation and identification of seed-borne fungi from rice

Rice seed samples were offered by Assoc. Prof. Dr. Kasem Soytong. The varieties of rice were as follows:

1. Sanpatong sticky rice (CM);
2. Sanpatong-Napi 58 sticky rice (CM);
3. Kor-Khor 5 Napi 58 (SP);
4. Kor-Khor 6 Napi 58 (CM);
5. Kor-Khor 7 Napi 58 (CM);
6. Kor-Khor 9 Napi 58 (CM);
7. PSL2
8. Kor-Khor 11 (SP)
9. Kor-Khor 57 (SP)
10. Khao-hom-Supan Naprang 58 (SP);

Seed samples were placed in moistened 9.0 cm filter papers with sterilize distilled water in 9.0 cm petri dishes. Ten seeds per variety were arranged at the periphery of the plate. Total 200 seeds were incubated in the dark at room temperature in moist chamber for 7 days. Each individual incubated seed box was observed under stereomicroscope in order to isolate the seed-borne fungi. The fungi growing out from the seeds were transferred into potato dextrose agar (PDA) plates to isolate fungi and incubated for two to six days. Continuous plates were subcultured until get pure culture.

The isolates of seed borne fungi were firstly identified by the morphology of the fungal culture, including colony and medium color, colony characters, spore characters, mycelium characters, fruiting structures by following the appropriate keys (Ellis, 1971; Barnett and Hunter, 1987; Domsch and Games, 1993; Sutton, 1980; Nag, 1993). The sterile isolates were grown on PDA with decoction of rice leaves medium to observe sporulation. For tentative identification, microscopic slides of each isolates were prepared and examined under binocular compound microscope for morphological identification.

3.2 Study on morphological and molecular phylogeny of *Magnaporthe oryzae* causing rice blast and antagonistic fungi *Chaetomium* spp.

A total isolates of *Magnaporthe oryzae* (= *Pyricularia oryzae*) , antagonistic fungi *Chaetomium* spp. (*Chaetomium brasiliense, Chaetomium cochliodes, Chaetomium lucknowense and Chaetomium elatum*) were studied for morphological and molecular identification.

3.2.1 Morphological identification

Pathogen isolates of *Magnaporthe oryzae* from rice were grown on rice flour agar (RFA) media to observe sporulation and mycelia characters under compound microscope.

Antagonistic fungi *Chaetomium* spp. was offered from Assoc. Prof. Dr. Kasem Soytong. The growing colony on potato dextrose agar (PDA) media and characteristic of mycelia, ascocarp, asci, ascospores and hair under compound microscope were recorded.

3.2.2 Molecular identification

3.2.2.1 DNA extraction

In molecular identification, fungal genomic DNA was extracted and prepared from each *Magnaporthe oryzae* isolate and *Chaetomium* spp. Each fungus was cultured in potato dextrose broth (PDB) media for 3 days. The fungal mycelia were freeze-dried and the genomic DNA was extracted by the modified CTAB (Cetyl trimethyl ammonium bromide) method. Briefly, the fungal mycelia were cleaned with 25mM EDTA by centrifugal machine. 100 mg fungal mycelia were vigorously crushed in liquid nitrogen to make a fine powder and transferred into an eppendorf tube. The cells were lysed in CTAB buffer and β-mercaptoethanol, mixed thoroughly and incubated at 65°C for 1h with mixing tubes every 15 min. The lysate were extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 14,000 rpm in a microcentrifuge for 5 min at 4°C. The aqueous phase were transferred to a new sterile tube and added 2µl Rnase (20µg/ml), incubated 30min at 37°C. Add 50µl 10% CTAB, mix thoroughly. Repeatedly add an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 14,000 rpm in a microcentrifuge for 5 min at 4°C. Remove aqueous top layer and transfer in to new eppendorf tube. Add an equal volum of cold isoproponal, mix thoroughly and let tubes sit at -20°C for 20min. The genomic DNA was precipitated in isopropanol and centrifuged at 4°C for 20 min at 14,000 rpm. The resulting pellets were washed twice with 70% and 95% ethanol, air dried and dissolve in 100µl TE buffer at 37°C, overnight. The quality and quantity of extracted DNA samples were routinely monitored by electrophoresis in a 1% agarose gel. Quantification was performed through comparison with known dilutions of lambda phage DNA. DNAs were stored at -20°C for further use.

3.2.2.2 Polymerase chain reaction (PCR) amplification

Phylogenetic analysis of each fungus was carried out by the acquisition of three regions: ITS, *β*-tubulin and Calmodulin gene. The ITS ribosomal DNA regions were amplified by PCR using the universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990).

The 25µl reaction mixture contained 2.5µl 10 × PCR buffer, 0.625µl each dNTP (1.25mM), 0.5µl $MgCl^2$, 1µl of each primer (20pmol/µl), 2 ng of DNA and 0.2µl of Taq DNA polymerase (1 U). PCR condition for the ITS regions were programed as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 1 min., 56°C for 1min., 72°C for 2 min., and a final extension at 72°C for 5 min. The amplified products (5 µl) were visualized on 1% (w/v) agarose gel to confirm the presence of a single amplified band. And then, the PCR products were purified with PCR purified kit.

3.2.2.3 Molecular phylogenetic analysis

The amplified products were sequenced and aligned with the sequences in the GenBank by basic local alignment search tool (BLAST) analysis (Altschul *et al.*, 1997) in the National Center for Biotechnology Information (NCBI) databases to find out the sequence homology with closely related organisms. Sequences from the closely related organisms were downloaded to construct the phylogenetic trees. The closely related sequences obtained were aligned through CLUSTALW using MEGA version 6.0 software (Tamura *et al.*, 2007) and a phylogenetic tree was constructed by neighbor-joining method using the same software.

3.3 *In vitro* testing antagonistic fungi and their abilities to control *Magnaporthe oryzae*

3.3.1 Pathogenicity test

The isolates of *Magnaporthe oryzae* were tested for pathogenicity using detached leaf method in pot followed Koch's Postulate. With this, the test was conducted as described by Correll *et al.* (2009) with modifications that growing plants to the 3–4 leaf-stage (approximately 2–3 weeks) prior to inoculation. Conidia were collected from 8 - 10 days old cultures grown on RFA by washing the agar surface with sterile water. The concentration of conidia was adjusted to 1×10^5 conidia per milliliter. Plants were incubated in a dew chamber at 100% relative humidity (RH) for 24 h and scored for disease symptoms. Controls were sprayed with water.

Plants were evaluated for disease reaction 7 days after inoculation using a 0–9 scale (IRRI evaluation system and Xia *et al.*, 1993) where 0 =no infection observed;1 = small brown specks, pinpoint infections small < 1mm; 2 = small rounded infections with open centers, lesion small < 2mm; 3 = small infections with open centers beginning to expand but small < 3mm; 4 = typical susceptible blast lesions with expanding open centers > 3mm on < 10% of the leaf area evaluated; 5 = susceptible type lesions with expanding open centers on 10–25% of the leaf area evaluated; 6 = susceptible type lesions with expanding open centers on 26–50% of the leaf area evaluated; 7 = susceptible type lesions with expanding open centers on 51–75% of the leaf area evaluated; 8 = susceptible type lesions with expanding open centers on 76–90% of the leaf area evaluated; 9 = susceptible type lesions with expanding open centers on > 90% of the leaf area evaluated. A mean reaction of 0–3.5 was considered resistant, 3.5–4.5 intermediate and ≥ 4.6 susceptible. The differential cultivars were grouped based on their disease reaction (either resistance with a disease rating of 0–3 or susceptible with a disease rating of 4–9).

3.3.2 Bi-culture antagonistic tests

Chaetomium lucknowense, *Chaetomium brasiliense*, *Chaetomium cochliodes* and *Chaetomium elatum* were tested against *Magnaporthe oryzae* by bi-culture method. The experiment was conducted using a Completely Randomized Design (CRD) with 4 replications by the methods of Soytong (1992c), Sibounnavong *et al.* (2009a) and Charoenporn *et al.* (2010). The antagonistic fungi and pathogen were separately cultured on PDA with rice flour media at room temperature (30- 32 ºC) for 7 days. And 0.5 cm diameter sterilized cork borer was used to remove agar plugs from the actively growing edge of cultures of the antagonistic fungi and pathogen and then transferred onto the same sterilized 9 cm-diameter PDA plates, an agar plug of the pathogen was placed on one side of the plate which opposited an agar plug of an antagonistic fungus. The single plug of antagonistic fungi and pathogen were transferred into two separate PDA plates as the controls. Plates were incubated at room temperature (30-32 ºC) for 30 days. Data were collected regarding to diameter of colony (cm) and the number of conidia produced by the pathogen in the bi-culture plates and control plates. A haemacytometer was used to count the number of conidia of pathogen.

Percentage inhibition of pathogen colony growth and conidia production were calculated using the following formula: % inhibition = (A-B) / A ×100.

Where, A is the diameter of colony or number of conidia produced by the pathogen on the control plates and B is the diameter of colony or number of conidia produced by the pathogen in the bi-culture plate.

Analysis of variance was statistically computed and treatment means were compared using Duncan Multiple’s Range Test (DMRT) at P = 0.05 and 0.01.

3.3.3 Crude extraction of antagonists

The antagonists *Chaetomium* spp. were separately cultured in potato dextrose broth (PDB) at room temperature for 45 days. The fungal biomass of each fungus were removed from PDB, filtered through cheesecloth and air-dried overnight. Fresh weight and dry weight of fungal biomass were weighted. The fungal biomass were ground with electrical blender， and placed in conical flask. And then were extracted with equal volume hexane 3-5 days at room temperature and stirred oftentimes and then the biomass was separated by filtration through Whatman filter paper. The solvents were then evaporated *in vacuo* to yield crude hexane extract. The marc was further extracted with ethyl acetate and methanol respectively, using the same procedure as hexane and yield crude ethyl acetate (EtOAc) and crude methanol (MeOH) extracts. Extraction method is shown in the Figure 3.1 as follows:

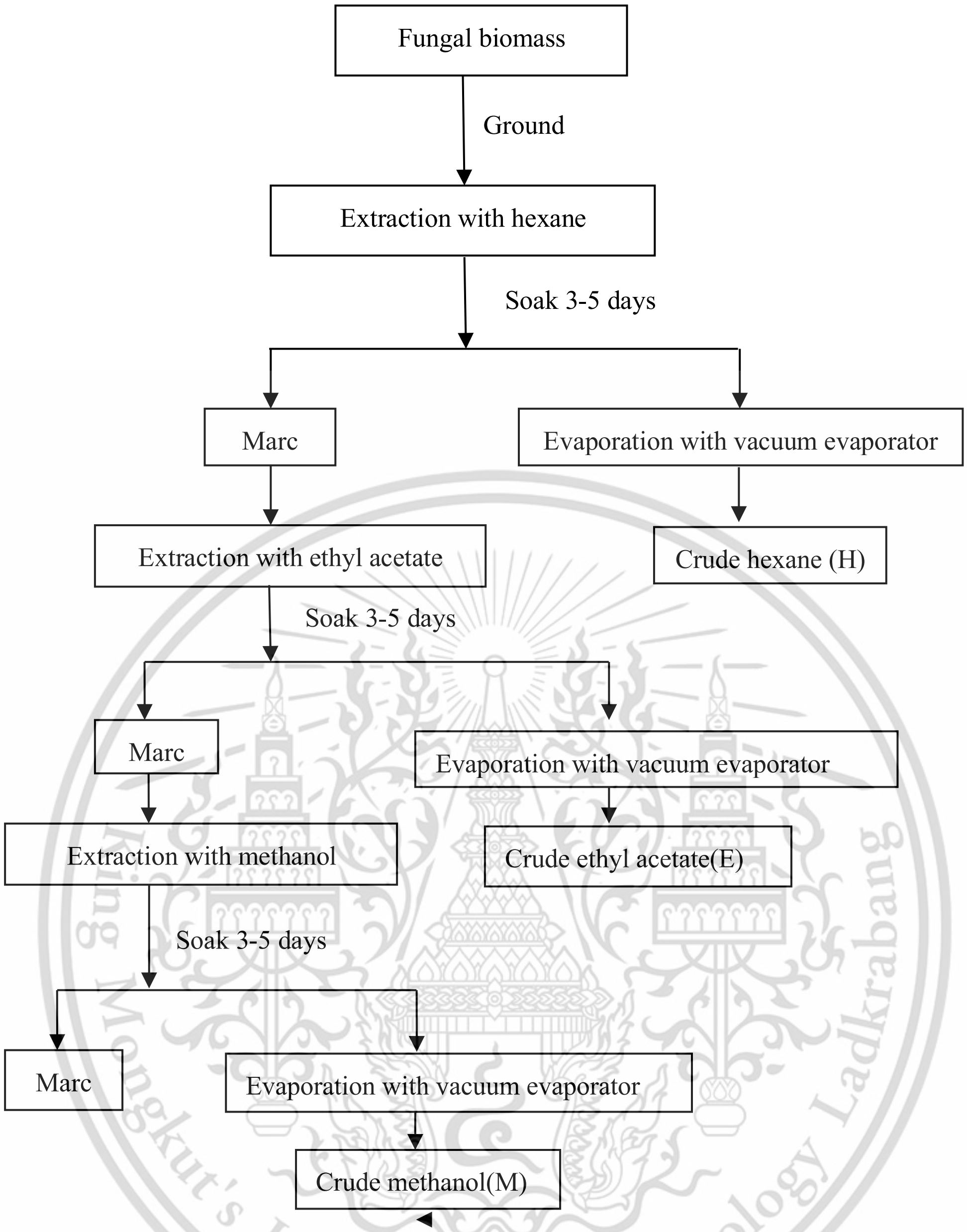


Figure 3.1 Flow chart of crude extraction of fungal biomass method

3.3.4 Testing biological activity of crude extracts from antagonistic fungi against *Magnaporthe oryzae*

The crude extracts of antagonist were tested for inhibition of *Magnaporthe oryzae.* The experiment was conducted by using 3×6 factorial in completely Randomized Design (CRD) with four replications. Factor A was represented crude extracts (including hexane extract, ethyl acetate extract and methanol extract) and factor B was represented the different concentrations of crude extracts: 0, 10, 50, 100, 500 and 1,000 ppm. Each crude extract was dissolved in one drop 2% dimethyl sulfoxide (DMSO), and then mixed into 30 ml RFA medium before autoclaving at 121℃,15P for 30 mins. The tested pathogen was cut at the edge of colony with sterilized suction tubes (3 mm), and the agar plug of pathogen were transferred to the middle of RFA medium (amending with each crude extract) plate (5.0 mm diameter) in each concentration and incubated at room temperature until the pathogen on the control plates growing full. Observation of abnormal spores and normal spore of pathogen from each treatment was observed under compound microscope and take photograph for comparison. The data were collected as colony diameter and the number of conidia. Analysis of variance was statistically computed and treatment means were compared using Duncan Multiple's Range Test (DMRT) at P = 0.05 and 0.01. The effective dose (ED_{50}) were calculated using probit analysis.

3.4 *In vitro* bioassay test using nano-particles of antagonistic fungi against *Magnaporthe oryzae*

Preparation of nano particles of antagonist fungi was followed the method of Dar and Soytong (2014). The nano-products from crude extracts (Nano-H, Nano-E, Nano-M) were assayed for their ability to control *M. oryzae*. Experiment was designed by using two factors factorial experiment in CRD with four replications.

The treatment combination was designed as follows:

Factor A: nano-particles	**Factor B:** concentration
A1= nano-H	B1= 0 ppm
A2= nano- E	B2= 3 ppm
A3= nano-M	B3= 5 ppm
	B4= 10 ppm
	B5= 15 ppm

Each Nano-particle was dissolved in one drop 2 % dimethyl sulfoxide (DMSO), and then mixed into 30 ml RFA medium before autoclaving at 121 ℃, 15 lbs/inch^2 for 30 min. The culture of *M. oryzae* was cut at the edge of colony with sterilized cock borer (3 mm). Agar plug of pathogen were transferred to the middle of

RFA media in plate (5.0 mm diameter) incorporated with each nano-particles. The transferred plates were incubated at room temperature until the pathogen in control plates growing full. Abnormal and normal spores of pathogen from each treatment were observed under binocular compound microscope and taken photograph for comparison. The data were collected as colony diameter and the number of spores that counted by using Haemacytometer. Analysis of variance was statistically computed and treatment means were compared using Duncan Multiple's Range Test (DMRT) at P = 0.05 and 0.01. The effective dose (ED_{50}) were also calculated using probit analysis.

3.5 *In vivo* nanoparticles constructed from antagonist against rice blast disease

3.5.1 Nanoparticles from mixture crude metabolites of *C. cochliodes* against rice blast disease

The experimental design was used as randomized complete block (RCB) with four replicates. The treatments were performed as follows: Treatment 1 was the non-inoculated control, Treatment 2 was the inoculated control, Treatment 3 was the nanoparticles from a crude extract mixture of *C. cochliodes* CTh05 at 10 ppm, and Treatment 4 was the chemical fungicide (tricyclazole) at the recommended rate of 2.25g L. The data were recorded as the fresh and dry weight of the stems at 90 d, and computed analysis of variance. Mean comparison in each treatment was done by SPSS software, ver. 21.0, and significance was declared at $P \leq 0.05$ and 0.01. Plants were assigned a disease index at 7 d post-inoculation using a scale of 0–9 (modified from Xia *et al*., 1993) where 0 = no infection, 1 = small brown spot infection < 1 mm; 2 = small rounded spot infection < 2 mm; 3 = small spot infection with open centres < 3 mm; 4 = lesions with expanded open centres > 3 mm on < 10% of the leaf area; 5 = lesions with expanded open centres on 10–25% of the leaf area; 6 = lesions with expanded open centres on 26–50% of the leaf area; 7 = expanded lesions with open centres on 51–75% of the leaf area; 8 = expanded lesions with open centres on 76–90% of the leaf area; 9 = expanded lesions with open centres on > 90% of the leaf area.

3.5.2 Nanoparticles from each crude metabolite of *C. cochliodes* against rice blast disease

This experiment was designed as a RCBD with four replications. Nanoparticles derived from hexane, ethyl acetate and methanol crude extracts of *C. cochliodes* CTh05 were separately applied at a concentration of 7 ppm to the rice seedlings inoculated with *M. oryzae* PO1. The treatments were done as follows: non-inoculated control (T1), inoculated with *M. oryzae* PO1 (T2), nano-CCoH (T3), nano-CCoE (T4), nano-CCoM (T5) and tricyclazole (T6). The disease index (DI) was evaluated as described above and disease reduction (DR) was calculated as follows:

$$\text{DR} = \text{averaged DI in treatment} - \frac{\text{averaged DI in inoculated control}}{\text{averaged DI in treatment}}$$

3.6 Testing nano-elicitors for phytoalexin production

Determination of phytoalexin in rice was preliminary investigated by thin layer chromatography (TLC). Phytoalexin production was investigated using the treated nanoparticles CB and the not-treated served as controls. Twenty-day old rice seedlings of var PSL 2 were inoculated with *M. oryzae* and sprayed with nano-CBH at a concentration of 15 ppm. The leaf samples were taken at 3, 6 and 9 days after inoculation. Each fresh leaf sample was weighted at 1.2 g and cleaned in methanol, then cut into small pieces and ground, soaked in 10 mL methanol, put in waterbath at 50 °C for 10 minutes, passed through Whatman filter paper No. 4. The filtrate was then evaporated with rotary vacuum evaporator to get crude extract. The crude extracts were added 3 mL of methanol, 1 mL ethyl acetate and kept for the trials. Detection of phytoalexin by TLC was done by preparing solvent of benzene and ethyl acetate at the ratio of 10:1. The TLC tank was added 2 mL TLC solvent. TLC plate was spotted with a crude extract sample and standard for comparison, then observed under UV light at 365 nm, soaked in anisaldehyde solvent, then dried and heated until spots appeared. R_f value was calculated and compared with standard. The experiment was repeated three times. The R_f value is calculated as: R_f = distance spot travels/ distance mobile phase travels.

CHAPTER 4

RESULTS

4.1 Isolation and identification of seed-borne fungi from rice and rice leaf blast pathogen

Rice seeds of 10 varieties were used and yielded 53 isolates as seed-borne fungi. There were firstly morphology identified as *Curvularia* spp. 9 isolates, *Fusarium* spp. 18 isolates, *Rhizoctonia* spp. 4 isolates, *Alternaria* spp. 6 isolates, *Cordana pauciseptata* 3 isolates, *Penicilium* spp. 6 isolates, *Trichoderma* spp. 6 isolates and *Magnaporthe oryzae* 1 isolate PO1 (Figure 4.1) as seen in Table 4.1.

Table. 4.1 Seed borne fungi from 10 rice varieties

Rice varieties	***Cuvularia* spp.**	***Fusarium* spp.**	***Rhizoctonia* spp.**	***Alternaria* spp.**	***Cordana pauciseptata***	***Penicilium* spp.**	***Trichoderma* spp.**	***Magnaporthe oryzae***
Sanpatong (R 1)	1	2	2	-	-	-	1	-
Sanpatong-Napi58(R 2)	-	3	-	1	1	-	-	-
Kor-Khor 5 (R 3)	2	-	-	1	-	2	1	-
Kor-Khor 6 (R 4)	1	1	1	-	-	-	1	-
Kor-Khor 7 (R 5)	1	2	-	-	-	-	1	-
Kor-Khor 9 (R 6)	1	3	-	1	1	-	-	-
PSL2(R 16)	1	2	-	1	-	1	-	-
Kor-Khor 11(R 17)	-	2	1	-	-	-	1	-
Kor-Khor 57(R 18)	1	2	-	1	1	1	1	1
Khao-hom-supan(R19)	1	1	-	1	-	2	-	-

The pathogen isolated from symptomatic leaves of rice var. PSL 2, Kor-Khor 57 (RD 57) and Kor-Khor 9 (RD 9) were firstly morphologically identified as *Magnaporthe oryzae*, isolate PO2 (Figure 4.2) from rice var. PSL 2, isolate PO3 (Figure 4.3) from Kor-Khor 57 (RD 57) and isolate PO4 (Figure 4.4) from Kor-Khor 9 (RD 9). The isolates were cultured on RFA which covered the plate (9 cm) in 10 d. The mycelia were observed to be septate and hyaline, producing conidiophores and three-celled obpyriform conidia.

4.2 Study on morphological and molecular phylogeny of *Magnaporthe oryzae* causing rice blast and antagonist fungi *Chaetomium* spp.

Magnaporthe oryzae isolates PO1, PO2, PO3, PO4 and antagonistic fungi *Chaetomium* spp. (*Chaetomium brasiliense, Chaetomium cochliodes, Chaetomium lucknowense and Chaetomium elatum*) were studied for morphological and molecular identification.

4.2.1 Morphological identification

Magnaporthe oryzae species were isolated from rice seed and rice leaf with blast symptom which coded as PO1, PO2, PO3 and PO4 as seen in Figures 4.1, 4.2, 4.3 and 4.4. The isolates were grown on rice flour agar (RFA) media to observe sporulation and mycelia characters under compound microscope. Colonies effuse, thinly hairy, greyish brown or olivaceous brown. Mycelia immersed, stroma none. Conidiophores mononematous, slender, thin-walled and usually emerging in small groups through stomata, mostly unbranched. Conidia solitary, dry, simple, obpyriform or obclavate, hyaline to pale olivaceous brown, smooth, mostly 2 septate, 3 celled.

