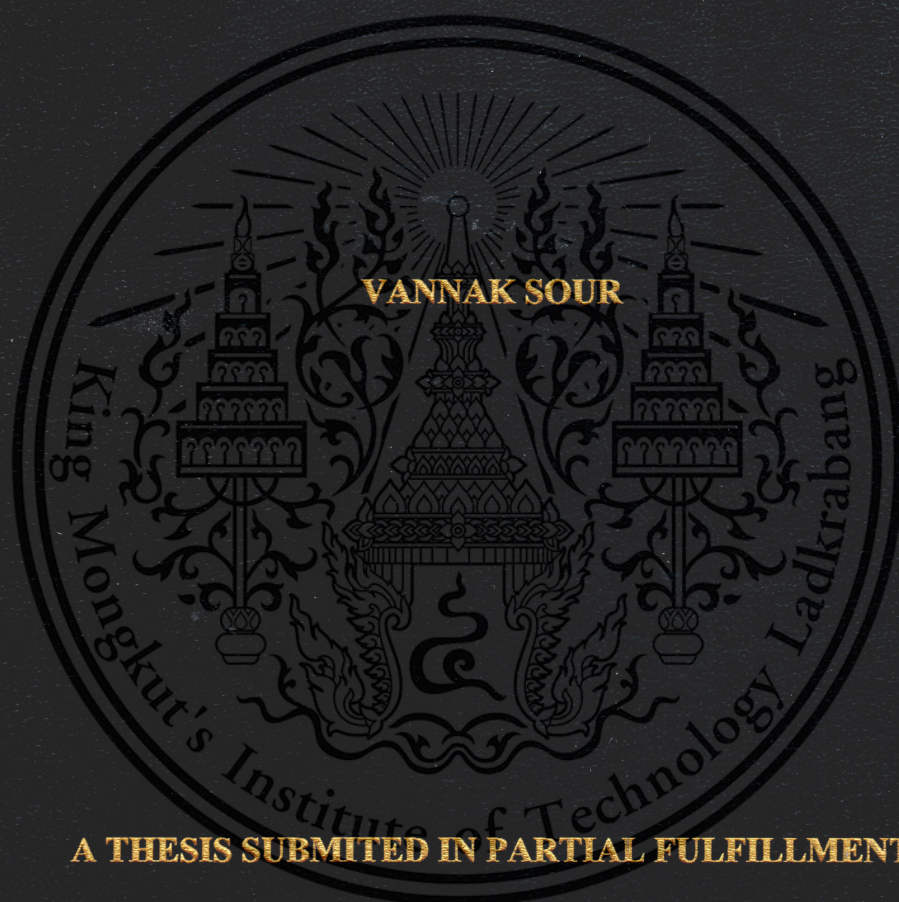


**STUDY OF ENDOPHYTIC FUNGI FROM ORCHIDS AND
SCREENING FOR PLANT DISEASE CONTROL**



**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENT FOR MASTER DEGREE OF
MASTER OF SCIENCE IN AGRICULTURE**

FACULTY OF AGRICULTURAL TECHNOLOGY

KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

2016

KMITL-2016-AG-M-065-214

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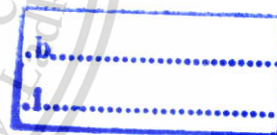
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ชื่อวิทยานิพนธ์:	การศึกษาเชื้อราเอนโดไฟต์จากกล้วยไม้และการคัดเลือกเชื้อที่มีประสิทธิภาพในการป้องกันเชื้อโรค
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บทคัดย่อ

ในการศึกษาครั้งนี้ เชื้อราเอนโดไฟต์จำนวน 11 สปีชีส์แยกได้จากกล้วยไม้ 9 สายพันธุ์ ได้แก่ *Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium hercoglossum*, *Dendrobium palpebrae*, *Dendrobium fimbriatum*, *Doritis pulcherrima*, *Dendrobium crumenatum*, *Dendrobium friedericksianum* และ *Grammatophyllum specinocum* ซึ่งเชื้อเหล่านี้ได้ทำการระบุชนิดตามแบบ Morphological identification มีดังนี้ *Chaetomium cochliodes*, *Chaetomium cupreum*, *Xylaria* sp., *Nigrospora* sp., *Fusarium* sp., *Curvularia* sp., *Achaetomium* sp., *Phoma* sp., *Colletotrichum* sp., *Pestalotiopsis* sp. และ *Corynascus* sp.

เชื้อ *Colletotrichum* sp. ที่เป็นสาเหตุการเกิดโรคแอนแทรคโนสของกล้วยไม้ ได้ทำการแยกจากใบของ *Grammatophyllum specinocum* และได้ทำการทดสอบ pathogenicity test เพื่อยืนยันว่าเป็นเชื้อที่ก่อโรคให้กับกล้วยไม้จริง เชื้อราเอนโดไฟต์ 3 สปีชีส์ได้ทำการคัดเลือกเพื่อใช้ในการทดสอบประสิทธิภาพในการป้องกันเชื้อโรคโดยใช้วิธี Bi-culture ซึ่งเชื้อทั้ง 3 สปีชีส์นี้ได้แก่ *Chaetomium cochliodes*, *Chaetomium cupreum* และ *Achaetomium* sp. ผลการทดลองพบว่าเชื้อทั้ง 3 สปีชีส์มีประสิทธิภาพในการยับยั้งการเจริญเติบโตของเชื้อ *Colletotrichum* sp. ได้ดีทั้งการเจริญเติบโตของโคโลนีและการสร้างสปอร์ ในการยับยั้งการเจริญเติบโตของโคโลนีพบว่าเชื้อ *Chaetomium cochliodes* ยับยั้งได้ดีที่สุด รองลงมาก็คือ *Chaetomium cupreum* และ *Achaetomium* sp. การยับยั้งการสร้างสปอร์พบว่าเชื้อ *Chaetomium cupreum* ยับยั้งได้ดีที่สุด รองลงมาคือ *Chaetomium cochliodes* และ *Achaetomium* sp.

Crude methanol จากเชื้อเอนโดไฟต์ *Chaetomium cochliodes* มีฤทธิ์ควบคุมการเจริญเติบโตของเชื้อสาเหตุโรคแอนแทรกโนสในกล้วยไม้ *Colletotrichum* sp. ที่ระดับ ED₅₀ 712 µg/ml ส่วน Crude hexane, crude ethyl acetate และ crude methanol จากเชื้อเอนโดไฟต์ *Chaetomium cupreum* สามารถควบคุมการเจริญเติบโตของ *Colletotrichum* sp. อยู่ที่ระดับ ED₅₀ ที่ 794, 624 และ 879 µg/ml ตามลำดับ.

งานวิจัยครั้งนี้ยังพบว่าสารนาโนจากเชื้อเอนโดไฟต์ *Chaetomium cochliodes* และ *Chaetomium cupreum* มีประสิทธิภาพในการยับยั้งการเกิดโรค *Colletotrichum* sp. ที่เป็นเชื้อสาเหตุการเกิดโรคแอนแทรกโนสใน *Grammatophyllum specinocum* ได้ดี ในการวิจัยครั้งต่อไป ควรศึกษาเกี่ยวกับการพัฒนาสารนาโนให้เป็นสารควบคุมเชื้อโรคที่เกิดจากเชื้อรา



Thesis:	Study of Endophytic Fungi from Orchids and Screening for Plant Disease Control
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Program:	Agriculture
Year:	2016
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ABSTRACT

In this study, eleven endophytic fungi was isolated from 9 orchid species of *Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium hercoglossum*, *Dendrobium palpebrae*, *Dendrobium fimbriatum*, *Doritis pulcherrima*, *Dendrobium crumenatum*, *Dendrobium friedericksianum* and *Grammatophyllum specinocum*. They were morphological identified as *Chaetomium cochliodes*, *Chaetomium cupreum*, *Xylaria* sp., *Nigrospora* sp., *Fusarium* sp., *Curvularia* sp., *Achaetomium* sp., *Phoma* sp., *Colletotrichum* sp., *Pestalotiopsis* sp., and *Corynascus* sp.