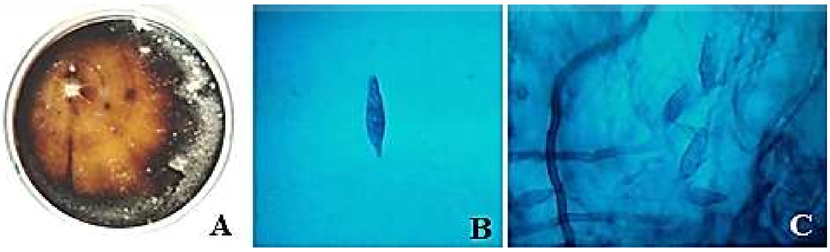


Figure 4.1 *Magnaporthe oryzae* PO1, A = pure culture in RFA; B = conidium; C = mycelia and conidia

Figure 4.2 *Magnaporthe oryzae* PO2, A = pure culture in RFA; B = conidia; C = mycelia and conidia

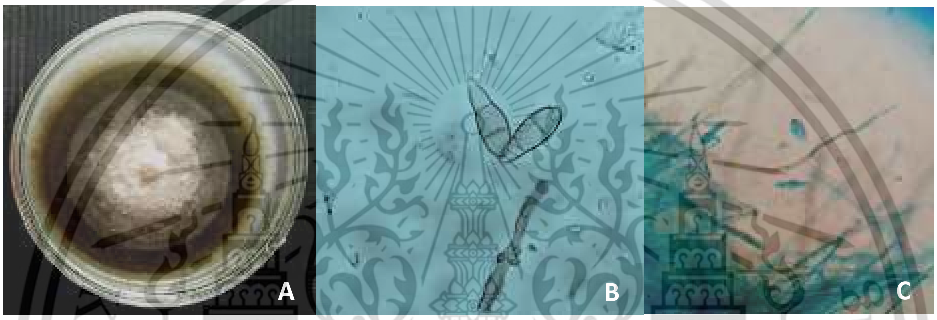


Figure 4.3 *Magnaporthe oryzae* PO3, A = pure culture in RFA; B = conidia; C = mycelia and conidia

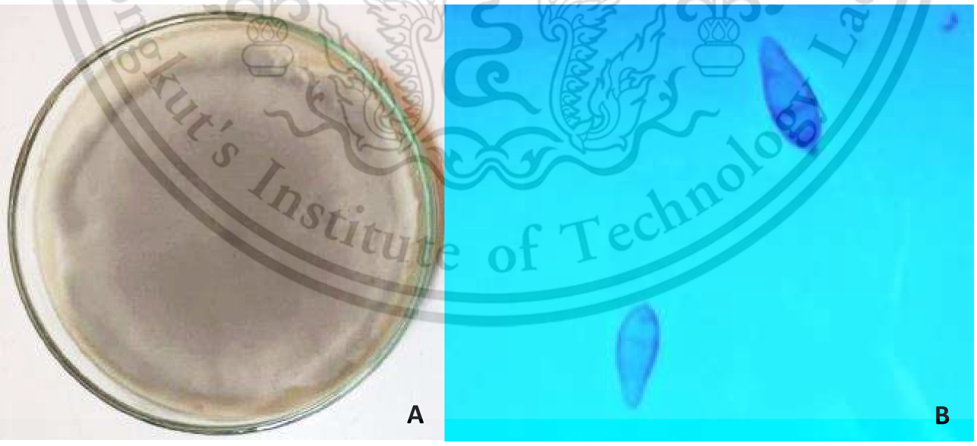


Figure 4.4 *Magnaporthe oryzae* PO4, A = pure culture in RFA; B = conidia

Antagonistic fungi *Chaetomium* spp. were grown to produce colony on potato dextrose agar (PDA) for 3 weeks to record the characteristic of colony and spores.

Chaetomium species were observed mycelia, ascocarp, asci, ascospores and hair under compound microscope.

Chaetomium elatum with dark olive-brown ascocarp, simple unbranched lateral hair, branched terminal hairs, club-shaped asci with 8 spores, ascospores dark olive-brown, lemon shaped as seen in Figure 4.5.

Chaetomium brasiliense was brown to black ascocarp, clothed with grey hairs, dark olive-brown terminal and lateral hairs, cyclindrical asci with 8 spores, ascosppores uniseriate light olive-brown, broadly ovate (Figure 4.6).

Chaetomium cochliodes was olive-green to brown, producing perithecia and subglobose asci, one asci containing eight ascospores as showed in Figure 4.7.

Chaetomium lucknowense was olive-green to brown, producing perithecia and subglobose asci, one asci containing eight ascospores, ascospores dark olive-brown, lemon shaped (Figure 4.8).

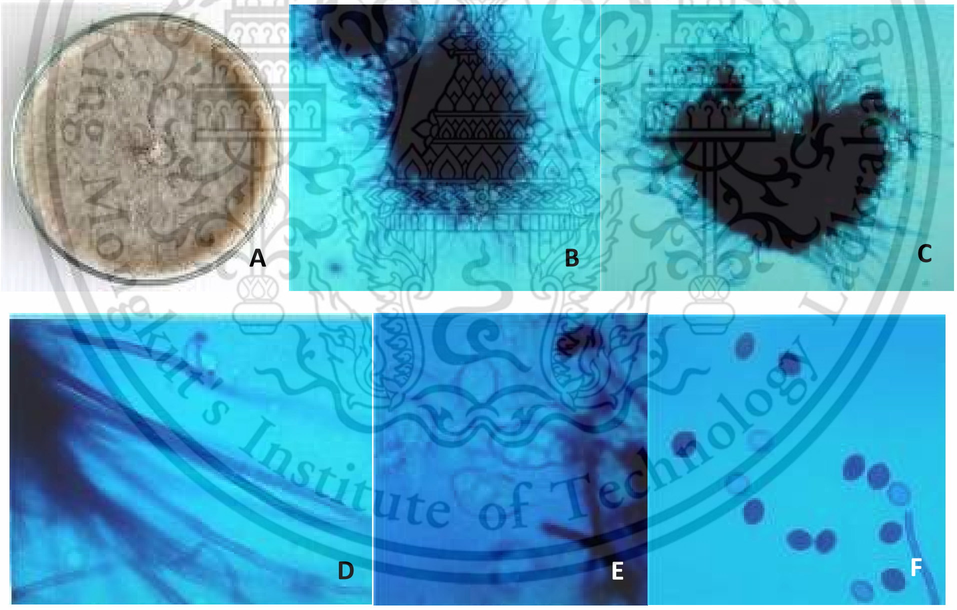


Figure 4.5 *Chaetomium elatum* , A= colony, B, C=Ascocarps, D= terminal hairs; E = asci; F= ascospores

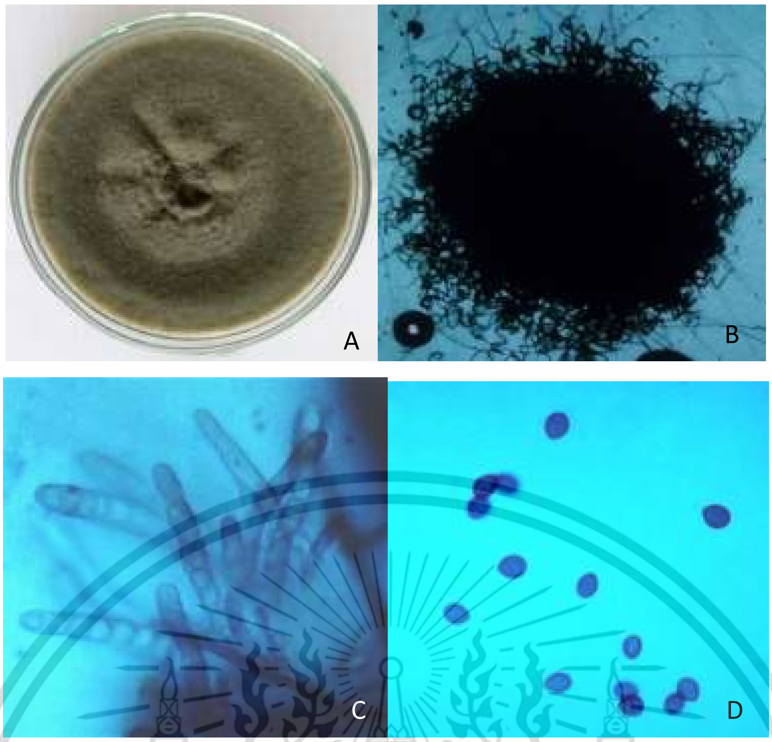


Figure 4.6 *Chaetomium brasiliense* A=colony, B=ascocarps, C=asci, D=ascospores

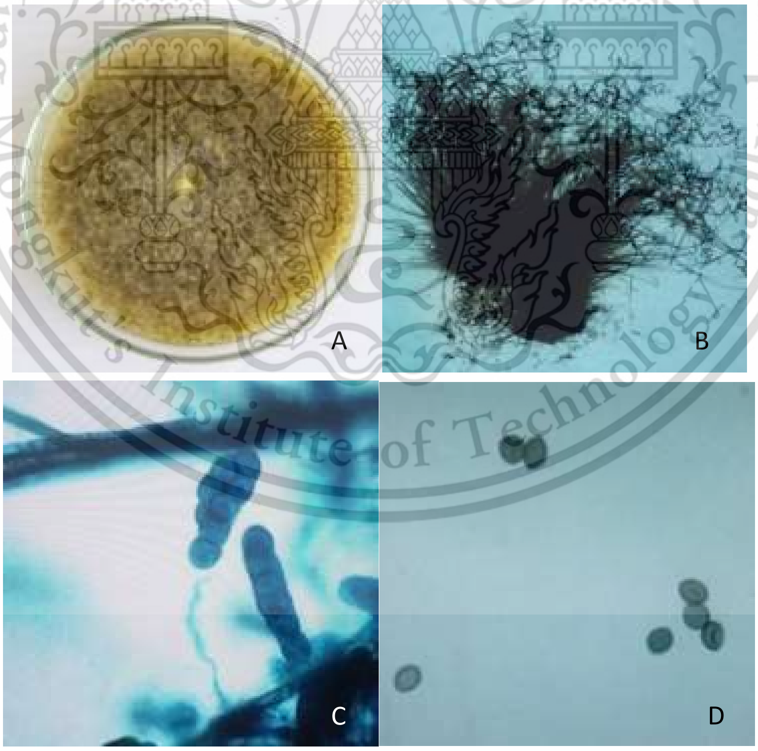


Figure 4.7 *Chaetomium cochliodes* A=colony, B=ascocarps, C=asci, D=ascospores

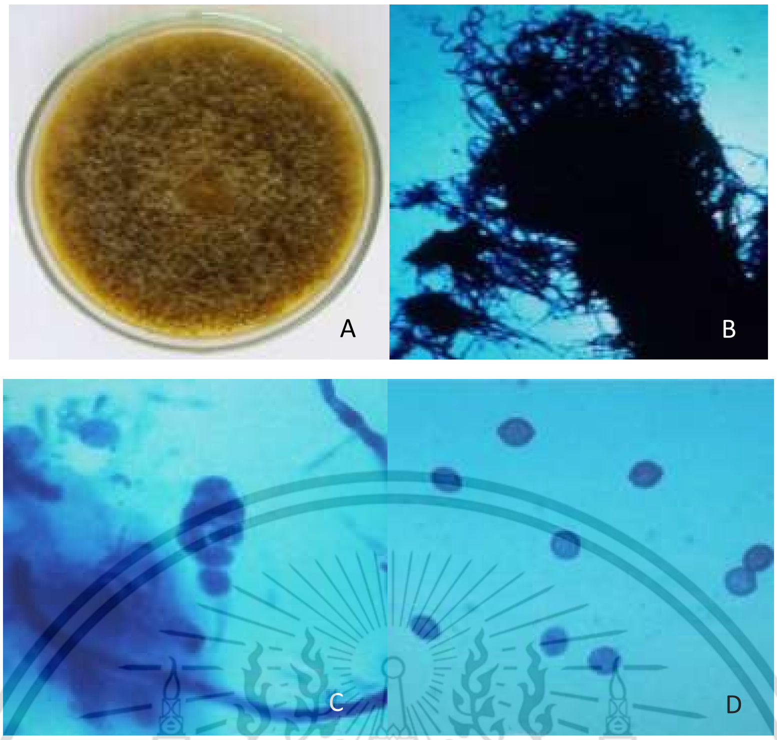


Figure 4.8 *Chaetomium lucknowense* A=colony, B=ascocarps, C=asci, D=ascospores

4.2.2 Molecular identification

Pathogen isolates *Magnaporthe oryzae* PO1 and PO2 were separately cultured in potato dextrose broth (PDB) and incubated at room temperature (30 ºC) for 5 days and confirmed species by molecular phylogenetic identification. Primers ITS1 and ITS4 were used to amplify internal transcribed spacer (ITS) regions 5.8S ribosomal DNA fragments of all 3 pathogen isolates. Both PCR products of isolates were about 530 kb. 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 PO1 shared 99% identity with those of *Magnaporthe oryzae* under accession number MH895782, MH443357(Figure 4.9). Isolate PO2 shared 99% identity with those of *Magnaporthe oryzae* accession number MH669475, MF678836, MF668692 and MF 668691. The phylogenic tree clearly identified the rice blast pathogen isolates Po1 and PO2 as *Magnaporthe oryzae* based upon the GenBank database as showed in Figure 4.9. Furthermore, nucleotide sequences of the ITS ribosomal DNA fragments of isolates PO1 were then determined and deposited in the GenBank under accession number MH590369 (Figure 4.9).

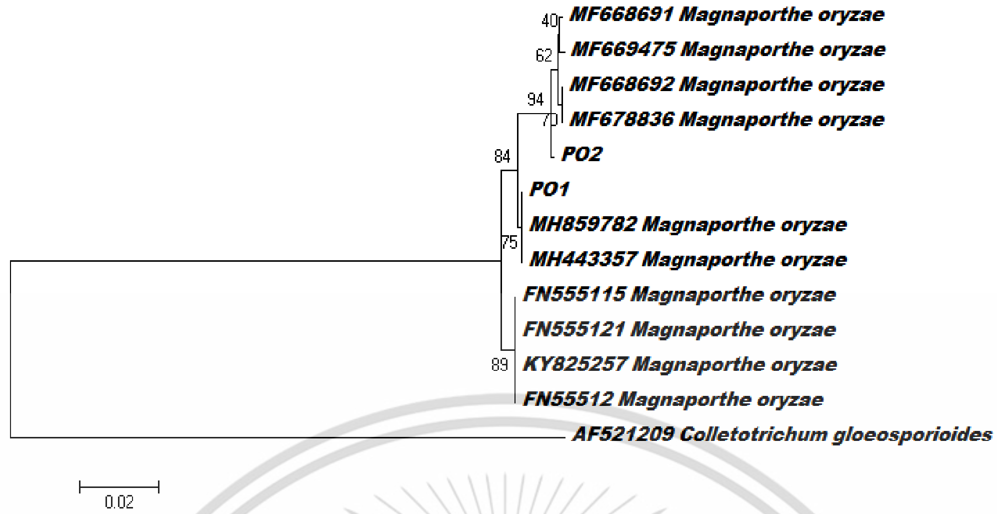


Figure 4.9 Phylogenetic tree of *Magnaporthe oryzae* from GenBank of *Magnaporthe oryzae* PO1 and PO2, constructed based upon the distance-based analysis of the ITS1 and 5.8S regions of rDNA. The numbers at the branches indicate the percentage of bootstrap values after 1000 replications

Meanwhile, the antagonistic fungi *Chaetomium* spp. were also investigated to confirm species by molecular method. Same primers ITS1 and ITS4 were used for amplifying the ITS regions of *Chaetomium* spp. The cluster revealed that the phylogenetic confirmations of *Chaetomium elatum* (CE) is closely related to MH856469, MH859003, MH864188, MN534815, MN534813, MN534809, which database supporting by 100% bootstrap value. *Chaetomium lucknowense* (CL) is closely related to MH080360, JX280827, which database supporting by 99% bootstrap value and *Chaetomium brasiliense* (CB) is closely related to KF 680267, KT357687, KF680266, KX146504, KT357682, KT357646, JX966545, which database supporting by 100% bootstrap value. Data from the GenBank reliably confirmed *Chaetomium cochliodes* (CCo) with a high degree of relatedness to HQ316565, followed by KT895345, JN209868 and JN209864 (Figure 4.10).

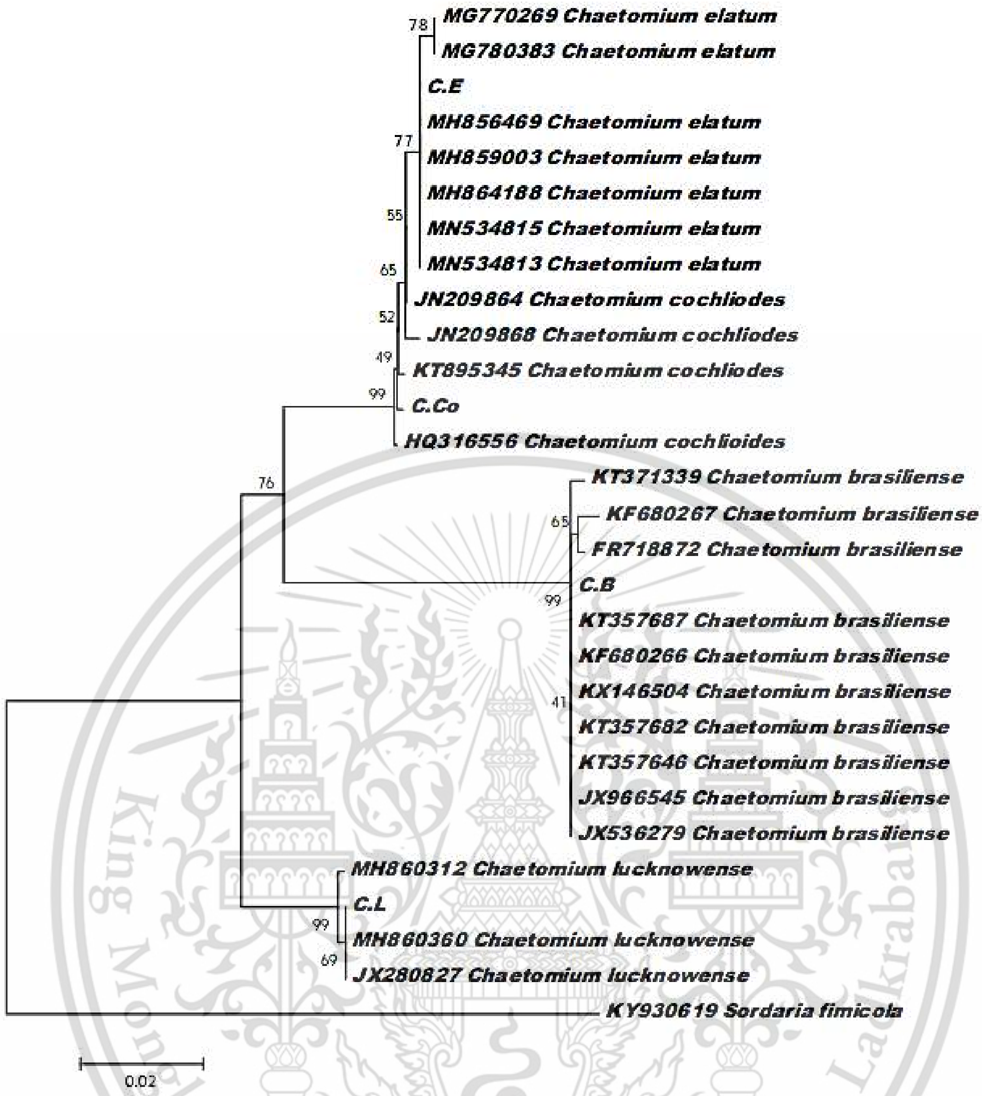


Figure 4.10 Phylogenetic relationships between *Chaetomium* spp. and related taxa inferred using a neighbor joining method with internal transcribed spacer (ITS) rDNA sequence, Bootstrap value based on 1,000 replications is shown above the branch

4.3 *In vitro* testing antagonistic fungi and their abilities to control *Magnaporthe oryzae*

4.3.1 Pathogenicity test

The isolate *Magnaporthe oryzae* PO1 was selected to confirm pathogenic isolate from pathogenicity test in vivo on rice var. RD57. The result showed that isolate PO1 infected in the rice leaves of Kor-Khor 57 (RD57) and caused blast symptoms with disease index of level 5 (Figure 4.11). *Magnaporthe oryzae* isolate

PO1 was the highest pathogenicity and rice var. Kor-Khor 57 (RD57) also showed high susceptibility to pathogen PO1, then the *M. oryzae* isolate PO1 and rice var. Kor-Khor 57 (RD57) were selected for the future studies.

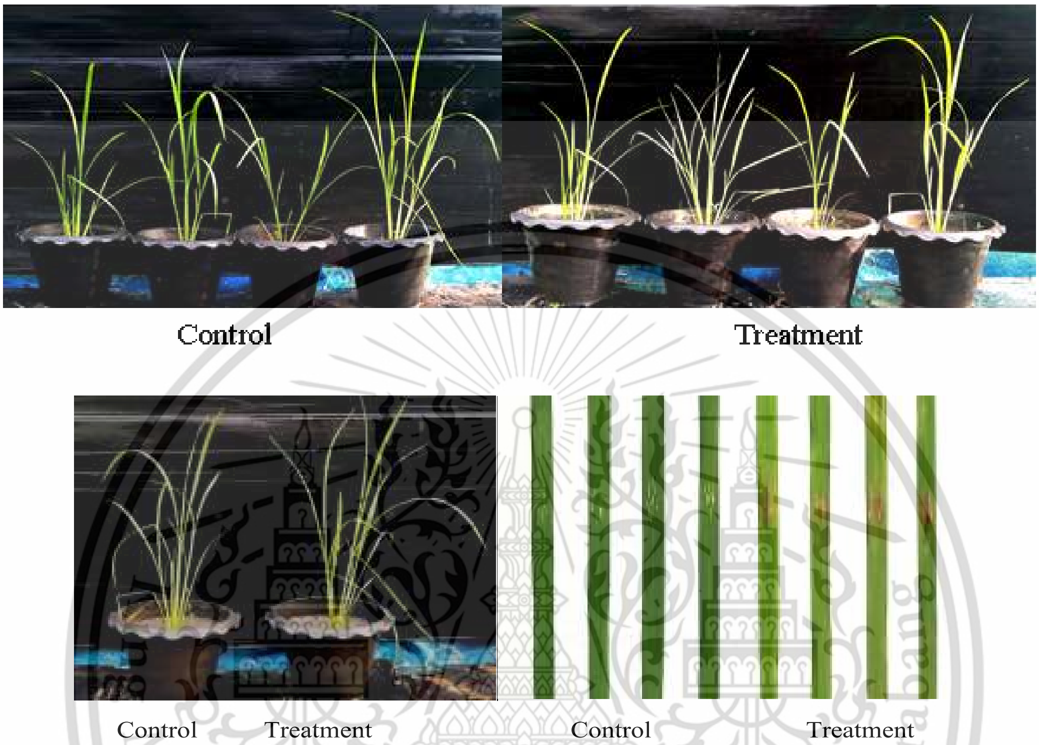
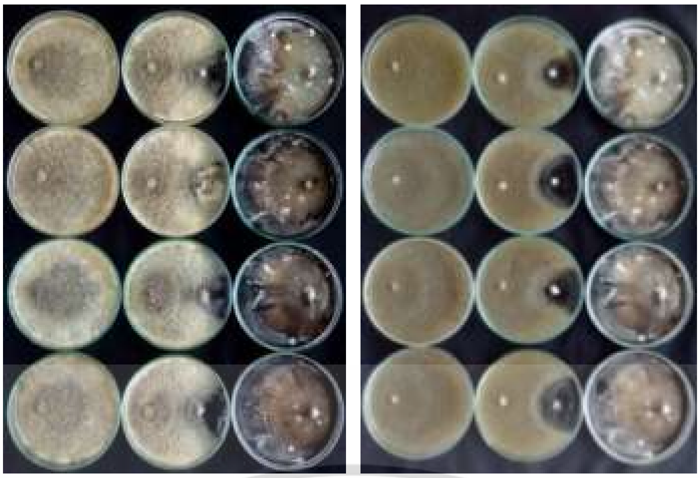


Figure 4.11 Pathogenicity test of *Magnaporthe oryzae* isolate PO1 on rice leaf of RD57

4.3.2 Bi-culture antagonistic tests

The antagonists were tested *in vitro* for proved its abilities to inhibit the growth of *Magnaporthe oryzae* isolate PO1 by using bi-culture technique (Figure 4.12). The antagonistic plates, bi-culture plates and pathogen plates were cultured 10 days and the pathogens colony diameter were measured for data analysis by DMRT (Table 4.2).



Antagonist Bi-culture Pathogen Antagonist Bi-culture Pathogen

***Chaetomium elatum* (CE) *Chaetomium cochliodes* (CCo)**

Antagonist Bi-culture Pathogen Antagonist Bi-culture Pathogen

***Chaetomium brasiliense* (CB) *Chaetomium lucknowense* (CL)**

Figure 4.12 Bi-culture tests between antagonists *Chaetomium elatum* (CE), *Chaetomium cochliodes* (CCo), *Chaetomium brasiliense* (CB), *Chaetomium lucknowense* (CL) and pathogen *Magnaporthe oryzae* isolate PO1

As the results showed in Table 4.2 and Figure 4.13, after culture 10 days, antagonist fungi *Chaetomium elatum* (CE) gave the highest colony growth inhibition of pathogen *Magnaporthe oryzae* isolate PO1 with 60.40% inhibition percentage when compared with control, followed by *Chaetomium cochliodes* (CCo) gave the higher inhibition percentage of pathogen colony growth as 51.66% than *Chaetomium brasiliense* (CB) and *Chaetomium lucknowense* (CL). When the incubation period was extended to 30 days, the colony grown over the pathogen and pathogen growth inhibition more over 50 %, even higher 90% in *Chaetomium elatum* (CE) bi-culture plates as seen in Figure 4.12.

Table 4.2 Colony growth of *Magnaporthe oryzae* on bi-culture tests at 10 days

Antagonist fungi	***Magnaporthe oryzae***	
	Colony diameter(cm)	inhibition of colony(%)
Control	$9.00^{a1/}$	-
Chaetomium elatum	3.56^{e}	$60.40^{a2/}$
Chaetomium cochliodes	4.35^{d}	51.66^{b}
Chaetomium brasiliense	6.63^{b}	26.24^{d}
Chaetomium lucknowense	5.83^{c}	35.13^{c}
CV%	1.20	2.11

1/: Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01

2/Inhibition(%)=R1-R2/R1x100, where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in bi-culture plates.

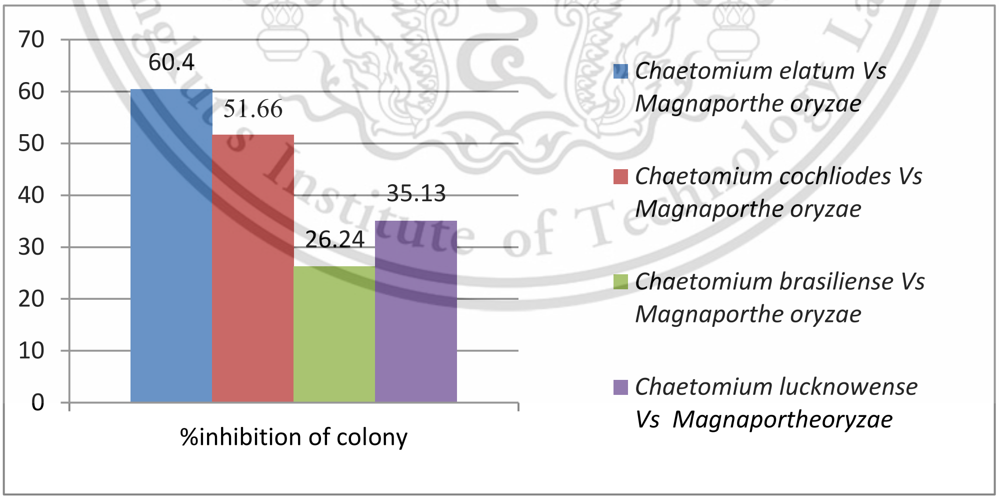


Figure 4.13 Colony growth inhibition percentage of pathogen PO1 after culture for 10 days

4.3.3 Crude extraction of antagonists

Each antagonist was cultured 500 plates in PDB for 30 days and gathered dry biomass around 100 to 150 grams, then yielded crude extracts with hexane, ethyl acetate and methanol as 0.57, 7.25 and 8.21 g, respectively for *Chaetomium brasiliense* (CB); 0.75, 6.43 and 9.55 g, respectively for *Chaetomium lucknowense* (CL), 0.66, 5.58 and 88.03 g, respectively for *Chaetomium elatum* (CE), 0.85, 8.22 and 10.32 g, respectively for *Chaetomium cochliodes* (CCo). The colour and texture of each crude extracts from antagonist metabolites were showed in Figure 4.14.

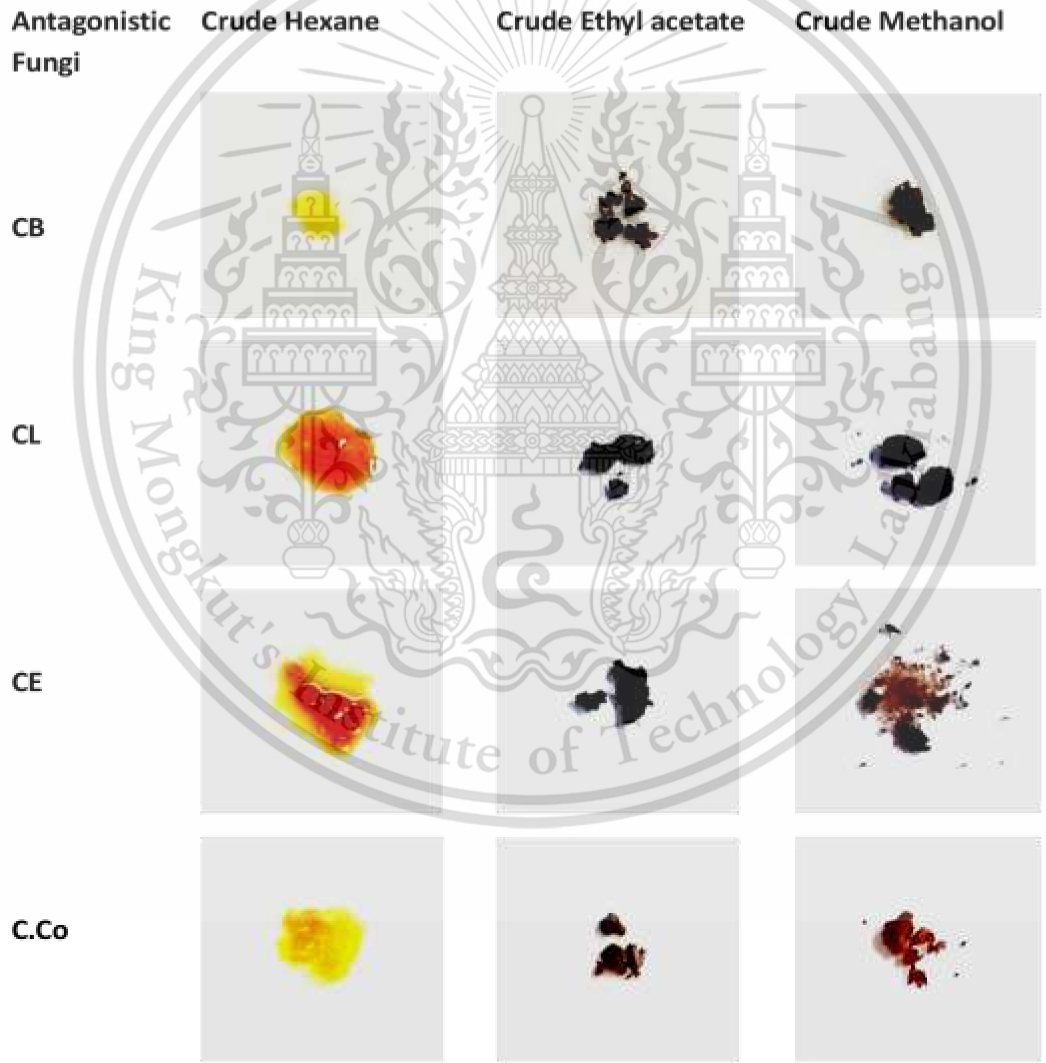


Figure 4.14 Crude extracts of bioactive metabolites from each antagonis; CB=*Chaetomium brasiliense*, CL=*Chaetomium lucknowense*, CE=*Chaetomium elatum*, CCo=*Chaetomium cochliodes*

4.3.4 Testing biological activity of crude extracts from antagonistic fungi against *Magnaporthe oryzae*

The results demonstrated that EtOAc crude extracts of *C. elatum* (CEE) resulted in the highest spore inhibition of 85.74 %, followed by crude methanol (CEM) and crude hexane (CEH), which resulted in a spore inhibition of 75.55 % and 66.66 %, respectively, in 12 d at 1,000 ppm. The fungal metabolites of CEH, CEE and CEM exhibited active antifungal activity against the *M. oryzae* isolate PO1 with ED_{50} values of 266.82, 106.98 and 188.34 ppm (Table 4.3 and Figure 4.15). Hexane crude extract of *C. brasiliense* (CBH) gave highly significant inhibition of spore production as 93.20 %, which was higher than EtOAC crude exrtract (CBE) and MeOH (CBM) crude extract with the spore production inhibition as 92.46, and 84.98, respectively. The fungal metabolites of CBH, CBE and CBM exhibted active antifungal activity against the *M. oryzae* isolate PO1 with ED_{50} values of 35.49, 55.91 and 119.06 ppm (Table 4.4 and Figure 4.6). EtOAC crude extract of *C. lucknowense* (CLE) gave significantly highest spore production inhibition as 84.92 % with the ED_{50} value of 57 ppm, followed by Hexane crude extract (CLH) and MeOH crude extract (CLM) with ED_{50} values of 103 and 422 ppm, respectively (Table 4.5 and Figure 4.17). EtOAC crude extracts of *C. cochliodes* (CCoE) resulted in the highest spore inhibition of 88 %, followed by crude methanol (CCoM) and crude hexane (CCoH), which resulted in a spore inhibition of 80.74 % and 67.57 %, respectively, in 12 d at 1,000 ppm. The fungal metabolites of CCoH, CCoE and CCoM exhibted active antifungal activity against the *M. oryzae* isolate PO1 with ED_{50} values of 85, 144, and 374 ppm (Table 4.6 and Figure 4.18).

Table 4.3 Crude extracts of *Chaetomium elatum* testing for growth inhibition of *Magnaporthe oryzae* at 8 days, spore production inhibition at 12 days and effective dose (ED$_{50}$) values

Crude extracts	Concentration (ppm)	Colony diameter (cm)$^{/1}$	Growth inhibition (%)$^{/2,3}$	Number of spores $^{/1,3}$(10^{5})	Spore Inhibition (%)$^{/2,3}$	ED$_{50}$(ppm)
CEH	0	5.00^{a}	-	57.25^{a}	-	266.82
	10	4.73^{b}	5.25^{l}	49.00^{b}	14.87^{j}	
	50	4.47^{c}	10.50^{k}	42.25^{cd}	27.83^{h}	
	100	4.27^{d}	14.50^{j}	33.75^{fg}	40.43^{f}	
	500	3.67^{f}	26.50^{h}	24.75^{h}	57.09^{d}	
	1000	2.73^{i}	45.25^{e}	19.50^{i}	66.66^{c}	
CEE	0	5.00^{a}	-	57.25^{a}	-	106.98
	10	4.36^{cd}	12.75^{jk}	45.75^{bc}	20.80^{i}	
	50	3.57^{f}	28.50^{h}	36.75^{ef}	36.29^{fg}	
	100	3.02^{h}	39.50^{f}	29.75^{g}	48.82^{e}	
	500	2.00^{k}	60.00^{b}	17.50^{ij}	70.24^{bc}	
	1000	1.54^{l}	69.00^{a}	8.75^{k}	85.74^{a}	
CEM	0	5.00^{a}	-	57.25^{a}	-	188.34
	10	4.64^{b}	7.00^{l}	45.75^{bc}	20.12^{ij}	
	50	4.12^{e}	17.50^{i}	39.25^{de}	31.48^{gh}	
	100	3.23^{g}	35.25^{g}	33.25^{fg}	41.66^{f}	
	500	2.13^{k}	57.25^{c}	25.00^{h}	58.27^{d}	
	1000	2.29^{j}	54.00^{d}	14.50^{j}	75.55^{b}	
C.V.(%)		1.70	4.23	8.19	5.17	

^{1}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01; ^{2}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01; ^{3}/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

CEH=Crude Hexane from *Chaetomium elatum*, CEE= Crude Ethyl acetate from *Chaetomium elatum,* CEM= Crude Methanol from *Chaetomium elatum*

Table 4.4 Crude extracts of *Chaetomium brasiliense* testing for growth inhibition of *Magnaporthe oryzae* at 8 days, spore production inhibition at 12 days and effective dose (ED_{50}) values

Crude extracts	Concentration (ppm)	Colony diameter (cm)$^{/1}$	Growth inhibition (%)$^{/2,3}$	Number of spores $^{/2,3}$(10^{5})	Spore Inhibition (%)$^{/2,3}$	ED_{50}(ppm)
CBH	0	5.00^{a}	-	39.00^{a}	-	35.49
	10	4.22^{bc}	15.50^{ij}	22.50^{cd}	42.28^{h}	
	50	3.98^{de}	20.25^{gh}	19.50^{de}	50.08^{g}	
	100	3.89^{ef}	22.00^{fg}	17.75^{ef}	54.55^{fg}	
	500	2.22^{j}	55.50^{b}	9.00^{hi}	77.01^{c}	
	1000	1.65^{k}	67.00^{a}	2.75^{j}	93.20^{a}	
CBE	0	5.00^{a}	-	39.00^{a}	-	55.91
	10	4.10^{cd}	18.00^{hi}	26.00^{c}	33.26^{i}	
	50	3.93^{de}	21.25^{fgh}	22.00^{cd}	43.65^{h}	
	100	3.76^{fg}	24.75^{ef}	16.25^{efg}	58.57^{ef}	
	500	2.33^{j}	53.25^{b}	12.50^{gh}	68.10^{d}	
	1000	1.68^{k}	66.25^{a}	3.00^{j}	92.46^{a}	
CBM	0	5.00^{a}	-	39.00^{a}	-	119.06
	10	4.37^{b}	12.50^{j}	34.00^{b}	12.87^{j}	
	50	3.98^{de}	20.25^{gh}	24.75^{c}	36.53^{i}	
	100	3.62^{g}	27.50^{e}	18.50^{de}	52.50^{g}	
	500	3.19^{h}	36.00^{d}	14.00^{fg}	64.14^{de}	
	1000	2.51^{i}	49.75^{c}	6.00^{ij}	84.98^{b}	
C.V.(%)		2.25	5.21	11.26	5.07	

^{1}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01; ^{2}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01; ^{3}/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

CBH=Crude Hexane from *Chaetomium brasiliense*, CBE= Crude Ethyl acetate from *Chaetomium brasiliense,* CBM= Crude Methanol from *Chaetomium brasiliense*

Table 4.5 Crude extracts of *Chaetomium lucknowense* testing for growth inhibition of *Magnaporthe oryzae* at 8 days, spore production inhibition at 12 days and effective dose (ED_{50}) values

Crude extracts	**Concentration (ppm)**	**Colony diameter (cm)$^{/1,3}$**	**Growth inhibition (%)$^{/1,3}$**	**Number of spores $^{/2,3}(10^{6})$**	**Spore Inhibition (%)$^{/2,3}$**	**ED_{50}(ppm)**
CLH	0	5.00^{a}	-	52.00^{a}	-	103.70
	10	4.53^{cd}	9.25^{gh}	40.25^{bc}	22.49^{hi}	
	50	4.44^{d}	11.00^{fg}	27.25^{ef}	47.59^{ef}	
	100	4.21^{g}	15.75^{d}	24.25^{fg}	53.31^{de}	
	500	3.84^{h}	23.00^{c}	19.75^{gh}	62.02^{c}	
	1000	3.71^{ij}	25.75^{ab}	14.75^{h}	71.82^{b}	
CLE	0	5.00^{a}	-	52.00^{a}	-	57.17
	10	4.58^{bc}	8.25^{h}	35.75^{cd}	31.22^{g}	
	50	4.36^{e}	12.75^{ef}	28.00^{ef}	46.10^{f}	
	100	4.17^{g}	16.50^{d}	21.25^{fgh}	59.04^{cd}	
	500	3.77^{hi}	24.50^{bc}	15.50^{h}	70.22^{b}	
	1000	3.61^{k}	27.75^{a}	8.00^{i}	84.92^{a}	
CLM	0	5.00^{a}	-	52.00^{a}	-	422.00
	10	4.63^{b}	7.25^{h}	43.00^{b}	17.03^{i}	
	50	4.47^{d}	8.50^{h}	39.25^{bcd}	24.24^{h}	
	100	4.33^{ef}	13.25^{ef}	33.25^{de}	35.86^{g}	
	500	4.26^{fg}	14.75^{de}	27.25^{ef}	47.59^{ef}	
	1000	3.64^{jk}	27.00^{a}	19.00^{gh}	63.53^{c}	
C.V.(%)		1.42	9.37	10.91	6.45	

[1]/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.05; [2]/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01; [3]/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

CLH=Crude Hexane from *Chaetomium lucknowense*, CLE= Crude Ethyl acetate from *Chaetomium lucknowense,* CLM= Crude Methanol from *Chaetomium lucknowense*

Table 4.6 Crude extracts of *Chaetomium cochliodes* testing for growth inhibition of *Magnaporthe oryzae* at 8 days, spore production inhibition at 12 days and effective dose (ED_{50}) values

Crude extracts	**Concentration (ppm)**	**Colony diameter (cm)$^{/2,3}$**	**Growth inhibition (%)$^{/2,3}$**	**Number of spores $^{/2,3}$(10^{5})**	**Spore Inhibition (%)$^{/2,3}$**	**ED_{50}(ppm)**
CCoH	0	5.00^{a}	-	20.75^{a}	-	374.43
	10	4.88^{bc}	2.25^{ij}	20.25^{a}	2.56^{j}	
	50	4.73^{d}	5.25^{gh}	17.25^{b}	16.86^{h}	
	100	4.43^{f}	11.25^{e}	14.25^{c}	31.23^{g}	
	500	3.91^{i}	21.75^{b}	10.00^{e}	52.18^{de}	
	1000	3.68^{j}	26.25^{a}	6.75^{fg}	67.57^{c}	
CCoE	0	5.00^{a}	-	20.75^{a}	-	85.87
	10	4.94^{ab}	1.00^{j}	17.25^{b}	16.70^{h}	
	50	4.82^{c}	3.50^{i}	12.75^{cd}	38.64^{f}	
	100	4.67^{d}	6.50^{g}	9.00^{ef}	56.69^{d}	
	500	4.02^{h}	19.50^{c}	5.00^{gh}	76.01^{b}	
	1000	3.64^{j}	27.00^{a}	2.50^{h}	88.03^{a}	
CCoM	0	5.00^{a}	-	20.75^{a}	-	144.23
	10	4.86^{c}	2.75^{i}	18.75^{ab}	9.68^{i}	
	50	4.73^{d}	4.00^{hi}	14.00^{c}	32.58^{g}	
	100	4.53^{e}	9.25^{f}	11.00^{de}	46.95^{e}	
	500	4.11^{g}	17.75^{d}	7.00^{fg}	66.40^{c}	
	1000	3.91^{i}	21.75^{b}	4.00^{h}	80.74^{b}	
C.V.(%)		0.74	7.22	10.79	6.27	-

[1]/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.05; [2]/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01; [3]/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

CCoH=Crude Hexane from *Chaetomium cochliodes*, CCoE= Crude Ethyl acetate from *Chaetomium cochliodes,* CCoM= Crude Methanol from *Chaetomium cochliodes*

		
0 10 50 100 500 1000 (ppm)	0 10 50 100 500 1000 (ppm)	0 10 50 100 500 1000 (ppm)
CEH	**CEE**	**CEM**

Figure 4.15 Crude extracts of *Chaetomium elatum* testing for colony growth and spore production inhibition of *Magnaporthe oryzae* at 8 days

CEH=Crude Hexane from *Chaetomium elatum*, CEE= Crude Ethyl acetate from *Chaetomium elatum,* CEM= Crude Methanol from *Chaetomium elatum*

		
0 10 50 100 500 1000 (ppm)	0 10 50 100 500 1000 (ppm)	0 10 50 100 500 1000 (ppm)
CBH	**CBE**	**CBM**

Figure 4.16 Crude extracts of *Chaetomium brasiliense* testing for colony growth and spore production inhibition of *Magnaporthe oryzae* at 8 days

CBH=Crude Hexane from *Chaetomium brasiliense*, CBE= Crude Ethyl acetate from *Chaetomium brasiliense,* CBM= Crude Methanol from *Chaetomium brasiliense*

		
0 10 50 100 500 1000 (ppm)	1 10 50 100 500 1000 (ppm)	2 10 50 100 500 1000 (ppm)
CLH	**CLE**	**CLM**

Figure 4.17 Crude extracts of *Chaetomium lucknowense* testing for colony growth and spore production inhibition of *Magnaporthe oryzae* at 8 days

CLH=Crude Hexane from *Chaetomium lucknowense*, CLE= Crude Ethyl acetate from *Chaetomium lucknowense,* CLM= Crude Methanol from *Chaetomium lucknowense*

0 10 50 100 500 1000 (ppm)	1 10 50 100 500 1000 (ppm)	2 10 50 100 500 1000 (ppm)
CCoH	**CCoE**	**CCoM**

Figure 4.18 Crude extracts of *Chaetomium cochliodes* testing for colony growth and spore production inhibition of *Magnaporthe oryzae* at 8 days

CCoH=Crude Hexane from *Chaetomium cochliodes*, CCoE= Crude Ethyl acetate from *Chaetomium cochliodes,* CCoM= Crude Methanol from *Chaetomium cochliodes*

4.4 *In vitro* bioassay test using nano-particles of antagonistic fungi against *Magnaporthe oryzae*

4.4.1 Nanoparticles derived from antagonists

The nanoparticles loaded with crude extracts from each antagonist were visually characterized as shown in Figure 4.19. Nano-particles derived from *Chaetomium elatum* which coded as Nano-CEH, Nano-CEE and Nano-CEM were light yellow to yellow in color. Nano-particles loaded with crude extract of *Chaetomium brasiliense* which coded as nano-CBH, nano-CBE and nano-CBM were white to light orange colors. Nano particles of *Chaetomium lucknowense* which coded as Nano-CLH, Nano-CLE and Nano-CLM were light yellow to yellow colors. The nanoparticles Nano-CCoH, Nano-CCoE and Nano-CCoM, loaded with crude extracts from *Chaetomium cochliodes*, were visually characterized as white, yellow and light yellow in colour, respectively. Interestingly, the scanning electron images showed the range of particles size of Nano-CCoH, Nano-CCoE and Nano-CCoM ranged between 567-611, `422-566 and 415-472 nm, respectively.

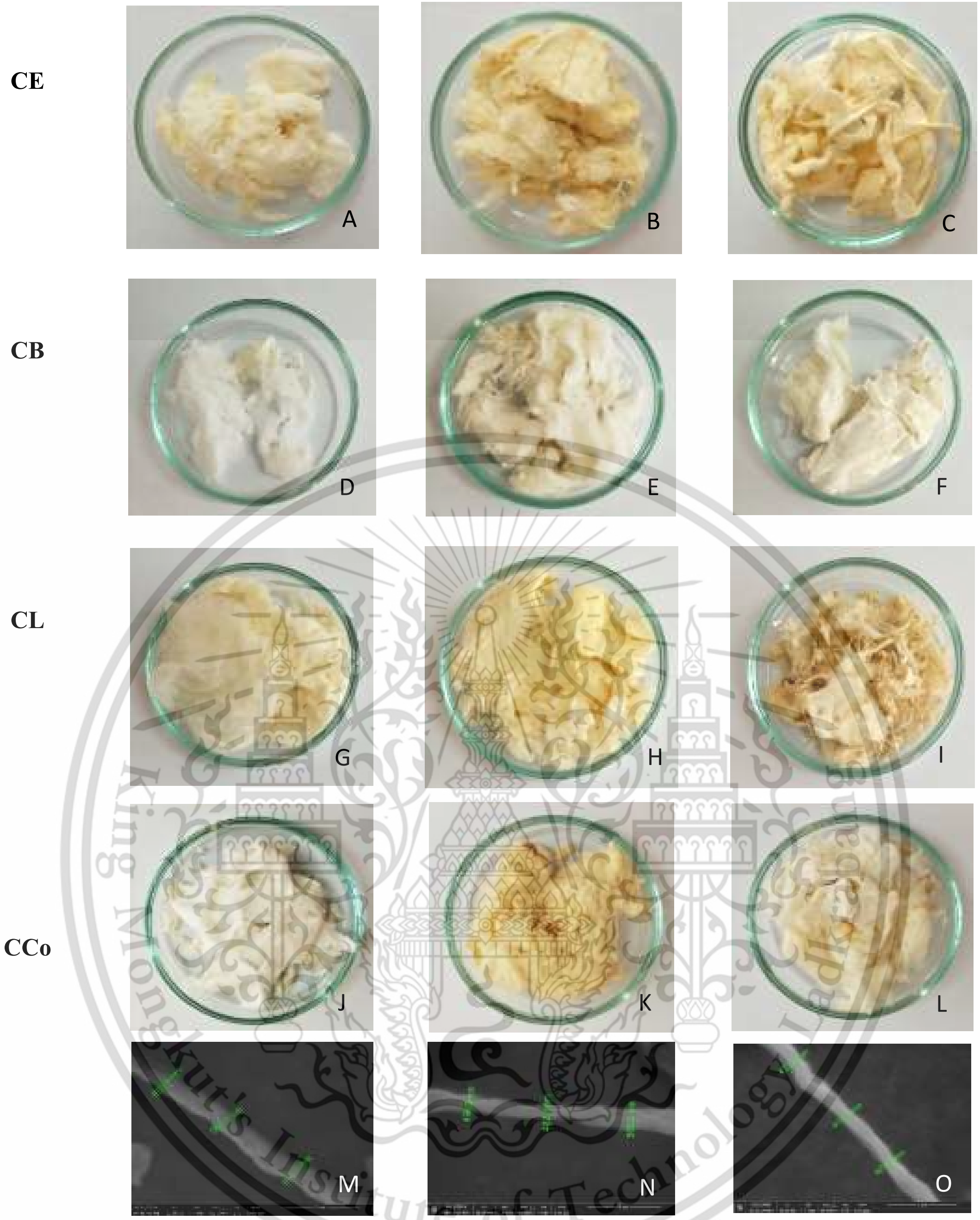


Figure 4.19 Characteristics of nanoparticles from *Chaetomium elatum* (CE), *Chaetomium brasiliense* (CB), *Chaetomium lucknowense* (CL) and *Chaetomium cochliodes* (CCo): A=Nano-CEH, B=Nano-CEE, C=Nano-CEM, D=Nano-CBH, E=Nano-CBE, F=Nano-CBM, G=Nano-CLH, H=Nano-CLE, I=Nano-CLM, J&M=Nano-CCoH, K&N=Nano-CCoE, L&O=Nano-CCoM

4.4.2 Bioassay test of nanoparticles from antagonist fungi against *M. oryzae*

The resulted in Nano-CEE expressed antifungal activity against *M. oryzae* causing rice blast, highly significant spore inhibition with ED_{50} value of 7.89 ppm, followed by nano-CEM and nano-CEH with the ED_{50} values of 8.66 ppm and 16.7 ppm, respectively (Table 4.7 and Figure 4.20). Nano-CBH gave the highest significance in spore production with the ED_{50} value of 6.86 ppm, followed by nano-CBE and nano-CBM with the ED50 values of 9.76 and 13.42 ppm., respectively (Table 4.8 and Figure 4.21). Nano particles of *C. lucknowense* exhibited antifungal activity against the conidial stage of *M. oryzae* causing rice blast. Nano-CLM gave highly significant spore inhibition with an ED_{50} value of 5.24 ppm, followed by nano-CLE and nano-CLH with the ED_{50} values of 7.01 and 10.72 ppm., respectively (Table 4.9 and Figure 4.22).