Colletotrichum sp. which caused anthracnose in orchids was isolated from leaf of *Grammatophyllum specinocum* and tested for pathogenicity for ensure the pathogen, 3 endophytic fungi were chosen for bio-control by bi-culture test, *Chaetomium cochliodes*, *Chaetomium cupreum* and *Achaetomium* sp. The result of bi-culture test showed that all antagonists showed ability to inhibit both colony growth and spore production of *Colletotrichum* sp. significantly different when compared to control. Colony inhibition showed that *Chaetomium cochliodes* was the most inhibition and followed by *Chaetomium cupreum* and *Achaetomium* sp. Spores inhibition showed that *Chaetomium cupreum* was the best antagonist and followed by *Chaetomium cochliodes* and *Achaetomium* sp.

It is shown that crude methanol from *Chaetomium cochliodes* expressed antifungal activity against *Colletotrichum* sp. causing leaf anthracnose of orchid, *Grammatophyllum specinocum* at the ED₅₀ value of 712µg/ml. Crude hexane, crude ethyl acetate and crude

methanol of *Chaetomium cupreum* showed antifungal against leaf anthracnose of orchid at ED_{50} values of 794, 624 and 879 $\mu\text{g/ml}$.

The research findings are also found that formulation of nano-products from antifungal metabolites from *Chaetomium cochliodes* and *Chaetomium cupreum* gave a good control leaf anthracnose of orchid, *Grammatophyllum specinocum*. Further research finding would be investigated to develop the nano-products as a biological fungicide.



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Chaper I

Introduction

Plants are usually associated with diverse microorganisms. Endophyte microorganisms are those that colonize the healthy plant internal tissue (Stone *et al.* 2004). The meaning of term “endophyte” is as broad as its literal definition and spectrum of potential hosts and inhabitants. Endophytes are used for both bacteria and fungi (Schulz and boyle. 2006). Modern usage of the term endophytic fungi in mycology refers to those fungi which live within leaves stems roots and other part of apparently healthy host plants and can not be seen in visually signs of infection (Stone *et al.* 2004). Dreyfuss and Chapela (1994) estimated that there may be at least one million species of endophytic fungi alone. It means that almost all plant species are usually infected with endophytic fungi.

Recently endophytic fungi have attracted the attention from many scientists in the world as estimated that such species may useful as sources of anticancer, antidiabetic, insecticidal and immunosuppressive compounds (Strobel and Daisy. 2003). Endophytic fungi from medicinal plants can be used for the development of drugs. For example *Fusarium oxysporum* isolated from *Catharanthus roseus* can produce Vinblastine and Vincristine, excellent anti-cancer drugs (Kumar *et al.* 2013). Endophytic fungi from Chinese medicinal plant *Actinidia macrosperma* can inhibit the tumour cells (Yin *et al.* 2012). The differences in diversity and types of endophytic fungi depend on their host plants and changes in environment (Gange *et al.* 2007). For the beneficial role of endophyte in plants can be divided into two categories based on types of activity: growth promotion and disease control (Anu. 2012). It can directly enhance plant growth, increase the host plants' adaptability to such diverse conditions as dry, cold, and high-salt environments, and help to resist pathogen damage (Waller. 2005) as well as being able to alter the community structure (Purahong and Hyde. 2011) and antioxidant activity of the host plants (Hamilton. 2012). For example fungal endophyte *Scolecobasidium humicola* isolated from roots of tomato can increase the tomato biomass with the organic nitrogen sources (Rola and Kazuhiko. 2013). They also can produce gibberellin and indoleacetic acid and promote host plant during stress condition (Waqas *et al.* 2012).

Orchids are monocotyledonous plant and taxonomically belong to Orchidales and Orchidaceae. There are 15,000 to 25,000 of orchid species distributed all around the world, mostly can found in tropical environment. They all can divide in 2 classifications as epiphytic

orchids and terrestrial orchids. In Thailand there were about 500 genus and 800 orchid species. The special hereditary characteristic of orchid species was having many tiny seed in the pod and there were not growing and no endosperm in embryo. So to be germinated, the orchids had to depend on some fungi species to be the energy and food sources such as many nutrients that important for growth (Thamasiri. 2005).

Orchids are among the plant groups that had aroused most widespread interest among scientists and horticulturists, both for their study and use. They have been subject to particularly high commercial demand over the past 40 years for the beauty of their structure and their vividly colored flowers, steeped in symbolism and mystery. Some countries had declared orchid species as their national flowers (Clemente. 2009). In Thailand orchids are the most valuable product for export and make money for country. Last years (2013) the export of orchids value were 563,134,221 Bart, and the country that most import Thai orchids is China, Japan, USA, India and Vietnam, respectively (Source: Office of Agriculture Economics). It took many interested in planting orchid for commercial from businessman and horticulturists. So when there were plentiful culture of orchids that's mean it's following by the distributed of diseases. There are many study that compiled the pathogen fungus on orchids include *Alternaria* sp., *Botrytis cineria*, *Cercospora* sp., *Cladosporium* sp., *Colletotrichum gloeosporioides*, *Curvularia eragrostidis*, *Diplodia* sp., *Drechslera* sp., *Fusarium* sp., *Nectria* sp., *Phyllosticta pyriformis*, *Pseudocercospora dendrobii*, *Phytophthora nicotiana* var. *parasitica*, *Phytophthora palmivora*, *Rhizotonia* sp., *Schlerotium rolfsii* and *Volutela* sp. (Thamasiri. 2005; Sutthiwari. 2006).

Fungal endophytes had investigated in a large number of orchid species from around the world. The traditional approached to identify the fungal endophytes of orchids were to isolated pelotons from orchid tissues and to maintain fungal colonies in pure culture. The fungi were then identified on the basis of anatomy and morphology including such features as nucleus number, hyphal cross wall structure and spore dimensions (Perkins *et al.* 1995). Worldwide, the fungi involved with orchids were almost all members of the phylum Basidiomycota group, however many do not produce sexual spores, and are consequently assigned to the form genus *Rhizoctonia* (Rasmussen. 2002). Although results may vary with the species of orchid examined, it appears that under natural conditions a particular orchid species may associate with only a few or a single fungal species. For example the *Xylaria* sp. and *Rhizotonia-like* fungi were isolated from root and leaves of genus *Lepanthes* (Bayman *et al.* 1997). Bougoure and Dearnaley (2005) isolated

endophytic fungi from *Dipodium Variegatum* (Orchidaceae) found the *Russula* sp. and non-mycorrhizal Deuteromycetes.

This study aimed to investigate the diversity of endophytic fungi from root and leaves of various orchid plants and screen for their ability to control Anthracnose in orchids.

State of Problem

At present the most farmers always use chemical matter such as chemical pesticide and fertilizers which cause effect to farmer's health and many toxic matter contaminate to the agricultural products. To investigate for bioactive from nature is the main project of many scientists, to replace of chemical usage. This research project was to isolate endophytic fungi from various orchid plants and to screen for bioactive substances for plant disease control.

Objective

1. To isolate the endophytic fungi from various orchid varieties
2. To Isolate *Colletotrichum gloeosporioides* from orchid and to perform pathogenicity test
3. Bi-culture antagonistic test
4. Test biological activity in vitro
5. In vivo test

Location of Research

Biocontrol research Laboratory and Mycology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand.

Strong crops inter. Co. Ltd. Samut Prakarn province, Thailand.

During of the Research

The research was conducted from February 2014 to March 2015.