It was found that the tested nano-CCoH, nano-CCoE and nano-CCoM at 15 ppm showed significantly inhibited spore production ($P \leq 0.01$) in comparison to the non-treated control (0 ppm). Nano-CCoE resulted in significantly ($P \leq 0.05$) greater spore inhibition in comparison to the non-treated control. Nano-CCoE inhibited spore production significantly ($P \leq 0.05$; 68 %), followed by nano-CCoM (47 %) and nano-CCoH (34 %) in 12 d (Table 4.10 and Figure 4.23). Nano-CCoE, nano-CCoM and nano-CCoH inhibited *M. oryzae* PO1 (rice blast) with ED_{50} values of 9.47, 16.51 and 33.41 ppm, respectively (Table 4.10).

Table 4.7 Nanoparticles of *Chaetomium elatum* testing for growth inhibition of *Magnaporthe oryzae* at 8 days, spore production inhibition at 12 days and effective dose (ED_{50}) values

Nanoparticles	**Concentration (ppm)**	**Colony diameter $(cm)^{/1}$**	**Growth inhibition $(\%)^{/2,3}$**	**Number of spores $^{/1,3}(10^{5})$**	**Spore Inhibition $(\%)^{/2,3}$**	**ED_{50}(ppm)**
Nano-CEH	0	5.00^{a}	-	32.50^{a}	-	16.70
	3	4.79^{b}	4.00^{i}	30.50^{ab}	5.69^{g}	
	5	4.60^{de}	8.00^{fg}	27.00^{bc}	16.45^{ef}	
	10	4.00^{g}	20.00^{d}	22.50^{de}	30.63^{cd}	
	15	3.65^{h}	27.00^{c}	15.75^{f}	51.85^{b}	
Nano-CEE	0	5.00^{a}	-	32.50^{a}	-	7.89
	3	4.64^{cd}	7.00^{gh}	29.75^{ab}	7.91^{fg}	
	5	4.30^{f}	14.00^{e}	20.50^{e}	37.06^{c}	
	10	3.60^{h}	28.00^{c}	15.50^{f}	52.73^{b}	
	15	3.00^{j}	40.00^{c}	6.50^{g}	80.46^{a}	
Nano-CEM	0	5.00^{a}	-	32.50^{a}	-	8.66
	3	4.75^{bc}	5.00^{hi}	30.25^{ab}	6.80^{fg}	
	5	4.50^{e}	10.00^{f}	25.00^{cd}	23.01^{de}	
	10	3.95^{g}	21.00^{d}	15.50^{f}	52.43^{b}	
	15	3.40^{i}	32.00^{b}	7.25^{g}	77.78^{a}	
C.V.(%)		1.17	7.17	12.45	19.01	

[1]/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.05.

[2]/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01.

[3]/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

Nano-CEH=Nanoparticles from crude hexane of *Chaetomium elatum*, Nano-CEE= Nanoparticles from crude ethyl acetate of *Chaetomium elatum,* Nano-CEM= Nanoparticles from crude methanol from *Chaetomium elatum.*

Table 4.8 Nanoparticles of *Chaetomium brasiliense* testing for growth inhibition of *Magnaporthe oryzae* at 8 days, spore production inhibition at 12 days and effective dose (ED_{50}) values

Nanoparticles	Concentration (ppm)	Colony diameter (cm)$^{/1}$	Growth inhibition (%)$^{/2,3}$	Number of spores $^{/1}$(10^{5})	Spore Inhibition (%)$^{/2,3}$	ED_{50} (ppm)
Nano-CBH	0	5.00	-	17.25^{a}	-	6.86
	3	4.95	1.00	14.50^{bc}	15.75^{e}	
	5	4.89	2.25	10.75^{d}	37.57^{d}	
	10	4.84	3.25	5.50^{f}	68.05^{b}	
	15	4.83	3.50	3.50^{g}	79.74^{a}	
Nano-CBE	0	5.00	-	17.25^{a}	-	9.76
	3	5.00	0.00	14.00^{bc}	18.47^{e}	
	5	4.96	0.75	11.75^{d}	31.72^{d}	
	10	4.92	1.50	7.50^{e}	56.36^{c}	
	15	4.88	2.50	4.50^{fg}	73.89^{ab}	
Nano-CBM	0	5.00	-	17.25^{a}	-	13.42
	3	5.00	0.00	15.75^{ab}	8.34^{f}	
	5	4.97	0.50	13.75^{c}	20.03^{e}	
	10	4.92	1.50	11.50^{d}	33.29^{d}	
	15	4.88	2.50	7.25^{e}	57.92^{c}	
C.V.(%)		0.64	44.38	10.23	8.14	

^{1}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.05.

^{2}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01.

^{3}/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

Nano-CBH=Nanoparticles from crude hexane of *Chaetomium brasiliense*, Nano-CBE= Nanoparticles from crude ethyl acetate of *Chaetomium brasiliense,* Nano-CBM= Nanoparticles from crude methanol from *Chaetomium brasiliense.*

Table 4.9 Nanoparticles of *Chaetomium lucknowense* testing for growth inhibition of *Magnaporthe oryzae* at 8 days, spore production inhibition at 12 days and effective dose (ED_{50}) values

Nanoparticles	**Concentration (ppm)**	**Colony diameter (cm)$^{/1}$**	**Growth inhibition (%)$^{/1,3}$**	**Number of spores $^{/1}(10^5)$**	**Spore Inhibition (%)$^{/1,3}$**	**ED_{50} (ppm)**
Nano-CLH	0	5.00^{a}	-	40.50^{a}	-	10.72
	3	4.96^{ab}	0.75^{e}	34.00^{b}	15.32^{j}	
	5	4.91^{bc}	1.75^{cde}	28.75^{cd}	28.53^{h}	
	10	4.88^{cd}	2.25^{bcd}	23.50^{ef}	41.81^{f}	
	15	4.83^{de}	3.25^{b}	14.50^{g}	64.18^{cd}	
Nano-CLE	0	5.00^{a}	-	40.50^{a}	-	7.01
	3	4.97^{a}	0.50^{e}	31.25^{bc}	22.41^{i}	
	5	4.94^{ab}	1.00^{de}	25.00^{def}	37.82^{fg}	
	10	4.85^{de}	3.00^{cd}	15.00^{g}	63.00^{d}	
	15	4.71^{g}	5.75^{a}	10.50^{gh}	73.87^{ab}	
Nano-CLM	0	5.00^{a}	-	40.50^{a}	-	5.24
	3	4.91^{bc}	1.75^{cde}	26.50^{de}	34.25^{g}	
	5	4.82^{e}	2.75^{bc}	21.25^{f}	47.53^{e}	
	10	4.76^{f}	4.75^{a}	12.75^{gh}	68.65^{bc}	
	15	4.69^{g}	6.00^{a}	8.75^{h}	78.52^{a}	
C.V.(%)		0.70	31.02	11.75	7.75	

^{1}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.05.

^{2}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01.

^{3}/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

Nano-CLH=Nanoparticles from crude hexane of *Chaetomium lucknowense*, Nano-CLE= Nanoparticles from crude ethyl acetate of *Chaetomium lucknowense,* Nano-CLM= Nanoparticles from crude methanol from *Chaetomium lucknowense.*

Table 4.10 Nanoparticles of *Chaetomium cochliodes* testing for growth inhibition of *Magnaporthe oryzae* at 8 days, spore production inhibition at 12 days and effective dose (ED_{50}) values

Nanoparticles	**Concentration (ppm)**	**Colony diameter (cm)$^{/1}$**	**Growth inhibition (%)$^{/1,3}$**	**Number of spores $^{/2,3}$(10^5)**	**Spore Inhibition (%)$^{/1,3}$**	**ED_{50} (ppm)**
Nano-CCoH	0	5.00^{a}	-	69.00^{a}	-	33.41
	3	4.96^{ab}	0.75^{e}	67.00^{ab}	2.78^{i}	
	5	4.88^{bc}	2.25^{de}	56.00^{bcd}	18.98^{f}	
	10	4.82^{cd}	3.50^{cd}	50.75^{cd}	26.63^{e}	
	15	4.76^{d}	4.75^{c}	45.00^{de}	34.87^{cd}	
Nano-CCoE	0	5.00^{a}	-	69.00^{a}	-	9.47
	3	4.93^{ab}	1.25^{e}	60.00^{abc}	13.23^{fg}	
	5	4.87^{bc}	2.50^{de}	49.00^{cd}	28.99^{de}	
	10	4.78^{d}	4.25^{cd}	36.00^{e}	47.99^{b}	
	15	4.58^{e}	8.25^{b}	22.00^{f}	68.26^{a}	
Nano-CCoM	0	5.00^{a}	-	69.00^{a}	-	16.51
	3	4.87^{bc}	2.50^{de}	64.00^{ab}	7.36^{gh}	
	5	4.77^{d}	3.75^{cd}	51.25^{cd}	26.00^{e}	
	10	4.56^{e}	8.75^{b}	44.00^{de}	36.40^{c}	
	15	3.88^{f}	22.25^{a}	36.00^{e}	47.97^{b}	
C.V.(%)		1.18	25.04	11.03	13.91	-

^{1}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.05.

^{2}/Average of four replications. Means followed by a common letter are not significantly differed by DMRT at P=0.01.

^{3}/Inhibition(%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

Nano-CCoH=Nanoparticles from crude hexane of *Chaetomium cochiliodes*, Nano-CCoE= Nanoparticles from crude ethyl acetate of *Chaetomium cochiliodes,* Nano-CCoM= Nanoparticles from crude methanol from *Chaetomium cochiliodes.*

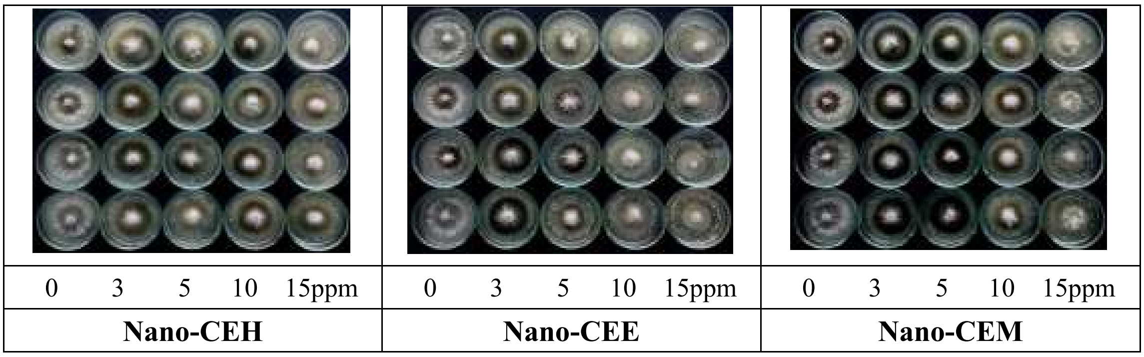


Figure 4.20 Nanoparticles of *Chaetomium elatum* testing for colony growth and spore production inhibition of *Magnaporthe oryzae* at 8 days

Nano-CEH=Nanoparticles from crude hexane of *Chaetomium elatum*, Nano-CEE= Nanoparticles from crude ethyl acetate of *Chaetomium elatum,* Nano-CEM= Nanoparticles from crude methanol from *Chaetomium elatum*

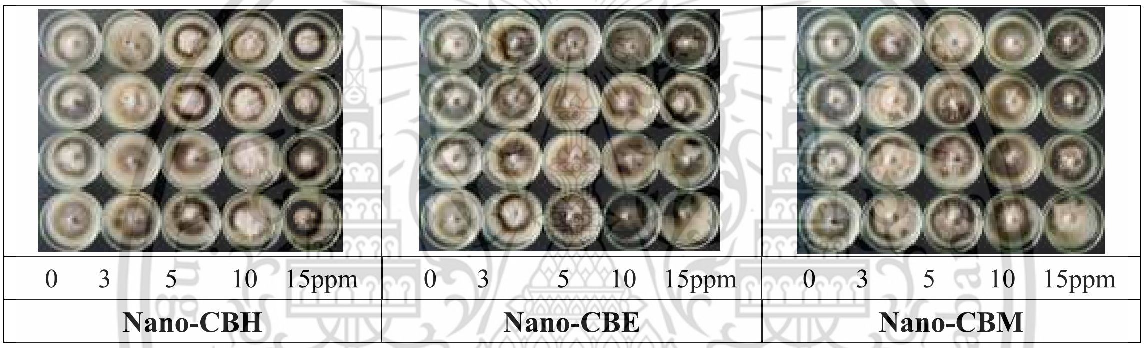


Figure 4.21 Nanoparticles of *Chaetomium brasiliense* testing for colony growth and spore production inhibition of *Magnaporthe oryzae* at 8 days

Nano-CBH=Nanoparticles from crude hexane of *Chaetomium brasiliense*, Nano-CBE= Nanoparticles from crude ethyl acetate of *Chaetomium brasiliense,* Nano-CBM= Nanoparticles from crude methanol from *Chaetomium brasiliense.*

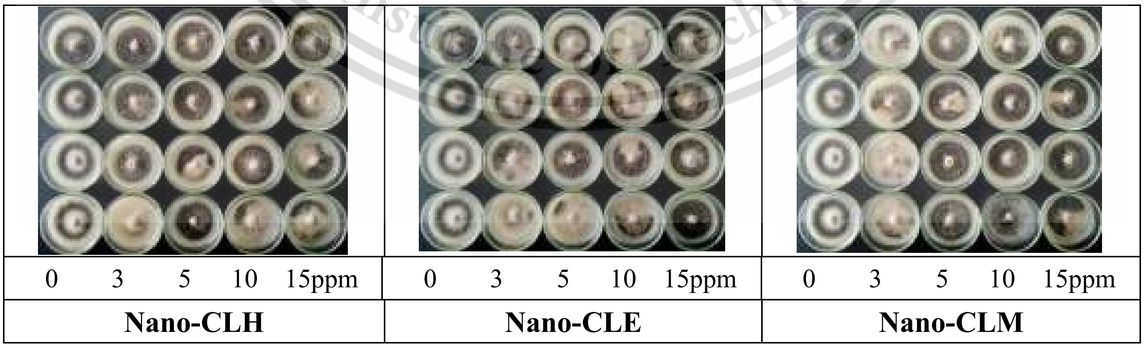


Figure 4.22 Nanoparticles of *Chaetomium lucknowense* testing for colony growth and spore production inhibition of *Magnaporthe oryzae* at 8 days

Nano-CLH=Nanoparticles from crude hexane of *Chaetomium lucknowense*, Nano-CLE= Nanoparticles from crude ethyl acetate of *Chaetomium lucknowense,* Nano-CLM= Nanoparticles from crude methanol from *Chaetomium lucknowense*

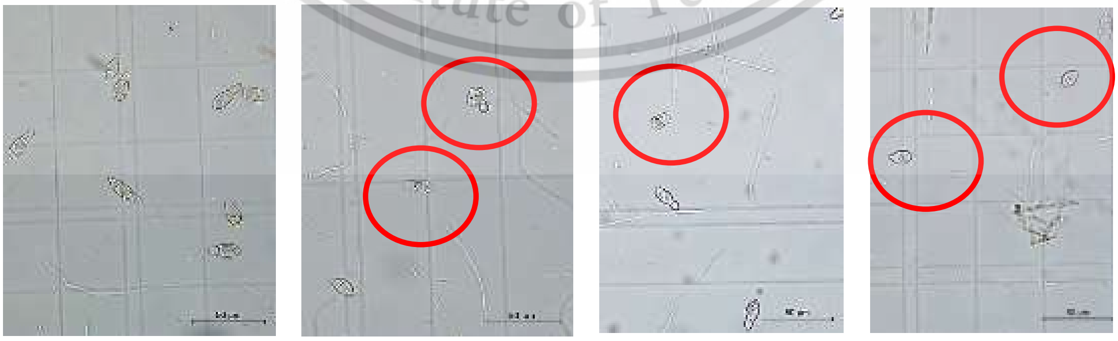
		
0 3 5 10 15ppm	0 3 5 10 15ppm	0 3 5 10 15ppm
Nano-CCoH	**Nano-CCoE**	**Nano-CCoM**

Figure 4.23 Nanoparticles of *Chaetomium cochliodes* testing for colony growth and spore production inhibition of *Magnaporthe oryzae* at 8 days

Nano-CCoH=Nanoparticles from crude hexane of *Chaetomium cochiliodes*, Nano-CCoE= Nanoparticles from crude ethyl acetate of *Chaetomium cochiliodes,* Nano-CCoM= Nanoparticles from crude methanol from *Chaetomium cochiliodes.*

4.4.3 Control mechanisms

The effect of nanoparticles, nano-CE from *C. elatum*, nano-CL from *C. lucknowense* and nano-CB from *C. brasiliense* against *M. oryzae* (rice blast pathogen) showed the control mechanism as lysis. The spores treated with nanoparticles showed broken and abnormal cells as seen in Figure 4.24. The spore suspensions of *M. oryzae* that were separately treated with Nano-CE, Nano-CL and Nano-CB lost their pathogenicity thereafter; with those inoculated to rice seedlings var PSL2, there were no signs and symptoms of rice blast. It was observed that the spores were abnormally shaped, and cells were broken after treatment with nanoparticles of *C. cochliodes*; in contrast, the spores were normally shaped in the non-treated control (Figure 4.25).



Normal spore **Abnormal spore (Nano-CB)** **Abnormal spore (Nano-CL)** **Abnormal spore (Nano-CE)**

Figure 4.24 The effect of nanoparticles, Nano-CE from *C. elatum*, Nano-CL from *C. lucknowense* and Nano-CB from *C. brasiliense* against *M. oryzae*

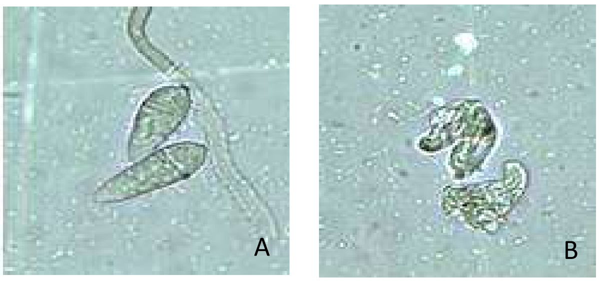


Figure 4.25 Normal spores of the rice blast pathogen (A) and abnormal spores (B) of the *Magnaporthe oryzae* isolate PO1 after treatment with nanoparticles derived *Chaetomium cochliodes*

4.5 *In vivo* nanoparticles constructed from antagonist against rice blast disease

4.5.1 Nanoparticles from mixture crude metabolites of *C. cochliodes* against rice blast disease

The crude extract mixture nano-particles from *C. cochliodes* revealed that the blast incidence caused by the *M. oryzae* isolate PO1 was significantly reduced ($P \leq 0.01$) by 38 %, after the application of nanoparticles from *C. cochliodes*, followed by the chemical fungicide treatment (tricyclazole), which reduced blast incidence by 29 % when compared to the inoculated control with *M. oryzae* (Table 4.11). In the *in-vivo* experiment, all treatments resulted in significant differences ($P \leq 0.01$) in plant height at 15 d post-treatment (Figure 4.26). The application of nanoparticles of *C. cochliodes* resulted in the greatest plant height (84.7 cm), which was significant at $P \leq 0.01$, followed by the chemical fungicide (tricyclazole), which resulted in a plant height of 74.9 cm; the non-inoculated control and the control inoculated with *M. oryzae* exhibited heights of 77.7 and 77.6 cm, respectively. The application of nanoparticles from *C. cochliodes* and tricyclazole were not significant differences in stem fresh weight, 64.7 and 66.7 g, respectively, but they were significantly differed ($P \leq 0.01$) from the non-inoculated control and the inoculated with *M. oryzae*, 57.2 and 45.5 g, respectively. The root fresh weight showed the highest (79.4 g) in plants which treated with nanoparticles of *C. cochliodes*, followed by the non-inoculated control, the inoculated with *M. oryzae*, and the plants treated with tricyclazole treatments: 66.6, 58.0 and 44.2 g, respectively. The nanoparticles of *C. cochliodes* resulted in significantly ($P \leq 0.01$) higher stem dry weight (14.1 g) compared with tricyclazole (11.1 g) and inoculation with *M. oryzae* alone 12.0 g (Table 4.11). The root dry weight showed the highest after treatment with tricyclazole (11.3 g), followed by the nanoparticles of *C. cochliodes* (10.5 g) and inoculated with *M. oryzae* alone (6.4 g).

Table 4.11 Effect of crude extract mixture nano-particles on plant height, fresh and dry weight of stems and roots and disease reduction of blast in rice var RD 57

Treatments	**Plant height (15 d) (cm)**	**Plant height (30d) (cm)**	**Stem Fresh weight (g)**	**Root Fresh weight (g)**	**Stem Dry Weight (g)**	**Root Dry Weight (g)**	**Disease Reduction (%)**
T1(Non-inoculated control)	62.0^{a}	77.74^{b}	57.23^{b}	66.55^{b}	17.81^{a}	8.82^{b}	-
T2 (Inoculated with *M. oryzae*)	62.6^{a}	77.58^{b}	45.53^{c}	58.03^{c}	12.01^{c}	6.42^{b}	-
T3 (*M. oryzae* + nanoparticles of *C. cochliodes*)	63.3^{a}	84.67^{a}	64.70^{a}	79.41^{a}	14.05^{b}	10.51^{a}	37.5
T4 (*M. oryzae* + Tricyclazole)	62.1^{a}	74.91^{b}	66.66^{a}	44.20^{d}	11.12^{cd}	11.28^{a}	29.1
CV(%)	2.52	12.48	22.82	29.87	29.29	28.96	-

Means followed by a common letter are not significantly different by DMRT at $P \leq 0.05$.