Chapter II

Review literatures

2.1 Endophytic fungi

The living together of unlike organisms is known as symbiosis. The term symbiosis (from the Greek: *syn* "with" and *biosis* "living") can play a roll in different kind of associations. In natural ecosystems plants are potential hosts for a broad spectrum of microbes (usually bacteria and fungi), the microbes that live on surface of plants call as epiphytes and microbes that live inside tissue of plant call as endophytes. They can be broadly categorized into mutualism, commensalism and parasitism according to benefits for both partners (mutualistic symbiosis), microbial benefit not affecting the host plant (commensalistic symbiosis) or negative impact on host fitness (pathogenic or parasitic interaction) (Paszowski. 2006). Endophytes are organisms that live inside the plant without apparent any symptom of infection (Gimenez *et al.* 2007).

2.1.1. Definition and classification of endophytic fungi

"Endophyte" was named by Debary (1866) to describe fungi ranging from virulent foliar pathogens to mycorrhizal root symbionts. The term endophyte comes from the Greek (*endo* "within" and *phyton* "plant") that include a broad spectrum of plant endosymbionts from bacteria to insects colonizing inside any organ of the plant with variable life styles (Schulz and Boyle. 2005). Endophytes include an assemblage of microorganisms with different life style: some endophyte perform as saprophyte on dead or senescing tissue, mutualisms that can protect the host plant from pathogen or can promote growth the host plant, but also can be the latent pathogens and virulent pathogens at early stages of infection. These parasitic interactions may vary from mutualistic to commensalistic to latently pathogenic and exploitive. Phenotypes of the interactions are often plastic, depending on the genetic dispositions of the two partners, their developmental stage and nutritional status, but also on environmental factors (Schulz and Boyle. 2006).

Fungal endophytes are fungi that grow inside living plant tissues for at least part of their life cycle without causing disease symptoms (Stone *et al.* 2004). There was some debate over where the boundaries of this definition should exist (Schulz and Boyle. 2005). Generally, an endophytic relationship refers to a mutualistic relationship with a positive impact on the fitness of both organisms; however, antagonisms in species specific interactions have been demonstrated (Saikkonen. 2004).

Based on phylogeny and life history traits, endophytic fungi were originally organized into two broad groups – clavicipitaceous endophytes (CEs) colonize shoots and rhizomes of a narrow host range of cool and warm season grasses (*Poaceae*) and non-clavicipitaceous endophytes (NCEs) have been isolated from shoots and/or roots of almost all sampled plants and are plentifully diverse. More recently, these groups have been divided into four distinct functional classes based on a number of different criterion related to life history. The classification into one of the four classes based on: host range (single species to highly ubiquitous), tissue colonization (localized to general), extent in plant colonization (limited to extensive) and biodiversity (low to high), mode of transmission (vertical to horizontal) and host, most of them belonging to Ascomycota. (Rodriguez *et al.* 2009).

The CEs (class 1 endophytes) belong to the fungal teleomorphic genera *Epichloe* and *Balansia* correspond to the anamorphs *Neotyphodium* and *Ephelis*, respectively. They form systemic intercellular infections with a hyphal gradient along the plant axis and primarily transmitted vertically by seeds (Schulz and Boyle. 2005; Kuldau and Bacon. 2008).

The NCEs divided into three functional groups, class 2 class 3 and class 4. Class 2 endophytes broadly colonize both above and below ground tissues, but with limited biodiversity in individual plant hosts. They are both transmitted vertically and horizontally, sometime colonize non-habitat or habitat adapted benefits to the plant host. Endophytic fungi in this class belong to a few members of the *Agaricomycotina*, *Pucciniomycotina*, *Basidiomycota* and also include *Pezizomycotina* (Ascomycota) such as *Phoma* sp., *Arthrotrrys* sp., *Fusarium culmorum*, *Colletrichum* sp. and *Curvularia protuberata* that can also found in roots, rhizomes, stems and leaves (Rodriguez *et al.* 2009).

NCEs class 3 which colonization has restricted to shoots, but with high biodiversity in individual tissues. They have been isolated broadly from tropical forest to boreal and Arctic plant populations. They are horizontally transmitted by wind, rain and insects (Higgins *et al.* 2007; Arnold. 2008; Feldman *et al.* 2008). The Ascomycota that isolated from leaves the most belonging to the *Sordariomycetes*, *Dothidiomycetes*, *Pezizomycetes*, *Leotiomycetes* and *Eurotiomycetes*, while *basidiomycetous* isolates belonging to *Agaricomycotina*, *Pucciniomycotina*. Their benefits to the plant seem to be more complex and non-habitat-adapted (Arnold *et al.* 2007; Higgins *et al.* 2007; Rodriguez *et al.* 2009; Vega *et al.* 2010).

Class 4 endophytic fungi colonize only roots. They also known as Dark Septate Endophytes (DSEs), are easily distinguished by their highly melanized septate hyphae. These

fungi are limited to the roots of their host, coexisting with mycorrhizal fungi but not growing outward into the rhizosphere. They have a broad host range and belong to different phylogenetic groups among Ascomycota and among non-mycorrhizal members of the order *Sebacinales* (Basidiomycota) (Addy *et al.* 2005; Selosse *et al.* 2009; Schäfer and Kogel. 2008).

2.1.2 Ecology of fungal endophyte

Fungal endophyte species are found across diverse habitats in the majority of plant species (Vega. 2008). They can reside in root, stem, leaf or multiple tissues (Stone *et al.* 2006; Yuan *et al.* 2009). As refer to many study of their diversity in many plants as follow:

Dandu *et al.* (2013) isolated endophytic fungi from 5 medicinal plants viz *Boswellia ovalifoliolata*, *Pterocarpus santalinus*, *Shorea thumbuggaia* and *Syzygium alternifolium*. Fourteen fungal species viz *Fusarium oxysporum*, *Colletotrichum falcatum*, *Pestalotiopsis sp.*, *Aspergillus fumigatus*, *Aspergillus flavipes*, *Sterile mycelia*, *Penicillium senticosum*, *Gliocladium roseum*, *Phomopsis jacquiniana*, *Nigrospora sphaerica*, *Leptosphaeria sp.*, *Phomopsis archeri*, *Alternaria alternata*, *Aspergillus niger* were isolated. They found that fungi were most diverse in leaves and root while in stems have low diverse of endophytic fungi. In opposite of this research and these plants, Sun *et al.* (2011) isolated endophytic fungi from *Suaeda* species, annual halophytes growing in soil with high salinity and high concentration of irons where non-halophytes are unable to thrive, found *Alternaria sp.*, Ascomycete *sp.*, *Bipolaris- sp.*, *Camarosporium sp.*, *Cladosporium sp.*, *Fusarium sp.*, *Penicillium sp.* and *Phoma sp.* The isolation frequency in stems was higher than in leaves.

Glenn and Bodri (2012) isolated endophytic fungi from 4 species of the carnivorous pitcher plant genus *Sarracenia*: *S. minor*, *S. oreophila*, *S. purpurea*, and *S. psittacina*. Twelve taxa of fungi, 8 within the Ascomycota and 4 within the Basidiomycota, were identified. They speculate that endophyte-infected *Sarracenia* may benefit from their fungal associates by their influence on nutrient availability from within pitchers and possibly by directly influencing the biota within pitchers.