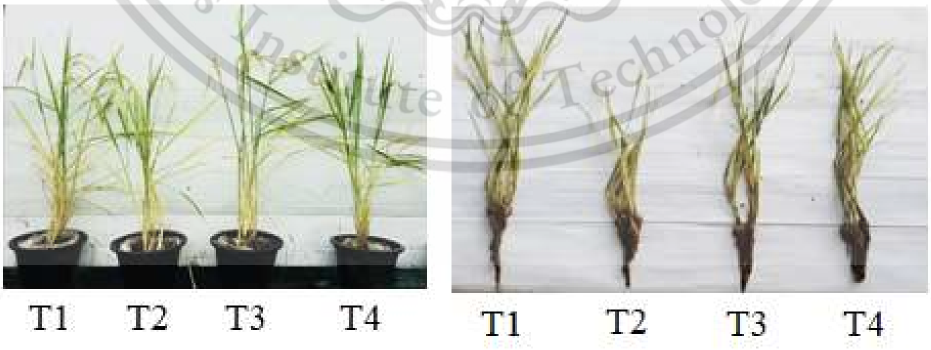


Figure 4.26 Testing the capacity for nanoparticles derived from *Chaetomium cochliodes* to inhibit rice blast, T1 = non-inoculated control, T2 = inoculated with *M. oryzae*, T3 = *M. oryzae* + nanoparticles of *C. cochliodes*, and T4 = *M. oryzae* + Tricyclazole

4.5.2 Nanoparticles from each crude metabolite of *C. cochliodes* against rice blast disease

Nanoparticles derived from hexane, ethyl acetate and methanol crude extracts from *C. cochliodes* CTh05 (Nano-CCoH, Nano-CCo-E and Nano-CCoM) were separately applied to the rice seedlings inoculated with *M. oryzae* PO1. The results demonstrated that 15 d after the treatments, Nano-CCoM resulted in ($P \leq 0.01$) greater plant height (43.0 cm) compared with Nano-CCoH and Nano-CCoE, which resulted in a plant height of 41.0 and 40.5 cm, respectively, and followed by tricyclazole (38.5 cm,. meanwhile, the inoculated control showed a plant height of 38.6 cm (Table 4.12 and Figure 4.27). However, 30 d after treatment, plant height did not significantly differ among plants treated with Nano-CCoM, Nano-CCoE and tricyclazole; for these groups, plant heights were 77.8, 76.3 and 75.0 cm, respectively, and followed by the Nano-CCoH (62.3 cm) treatment. The inoculated control had a plant height of 53.8 cm. The blast incidence was reduced after treatment with Nano-CCoM (60 %) more than Nano-CCoE (58 %), tricyclazole (56 %) or Nano-CCoH (50 %).

Table 4.12 Effect of nanoparticles derived from hexane, ethyl acetate and methanol extracts on plant height and disease reduction (%) on rice var. RD 57

Treatments	**15 (d)**	**30 (d)**	**Disease reduction (%)**
T1 (Non-inoculated control)	38.70^{c}	54.50^{c}	-
T2 (Inoculated with*M. oryzae*)	38.60^{c}	53.75^{c}	-
T3 (Nano-CCoH)	41.00^{ab}	62.25^{b}	50.2
T4 (Nano-CCoE)	40.50^{b}	76.25^{a}	57.5
T5 (Nano-CCoM)	43.00^{a}	77.75^{a}	59.8
T6 (Tricyclazole)	38.50^{c}	75.00^{a}	55.5

Means followed by a common letter are not significantly different by DMRT at P≤0.05;

Nano-CCoH=Nanoparticles from crude hexane of *Chaetomium cochiliodes*, Nano-CCoE= Nanoparticles from crude ethyl acetate of *Chaetomium cochiliodes,* Nano-CCoM= Nanoparticles from crude methanol from *Chaetomium cochiliodes*.

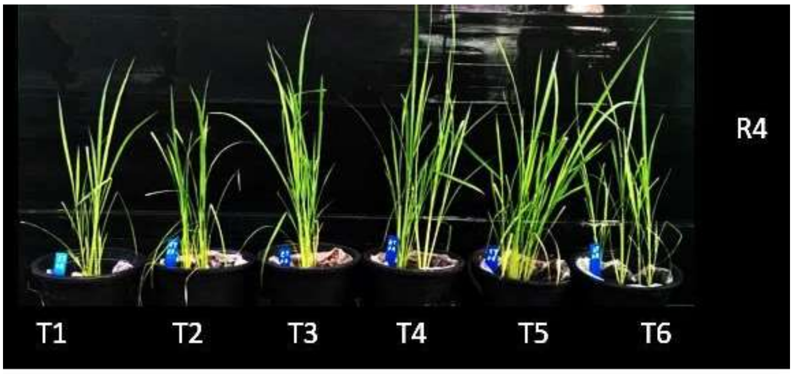


Figure 4.27 Testing the capacity of nanoparticles derived from *Chaetomium cochliodes* in inhibiting rice blast 15 days after treatments, non-inoculated control (T1), inoculated with *M. oryzae* (T2), Nano-CCoH (T3), Nano-CCoE(T4), Nano-CCoM (T5) and tricyclazole (T6)

4.6 Testing nano-CB for phytoalexin production

The rice leaves treated with nano-CB from *C. brasiliense* were examined for phytoalexin production by thin layer chromatography. The treated nano-CB found spot on TLC plates when using combination of benzene : ethyl acetate (10:1) under 365 nm UV light for leaves with the R_f value of 0.08 supporting to be Sakuranertin, and spot on TLC plates with the R_f values of 0.28 supposing to be Oryzalexin B. With the treated nano-CB to the inoculated rice var PLS2 with *M. oryzae,* the rice seedlings were expected to produce phytoalexins against rice blast disease (Figure 4.28).

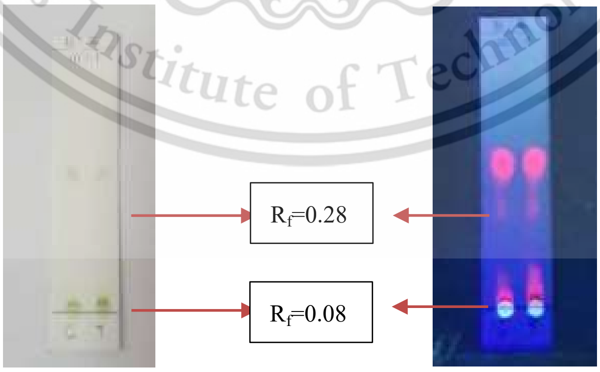


Figure 4.28 The presence of possible phytoalexins of Sakuranetin at R_f value 0.08 and Oryzalexin B at R_f value 0.28 in TLC plates when using combination of benzene : ethyl acetate (10:1) under 365 nm UV light for leaves

CHAPTER 5

DISCUSSION

Totally 53 seed borne fungi isolates were found from 10 rice varieties which identified as *Curvularia* spp., *Fusarium* spp., *Rhizoctonia* spp., *Alternaria* spp., *Cordana pauciseptata, Penicilium* spp., *Trichoderma* spp. and *Magnaporthe oryzae*. Four isolates of *Magnaporthe oryzae* were isolated from rice seed and leaf blast symptom. It was identified by morphology and confirmed by molecular method. *M. oryzae* is an aggressive isolate that causes blast disease of rice as reported by Ou (1985). The symptom normally is the presence of roundish to slightly elongated, necrotic, gray spots as reported in another study (Song and Soytong, 2016). The molecular phylogeny was confirmed as *M. oryzae,* which was in the same clade with sequences of *M. oryzae* MF669475, MF678836, MF668692, MF668691 recorded in the database supported by 99 % bootstrap value consistent with Jagadeesh *et al.* (2018). *C. elatum* (ChE01) is confirmed in phylogenetic tree that closely related to MH856469, MH859003, MH864188, MN534815, MN534813, MN534809, which database supporting by 100 % bootstrap value and *C. lucknowense* (CL) is closely related to MH080360, JX280827, which database supporting by 99 % bootstrap value. *C. brasiliense* (CB) is closely related to C. *brasiliense* JX966545 which database supporting by 100 % bootstrap value (Yew *et al.,* 2014). Pornsuriya *et al.* (2011) stated that phylogenetic analysis of ITS rDNA sequence data also supports *Chaetomium siamense* as distinct.

In the current study, *C. cochliodes* CTh05 demonstrated activity against the *M. oryzae* isolate PO1, which causes rice blast. The fungal isolate that caused blast symptoms on rice var. RD57 was morphologically and molecularly identified as *M. oryzae*. Morphology and molecular techniques confirmed the identity of *C. cochliodes* CTh05. *Magnaporthe oryzae* isolate PO1 found to be a virulent isolate causing blast of rice var. RD57. Biculture tests showed that *C. cochliodes* CTh05 inhibited the growth of *M. oryzae* PO1. A previous report by Soytong (2014) stated that *C. cochliodes* proved to be antagonistic to *Drechslera oryzae* (brown leaf spot of rice var Pittsanulok 2). *Chaetomium cochliodes* CTh05 significantly inhibited colony growth and spore production of the tested pathogen in biculture tests, as reported by Tann and Soytong (2017) for *Chaetomium cupreum* CC3003 inhibiting *C. lunata* (leaf spot of rice).

The tested *Chaetomium* spp. showed activity against *M. oryzae* in bi-culture with similar effects as antibiosis mechanism of control reported by Soytong (1992) that *Chaetomium cupreum* isolated from upland rice field soils in the Philippines, was antagonistic to the anamorph *Pyricularia oryzae* in Bi-culture test. Coating rice seed of blast susceptible rice line, IR442-2-58 resulted in control rice blast disease.

The crude EtOAC, crude MeOH and crude hexane extracts of *C. elatum* inhibited spore production with ED_{50} values of 106, 188 and 266 ppm, respectively. Results were similar to previous work (Song and Soytong 2016). The crude hexane extract of *C. brasiliense* inhibited spore production of *M. oryzae* with the ED_{50} value of 35 ppm when compared to EtOAC and MeOH crude extracts with the ED50 values of 55 and 119 ppm, respectively. The research finding was similar to that of the work of Soytong (2014). Moreover, crude extracts from EtOAC of *C. lucknowense* inhibited the spore production of *M. oryzae* PO2 with the ED_{50} value of 57 ppm. This was a similar result with the work of Soytong *et al.* (2013). Hexane and MeOH crude extracts *C. lucknowense* inhibited the tested pathogen with ED_{50} values of 103 and 422 ppm, respectively.

The fungal metabolites of *C. cochliodes* CTh05 (CCoH, CCoE and CCoM) expressed antifungal activity against *M. oryzae* isolate PO1 and inhibited spore production with ED50 values of 374, 85, 144 ppm. Soytong (2014) reported that metabolites from *C. cochliodes* suppressed the spore production of *D. oryzae* (brown leaf spot of rice) significantly, with an ED50 value of 66.45 ppm. Tann and Soytong (2017) reported that the hexane-crude extract, ethyl acetate-crude extract and methanol-crude extract of *C. cupreum* C3003 inhibited the spore production of *C. lunata* (leaf spot of rice) with ED_{50} values of 6.41, 0.83 and 7.81 ppm, respectively. *Chaetomium cochliodes* CTh05, which was tested in the current study, was previously reported to produce chaetoviridines E, chaetochalasin A and 24(R)-5a, 8a-epidioxyergosta-6-22-diene-3b-ol which those compounds inhibited *Plasmodium falciparum*. The cochliodones C, chaetoviridines E, chaetoviridines F and chaetochalasin A exert antimycobacterial activity. Moreover, chaetoviridines E and F inhibited cytotoxicity against the KB, BC1 and NCI-H187 cell lines (Phonkerd *et al.*, 2008). The current study revealed that the fungal metabolites from *C. cochliodes* CTh05 suppressed *M. oryzae* PO1, which causes rice blast.

The highlight of the research findings is interesting in that nanoparticles constructed from *Chaetomium* spp. can be used at lower concentrations than crude extracts to inhibit *M. oryzae* causing rice blast. Previous research on nanoparticles from *Chaetomium* sp. were characterized to be nano-materials loaded with active compounds. Crude extracts from *Chaetomium* spp. were incorporated into polyacetic acid and electrospin at 25-30 kv. Scanning electron microscope images revealed that the nano materials from *Chaetomium* spp. measured as 171 nanometers (Tan and Soytong, 2016). Results showed that nano-CEE, nano-CBH and nano-CEM inhibited spore production of rice blast pathogen, *M. oryzae* with the ED_{50} values of 7.89, 8.66 and 16.7 ppm, respectively. Nanoparticles of *C. brasiliense,* nano-CBH, nano-CBE and nano-CBM suppressed spore production of *M. oryzae* with the ED_{50} values of 6.86, 9.76 and 13.42 ppm, respectively. Nano-CLM, nano-CLE and nono-CLH resulted in spore inhibition of *M. oryzae* with the ED_{50} values of 5.24 ,7.01 and 10.72 ppm, respectively. Tan and Soytong (2016, 2017) reported that nanoparticles loaded with *Chaetomium globosum* KMITL0805 actively worked against *Curvularia lunata* causing leaf spot of rice var Sen Pidoa, and the nanoproduct from *Chaetomium cupreum* CC3003 could control leaf spot of rice var. Sen Pidoa. Moreover, in vivo test showed these nano-particles treated to rice seedlings could control leaf spot of rice var. Sen Pidoa and decreased leaf spot disease of rice which supporting the stability of nanoparticles after apply to the rice seedlings.

Control mechanism of nanoparticles derived from *Chaetomium* spp. was proven to be antibiosis and as lysis as previous reports by Tan and Soytong (2016) and

Vilavong and Soytong (2017). It was demonstrated that rice seedlings inoculated with the treated spores of *M. oryzae* with nano-CE, nano-CL and nono-CB resulted in loss of pathogenicity. Similar result was reported by Sibounnavong *et al.* (2011).

Nanoparticles derived from *C. cochliodes* CTh05 (nano-CCoH, nano-CCoE and nano-CCoM) demonstrated to inhibit *M. oryzae* PO1 with ED_{50} values of 9, 16 and 33 ppm, respectively. *Chaetomium cochliodes* CTh05 has been found to produce the active compounds against human pathogens (Phokerd *et al*., 2008). Those active compounds may possible serve as a control mechanism.

The crude extracts derived from *C. cochliodes* CTh05, CCoH, CCoE and CCoM found to inhibit *M. oryzae* at ED_{50} values of 374, 85 and 144 ppm, respectively. The nanoparticles of nano-CCoH, nano-CCoE and nano-CCoM gave high potential at low application, with ED_{50} values of 33, 9 and 16 ppm, respectively. It is suggested that applying nanoparticles would be less costly than metabolites. A similar report by Tan and Soytong (2016) stated that nano-CGH, nano-CGE and nano-CGM derived from *C. globosum* KMITL-N0805 exhibited antifungal activity against *C. lunata* (leaf spot of rice) with ED_{50} values of 1.21, 1.19 and 1.93 ppm, respectively.

The current study revealed that the spores of *M. oryzae* exhibited abnormal morphology after treatment with nanoparticles from *C. cochliodes* CTh05. It was also reported by Tan and Soytong (2017) that nanoparticles from *C. globosum* can disrupt and distort the spores of *C. lunata* and cause a loss of pathogenicity. Singh *et al*. (2015) reported that nanotechnology in agriculture is being revolutionized by innovative new techniques for disease control. The current research revealed that nanoparticles constructed from fungal metabolites are promising for plant disease control. Dar and Soytong (2014) reported that the fungal metabolites from *C. globosum* and *C. cupreum*, generated to be nanomaterials at the averaged size of 241 and 171 nm, respectively. Tan and Soytong (2016) also reported that nanoparticles from *C. globosum* can disrupt and distort the pathogen cells and cause a loss of pathogenicity. Nano-CCoH, nano-CCoE and nano-CCoM constructed from *C. cochliodes* CTh05 may help to increase the efficiency of application at lower doses to inhibit rice blast pathogen as stated by Sharon *et al*. (2010).

In the current work, blast incidence in rice seedlings treated with nano-particles constructed from a crude extract mixture at 10 ppm was reduced by 38 %, and followed by the chemical fungicide treatment (tricyclazole) which reduced blast incidence by 29 %. Similar results were reported by Soytong (2014), who stated that bioproducts from *C. cochliodes* CTh05 can control the rice leaf spot caused by *C. lunata*, but at a high application rate of 50 g per 20 L of water.

In vivo evaluation of nano-CCoM, nano-CCoE and nano-CCoH constructed from crude hexane, ethyl acetate and methanol extracts of *C. cochliodes* CTh05 reduced blast incidence at concentrations of 7 ppm. It suggested that application of nanoparticles constructed from each crude extract (nano-CCoM , nano-CcoE and nano-CCoH) reduced blast incidence at a lower rate of application than nanoparticles derived from a crude extract mixture from *C. cochliodes* CTh05. Further research should investigate these nanoparticles as elicitors for rice immunity to blast disease.

Result from TLC plates found Sakuranetin at Rf value 0.08 which similar to

Hasegawa (2014) who stated that sakuranetin extracted from rice leaves showed spot in TLC to detect the presence of sakuranetin at R_f 0.09 under 365 nm UV light. It is indicated to be Oryzalexin B the spot on TLC with the R_f value of 0.28 when using combination of benzene : ethyl acetate (10:1) under 365 nm UV light for leaves. The research finding revealed the same as Akatsuka (1983) who found Oryzalexin B as the phytoalexin against rice blast in TLC at the same R_f value.

It is concluded that the research findings are reported to be the first time on discovery of nanoparticles construced from natural product fungal metabolites from *Chaetomium* spp that could be used to control rice blast disease and induced phytoalexin production against blast disease.

CHAPTER 6

CONCLUSION

Magnaporthe oryzae (= *Pyricularia oryzae*) were isolated from different varieties of rice seeds and leaf lesion, and proved to be pathogenic to cause the blast in rice var. RD 57 and PSL 2. *Chaetomium elatum*, *C. lucknowense, C. brasiliense* and *C. cochliodes* were proved to be antagonized to *M. oryzae*. All isolates of *M. oryzae* and *Chaetomium* spp. were taxonomic confirmation by morphology and molecular phylogeny. Biculture tests proved that all *Chaetomium* spp. gave a good control the rice blast pathogen. The metabolites extracted from the tested *Chaetomium* spp. were actively against *M. oryzae.* Hexane extract of *C. brasiliense* showed the highest spore inhibition with ED_{50} of 35 ppm and follwed by EtOAC extract of *C. lucknowense* and EtOAC extract of *C. elatum* with ED_{50} of 57 and 106 ppm. The metabolites of *C. cochliodes* (CCoH, CCoE and CCoM) showed antifungal activity against the rice blast pathogen with ED_{50} of 85, 144 and 374 ppm, respectively.

Nanoparticles constructed from crude metabolites of *C. elatum*, *C. lucknowense, C. brasiliense* and *C. cochliodes* were expressed antifungal activity against *M. oryzae*. Nano-CLM (*C. lucknowense*, Nano-CBH (*C. brasiliense*) and Nano-CEE (*C. elatum*) inhibited spore production with ED_{50} of 5.24 , 6.86 and 7.89 ppm. The nanoparticle sizes of nano-CCoH, nano-CCoE and nano-CCoM were ranged between 567-611, `422-566 and 415-472 nm., respectively. The nano-CCoH, nano-CCoE and nano-CCoM derived from *C. cochliodes* at concentrations of 3-15 ppm inhibited spore production of *M. oryzae* in with ED_{50} of 9, 16 and 33 ppm, respectively. The rice seedlings var. PSL2 treated with nano-CBH from *C. brasiliense* found to produce Sakuranertin and Oryzalexin B which the R_f value was 0.08 assumed to be Sakuranertin, and Rf values of 0.28 is Oryzalexin B. It is found to be the first time that nano-CBH from *C. brasiliense* act as elicitor to induce plant immunity against rice blast. *In vivo* experiments showed a reduction of 38% in blast disease after application of crude extract mixture of nanoparticles from *C. cochliodes* at 10 ppm for 30 days. But the Tricyclazole reduced the blast incidence by 29%. Rice blast was decreased after applying nano-CCoM at 7 ppm reduced the blast of 60 % and followed by nanoCCoE, nanoCCoH which reduced the blast of 58 and 50% respectively, when compared to chemical tricyclazole resulted in a 56% blast reduction.

It is a new discovery of nanotechnology for disease control which construced from natural product of *Chaetomium* spp to induce phytoalexin production against blast disease.

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APPENDIX

PUBLICATION

Publications:

1. Song, J. J., Kanokmedhakul, S., Kanokmedhalkul, K. and Soytong, K. (2018). Application of nano-particles derived from *Chaetomium elatum* ChE01 to control *Pyricularia oryzae* causing rice blast. International Journal of Agricultural Technology 14(6):923-932.
2. Song, J. J., Soytong, K., Kanokmedhakul, S. and Kanokmedhakul, K. (2018). Nano-particles from *Chaetomium lucknowense* to inhibit rice blast pathogen causded by *Pyricularia oryzae* in pot experiment. International Journal of Agricultural Technology 14(7): 1961-1968.
3. Song J J, K Soytong, S Kanokmedhakul, K Kanokmedhkul, S Poeaim (2020). Natural product of nano-particles constructed from *Chaetomium* spp. to control rice blast disease caused by *Magnaporthe oryzae*. *Intl J Agric Biol* 23:1013–1020. (ISI, IF=0.8)
4. Song JJ, K Soytong, S Kanokmedhakul, K Kanokmedhkul, S Poeaim (2020). Antifungal activity of microbial nanoparticles derived from *Chaetomium* spp against *Magnaporthe oryzae* causing rice blast. Plant Protection Science. (ISI, IF=1.4, In press).

APPENDIX

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Full Length Article

Natural Product of Nano-Particles Constructed from *Chaetomium* spp. to Control Rice Blast Disease Caused by *Magnaporthe oryzae*

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Abstract

Nanoparticles containing active compounds derived from *Chaetomium cochliodes* (CTh05) was tested to control rice blast disease caused by *Magnaporthe oryzae* isolate PO1. The causal agent of rice blast on leaves of rice var. RD57 was isolated. *M. oryzae* and *C. cochliodes* were identified morphologically and confirmed by molecular phylogenetic. *M. oryzae* was demonstrated to be pathogenic, causing blast of rice var. RD57. Biculture tests demonstrated that *C. cochliodes* could suppress the growth of *M. oryzae*. The crude metabolites of *C. cochliodes* (CCoH, CCoE and CCoM) at the concentrations of 10–1000 ppm expressed antifungal activity against the *M. oryzae*, resulting in inhibited spore production in 12 days with effective dose (ED_{50}) values of 85, 144 and 374 ppm, respectively. The nanoparticles sizes of nano-CCoH, nano-CCoE and nano-CCoM were ranged between 567–611, `422–566 and 415–472 nm, respectively. Nanoparticles derived from *C. cochliodes* (nano-CCoH, nano-CCoE and nano-CCoM) at concentrations of 3–15 ppm significantly inhibited *M. oryzae* in 12 days with ED_{50} values of 9, 16 and 33 ppm, respectively. *In vivo* experiments revealed a reduction of 38% in blast disease after application of nanoparticles constructed from crude extract mixtures from *C. cochliodes* at 10 ppm for 30 days. Tricyclazole resulted in reduction of blast disease by 29%. Rice blast disease was decreased in 30 days after applying nano-CCoM at the concentration of 7 ppm (60% disease reduction), followed by nanoCCoE and nanoCCoH with disease reduction of 58 and 50% respectively, and tricyclazole resulted in a 56% reduction in rice blast. © 2020 Friends Science Publishers

Keywords: *Chaetomium* spp.; *Magnaporthe oryzae*; Nanoparticles; Rice blast

Introduction

Rice blast is a disease caused by *Magnaporthe oryzae* (Hebert) Barr. This disease is the first recorded of rice (*Oryza sativa* L.) and it was noted as rice fever disease in China as early as in 1637 (Wang and Valent 2009). Rice blast has spreaded through out in Asia, Latin America and Africa, and is now reported in over 85 countries worldwide (TeBeest *et al*. 2007). *M. oryzae* can infect all parts of the plant, which resulted in yield losses in many developing countries in recent years (Wang and Valent 2009). Rice blast has become the most common rice disease due to its wide distribution and high infection level under favourable conditions. Valent (2004) stated that the disease has already caused epidemics in all continents where rice is grown, and yield loss due to blast can be as high as 50% (Gnanamanickam 2009). The effective disease control strategies are needed to reduce or eliminate the use of chemical fungicides, which damaging to the surroundingenvironments and residue in agricultural products. Nanotechnology in agriculture has emerged as a new tool to create and re-structure the materials at the molecular level. Molecular nanotechnology involves in constructing organic materials into defined structures, atom by atom or molecule by molecule (Soutter 2013). The application of nanotechnology in agriculture has gained an interestingattention in recent years (Li *et al*. 2011). Researchers have actively investigated the synthesis of organic nanomaterials in various types andtested their biological properties (Elibol *et al*. 2003; Salata 2004). Nanotechnology in agriculture is being explored for crop production (Soutter 2013) and may bepotentially provided the solutions for various challenges faced in agriculture (Ditta 2012). Nanoparticles contain bioactive substances from natural products that can rapidly and effectively penetrate through plant cuticles and tissues and can increase

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the stability of active compounds to decrease leaching (Perlatti *et al*. 2013). Thus, it can provide an efficient pest management strategy in agriculture (Rai and Ingle 2012). These can be formulated in colloidal suspension or powder for application (Ditta 2012).