Saithong *et al.* (2010) from Thailand isolated the endophytic fungi from the bark of *Cephalotaxus mannii* (plum-yew) found 13 genera, viz. *Cladosporium sp.*, *Acremonium sp.*, *Trichoderma sp.*, *Monilia sp.*, *Fusarium sp.*, *Spicaria sp.*, *Humicola sp.*, *Rhizoctonia sp.*, *Cephalosporium sp.*, *Botrytis sp.*, *Penicillium sp.*, *Chalaropsis sp.* and *Geotrichum sp.*

Kumar and Hyde (2004) reported that 60 taxa including 30 morphotypes were isolated from the different parts of the Chinese medicinal plant, *Tripterygium wilfordii*. The endophytic

assemblages of *T. wilfordii* comprised a number of cosmopolitan species such as *Colletotrichum gloeosporioides*, *Guignardia* sp., *Glomerella cingulata*, *Pestalotiopsis* sp., *Phomopsis* sp. and *Phyllosticta* sp. The overall fungal community of *T. wilfordii* was moderately diverse. The fungal community from the twig xylem parts was most diverse, followed by leaves, twig bark, root xylem and flowers.

As above mentioned, we can indicated that endophytic fungi ecology were very diverse, but there was a question that how can endophytic fungi colonize in plant tissue from one generation to one generation. About this question, Yuan *et al.* (2009) concluded that endophytic fungi transfer from each plants by 2 ways. The first one is vertical transfer that direct transfer by seeds. The second is horizontal transfer that can transfer by air, rain and insect in environment who have high taxonomic and host diversity, and can broadly colonize all plants in an ecosystem.

2.1.3 Secondary metabolites produced by endophytic fungi

Endophytes may produce new bioactive substances because of a continual metabolic interaction with their hosts and the environment and this metabolic interaction is important for the symbiosis of both partners (Schulz and Boyle, 2005; Suryanarayanan *et al.* 2009). Many researchers tried to indicate for antimicrobial from many plants. Below, there are many researchs about isolate antimicrobial substance from endophytic fungi.

From China, Su *et al.* (2013) investigated the dark septate endophyte (DSE) *Harpophora oryzae* in rice roots and its biocontrol potential in rice blast disease caused by *Magnaporthe oryzae*. The result showed that the colonization pattern of *Harpophora oryzae* was consistent with the typical characteristics of DSEs. *Harpophora oryzae* enhanced local resistance by reactive oxygen species (ROS) and high antioxidative level and induced OsWRKY45-dependent SA-mediated systemic resistance against rice blast. Yin *et al.* (2012) isolated Endophytic fungi from *actinidia macrosporma* and investigated for their bioactivity indicated that fungi from this plant exhibited toxicity against brine shrimp and antitumour. Liang *et al.* (2012) indicated that endophytic fungi from ornamental plant *Ophiopogon japonicus* (Liliaceae) can produced metabolite inhibited *Staphylococcus aureus* and *Cryptococcus neoformans*. Gao *et al.* (2011) reported that 4 strains of endophytic fungi from roots of Pigeon Pea (*Cajanus cajan* (L.) Millsp.) were producing Cajaninstilbene acid (CSA, 3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid), CSA is responsible for the prominent pharmacological activities in pigeon pea. The five endophytic isolates were characterized by analyzing the internal transcribed spacer (ITS) rRNA

and b-tubulin genes. The 4 strains isolated from pigeon pea roots were found to be closely related to the species *Fusarium oxysporum* and another one was identified as *Neonectria macrodidym*.

While in Philippine Jeremy and Thomas. (n.d.) indicate that crude isolated endophytic fungi from *Canarium ovatum* show that *Colletotrichum* sp. PFE31 crude extract exhibited broad spectrum antibacterial activity against representative Gram-positive, Gram-negative, and spore forming bacilli. Melfei *et al.* (2013) isolated Endophytic fungi from *Pandanus amaryllifolius*. The genera *Colletotrichum*, *Chaetomium*, *Diaporthe*, *Glomerella*, *Guignardia*, *Lasiodiplodia*, *Lulworthia*, *Phoma*, *Phyllosticta*, *Trichoderma*, and *Truncatella* were identified by morphology. The selected species was use for antimicrobial, show that can against *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Eschericia coli*, and *Gordonia terrae*.

Oil seed crop, *Jatropha curcas*, was selected for endophytic fungi in India by Susheel and Nutan. (2013). *Colletotrichum truncatum*, *Nigrospora oryzae*, *Fusarium proliferatum*, *Guignardia cammillae*, *Alternaria destruens*, and *Chaetomium* sp. were selected from leaf of *Jatropha curcas*. Dual plate culture bioassays and bioactivity assays of solvent extracts of fungal mycelia showed that isolates of *Colletotrichum truncatum* were effective against plant pathogenic fungi *Fusarium oxysporum* and *Sclerotinia sclerotiorum*. Susheel *et al.* (2013) isolated *Chaetomium globosum* from *Withania somnifera*. They extracted this species by Ethyl acetate, hexane and methanol extracts method and the result showed that Ethyl acetate and methanol extracts were more effective than hexane extract. Qadri *et al.* (2013) reported that 72 strains of endophytic fungi which isolated from selected plants at Western Himalayas, India were isolated. Several strains of these endophytic fungi showed the abilities to against *E. coli* and *S. aureus*. Twenty four strains can inhibit three or more phytopathogens 17 fungi possessed immunomodulatory activities with five of them showing significant immune suppression as demonstrated by the in vitro lymphocyte proliferation assay. Kumar *et al.* (2013) isolated endophytic fungi from *Catharanthus roseus* and found a fungus that can produce vinblastine and vincristine, anti-cancer drugs, in appreciable amounts. Those endophytic fungi have identified as *Fusarium-oxysporum*.

In Thailand, Tiamphet and Nuangmek. (2013) isolated endophytic fungi from Eucalyptus and another 29 species of herbs. Two hundred and seventy endophytic fungi were isolated. Unknown, *Phomopsis* sp. and *Xylaria* sp. were the most common species. Dual culture method was used to screening for evaluation on the potential effectiveness of candidate biological control agents against rot disease of eucalyptus (K7) seedling caused by *Rhizoctonia solani*. Revealed

that *Nodulisporium* sp. and *Muscodor* sp. were strong anti-fungal activity against *R. solani* and also have good result in green house. In the same year in southern Thailand, Supaphon *et al.* (2013) reported that 7 isolates of endophytic fungi from three seagrasses species, *Cymodocea serrulata*, *Halophila ovalis* and *Thalassia hemprichii* including *Hypocreales* sp. PSU-ES26 from *C. serrulata*, *Trichoderma* sp. PSU-ES8 and PSU-ES38 from *H. ovalis*, and *Penicillium* sp. PSU-ES43, *Fusarium* sp. PSU-ES73, *Stephanonectria* sp. PSU-ES172 and an unidentified endophyte PSU-ES190 from *T. hemprichii* exhibited strong antimicrobial activity against human pathogens with minimum inhibitory concentrations (MIC) of less than 10 µg/ml. The inhibitory extracts at concentrations of 4 times their MIC destroyed the targeted cells as observed by scanning electron microscopy. These results showed the antimicrobial potential of extracts from endophytic fungi from seagrasses.

In Taiwan, Min *et al.* (2012) isolated endophytic fungi from medical herbs of *Lauraceae* and *Rutaceae*. One hundred and fifty six isolates of endophytic fungi were collected from twigs of medicinal plants, *Lauraceae* (67 isolates) and *Rutaceae* (89 isolates). The most common endophytes were in the taxa of *Colletotrichum*, *Guignardia*, *Hypoxyylon*, *Nigrospora*, *Phomopsis* and *Xylaria*, and the most common hosts were *Citrus* and *Zanthoxylum* of *Rutaceae* and *Cinnamomum* of *Lauraceae*. Endophytic fungi of *Lasmenia* sp. showed significantly reduced severity of anthracnose of Chinese cabbage caused by *Colletotrichum higginsianum* under greenhouse conditions.

In Korea, Paul *et al.* (2011) reported that total of 481 isolates were recovered from the surface sterilized tissues of leaves, stems and roots of chili pepper (*Capsicum annum* L.) in seedling, flowering and fruiting stages. Twenty one fungal genera were characterized, belonging to 16 Ascomycota and 5 Basidiomycota. *Penicillium* in seedling stage, *Fusarium* in flowering stage, *Colletotrichum* followed by *Fusarium*, *Alternaria* and *Xylaria* in fruiting stage was predominant and *Alternaria*, *Cladosporium* and *Fusarium* were common in all growth stages. Ninety phenotypes were evaluated for the antimicrobial activity against three major pathogens (*Phytophthora capsici*, *Colletotrichum acutatum* and *Fusarium oxysporum*) of chili pepper. Among them 16 isolates inhibited the growth of at least one test microorganisms. Three strains showed a broad spectrum antifungal activity and displayed strong inhibition against chili pepper pathogenic fungi.

In Brazil, Hanada *et al.* (2010) isolated endophytic fungi from stems and branches of *Theobroma cacao* (cacao) and *Theobroma grandiflorum* which growing in the Amazon region of

Brazil. One hundred and three isolates that could be classified at least up to genus level were tested against *P. palmivora* in pods attached to cacao trees in the field. Results indicated that 70% of isolates showed biocontrol effects to a certain extent, suggesting that culturable endophytic fungal biodiversity in this system was mostly mutualistic type of interaction with the host.

In Panama of USA, Herre *et al.* (2007) isolated endophytic fungi from *Theobroma cacao* and showed that the endophytic fungi showed abilities against *Phytophthora palmivora*.

Endophytic fungi also can promote plant growth to growing up better. They were able to produce phytohormones such as Gibberelin (GAs) and indoleacetic acid (IAA) and mitigate abiotic stresses like salinity and drought.

Rola and Kazuhiko. (2013) Isolated endophytic fungi from roots of tomatoes and cabbages. Two hundred and five isolates have showed that the most fungi dominant by *Fusarium sp.* and a new fungal endophyte, *Scolecobasidium humicola*, was identified as a common dark septate endophytic fungal (DSE) species under both natural and agricultural conditions. This fungus was found to grow endophytically in the roots of tomato seedlings. Two isolates of *S. humicola* showed ability to increase plant biomass with an organic nitrogen source. This research was the first report of *S. humicola* as an endophyte and could help to improve plant growth with organic nitrogen sources.

Waqas *et al.* (2012) isolated endophytic fungi from the root of cucumber. Two strains of fungal endophyte, *Phoma glomerata* LWL2 and *Penicillium sp.* LWL3, were selected and further to examine for their production of gibberelins (GAs) and indoleacetic acid (IAA). The result showed that both fungi strain can promote shoot length of two rice varieties, *Waito-C* and *Dongjin-beyo* rice. The cultures of *P. glomerata* and *Penicillium sp.* also contained IAA. The culture application and endophytic association with host-cucumber plants increased the plant biomass and related growth parameters under sodium chloride and polyethylene glycol induced salinity and drought stress as compared to control plants. The endophytic symbiosis resulted in higher assimilation of essential nutrients like potassium, calcium and magnesium as compared to control plants during salinity stress. Endophytic association reduced the sodium toxicity and promoted the host-benefit ratio in cucumber plants as compared to non-inoculated control plants. The symbiotic association mitigated stress by compromising the activities of reduced glutathione, catalase, peroxidase and polyphenol oxidase. Under stress conditions, the endophyte infection significantly modulated stress through down-regulated abscisic acid, altered jasmonic acid, and

elevated salicylic acid contents as compared to control. In conclusion, the two endophytes significantly reprogrammed the growth of host plants during stress conditions.

Khan *et al.* (2012) investigated plant growth promoting activity of roots inhabiting endophytic fungi in order to evaluate their role in the survival of host plants under extreme sand dune environment of coastal regions. One hundred and twenty two fungal isolates were collected from the roots of 9 sand dune plants and were screened for growth promoting secondary metabolites. The results showed that 101 fungal isolates (82.7%) promoted plant height and shoot length of *Waito-C* rice, while 21 fungal isolates (17.2%) inhibited growth attributes. The fungal isolate *Gibberella fujikuroi* along with distilled water and Czapek broth medium were used as control during the experiment. It was concluded that a major proportion of endophytic fungi inhabiting sand dune plants produce metabolites, which are helpful in plant growth and development.

Hamayun *et al.* (2010) isolated endophytic fungi from cucumber (*Cucumis sativus* L.) found 19 strains of fungi. Pure cultures of 19 endophytic fungi were tested for shoot length promotion of *Waito-C* rice to identify the GA production capacity of these fungal isolates. One of these isolate showed that increased shoot length of *Waito-C* in comparison to control treatments.

2.1.4 Host benefit of infection endophytic fungi

Fungal endophytes, and their effects on plant metabolism, play a large role in phenotypic plasticity and environmental adaptability and likely played a unique role in selection and speciation in many host species (Rudgers *et al.* 2009). Plant can supply nutrients to endophyte, and endophyte then release active metabolites through metabolic activities. Those active metabolites can enhance the roots development and assist solubilizing phosphorus, with which would increase the ability of plants to absorb nutrients from the soil (Li *et al.* 2008). Because of those activities, endophyte can promote host growth and supply resistance mechanism of host. As the study of Dai *et al.* (2008) who inoculated the endophytic fungi *fusarium sp.* strain E4 and E5 which isolated from medicinal plant, *Euphorbia pекinensis*, to the host plant in pot culture. The result exhibited significant increase in growth and all tested growth parameters, as compared to the control.

There is a wide spread phenomenon about the symbiotic relationships between endophytes and plants. But this relationships can switch between mutualistic and parasitic (Kogel *et al.* 2006). While a single gene changes can alter the mechanism of endophyte. For example, *E. festucae* which is a wild-type grows systemically in intercellular spaces, can promote the

growth and enhance the resistance of plant, but while a single-copy plasmid of a NADPH oxidase gene, *noxA*, was inserted in the coding region, the plant would lose its apical dominance, become severely stunted, show precocious senescence, and eventually die (Tanaka *et al.* 2006). The same endophyte colonized in different host can react in the different lifestyles. *Colletotrichum magna* can cause anthracnose in cucurbit plants, but can grow asymptotically when colonized in various non-cucurbit species (Kogel *et al.* 2006).

2.2 Orchids

2.2.1 Botanical of orchids

Orchids are the monocotyledon taxonomy in Orchidales, Orchidaceae. There are 15000 to 25000 of orchid species distributed all around the world, mostly can found in tropical environment. They all can divide in 2 classifications as epiphytic orchids and terrestrial orchids. In Thailand found 500 genus and 800 orchid species. The special hereditary characteristic of orchid species is having many tiny seed in the pod and there are not growing and no endosperm in embryo. So to be germinated the orchids have to depend on some fungi species to be the energy and food sources such as many nutrients that important for growth (Thamasiri. 2005).

2.2.1.1 Growth habits

Many tropical orchids discovered and collected in the eighteenth and nineteenth centuries were found growing on the trunks or branches of trees. This growth situation spawned the erroneous but persistent belief that orchids were parasites. Actually these plants utilize the trees only for support, receiving their nutrients from whatever the rain washes their way, as well as from bird droppings and from organic debris that may collect around their roots. Such plants are called epiphytes.

Orchids may be considered to be two basic growth habits: epiphytic (those that grow on trunks or branches of trees) or terrestrial. The terrestrial species grow with their roots in soil.

Variations on this general theme do occur. There are also orchids that cling to rocks for the foothold: these are generally treated as epiphytes since they usually depend upon natural forces to carry nutrients to them. Some species can not be absolutely classed as either epiphytic or terrestrial. There are those that begin life growing in soil but become vinelike and cling to tree trunks or other convenient supports; then in later life the soil-rooted part die away, leaving the plant a true epiphyte (Gustav. 1993; Brian and Wilma. 1999).