In recent year, the natural products from the fungus *Chaetomium* spp. reported to be antifungal activity against several plant pathogens (Soytong *et al*. 2001). Nanoparticles were constructed from crude hexane, ethyl acetate and methanol extracts of *Chaetomium globosum* KMITL-N0805, whichcoded as Nano-CGH, nano-CGE and nano-CGM, actively inhibited *Curvularia lunata* (Wakker) Boedijn in rice var. Sen Pidoa, with ED_{50} values of 1.21, 1.19 and 1.93 ppm, respectively (Tann and Soytong 2016). The findings are demonstrated *in vivo* tests that nano-CGH, nano-CGE and nano-CGM from*C. Globosum* can be controlled leaf spot of rice at 60 days; nano-CGH and nano-CGM decreased disease of 61.54% and nano-CGE decreased disease incidence by 53.83% (Tann and Soytong 2016). Furthermore, Tann and Soytong (2017) reported that nano-product derived from *C. cupreum* L.M Ames reduced the rice leaf spotranging from 41.7–58.3% compared to the non-treated controlin pot experiment.

The objective of the current research was to evaluate the nanoparticles constructed from natural products of *C. cochliodes* CTh05 for their biological activities against *M. oryzae*, which causes rice blast and their application for disease control.

Materials and Methods

Isolation of pathogens and pathogenicity test

The blast specimens were collected from the symptomatic leaves of rice var. RD 57 in rice fields at the Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand on 12 June 2017. Isolation was performed using a tissue transplanting technique (Abed-Ashtiani *et al*. 2016) and a pure culture was maintained in rice flour agar (RFA) (rice flour 25 g, yeast 2 g and agar 15 g) media. Morphological identification using a binocular compound microscope was done according to the work of Ou (1985). The pathogen was deposited as culture collection No. KMILT 001/2017 at the Biocontrol Research Unit, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand.

Antagonistic fungus

C. cochliodes CTh05, used in the current study, has been reported by Phonkerd *et al*. (2008) to produce four dimeric spiro-azaphilones; (cochliodones A to D), two azaphilones; (chaetoviridines E and F) and an epi-chaetoviridin A, which expressed antimicrobial activity against malaria disease (*Plasmopara falciparum* Welch), tuberculosis (*Mycobacterium tuberculosis* Zopf) and cancer cell lines. *C. cochliodes* CTh05 was cultured in potato dextrose agar (PDA) and incubated at room temperature (27–30°C). The morphological identification was performed according to Arx *et al*. (1986) and Soytong (1989).

Molecular identification

The fungal genomic DNA was separately extracted from *M. oryzae* PO1 and *C. cochliodes* CTh05. Each fungus was cultured for 3 d in potato dextrose broth (PDB). The genomic DNA was done from freeze-dried mycelia using the modified cetyl trimethyl ammonium bromide (CTAB) method. The mycelia were cleaned with 25 m*M* ethylene diamine tetraacetic acid (EDTA) by centrifugation at 14 000 rpm at 4°C for 5 min, then, 100 mg of mycelia were crushed in liquid nitrogen, and lysed in CTAB buffer containing β-mercaptoethanol (2 μL). The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and then centrifuged at 14 000 rpm at 4°C, for 5 min then, 2 μL of Rnase (20 μg mL) was added to the aqueous phase and incubated at 37°C for 30 min. The samples were mixed with 50 μL of 10% CTAB and centrifuged. The pellets were washed twice with 70% and 95% ethanol and dissolved in 100 μL TE (Tris-EDTA) buffer at 37°C overnight. The quality and quantity of extracted DNA were monitored by electrophoresis on a 1% agarose gel. Quantification of the DNA was performed by comparing the intensity of the bands to known dilutions of lambda phage DNA. Polymerase chain reaction (PCR) was done to amplify the internal transcribed spacer (ITS) ribosomal DNA regions using the universal primers ITS1 and ITS4, according to the method of White *et al*. (1990).

The amplified products were sequenced and aligned with sequencing in the GeneBank by the basic local alignment search tool (BLAST) (Altschul *et al*. 1997) at the National Centre for Biotechnology Information (NCBI) database. The sequences of closely related organisms were downloaded to construct the phylogenetic trees, which were aligned through CLUSTALW using MEGA version 6.0 software (Tamura *et al*. 2007). The phylogenetic tree was done according to the neighbour-joining method.

Biculture test

The biculture test was conducted by following the method described by Soytong and Quimio (1989). The experimental design was done by completely randomized design (CRD) with four repeated experiments. *C. cochliodes* CTh05 and *M. oryzae* were transferred separately on agar plugs (0.3 cm diameter) to RFA at opposite sites in bi-culture plates. *C. cochliodes* CTh05 and the rice blast pathogen were separately cultured and each isolate on RFA served as the control. All plates were maintained at room temperature for 30 d. Data collection were recorded on colony diameter (cm), number of spores and were computed analysis of variance (ANOVA) by the Statistical Package for Social

Sciences (IBM SPSS Statistics, ver. 21.0) software (Titone *et al*. 2015). Significance was declared at $P \leq 0.05$ and 0.01.

Bioactivity tests of crude metabolites from *C. cochliodes* CTh05

Crude extracts from *C. cochliodes* CTh05 were cultured in PDB at room temperature (30°C) for 30 d. The dried fungal biomass culture was separatelyextracted with hexane, ethyl acetate and methanol following the method described by Phonkerd *et al*. (2008). The experiment was designed as a two-factor factorial experiment with a CRD and four replications. Factor A represented the crude extracts hexane, ethyl acetate and methanol, and factor B represented theconcentrations including 0, 10, 50, 100, 500 and 1000 ppm. A culture agar plug of 3 mm was transferred to the middle of RFA plate in each treatment and then incubated at room temperature (27–30°C) for 15 d. The data were presented as the colony diameter (cm), and the number of spores was determined using a hemocytometer. The data were analysed using ANOVA by SPSS software ver. 21.0 (Titone *et al*. 2015). Significance was declared at $P \leq 0.05$ and 0.01. The effective dose (ED_{50}) was done using the probit analysis program (Titone *et al*. 2015).

Bioactivity tests of crude metabolite nanoparticles from *C. cochliodes* CTh05

The morphological characteristics of the nano-CCoH, nano-CCOE, and nano-CCoM were viewed under a scanning electron microscope. The crude extracts from *C. cochliodes* CTh05, including crude hexane, ethyl acetate and methanol extracts, were separately incorporated into polylactic acid-based nanoparticles through electrospinning, following the method described by Dar and Soytong (2014) and Tann and Soytong (2016) to yield nanoparticles from crude hexane, ethyl acetate and methanol extrtacts of *C. cochliodes* CTh05, coded as nano-CCoH, nano-CCoE and nano-CCoM. The nanoparticle products were collected and stored in capped bottles after electrospinning. The characteristics of the nano-CCoH, nano-CCoE and nano-CCoM were observed by the naked eye and viewed under a scanning electron microscope and the properties were analysed by Fourier-transform infrared spectroscopy (FTIS).

The nano-CCoH, -CCoE and -CCoM were tested for their abilities to inhibit the rice blast pathogen. The experiment was performed using a two-factor factorial CRD with four replications. Factor A represented the type of nanoparticles and factor B the concentrations (0, 3, 5, 7, 10 and 15 ppm). The experiment was repeated four times. The data presented as colony diameter (cm) and the number of spores. Statistical significance was determined using ANOVA by SPSS software, ver. 21.0 (Titone *et al*. 2015). Significance was declared at $P \leq 0.05$ and 0.01. The effective dose (ED50) was calculated using the probit analysis program (Titone *et al*. 2015).

In vivo nanoparticles constructed from *C. cochliodes* CTh05 against rice blast disease

The experimental design was used as randomized complete block (RCB) with four replicates. The treatments were performed as follows: Treatment 1 was the non-inoculated control, Treatment 2 was the inoculated control, Treatment 3 was the nanoparticles from a crude extractmixture of *C. cochliodes* CTh05 at 10 ppm, and Treatment 4 was the chemical fungicide (tricyclazole) at the recommended rate of 2.25 g L^{-1}. The data were recorded as the fresh and dry weight of the stems at 90 d and computed analysis of variance. Mean comparison in each treatment was done by SPSS software, ver. 21.0, and significance was declared at $P \leq 0.05$ and 0.01. Plants were assigned a disease index at 7 d post-inoculation using a scale of 0–9 (modified from Xia *et al*. 1993) where 0 = no infection, 1 = small brown spot infection < 1 mm; 2 = small rounded spot infection < 2 mm; 3 = small spot infection with open centres < 3 mm; 4 = lesions with expanded open centres > 3 mm on < 10% of the leaf area; 5 = lesions with expanded open centres on 10–25% of the leaf area; 6 = lesions with expanded open centres on 26–50% of the leaf area; 7 = expanded lesions with open centres on 51–75% of the leaf area; 8 = expanded lesions with open centres on 76–90% of the leaf area; 9 = expanded lesions with open centres on > 90% of the leaf area.

In vivo nano-particles from *C. cochliodes* CTh05 against rice blast disease

This experiment was designed as a RCBD with four replications. Nanoparticles derived from hexane, ethyl acetate and methanol crude extracts from *C. cochliodes* CTh05 were separately appliedat a concentration of 7 ppm to the rice seedlings inoculated with *M. oryzae* PO1. The treatments were done as follows: non-inoculated control (T1), inoculated with *M. oryzae* PO1 (T2), nano-CCoH (T3), nano-CCoE (T4), nano-CCoM (T5) and tricyclazole (T6). The disease index (DI) was evaluated as described above and disease reduction (DR) was calculated as follows:

$$\text{DR} = \text{averaged DI in treatment} - \frac{\text{averaged DI in inoculated control}}{\text{averaged DI in treatment}}$$

Results

Isolates of rice blast pathogen and *Chaetomium* spp.

The pathogen isolated from symptomatic leaves of rice var. RD57 was morphologically identified as *M. oryzae* isolate PO1 (Fig. 1). The fungus was cultured on RFA which covered the plate (9 cm) in 10 d. The mycelia were observed to be septate and hyaline, producing conidiophores and three-celled conidia. *C. cochliodes* CTh05, from a previous study by Phonkerd *et al*. (2008), was cultured on PDA for 3 weeks and was olive-green to brown, producing perithecia

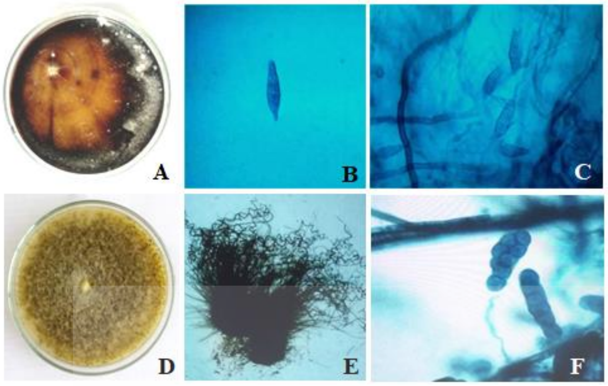

Fig. 1: *Magnaporthe oryzae* PO1, **A** = pure culture in RFA; **B** = conidium; **C** = mycelia and conidia and *Chaetomium cochliodes* CTh05, **D** = Colony, **E** = Perithecia, **F** = Asci and ascospores

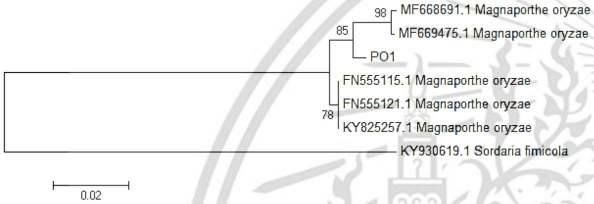

Fig. 2: Phylogenetic tree of *Magnporthe oryzae* from GenBank, including *Magnporthe oryzae* PO1, constructed based upon the distance-based analysis of the ITS1 and 5.8S regions of rDNA. The numbers at the branches indicate the percentage of bootstrap values after 1000 replications. The outgroup taxon is *Sordaria fimicola*

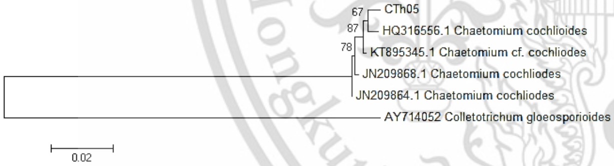

Fig. 3: Phylogenetic tree of *C. cochliodes* from GenBank, including *C. cochliodes* CTh05, constructed based upon the distance-based analysis of the ITS1 and 5.8S regions of rDNA. The numbers at the branches indicate the percentage of bootstrap values after 1000 replications. The outgroup taxon is *Colletotrichum gloeosporioides*

and subglobose asci; one ascus containing eight ascospores (Fig. 1).

Molecular phylogenic identification was performed to confirm the species. The phylogenetic treewasclearly identified the rice blast pathogen as *M. oryzae* MH590369, based upon the GeneBank database (Fig. 2). Data from the GeneBank reliably confirmed CTh105 as *C. cochliodes* MH590621 (Fig. 3). The pathogenicity of *M. oryzae* isolate PO1 to rice var. RD57 proved to be the blast pathogen. The inoculated wounds exhibited lesions inroundish to elongated grey necrotic spots, approximately 2–5 mm in diameter within 10 d.

Biculture test

M. oryzae isolate PO1 was inhibited by *C. cochliodes* CTh05. The colony diameter of *C. cochliodes* CTh05 averaged 4.4 cm in bicultureplate whereas the control platewas 9.0 cm. The colony growth inhibition was 52% after 10 d but when the incubation period was extended to 30 d, the colony grew over the pathogen and inhibition averaged above 90%.

Characterization of the nano-particles

The nanoparticles nano-CCoH, nano-CCoE and nano-CCoM, loaded with crude extracts from *C. cochliodes* CTh05, were visually characterized. Nano-CCoH, nano-CCoE and nano-CCoM were white, yellow and light yellow in colour, respectively (Fig. 4). Interestingly, the scanning electron images showed the range of particles size of nano-CCoH, nano-CCoE and nano-CCoM ranged between 567–611, 422–566 and 415–472 nm, respectively (Fig. 4).

Bioactivity test of crude metabolites from *C. cochliodes* CTh05

The results demonstrated that CCoE resulted in the highest spore inhibition of 88%, followed by CCoM and CCoH, which resulted in a spore inhibition of 81 and 68%, respectively, in 12 d at 1000 ppm (Table 1). The fungal metabolites of CCoH, CCoE and CCoM exhibited active antifungal activity against the *M. oryzae* isolate PO1 with ED_{50} values of 85, 144 and 374 ppm (Table 1).

Bioactivity tests of crude metabolite nanoparticles from *C. cochliodes* CTh05

The nano-CCoH, nano-CCoE and nano-CCoM at 15 ppm showed significantly inhibited spore production ($P \leq 0.01$) in comparison to the non-treated control (0 ppm). Nano-CCoE resulted in significantly ($P \leq 0.05$) greater spore inhibition in comparison to the non-treated control. Nano-CCoE inhibited spore production significantly ($P \leq 0.05$; 68%), followed by nano-CCoM (47%) and nano-CCoH (34%) in 12 d (Table 2). Nano-CCoE, nano-CCoM and nano-CCoH inhibited *M. oryzae* PO1 (rice blast) with ED_{50} values of 9.47, 16.51 and 33.41 ppm, respectively (Table 2). It was observed that the spores were abnormally shaped, and cells were broken after treatment with nanoparticles of *C. cochliodes* CTh05; in contrast, the spores were normally shaped in the non-treated control (Fig. 5).

In vivo nanoparticles constructed from *C. cochliodes* CTh05 against rice blast disease

The result revealed that the blast incidence caused by the *M. oryzae* isolate PO1 was significantly reduced ($P \leq 0.01$) by 38%, after the application of nanoparticles from *C. cochliodes* CTh05, followed by the chemical fungicide

Table 1: The effects of fungal metabolites of *C. cochliodes* CTh05 against *M. oryzae* PO1 at 12 d

Metabolites	Concentration (ppm)	Colony diameter (cm)	Growth inhibition (%)	Number of spores (10^5)	Spore Inhibition (%)	ED_{50}(ppm)
CCoH	0	5.00^{a}	-	20.75^{a}	-	
	10	4.88^{bc}	2.25^{ij}	20.25^{a}	2.56^{j}	
	50	4.73^{d}	5.25^{gh}	17.25^{b}	16.86^{h}	374.43
	100	4.43^{f}	11.25^{e}	14.25^{c}	31.23^{g}	
	500	3.91^{i}	21.75^{b}	10.00^{e}	52.18^{de}	
	1000	3.68^{j}	26.25^{a}	6.75^{fg}	67.57^{c}	
CCoE	0	5.00^{a}	-	20.75^{a}	-	
	10	4.94^{ab}	1.00^{j}	17.25^{b}	16.70^{h}	
	50	4.82^{c}	3.50^{i}	12.75^{cd}	38.64^{f}	85.87
	100	4.67^{d}	6.50^{g}	9.00^{ef}	56.69^{d}	
	500	4.02^{h}	19.50^{c}	5.00^{gh}	76.01^{b}	
	1000	3.64^{j}	27.00^{a}	2.50^{h}	88.03^{a}	
CCoM	0	5.00^{a}	-	20.75^{a}	-	
	10	4.86^{c}	2.75^{i}	18.75^{ab}	9.68^{i}	
	50	4.73^{d}	4.00^{hi}	14.00^{c}	32.58^{g}	144.23
	100	4.53^{e}	9.25^{f}	11.00^{de}	46.95^{e}	
	500	4.11^{g}	17.75^{d}	7.00^{fg}	66.40^{c}	
	1000	3.91^{i}	21.75^{b}	4.00^{h}	80.74^{b}	
C.V.(%)		0.74	7.22	10.79	6.27	-

Means followed by a common letter are not significantly different by DMRT at $P \leq 0.05$

Table 2: The effects of nano particles derived from *C. cochliodes* CTh05 against *M. oryzae* PO1 at 12 d

Nano-particles	Concentration (ppm)	Colony diameter (cm)	Growth inhibition (%)	Number of spores (10^5)	Spore Inhibition (%)	ED_{50}(ppm)
Nano-CCoH	0	5.00^{a}	-	69.00^{a}	-	
	3	4.96^{ab}	0.75^{e}	67.00^{ab}	2.78^{i}	
	5	4.88^{bc}	2.25^{de}	56.00^{bcd}	18.98^{f}	33.41
	10	4.82^{cd}	3.50^{cd}	50.75^{cd}	26.63^{e}	
	15	4.76^{d}	4.75^{c}	45.00^{de}	34.87^{cd}	
Nano-CCoE	0	5.00^{a}	-	69.00^{a}	-	
	3	4.93^{ab}	1.25^{e}	60.00^{abc}	13.23^{fg}	
	5	4.87^{bc}	2.50^{de}	49.00^{cd}	28.99^{de}	9.47
	10	4.78^{d}	4.25^{cd}	36.00^{e}	47.99^{b}	
	15	4.58^{e}	8.25^{b}	22.00^{f}	68.26^{a}	
Nano-CCoM	0	5.00^{a}	-	69.00^{a}	-	
	3	4.87^{bc}	2.50^{de}	64.00^{ab}	7.36^{gh}	
	5	4.77^{d}	3.75^{cd}	51.25^{cd}	26.00^{e}	16.51
	10	4.56^{e}	8.75^{b}	44.00^{de}	36.40^{c}	
	15	3.88^{f}	22.25^{a}	36.00^{e}	47.97^{b}	
C.V.(%)		1.18	25.04	11.03	13.91	-

Means followed by a common letter are not significantly different by DMRT at $P \leq 0.05$

treatment (tricyclazole), which reduced blast incidence by 29% when compared to the inoculated controlwith *M. oryzae* PO1 (Table 3). In the *in vivo* experiment, all treatments resulted in significant differences ($P \leq 0.01$) in plant height at 15 d post-treatment (Fig. 6). The application of nanoparticles of *C. cochliodes* CTh05 resulted in the greatest plant height (84.7 cm), which was significant at $P \leq 0.01$, followed by the chemical fungicide (tricyclazole), which resulted in a plant height of 74.9 cm; the non-inoculated control and the control inoculated with *M. oryzae* exhibited heights of 77.7 and 77.6 cm, respectively. The application of nanoparticles from *C. cochliodes* CTh05 and tricyclazole were not significant differences in stem fresh weight, 64.7 and 66.7 g, respectively, but they were significantly differed ($P \leq 0.01$) from the non-inoculated control and the inoculated with *M. oryzae*, 57.2 and 45.5 g, respectively. The root fresh weight showed the highest (79.4 g) in plants which treated with nanoparticles of *C. cochliodes* CTh05, followed by the non-inoculated control, the inoculated with *M. oryzae*, and the plants treated with tricyclazole treatments: 66.6, 58.0 and 44.2 g, respectively (Table 3). The nanoparticles of *C. cochliodes* resulted in significantly ($P \leq 0.01$) higher stem dry weight (14.1 g) compared with tricyclazole (11.1 g) and inoculation with *M. oryzae* alone 12.0 g (Table 3). The root dry weight showed the highest after treatment with tricyclazole (11.3 g), followed by the nanoparticles of *C. cochliodes* (10.5 g) and inoculated with *M. oryzae* isolate PO1 alone (6.4 g).

In vivo nano-particles from *C. cochliodes* CTh05 against rice blast disease

The results demonstrated that 15 d after the treatments, nano-CCoM resulted in ($P \leq 0.01$) greater plant height (43.0 cm) compared with nano-CCoH and nano-CCoE, which resulted in a plant height of 41.0 and 40.5 cm, respectively, and followed by tricyclazole 38.5 cm, meanwhile, the inoculated control showeda plant height of 38.6 cm (Table 4 and Fig. 7). However, 30 d after treatment, plant height did not significantly differ among plants treated with nano-

Table 3: Effect of crude extract mixture nano-particles on plant height, fresh and dry weight of stems and roots and disease reduction of blast in rice var. RD 57

Treatments	Plant height (15 d) (cm)	Plant height (30d) (cm)	Stem Fresh weight (g)	Root Fresh weight (g)	Stem Dry Weight (g)	Root Dry Weight (g)	Disease Reduction (%)
T1(Non-inoculated control)	62.0^{a}	77.74^{b}	57.23^{b}	66.55^{b}	17.81^{a}	8.82^{b}	-
T2 (Inoculated with *M. oryzae)*	62.6^{a}	77.58^{b}	45.53^{c}	58.03^{c}	12.01^{c}	6.42^{b}	-
T3(*M. oryzae* + nanoparticles of *C. cochliodes*)	63.3^{a}	84.67^{a}	64.70^{a}	79.41^{a}	14.05^{b}	10.51^{a}	37.5
T4 (*M. oryzae* + Tricyclazole)	62.1^{a}	74.91^{b}	66.66^{a}	44.20^{d}	11.12^{cd}	11.28^{a}	29.1
CV(%)	2.52	12.48	22.82	29.87	29.29	28.96	-

Means followed by a common letter are not significantly different by DMRT at $P \leq 0.05$

Table 4: Effect of nanoparticles derived from hexane, ethyl acetate and methanol extraction plant height and disease reduction (%) on rice var. RD 57

Treatments	15 (d)	30 (d)	Disease reduction (%)
T1 (Non-inoculated control)	38.70^{c}	54.50^{c}	-
T2 (Inoculated with *M. oryzae*)	38.60^{c}	53.75^{c}	-
T3 (Nano-CCoH)	41.00^{ab}	62.25^{b}	50.2
T4 (Nano-CCoE)	40.50^{b}	76.25^{a}	57.5
T5 (Nano-CCoM)	43.00^{a}	77.75^{a}	59.8
T6 (Tricyclazole)	38.50^{c}	75.00^{a}	55.5

Means followed by a common letter are not significantly different by DMRT at $P \leq 0.05$

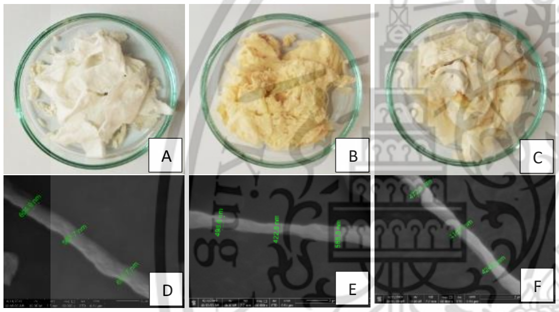

Fig. 4: Nanoparticles from *C. cochliodes* CTh05 (upper part: **A**= nano-CCoH, **B** = nano-CCoE, **C** = nano-CCoM) and scanning electron microscopy of nano-particles (lower part: **D** = nano-CCoH, **E** = nano-CCoE and **F** =nano-CCoM)

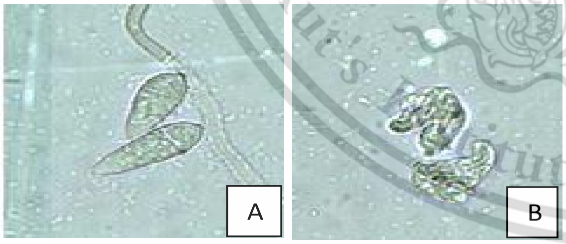

Fig. 5: Normal spores of the rice blast pathogen (**A**) and abnormal spores (**B**) of the *M. oryzae* isolate PO1 after treatment with nanoparticles derived *C. cochliodes* CTh05

CCoM, nano-CCoE and tricyclazole; for these groups, plant heights were 77.8, 76.3 and 75.0 cm, respectively and followed by the nano-CCoH (62.3 cm) treatment. The inoculated control had a plant height of 53.8 cm. The blast incidence was reduced after treatment with nano-CCoM (60%) more than nano-CCoE (58%), tricyclazole (56%) or nano-CCoH (50%).