2.2.1.2 Environmental factors

In the wild, orchids grow in competition with other plants, whether in the canopy of trees, among rocks or in open grasslands. An investigation of the details of their environment will reveal the condition that are best for them in a greenhouse: shading from direct sunlight, temperatures moderate to warm but not excessively high, good drainage, high relative humidity coupled with good air movement, and plenty of rainwater during the growing season. It is not always easy, in a greenhouse, to provide all these features at optimum levels in combination, but it is important to achieve the best possible balance of humidity, light, warmth and air movement for healthy growth and maximum flowering. A greenhouse full of plants is usually a well-balanced one, and there were many other plants whose growing requirements were compatible with those of orchids. Begonias, hoyas, columnneas, bromeliads, peperomias, ferns and other tropical plants can all find a place in a heated greenhouse and help to provide the right atmosphere. It is helpful to have an earth floor under the benches, in which some of these companion plants will thrive (Joyce. 1988).

2.2.1.3 The temperature/humidity/ factor

Orchids may be conveniently grouped into general categories according to the temperatures they prefer for their best growth. The following three groups cool, intermediate and warm are based on required winter night temperatures.

Cool-growing orchids prefer temperatures of 50° to 55°, rising to 60° to 70° during the day; this group includes Cymbidiums, Odontoglossums and some Paphiopedilums. Intermediate temperatures of 55° to 60° at night and 65° to 75° during the day are preferred for Cattleyas, Dendrobiums, some oncidiums, hybrid paphiopedilums and many species orchids. And night temperatures of 60° to 65°, 70° to 85° during the day, are ideal for warm-growing orchids such as Vandas, Phalaenopsis, and tropical Paphiopedilums.

Humidity is always easier to rise than it is to lower. Gravel-filled trays that catch excess water from routine watering will provide some humidity as the water evaporates from the surface of the stones. Although orchids are moisture loving plants, orchids always grow in places where periodic dryness is possible. High up in the trees the epiphytes become saturated in every storm, in enveloping clouds, or in heavy dew at night, but they dry out rapidly and always enjoy a buoyant atmosphere (Joyce. 1988).

2.2.2 Important diseases of orchids

Orchids are among the plant groups that have aroused most widespread interest among scientists and horticulturists, both for their study and use. They have been subject to particularly high commercial demand over the past 40 years for the beauty of their structure and their vividly colored flowers, steeped in symbolism and mystery. Some countries have declared orchid species as their national flowers (Clemente. 2009). In Thailand orchids are the most valuable product for export and make money for country. Last years (2013) the export of orchids value were 563,134,221 Baht, and the country that most import Thai orchids were China, Japan, U.S.A, India and Vietnam, respectively (Source: Office of Agriculture Economics). It takes many interested in planting orchid for commercial from businessman and horticulturists. So when there are plentiful culture of orchids that's mean it's following by the distributed of disease. There are many study that compiled the pathogen fungus on orchids include *Alternaria* sp., *Botrytis cineria*, *Cercospora* sp., *Cladosporium* sp., *Colletotrichum gloeosporioides*, *Curvularia eragrostidis*, *Diplodia* sp., *Drechslera* sp., *Fusarium* sp., *Nectria* sp., *Phyllosticta pyriformis*, *Pseudocercospora dendrobii*, *Phytophthora nicotianea* var. *parasitica*, *Phytophthora palmivora*, *Rhizotonia* sp., *Schlerotium rolfsii* and *Volutela* sp. (Thamasiri. 2005; Sutthiwari. 2006).

The orchid cultivation has many problems particularly, fungal diseases. Among these, anthracnose caused by *Colletotrichum gloeosporioides* is a most destructive disease and known to cause great losses to the orchid growers in terms of both quality and quantity. The anthracnose fungus, *Colletotrichum gloeosporioides*, can attack leaves, petioles and blooms during periods of prolonged leaf moisture and high humidity. The ability to cause latent or quiescent infections has grouped *Colletotrichum* among the most important post-harvest pathogens (Pallem *et al.* 2012).

2.3 Previous study about endophytic fungi in orchids

Chen *et al.* (2013) isolated endophytic fungi from 7 *dendrobium* sp. (*Orchidaceae*) searched for *Xylariaceous* fungi. The result showed that a total of 961 culturable endophytic fungi were isolated from seven species of *Dendrobium*. In the original 961 cultures, the xylariaceous taxa had the highest isolation frequency, 22.58% (217/961), followed by *Fusarium* at 10.71% (103/961), *Colletotrichum* at 6.55% (63/961), and *Phomopsis* at 4.37% (42/961).

Sawmya *et al.* (2013) compared the leaf and root endophyte assemblages of two orchids (*Bulbophyllum neilgherrense* and *Pholidota pallida*) from natural forest and greenhouse

conditions. The result found that *Xylariaceae* sp. was consistently associated with leaf and root tissues, while *Guignardia* and *Pestalotiopsis* were found predominantly in the leaf tissues of both orchids. The endophyte assemblages showed that the endophytes exhibited distinct organ but little host specificity. Endophytic fungi have more divers in green house condition more than in forest condition and the roots showed more diverse than in leaves.

Mangunwardoyo *et al.* (2012) were isolated endophytic fungi from *Dendrobium crumenatum* (Pigeon orchid) and investigated antimicrobial activity against 5 pathogens namely *Candida albicans* ATCC 2091, *Candida tropicalis* LIPIMC 203, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923. The result showed that 12 species of endophytic fungi were identified from 60 samples obtained from *D. crumenatum* namely *Cladosporium cladosporioides*, *C. sphaerospermum*, *Colletotrichum gloeosporioides*, *Colletotrichum* sp., *Curvularia brachyspora*, *Fusarium nivale*, *F. solani*, *Guignardia endophyllicola* (anamorf: *Phyllosticta capitalensis*), *Pestalotiopsis* sp., *Scolecobasidium* sp., *Westerdikella* sp., and *Xylohypha* sp. The dominant endophytic fungi were *G. endophyllicola*. For investigated the antimicrobial showed that *Fusarium nivale* was able to inhibit the growth of *C. albicans* and *C. tropicalis*. However, others specimens did not inhibit the tested bacterial, which are Gram-negative *E. coli* and Gram-positive *B. subtilis* and *S. aureus*.

Kasmir *et al.* (2011) isolated endophytic fungi from 4 species epiphytic orchids namely *Bulbophyllum kaitiense* Reichebt. *Gastrochilus acaulis* (Lindley) Kuntze *Dendrobium nanum*-Hook.f and *Geodorum densiflorum* (Lam). Schltr. Two important fungi belonging to tricomaceae members *Aspergillus terreus* and *Penicillium aculeatum*.

Tempesta *et al.* (2011) isolated endophytic fungi to screening for *Pestalotiopsis* from leaves of 2 orchid species. Twenty nine strains of *Pestalotiopsis* sp. was discovered such as *P. sydowiana*, *P. photinae*, *P. leucothoes*, *P. clavispora*, *P. crassiuscula*, *P. rhododendri*, *P. versicolor*, *P. photinae*, *P. gracilis*, *P. aquatic*, *P. palmarum*, *P. theae*, *P. maculans*, *P. funerea*, *P. neglecta*, *P. disseminata*, *P. adusta*, *P. microspore*, *P. bacilia* and *P. vismiae*.

Valdes *et al.* (2011) investigated fungi from root of terrestrial orchid *Cypripedium irapeanum* found 10 isolates 8 strains of fungi: 3 identified as endophytic fungi, *Fusarium* sp. and *Cylindrocarpon* sp., 3 as micorhiza, *Sistotrema* sp., *Rhizoctonia solani* and *Epulorhiza* sp., 3 as dark septate endophytes, MUT 885 and *Phomopsis* sp., 1 as biocontrol fungus, *Gliocladium catenulatum*.