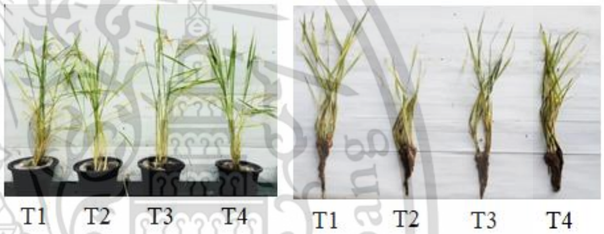

Fig. 6: Testing the capacity for nanoparticles derived from *C. cochliodes* CTh05 to inhibit rice blast, T1 = non-inoculated control, T2 = inoculated with *M. oryzae*, T3 = *M. oryzae* + nanoparticles of *C. cochliodes*, and T4 = *M. oryzae* + Tricyclazole

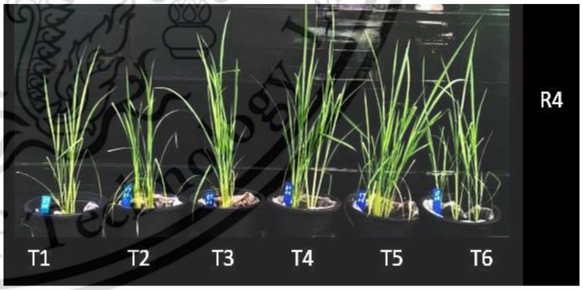

Fig. 7: Testing the capacity of nanoparticles derived from *C. cochliodes* CTh05 in inhibiting rice blast 15 days after treatments, non-inoculated control (T1), inoculated with *M. oryzae* (T2), nano-CCoH (T3), nano-CCoE (T4), nano-CCoM (T5) and tricyclazole (T6)

Discussion

In this study, *C. cochliodes* CTh05 demonstrated activity against the *M. oryzae* isolate PO1, which causes rice blast. The fungal isolate that caused blast symptoms on rice var. RD57 was morphologically and molecularly identified as *M.*

oryzae. Morphology and molecular techniques confirmed the identity of *C. cochliodes* CTh05. *M. oryzae* isolate PO1 found to be a virulent isolate causing blast of rice var. RD57. Biculture tests showed that *C. cochliodes* CTh05 inhibited the growth of *M. oryzae* PO1. A previous report by Soytong (2014) stated that *C. cochliodes* proved to be antagonistic to *Drechslera oryzae* (brown leaf spot of rice var. Pittsanulok 2). *C. cochliodes* CTh05 significantly inhibited colony growth and spore production of the tested pathogen in biculture tests, as reported by Tann and Soytong (2017) for *C. cupreum* CC3003 inhibiting *C. lunata* (leaf spot of rice).

The fungal metabolites of *C. cochliodes* CTh05 (CCoH, CCoE and CCoM) expressed antifungal activity against *M. oryzae* isolate PO1 and inhibited spore production with ED50 values of 374, 85, 144 ppm. Soytong (2014) reported that metabolites from *C. cochliodes* suppressed the spore production of *D. oryzae* (brown leaf spot of rice) significantly, with an ED50 value of 66.45 ppm. Tann and Soytong (2017) reported that the hexane-crude extract, ethyl acetate-crude extract and methanol-crude extract of *C. cupreum* C3003 inhibited the spore production of *C. lunata* (leaf spot of rice) with ED_{50} values of 6.41, 0.83 and 7.81 ppm, respectively. *C. cochliodes* CTh05, which was tested in the current study, was previously reported to produce chaetoviridines E, chaetochalasin A and 24(R)-5a, 8a-epidioxyergosta-6-22-diene-3b-ol which those compounds inhibited *Plasmodium falciparum*. The cochliodones C, chaetoviridines E, chaetoviridines F and chaetochalasin A exert antimycobacterial activity. Moreover, chaetoviridines E and F inhibitedcytotoxicity against the KB, BC1 and NCI-H187 cell lines (Phonkerd *et al*. 2008). The current study revealed that the fungal metabolites from *C. cochliodes* CTh05 suppressed *M. oryzae* PO1, which causes rice blast.

Nanoparticles derived from *C. cochliodes* CTh05 (nano-CCoH, nano-CCoE and nano-CCoM) demonstrated to inhibit *M. oryzae* PO1 with ED_{50} values of 9, 16 and 33 ppm, respectively. *C. cochliodes* CTh05 has been found to produce the active compoundsagainst human pathogens (Phokerd *et al*. 2008). Those active compounds may possible serve as a control mechanism.

The crude extracts derived from *C. cochliodes* CTh05, CCoH, CCoE and CCoM found to inhibit *M. oryzae* at ED_{50} values of 374, 85 and 144 ppm, respectively. The nanoparticles of nano-CCoH, nano-CCoE and nano-CCoM gave high potential at low application, with ED_{50} values of 33, 9 and 16 ppm, respectively. It is suggested that applying nanoparticles would be less costly than metabolites. A similar report by Tann and Soytong (2016) stated that nano-CGH, nano-CGE and nano-CGM derived from *C. globosum* KMITL-N0805 exhibited antifungal activity against *C. lunata* (leaf spot of rice) with ED_{50} values of 1.21, 1.19, and 1.93 ppm, respectively.

The current study revealed that the spores of *M. oryzae* exhibited abnormal morphology after treatment with nanoparticles from *C. cochliodes* CTh05. It was also reported by Tann and Soytong (2017) that nanoparticles from *C. globosum* can disrupt and distort the spores of *C. lunata* and cause a loss of pathogenicity. Singh *et al*. (2015) reported that nanotechnology in agriculture is being revolutionized by innovative new techniques for disease control. The current research revealed that nanoparticles constructed from fungal metabolites are promising for plant disease control. Dar and Soytong (2014) reported that the fungal metabolites from *C. globosum* and *C. cupreum*, generated to be nanomaterials at the averaged size of 241 and 171 nm, respectively. Tann and Soytong (2016) also reported that nanoparticles from *C. globosum* can disrupt and distort the pathogen cells and cause a loss of pathogenicity. Nano-CCoH, nano-CCoE and nano-CCoM constructed from *C. cochliodes* CTh05 may help to increase the efficiency of application at lower doses to inhibit rice blast pathogen as stated by Sharon *et al*. (2010).

In the current work, blast incidence in rice seedlings treated with nano-particles constructed from a crude extract mixture at 10 ppm was reduced by 38%, and followed by the chemical fungicide treatment (tricyclazole) which reduced blast incidence by 29%. Similar results were reported by Soytong (2014), who stated that bioproducts from *C. cochliodes* CTh05 can control the rice leaf spot caused by *C. lunata*, but at a high application rate of 50 g 20 L^{-1} of water.

Conclusion

In vivo evaluation of nano-CCoM, nano-CCoE and nano-CCoH constructed from crude hexane, ethyl acetate and methanol extracts of *C. cochliodes* CTh05 reduced blast incidence at concentrations of 7 ppm. It suggested that application of nanoparticles constructed from each crude extract (nano-CCoM, nano-CcoE and nano-CCoH) reduced blast incidence at a lower rate of application than nanoparticles derived from a crude extract mixture from *C. cochliodes* CTh05. Further research should investigate these nanoparticles as elicitors for rice immunity to blast disease.

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Antifungal activity of microbial nanoparticles derived from *Chaetomium* spp against *Magnaporthe oryzae* causing rice blast

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Abstract: *Magnaporthe oryzae* isolate PO2 was proved to cause rice blast var. PSL 2 in this study. *Chaetomimum elatum*, *Chaetomimum lucknowense* and *Chaetomimum brasiliense* were antagonized to *M. oryzae*. Hexane extract of *C. brasiliense* gave the highest inhibition of spore production with ED_{50} of 35 ppm, EtOAC extract of *C. lucknowense* inhibited spore production at 57 ppm. and EtOAC extracts of *C. elatum* inhibited spore production at 106 ppm. Nano-CLM (*C. lucknowense*) inhibited sprore production at 5.24 ppm, nano-CBH (*C. brasiliense*) inhibited spore production at 6.86 ppm and nano-CEE (*C. elatum*) inhibited spore production at 7.89 ppm.. The rice leaves treated with nano-CBH from *C. brasiliense* was produced Sakuranertin and Oryzalexin B as seen on the thin layer chromatography where the Rf value was 0.08 assuming to be Sakuranertin, and Rf values of 0.28 supposing to be Oryzalexin B. It found that nanoparticles act as elicitors to induce immunity in rice plants through the production of phytoalexin.

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Dear Dr. Jiaojiao Song,

I woulf like to inform you that your manuscript:

Antifungal activity of microbial nanoparticles derived from *Chaetomium* spp against *Magnaporthe oryzae* causing rice blast (No. 41/2019-PPS)

has been **accepted for publication in Plant Protection Science and will be published in Issue 3, Volume 56, 2020.**

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Abstract: *Magnaporthe oryzae* isolate PO2 was proved to cause rice blast var. PSL 2 in this study. *Chaetomimum elatum*, *Chaetomimum lucknowense* and *Chaetomimum brasiliense* were antagonized to *M. oryzae*. Hexane extract of *C. brasiliense* gave the highest inhibition of spore production with ED_{50} of 35 ppm, EtOAC extract of *C. lucknowense* inhibited spore production at 57 ppm. and EtOAC extracts of *C. elatum* inhibited spore production at 106 ppm. Nano-CLM (*C. lucknowense*) inhibited sprore production at 5.24 ppm, nano-CBH (*C. brasiliense*) inhibited spore production at 6.86 ppm and nano-CEE (*C. elatum*) inhibited spore production at 7.89 ppm.. The rice leaves treated with nano-CBH from *C. brasiliense* was produced Sakuranertin and Oryzalexin B as seen on the thin layer chromatography where the Rf value was 0.08 assuming to be Sakuranertin, and Rf values of 0.28 supposing to be Oryzalexin B. It found that nanoparticles act as elicitors to induce immunity in rice plants through the production of phytoalexin.

Keywords: microbial nanoparticles, *Chaetomium* spp, rice blast, *Magnaporthe oryzae*

Introduction

Rice blast caused by *Magnaporthe oryzae* is one of economic importance for the lost yields, especially in Asia (Ou 1985) and it is a crucial food staple for more than half of the world (FAO, 2009). Chemical fungicides have been applied to control the disease, but the pathogens often developed the resistance to those chemical fungicides (Soytong *et al*. 2001). Resistant varieties are usually broken down by resistant genes, none of these resistance genes, except Pi9, are durable broad-spectrum resistance. Those resistant genes often breaks down within 3–5 years due to the high variability and fast evolving populations of the fungus (Devi *et al.* 2015). In recent years, blast resistant genes were introgressed Luhui 17, G46B, Zhenshan 97B, Jin 23B, CO39, IR50, Pusa1602 and Pusa1603 lines through marker assisted selection. The genes for resistance induced the occurrence of new races of blast fungus (Miah *et al*. 2013). The use of chemical fungicides resulted to deposit in rice products and residues in soil, water and the surrounding environment (Soytong *et al.* 2001). Scientists are increasingly investigating the biological control of plant pathogens to find effective agents for disease control.

The alternative method is proposed to apply nanoproducts for plant disease control by inducing plant immunity. The application of nanotechnology in agriculture has come out and advanced in recent years (Li *et al.* 2011). Nanotechnology serves as a new effective alternative method of plant disease control to reduce pathogen inoculum and disease incidence. It involves the formation of organic materials into defined structures (Soutter 2012). Scientists are actively searching for organic nanomaterials to be used for biological properties (Elibol *et al*. 2003). Nano-particles can provide an efficient means for disease management in agriculture (Rai and Ingle 2012).

Phytoalexins were first discovered as phytoalexin-like substances in rice plants infected with anamorph *P. oryzae,* imperfect stage of *M. oryzae* using the drop-diffusate method by Uehara (1958). Fifteen phytoalexins were discovered in rice (*Oryza sativa* L.) that was infected by *M. oryzae* causing rice blast. Sakuranetin is a flavonoid and four groups of diterpenoids as phytocassanes A to E, oryzalexins A to F, momilactones A and B, and oryzalexin S including phytoalexins like momilactones A and B (Kodama *et al.* 1992), oryzalexins A-F, oryzalexin S, phytocassanes A-D (Dillon *et al.* 1997). The research finding was to evaluate fungal metabolites and nanoparticles derived from *C. elatum, C. brasiliense* and *C. lucknowense* to inhibit the growth of rice blast caused by *M. oryzae* isolate PO2, and testing for phytoalexin production in the infected rice leaves.

Materials and Methods

Pathogen and pathogenicity test

The blast symptoms on leaves of rice var PSL2 were cut into square pieces of 0.5 x 0.5 cm between advanced margin, soaked in 10 % sodium hypochlorite for 19 min, then cleaned with sterilize distilled water for 3 times, left to dry on sterilized tissue paper before being transferred to water agar. Hyphal tips from advanced margin tissues were transferred to potato dextrose agar until pure cultures were obtained. Each isolate was inoculated into wounded leaves of 20 rice seedlings planted in pots by making a puncture in 0.5 cm diameter for 5 times. Controls were the non-inoculated ones. Lesion size was measured as diameter (cm). The aggressive isolate was used for the experiment. The pathogenicity test was repeated four times following Koch's Postulate's Method. The isolate was confirmed identification by morphological and molecular phylogenetics that followed the instructions of Pornsuriya *et al.* (2011).

Biocontrol agents

Chaetomium elatum (ChE01), *Chaetomium brasiliense* (CB) and *Chaetomium lucknowense* (CL) were used as the biological control agents from previous works (Song and Soytong 2016, 2017). *C. elatum* ChE01 has been reported to produce a new chaetoglobosin V, prochaetoglobosin III and prochaetoglobosin III(ed). These compounds have been reported to cause cytotoxicity against the human breast cancer and cholangiocarcinoma cell lines (Thohinung *et al.* 2010). *C. brasiliense* have been reported to produce four new depsidones, mollicellins K−N (**1**−**4**), and six known depsidones, mollicellins B (**5**), C (**6**), E (**7**), F (**8**), H (**9**), and J (**10**). These pure compounds of **1**−**3**, **5**−**7**, and **10** have shown antimalarial activity against *Plasmodium falciparum*. Mollicellins K expressed antimycobacterial activity against *Mycobacterium tuberculosis* and antifungal activity against *Candida albicans*. Compounds **1**−**10** have exhibited cytotoxicity against the KB, BC1, NCI-H187, and mollicellins K against cholangiocarcinoma cell lines (Khumkomkhet *et al.* 2009). *C. lucknowense* has been reported to produce fungal metabolite, chaetomanone actively against *Fusarium oxisporum* f. sp. *lycopersici* causing tomato wilt (Sibounnavong *et al.* 2011). Bi-culture test was done by following the method of Song and Soytong (2016).

Molecular identification

The genomic DNA was separately extracted from *M. oryzae* isolate PO2, *Chaetomium elatum* (ChE01), *C. brasiliense* (CB) and *C. lucknowense* (CL). Each fungus was cultured in potato dextrose broth (PDB) for 5 days, then the mycelia were freeze-dried. DNA was extracted by a modified CTAB (Cetyl trimethyl ammonium bromide), then cleaned with 25mM EDTA by centrifugation. It was ground in liquid nitrogen to get a fine powder. The fungal cells were lysed in CTAB buffer, β-mercaptoethanol, and mixed, then incubated at 65°C in water bath for 1h. The lysates were extracted with chloroform/isoamyl alcohol (24:1), centrifuged at 14,000 rpm for 5 min at 4°C. The aqueous phase was added 2µl Rnase (20µg/mL) in a sterile tube for 30 min at 37°C, mixed with 50µl 10% CTAB. The mixture was then centrifuged at 14,000 rpm for 5 min at 4°C. DNA was precipitated in isopropanol and centrifuged at 4°C for 20 min at 14,000 rpm. The pellets were washed twice with 70% and 95% ethanol, air dried and dissolved in 100µl TE buffer at 37°C.

The extracted DNAs were monitored by electrophoresis in a 1% agarose gel to check DNA bands. Polymerase chain reaction (PCR) amplification of each fungal DNA was performed by ITS ribosomal DNA regions amplified by PCR using the universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The 25µl reaction mixture contained 2.5µl PCR buffer, 0.625µl of each dNTP (1.25mM), 0.5µl $MgCl^2$, 1µl of each primer (20 pmol/µl), 2 ng DNA and 0.2µl Taq DNA polymerase (1 U). PCR conditions for the ITS regions were programmed with initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C and 56°C for 1 min., 72°C for 2 min., and a final extension at 72°C for 5 min.

The amplified products (5µl) were visualized on 1% (w/v) agarose gel. PCR products were purified with the PCR purification kit. The amplified products were sequenced DNAs and aligned by comparison with the sequences at the GenBank and the basic local alignment search tool (BLAST) analysis (Altschul *et al.* 1997) in the National Center for Biotechnology Information (NCBI) databases. Sequences from closely related species were downloaded and aligned through CLUSTALW using MEGA version 6.0 software (Tamura *et al.* 2007). The phylogenetic trees were done by the neighbor-joining method using the same software.

Testing metabolites against pathogen

ChE01*,* CB and CL were separately cultured in PDB and incubated at 28 °C for 30 days to obtain the fungal biomass. The extraction of metabolites from each isolate was done using hexane, ethyl acetate and methanol as solvents following the method of Thohinung *et al.* (2010) and yielded the fungal substances as crude hexane, crude ethyl acetate and crude methanol extracts. The experiment was conducted by using factorial experiment in Completely Randomized Design (CRD), where factor A was crude extracts of fungal substances and factor B was the tested concentrations (0,10, 50, 100, 500 and 1000 ppm). The experiment was repeated four times. Colony diameter (cm) and sporulation were measured and counted using haemacytometer. Data were analyzed for variance and means were compared using Duncan Multiple Range Test (DMRT) at p=0.05 and 0.01 levels of significance.

Testing nanoparticles against pathogen

Nanoparticles were prepared from crude hexane, ethyl acetate, and methanol extracts of ChE01*,* CB and CL separately incorporated into polylactic acid (PLA)-based nanoparticles through electrospinning by followed the method of Dar and Soytong (2014). The crude extracts from ChE01*,* CB and CL were constructed nanoparticles to get nano-CEH, nano-CEE and nano-CEM. These nanoparticles were tested to inhibit *M. oryzae* isolate PO2 which was conducted by factorial experiment in CRD with four replications. Factor A was nanoparticles and factor B was the tested concentrations (0, 3, 5, 10 and 15 ppm). The experiment was repeated four times. Data were collected as colony diameter (cm) and sporulation was counted using a haemacytometer. Data were subjected to analysis of variance and means were compared using Duncan Multiple Range Test (DMRT) at p=0.05 and 0.01 levels of significance.

Control mechanisms

All nanoparticles treated to the pathogen cells were examined under binocular compound microscope. The normal and abnormal spores of *M. oryzae* were observed. The abnormal spores from treated nanoparticles were inoculated to wounded leaves in 20 days rice seedlings var PSL2, then disease incidence was recorded.

Testing nano-CB for phytoalexin production

Determination of phytoalexin in rice was preliminary investigated by thin layer chromatography (TLC). Phytoalexin production was investigated using the treated nanoparticles CB and the not-treated served as controls. Twenty-day old rice seedlings of var PSL 2 were inoculated with *M. oryzae* and sprayed with nano-CBH at a concentration of 15 ppm. The leaf samples were taken at 3, 6 and 9 days after inoculation. Each fresh leaf sample was weighted at 1.2 g and cleaned in methanol, then cut into small pieces and ground, soaked in 10 mL methanol, put in waterbath at 50 °C for 10 minutes, passed through Whatman filter paper No. 4. The filtrate was then evaporated with rotary vacuum evaporator to get crude extract. The crude extract was added 3 mL methanol and 1 mL ethyl acetate and kept for the trials. Detection of phytoalexin by TLC was done by preparing solvent of benzene and ethyl acetate at the ratio of 10:1. The TLC tank was added 2 mL TLC solvent. TLC plate was spotted with a crude extract sample and standard for comparison, then observed under UV light at 365 nm, soaked in anisaldehyde solvent, then dried and heated until spots appeared. Rf value was calculated and compared with standard. The experiment was repeated three times. The Rf value is calculated as: Rf = distance spot travels/ distance mobile phase travels.

Results

Pathogen and pathogenicity test

M. oryzae was isolated from rice leaves showing blast symptoms and proved to be pathogenic. The culture grown on PDA was greyish brown when mature, producing conidia in opera shape with 2 septa, on conidiophore as imperfect stage (Figure 1). The isolate was confirmed by molecular phylogenic identification as seen in Figure 2. *M. oryzae* isolate PO2 was confirmed to be a pathogenic isolate to cause rice blast in rice var PSL2. The pathogenecity test showed the blast symptoms on inoculated leaves which roundish to slightly elongated, necrotic and gray spots.

Biocontrol agents

C. elatum, C. brasiliense and *C. lucknowense* were subjected to morphological and molecular studies. *Chaetomium* spp were used as the biological control agents from previous works. These isolates were confirmed by morphological and molecular phylogenic identification. Antagonistic activities of *C. elatum*, *C. lucknowense* and *C. brasiliense* against *M. oryzae* gave preliminary results of the control mechanism. *C. elatum* expressed the highest growth inhibition of rice blast pathogen at 71.55 %, followed by *C. lucknowense* and *C. brasiliense*, which inhibited the pathogen fungal growth by 70.55% and 69.44 %, respectively (Table 1 and Figure 4).

Molecular phylogenic confirmation

The fungi were separately cultured in potato dextrose broth (PDB) and incubated at room temperature (30 ºC) for 5 days. *M. oryzae* isolate PO2 was confirmed by molecular phylogenetic identification.

The cluster revealed that *M. oryzae* was in the same clade with sequences of *M. oryzae* MH859782, MH859782(2), MH443357, MF669475, MF678836, MF668692, MF668691 recorded in the database supported by 99 % bootstrap value (Figure 2). The separated phylogenetic confirmations of *C. elatum* (ChE01) is closely related to MH856469, MH859003, MH864188, MN534815, MN534813, MN534809, which database supporting by 100% bootstrap value. *C. lucknowense* (CL) is closely related to MH080360, JX280827, which database supporting by 99% bootstrap value and *C. brasiliense* (CB) is closely related to KF 680267, KT357687, KF680266, KX146504, KT357682, KT357646, JX966545, which database supporting by 100% bootstrap value (Figure 3). All isolates were deposited at the Department of Plant Pest Management, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand.

Testing metabolites against pathogen

EtOAC crude extracts of *C. elatum* showed significantly higher spore inhibition with ED_{50} value of 106 ppm, followed by crude MeOH and crude Hexane with ED_{50} values of 188 and 266 ppm, respectively. Hexane crude extract of *C. brasiliense* gave highly significant inhibition of spore production with ED_{50} values of 35 ppm, which was higher than EtOAC crude exrtract and MeOH crude extract with the ED_{50} values of 55 and 119 ppm., respectively. EtOAC crude extract of *C. lucknowense* gave significantly highest spore production with the ED_{50} value of 57 ppm, followed by Hexane crude extract and MeOH crude extract with ED_{50} values of 103 and 422 ppm, respectively (Table 2).

Testing nanoparticles against pathogen

The characteristics of nanoparticles from *C. elatum*: Nano-CEH, Nano-CEE, Nano-CEM; *C. brasiliense*: Nano-CBH, Nano-CBE, Nano-CBM and *C. lucknowense*: Nano-CLH, Nano-CLE, and Nano-CLM are shown in Figure 5. The resulted in Nano-CEE expressed antifungal activity against *M. oryzae* causing rice blast, highly significant spore inhibition with ED_{50} value of 7.89 ppm, followed by nano-CEM and nano-CEH with the ED_{50} values of 8.66 ppm and 16.7 ppm, respectively. Nano-CBH gave the highest significance in spore production with the ED_{50} value of 6.86 ppm, followed by nano-CBE and nano-CBM with the ED50 values of 9.76 and 13.42 ppm., respectively. Nano particles of *C. lucknowense* exhibited antifungal activity against the conidial stage of *M. oryzae* causing rice blast. Nano-CLM gave highly significant spore inhibition with an ED_{50} value of 5.24 ppm, followed by nano-CLE and nano-CLH with the ED_{50} values of 7.01 and 10.72 ppm., respectively (Table 3).