Juntawong *et al.* (2010) investigated endophytic fungi from terrestrial orchids *Geodurum*'s roots in three different seasons in deciduous forest in northern Thailand. The result showed that 198 isolates comprising to 13 genera. The major genera were *Trichoderma* sp. followed by *Fusarium* sp., the least were *Aspergillus* sp., *Colletotrichum* sp., *Eupenicillium* sp. and *Helicoma* sp. In rainy season showed high diversity of fungi more than dry and cold season. This study indicated that changes of physical factors in different seasons had effect on endophytic fungi diversity.

Gezgin and Eltem. (2009) were investigated on orchids from the Aegean and Mediterranean regions. Four species of orchids as follow *Anacamptis pyramidalis* (L.) L.C.M.Richard, *Orchis sancta* L., *Ophrys fusca* Link., and *Serapias vomeracea* subsp. *Orientalis* Greuter were isolated for endophytic fungi. The result showed that a total of 47 isolates, having genus characterisations as 44 (94%) isolates belonging to the genus *Fusarium*, 2 (4%) isolates belonging to the *Rhizoctonia* DC. ex Fr.-like fungi, and 1 (2%) isolate belonging to the genus *Papulaspora preuss*, were found from the orchid root and tubers.

Tao *et al.* (2008) investigated endophytic fungi from terrestrial orchid *Bletilla ochracea*. There were many species of fungi had revealed in this study. Fourteen species were isolated from the leaves, *Alternaria* sp., *Ascomycete* sp., *Chaetothyriales* sp., *Cladosporium* sp., *Colletotrichum* sp., *Cryptococcus* sp., *Dioszegia* sp., *Epulorhiza* sp., *Gibberella* sp., *Herpotrichiellaceae* sp., *Leptosphaerulina* sp., *Mycosphaerella* sp., *Pestalotiopsis* sp. and *Phaeosphaeria* sp. Nine species were isolated from roots, *Cercospora* sp., *Cylindrocarpon* sp., *Exophiala* sp., *Nectria* sp., *Fusarium* sp., *Gibberella* sp., *Herpotrichiellaceae* sp., *Neonectria* sp. and *Sebacina* sp. As the result of this study micorrhiza fungi were isolated from leaves. The diversity within leaves was higher than that within roots. Fungal communities within leaf and root tissues were significantly different.

Ovando *et al.* (2005) screened endophytic fungi in roots of mature plants of epiphytic orchid *Cattleya skinneri* to test their potential to improve seed germination in *C. skinneri* and develop in acclimatizing plantlets of *C. aurantiaca* and *Brassavola nodosa*. One hundred and eight isolates belonging to 11 genera consist of *Trichoderma* sp., *Verticillium* sp., *Aspergillus* sp., *Epulorhiza* sp., *Fusarium* sp., *Monilliopsis* sp., *Pestalotiopsis* sp., *Botrytis* sp., *Penicillium* sp., *Penicillifer* sp. and *Tetracladium* sp. Eight strains in this result used for promote seed germination of *C. skinneri* and 3 strains used for promoted plant growth of *B. nodosa*. None of these strains can promote both of experiments.

Bougoure and Dearnaly. (2005) isolated endophytic fungi from roots of *Dipodium variegatum* found *Russula occidentalis*, *R. salaris*, *R. lepida*, *Verticillium* sp., *Tricoderma hamatum*, *Halocyphina villosa* and *Merismodes fasciculata*.



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Chapter III

Research Methodology

3.1 Isolation of endophytic fungi from orchid species

3.1.1 Sample collection

The samples were collected in greenhouse from Rajamangala University Technology Tawan-ok Chantaburi Campus, Chantaburi province, Thailand. Samples of Leaves and roots from 9 species of selected orchids were packed in sterile polythene bags to keep moisture and brought to laboratory over 12 hours after collecting. The 9 orchid species were collected the samples (Fig. 3.1) as follows.

1. *Grammatophyllum scriptum*
2. *Cymbidium dayanum*
3. *Dendrobium hercoglossum*
4. *Dendrobium palpebrae*
5. *Dendrobium fimbriatum*
6. *Doritis pulcherrima*
7. *Dendrobium crumenatum*
8. *Dendrobium friedericksianum*
9. *Grammatophyllum specinocum*

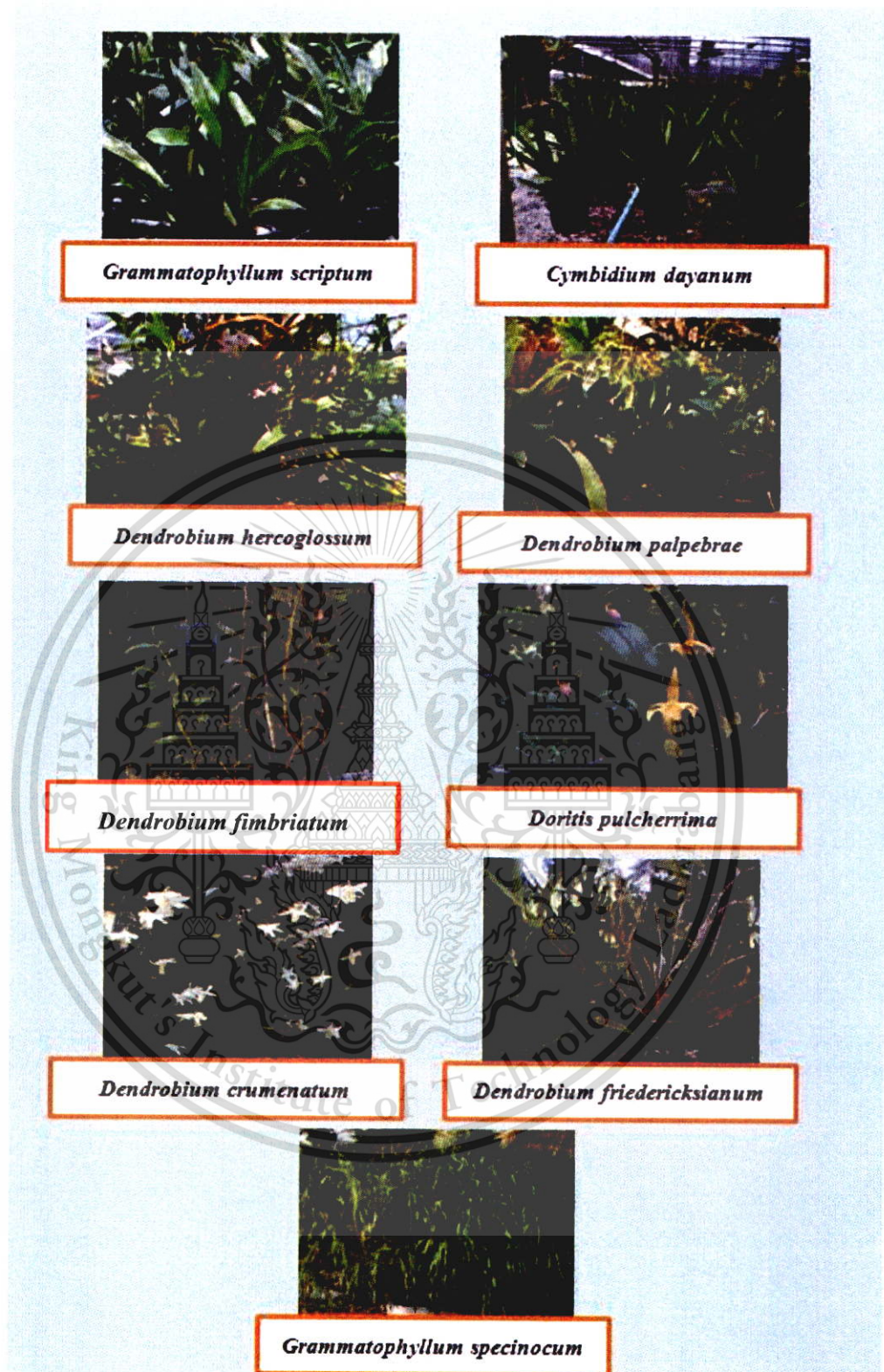


Fig. 3.1. The orchid species used for isolating endophytic fungi

3.1.2 Isolation of endophytic fungi

The surface sterilization was done by following the procedure of Helander *et al.* (2007) and Blumenstein (2010) with some modifications.