Control mechanisms

The effect of nanoparticles, nano-CE from *C. elatum*, nano-CL from *C. lucknowense* and nano-CB from *C. brasiliense* against *M. oryzae* (rice blast pathogen) showed the control mechanism as lysis. The spores treated with nanoparticles showed broken and abnormal cells as seen in Figure 6. The spore suspensions of *M. oryzae* that were separately treated with nano-CE, nano-CL and nono-CB lost their pathogenicity thereafter; with those inoculated to rice seedlings var PSL2, there were no signs and symptoms of rice blast.

Testing nano-CBH for phytoalexin production

The rice leaves treated with nano-CBH from *C. brasiliense* were examined for phytoalexin production by thin layer chromatography. The treated nano-CBH found spot on TLC plates when using combination of benzene : ethyl acetate (10:1) under 365 nm UV light for leaves with the Rf value of 0.08 supporting to be Sakuranertin, and spot on TLC plates with the Rf values of 0.28 supposing to be Oryzalexin B. With the treated nano-CBH to the inoculated rice var PLS2 with *M. oryzae,* the rice seedlings were expected to produce phytoalexins against rice blast disease (Figure 7).

Discussion

M. oryzae PO2 is an aggressive isolate that causes blast disease of rice as reported by Ou (1985). The symptom normally is the presence of roundish to slightly elongated, necrotic, gray spots as reported in another study (Song and Soytong 2016). The molecular phylogeny was confirmed as *M. oryzae,* which was in the same clade with sequences of *M. oryzae* MF669475, MF678836, MF668692, MF668691 recorded in the database supported by 99 % bootstrap value consistent with Jagadeesh *et al.* (2018). *C. elatum* (ChE01) is confirmed in phylogenetic tree that closely related to MH856469, MH859003, MH864188, MN534815, MN534813, MN534809, which database supporting by 100% bootstrap value and *C. lucknowense* (CL) is closely related to MH080360, JX280827, which database supporting by 99% bootstrap value. *C. brasiliense* (CB) is closely related to C. *brasiliense* JX966545 which database supporting by 100% bootstrap value (Yew *et al.* 2014). Pornsuriya *et al.* (2011) stated that phylogenetic analysis of ITS rDNA sequence data also supports *Chaetomium siamense* as distinct.

The tested *Chaetomium* spp. showed activity against *M. oryzae* in bi-culture with similar effects as antibiosis mechanism of control reported by Soytong (1992) that *Chaetomium cupreum* isolated from upland rice field soils in the Philippines, was antagonistic to the anamorph *Pyricularia oryzae* in Bi-culture test. Coating rice seed of blast susceptible rice line, IR442-2-58 resulted in control rice blast disease.

The crude EtOAC, crude MeOH and crude hexane extracts of *C. elatum* inhibited spore production with ED_{50} values of 106, 188 and 266 ppm, respectively. Results were similar to previous work (Song and Soytong 2016). The crude hexane extract of *C. brasiliense* inhibited spore production of *M. oryzae* with the ED_{50} value of 35 ppm when compared to EtOAC and MeOH crude extracts with the ED50 values of 55 and 119 ppm, respectively. The research finding was similar to that of the work of Soytong (2014). Moreover, crude extracts from EtOAC of *C. lucknowense* inhibited the spore production of *M. oryzae* PO2 with the ED_{50} value of 57 ppm. This was a similar result with the work of Soytong *et al.* (2013). Hexane and MeOH crude extracts *C. lucknowense* inhibited the tested pathogen with ED_{50} values of 103 and 422 ppm, respectively.

The highlight of the research findings is interesting in that nanoparticles constructed from *Chaetomium* spp. can be used at lower concentrations than crude extracts to inhibit *M. oryzae* causing rice blast. Previous research on nanoparticles from *Chaetomium* sp. were characterized to be nano-materials loaded with active compounds. Crude extracts from *Chaetomium* spp. were incorporated into polyacetic acid and electrospin at 25-30 kv. Scanning electron microscope images revealed that the nano materials from *Chaetomium* spp. measured as 171 nanometers (Tann and Soytong 2016). Results showed that nano-CEE, nano-CBH and nano-CEM inhibited spore production of rice blast pathogen, *M. oryzae* with the ED_{50} values of 7.89, 8.66 and 16.7 ppm, respectively. Nanoparticles of *C. brasiliense,* nano-CBH, nano-CBE and nano-CBM suppressed spore production of *M. oryzae* with the ED_{50} values of 6.86, 9.76 and 13.42 ppm, respectively. Nano-CLM, nano-CLE and nono-CLH resulted in spore inhibition of *M. oryzae* with the ED_{50} values of 5.24 ,7.01 and 10.72 ppm, respectively. Tan and Soytong (2016, 2017) reported that nanoparticles loaded with *Chaetomium globosum* KMITL0805 actively worked against *Cuvularia lunata* causing leaf spot of rice var Sen Pidoa, and the nanoproduct from *Chaetomium cupreum* CC3003 could control leaf spot of rice var. Sen Pidoa. Moreover, in vivo test showed these nano-particles treated to rice seedligs could control leaf spot of rice var. Sen Pidoa and decreased leaf spot disease of rice which supporting the stability of nanoparticles after apply to the rice seedlings.

Control mechanism of nanoparticles derived from *Chaetomioum* spp. was proven to be antibiosis and as lysis as previous reports by Tann and Soytong (2016) and Vilavong and Soytong (2017). It was demonstrated that rice seedlings inoculated with the treated spores of *M. oryzae* with nano-CE, nano-CL and nono-CB resulted in loss of pathogenicity. Similar result was reported by Sibounnavong *et al.* (2011). Result from TLC plates found Sakuranetin at Rf value 0.08 which similar to Hasegawa (2014) who stated that sakuranetin extracted from rice leaves showed spot in TLC to detect the presence of sakuranetin at Rf 0.09 under 365 nm UV light. It is indicated to be Oryzalexin B the spot on TLC with the Rf value of 0.28 when using combination of benzene : ethyl acetate (10:1) under 365 nm UV light for leaves. The research finding revealed the same as Akatsuka (1983) who found Oryzalexin B as the phytoalexin against rice blast in TLC at the same Rf value.

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Tables

Table 1. Colony growth of *Magnaporthe oryzae* on antagonistic bi-culture tests

Antagonists	*Magnaporthe oryzae*	
	Colony(cm)	% inhibition of colony
Control	9.00$^{a1/}$	-
Chaetomium elatum	2.56^{c}	71.55$^{a2/}$
Chaetomium brasiliense	2.75^{b}	69.44^{b}
Chaetomium lucknowense	2.65^{b}	70.55^{a}
CV%	1.20	2.11

[1] Average of four replications. Means followed by a common letter are not significantly different by DMRT at P=0.05

Table 2. Crude extracts of *Chaetomium elatum, Chaetomium brasiliense* and *Chaetomium lucknowense* testing for spore inhibition of *Magnaporthe oryzae*

Crude extracts	**Concentration (ppm)**	**Crude CE**			**Crude CB**			**Crude CL**		
		Number of spores $^{/1,3}$(10^{5})	**Spore Inhibition (%)$^{/2,3}$**	**ED$_{50}$ (ppm)**	**Number of spores $^{/2,3}$(10^{5})**	**Spore Inhibition (%)$^{/2,3}$**	**ED$_{50}$ (ppm)**	**Number of spores $^{/2,3}$(10^{6})**	**Spore Inhibition (%)$^{/2,3}$**	**ED$_{50}$ (ppm)**
Hexane	0	57.25^{a}	-	266	39.00^{a}	-	35	52.00^{a}	-	103
	10	49.00^{b}	14.87^{j}		22.50^{cd}	42.28^{h}		40.25^{bc}	22.49^{hi}	
	50	42.25^{cd}	27.83^{h}		19.50^{de}	50.08^{g}		27.25^{ef}	47.59^{ef}	
	100	33.75^{fg}	40.43^{f}		17.75^{ef}	54.55^{fg}		24.25^{fg}	53.31^{de}	
	500	24.75^{h}	57.09^{d}		9.00^{hi}	77.01^{c}		19.75^{gh}	62.02^{c}	
	1000	19.50^{i}	66.66^{c}		2.75^{j}	93.20^{a}		14.75^{h}	71.82^{b}	
EtOAc	0	57.25^{a}	-	106	39.00^{a}	-	55	52.00^{a}	-	57
	10	45.75^{bc}	20.80^{i}		26.00^{c}	33.26^{i}		35.75^{cd}	31.22^{g}	
	50	36.75^{ef}	36.29^{fg}		22.00^{cd}	43.65^{h}		28.00^{ef}	46.10^{f}	
	100	29.75^{g}	48.82^{e}		16.25^{efg}	58.57^{ef}		21.25^{fgh}	59.04^{cd}	
	500	17.50^{ij}	70.24^{bc}		12.50^{gh}	68.10^{d}		15.50^{h}	70.22^{b}	
	1000	8.75^{k}	85.74^{a}		3.00^{j}	92.46^{a}		8.00^{i}	84.92^{a}	
	0	57.25^{a}	-		39.00^{a}	-		52.00^{a}	-	

	10	45.75^{bc}	20.12^{ij}		34.00^{b}	12.87^{j}		43.00^{b}	17.03^{i}	
MeOH	50	39.25^{de}	31.48^{gh}	188	24.75^{c}	36.53^{i}	119	39.25^{bcd}	24.24^{h}	422
	100	33.25^{fg}	41.66^{f}		18.50^{de}	52.50^{g}		33.25^{de}	35.86^{g}	
	500	25.00^{h}	58.27^{d}		14.00^{fg}	64.14^{de}		27.25^{ef}	47.59^{ef}	
	1000	14.50^{j}	75.55^{b}		6.00^{ij}	84.98^{b}		19.00^{gh}	63.53^{c}	
C.V.(%)		8.19	5.17		11.26	5.07		10.91	6.45	

^{1}/Average of four replications. Means followed by a common letter are not significantly different by DMRT at P=0.01.

Table 3. Nanoparticles of *Chaetomium elatum, Chaetomium brasiliense* and *Chaetomium lucknowense* testing for spore inhibition of *Magnaporthe oryzae*

Nano particles	**Concentration (ppm)**	**Nano-CE**			**Nano-CB**			**Nano-CL**		
		Number of spores $^{/1,3}(10^5)$	**Spore Inhibition $(\%)^{/2,3}$**	**ED_{50} (ppm)**	**Number of spores $^{/1}(10^5)$**	**Spore Inhibition $(\%)^{/2,3}$**	**ED_{50} (ppm)**	**Number of spores $^{/1}(10^5)$**	**Spore Inhibition $(\%)^{/1,3}$**	**ED_{50} (ppm)**
	0	32.50^{a}	-		17.25^{a}	-		40.50^{a}	-	
	3	30.50^{ab}	5.69^{g}		14.50^{bc}	15.75^{e}		34.00^{b}	15.32^{j}	
Hexane	5	27.00^{bc}	16.45^{ef}	16.70	10.75^{d}	37.57^{d}	6.86	28.75^{cd}	28.53^{h}	10.72
	10	22.50^{de}	30.63^{cd}		5.50^{f}	68.05^{b}		23.50^{ef}	41.81^{f}	
	15	15.75^{f}	51.85^{b}		3.50^{g}	79.74^{a}		14.50^{g}	64.18^{cd}	
	0	32.50^{a}	-		17.25^{a}	-		40.50^{a}	-	
	3	29.75^{ab}	7.91^{fg}		14.00^{bc}	18.47^{e}		31.25^{bc}	22.41^{i}	
EtOAc	5	20.50^{e}	37.06^{c}	7.89	11.75^{d}	31.72^{d}	9.76	25.00^{def}	37.82^{fg}	7.01
	10	15.50^{f}	52.73^{b}		7.50^{e}	56.36^{c}		15.00^{g}	63.00^{d}	
	15	6.50^{g}	80.46^{a}		4.50^{fg}	73.89^{ab}		10.50^{gh}	73.87^{ab}	
	0	32.50^{a}	-		17.25^{a}	-		40.50^{a}	-	
	3	30.25^{ab}	6.80^{fg}		15.75^{ab}	8.34^{f}		26.50^{de}	34.25^{g}	
MeOH	5	25.00^{cd}	23.01^{de}	8.66	13.75^{c}	20.03^{e}	13.42	21.25^{f}	47.53^{e}	5.24
	10	15.50^{f}	52.43^{b}		11.50^{d}	33.29^{d}		12.75^{gh}	68.65^{bc}	
	15	7.25^{g}	77.78^{a}		7.25^{e}	57.92^{c}		8.75^{h}	78.52^{a}	
C.V.(%)		12.45	19.01		10.23	8.14		11.75	7.75	

^{1}/Average of four replications. Means followed by a common letter are not significantly different by DMRT at P=0.01.

Figures

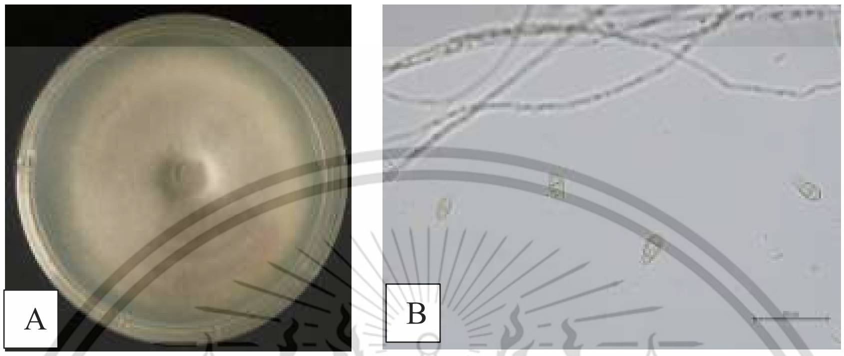

Figure 1. *Magnaporthe oryzae* isolate PO2: A= pure culture on RFA medium; B= Spore, bar: 50µm

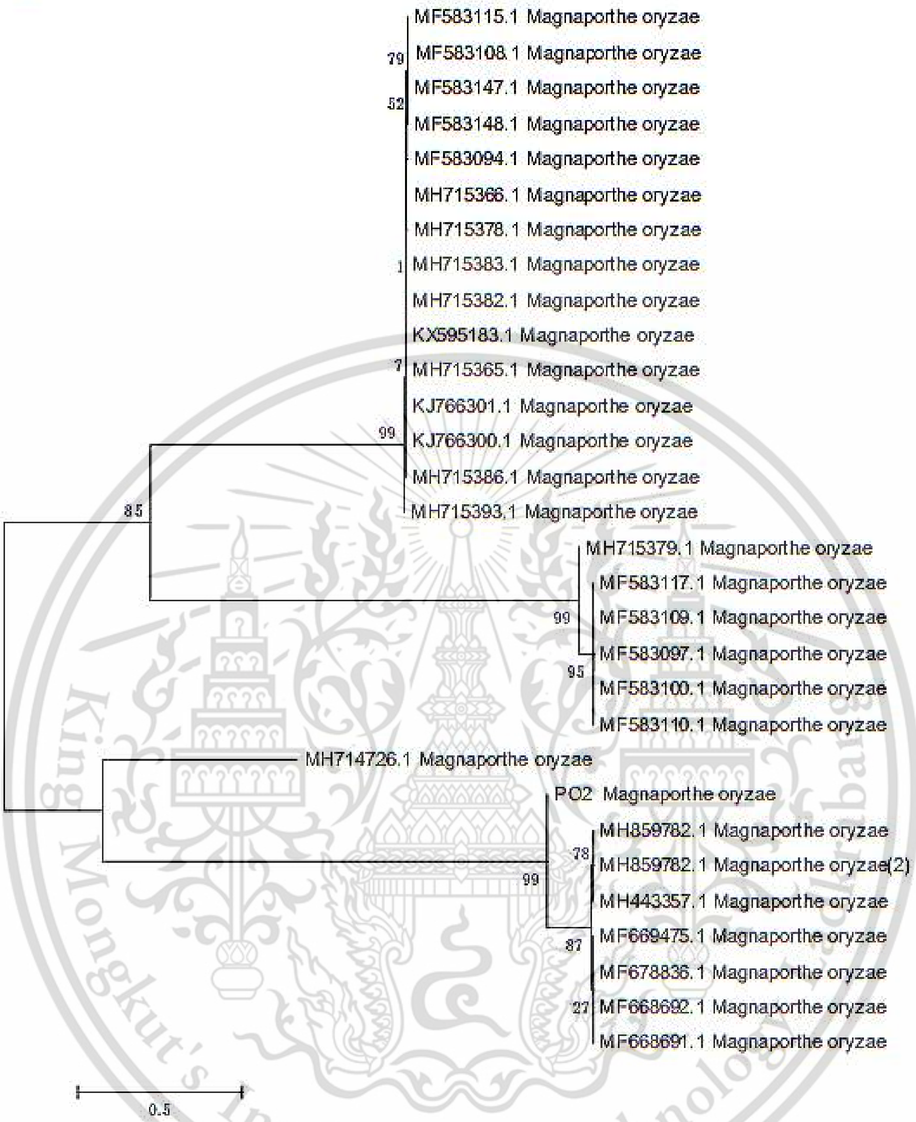

Figure 2. Phylogenetic tree of *Magnaporthe oryzae* from GenBank including *Magnaporthe oryzae* isolate PO2 constructed after distance-based analysis of ITS1, 4 and 5.8S regions of rDNA. Numbers at the branches indicate the percentage of bootstrap values after 1000 replications.

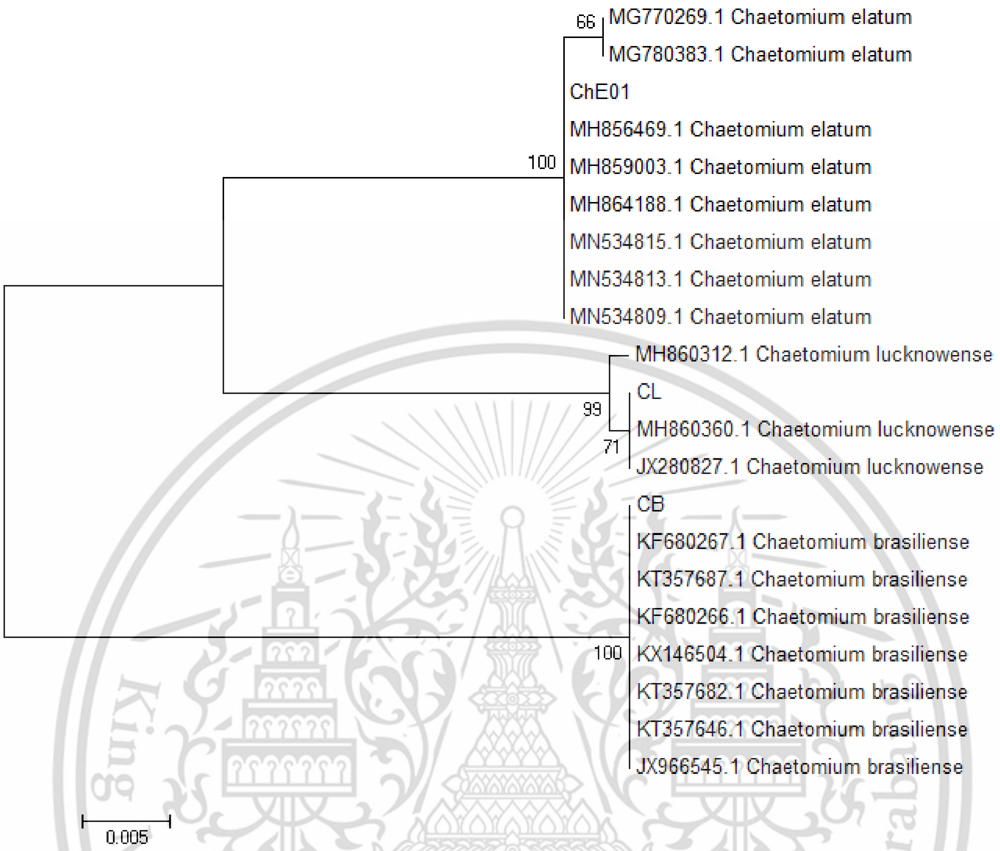

Figure 3. Phylogenetic tree of *Chaetomium* spp. from GenBank including *Chaetomium elatum*, *Chaetomium lucknowense* and *Chaetomium brasiliense* constructed after distance-based analysis of ITS1, 4 and 5.8S regions of rDNA. Numbers at the branches indicate the percentages of bootstrap values after 1000 replications.

Figure 4. Bi-culture of *Chaetomium elatum* against *Magnaporthe oryzae* isolate PO2 , *A=Chaetomium elatum,* B=Bi-culture plates, C= *Magnaporthe oryzae* isolate PO2

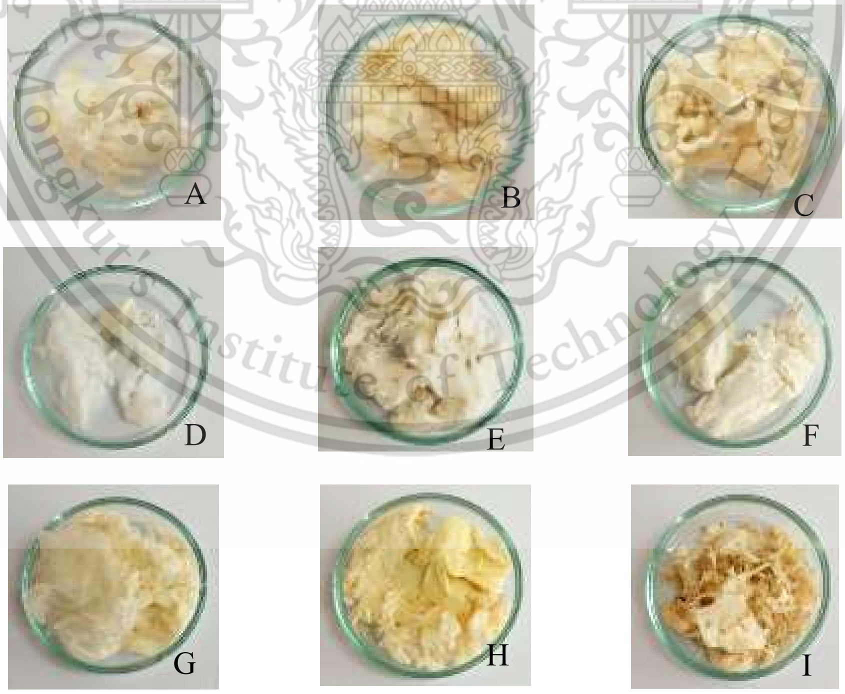

Figure 5. Characteristics of nanoparticles from *Chaetomium elatum, Chaetomium brasiliense* and *Chaetomium lucknowense*. A=Nano-CEH, B=Nano-CEE, C=Nano-CEM, D=Nano-CBH, E=Nano-CBE, F=Nano-CBM, G=Nano-CLH, H=Nano-CLE, I=Nano-CLM

Normal spore | **Abnormal spore (Nano-CB)** | **Abnormal spore (Nano-CL)** | **Abnormal spore (Nano-CE)**

Figure 6. The effect of nanoparticles, nano-CE from *C. elatum*, nano-CL from *C. lucknowense* and nano-CB from *C. brasiliense* against *M. oryzae* isolate PO2

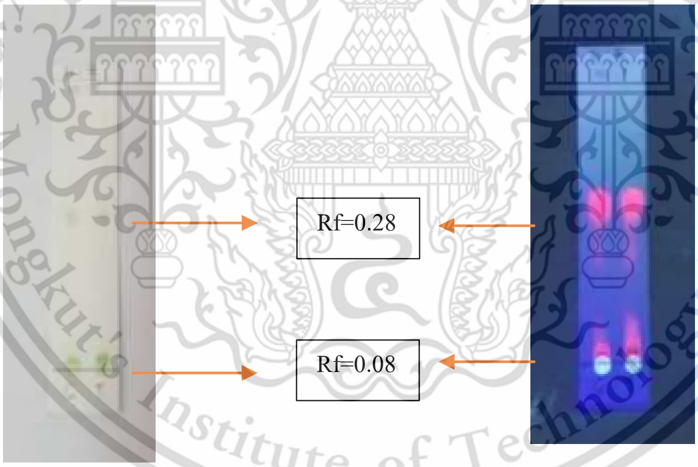

Figure 7. The presence of possible phytoalexins of Sakuranetin at Rf value 0.08 and Oryzalexin B at Rf value 0.28 in TLC plates when using combination of benzene : ethyl acetate (10:1) under 365 nm UV light for leaves

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