Firstly, cut the sample approximately 0.5cm² long piece from each leaf and root of orchids; removed epidermal tissues gently by razor blade from leaves and removed outer cuticle from root sections. Thoroughly washed in sterile water to remove dirt and then dipped the samples into 75% ethanol for 3 minutes. After that, took samples in 0.5% Na-hypochlorite solution for 2 minutes, then washed in sterile water for 3 times. Dried the samples in sterile tissue paper until dehydrate.

The procedure was carried out under a laminar flow in aseptic condition. After removing the excess water, the segments were placed on Petri plates (9 cm diam.) containing with WA (water agar). The plates were incubated at room temperature and fungal colonies were regularly observed. The Petri dishes were then periodically checked every day for fungal growth. Each time seen new colony from inner leaf tissue and root segments, it was sub-cultured on PDA (Potato Dextrose Agar) plates to make pure cultures.

3.1.3 Morphological identification

The morphology of fungal isolates, slides were prepared from cultures and stained with bromothymol blue reagent in lacto phenol and were examined with a binocular compound microscope. Identification based on morphological characteristics such as growth pattern, colony and hyphae, colour of colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and characteristics and size of the spore (Barnett and Hunter, 1956).

The morphological characteristics were followed Crous *et al.* (2009) that used as criterion to the group all fungi. Each fungus was observed and examined to the characteristics and assigned to a specific morphological group.

- Colour. The colony colours were observed to discriminate the fungi. If a fungal colony was more than one distinct colour, the description was explained from the middle towards the outer part.
- Agar colour. Some isolates may possible be released metabolites or substances to change the colour of the agar where the colony is growing.

- Liquid drops. The presence and the colour of the liquid drops that some fungi produced on their surface were recorded.
- Colony shapes. Each colony was observed to grow in a certain way forming different shapes. The colony shapes were distinct: crenate, entire edge, erose or dentate, fimbriate, lobate, radially striate with lobate edge, undulate, with rhizoids.
- Colony texture was distinct as follow: - woolly, velvety, hilly, spongy, dry, furry and creamy.
- Spore characteristics were noted. If the fungus was developed a clear accumulation of spores on their surfaces and the colour of the spores were observed.
- The growth rate was observed as an additional characteristic in order to increase the common characteristic within a group.

3.2 Isolation of *Colletotrichum* sp. from anthracnose of orchid and pathogenicity test

3.2.1 Isolation of *Colletotrichum* sp.

Colletotrichum sp. was isolated from leaf anthracnose of *Grammatophyllum specinocum* (Fig.3.2). Disease sample was taken to isolate the pathogen by using transplanting technique (Agrios. 2005). Disease sample was cut in to small pieces, washed by distilled water for one time, put in 70% ethanol for 3 minutes, poured in 0.5% sodium hypochlorite solution 2 minutes and washed in distilled water thrice, dried with autoclave tissue paper, then put onto water agar (WA) incubated in room temperature until mycelia emerge. The mycelia were transferred to Potato Dextrose Agar (PDA) to get pure culture.

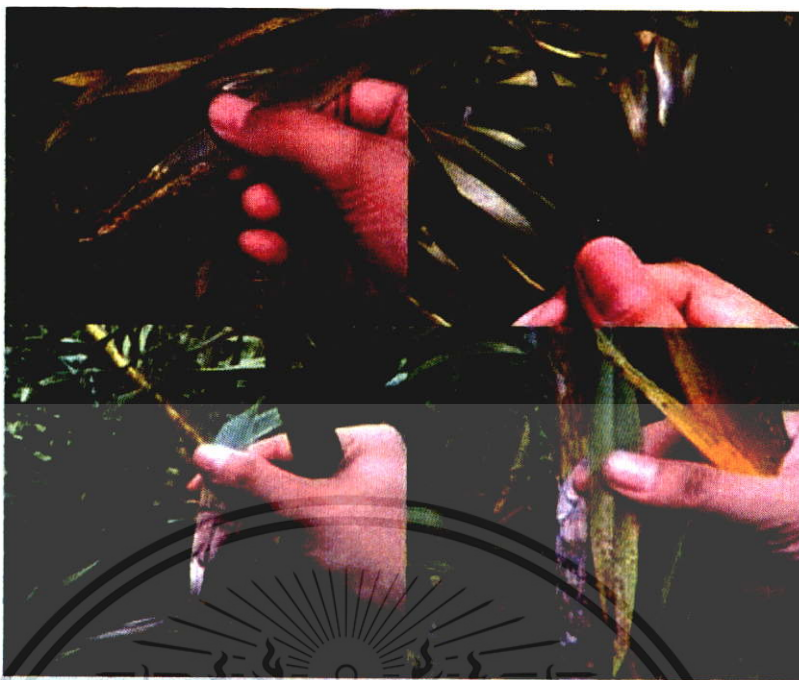


Fig. 3.2. Leaf anthracnose of *Grammatophylum specinocum*

3.2.2 Pathogenicity test

Colletotrichum sp. isolated from leaf anthracnose of *Grammatophylum specinocum* were tested for pathogenicity test by detached leaf method. The leaves were washed by distilled water for one time, dried with autoclave tissue paper. Two sterile moist chambers 10x20 cm layed with sterile wet tissue paper, arranged to put 4 leaves in each box, using sterile needle stabled on leave. The sterile water was contained with spore suspension of pathogen. Dropped pathogen suspension on scar of orchid leaves in one box. These 2 boxes were kept in room temperature and observed for disease symptom. The appeared symptoms were re-isolated to confirm the pathogen occurring anthracnose of orchid.

3.3 Bi-culture antagonistic test

Three endophytic antagonists were tested using bi-culture test which arranged in Completely Randomized Design (CRD) with 4 replications. The antagonistic fungi and pathogen were separately cultured on PDA at room temperature (30–32°C) for seven days. A 0.5 cm diameter sterilized cork borer was used to remove agar plugs from the actively growing edge of cultures of the pathogenic fungus and of the antagonistic fungus into 9cm diameter PDA plates; an

agar plug of the pathogen was placed on one the opposite site an agar plug of an antagonistic fungus. The plates inoculated with a single plug of an antagonistic fungus or of the pathogen acted as the controls. The plates incubated at room temperature for 30 days.

Observation of abnormal spores and normal spore of pathogen from each treatment was observed under compound microscope and took photograph for comparison.

Data were collected including colony diameter (cm) and the number of spore produced by the pathogen. The number of spore production was counted using by haemocytometer. Percentage inhibition of colony growth or spore production of pathogen was transformed according to the following formula:- % inhibition (colony diameter or spore production of pathogen in control plate - colony diameter or spore production of pathogen in bi-culture plate)/ colony diameter or spore production of pathogen in control plate x 100. Colony diameter and spore production were statistically computed analysis of variance, the treatment means were compared using Duncan's Multiple Range Test (DMRT) at $p = 0.05$ and 0.01 .

The endophytic fungi that give good result to control disease were taken for further experiment on fungal biological active substance test against plant pathogen.

3.4 Test biological activity *in vitro*

3.4.1 Extraction method

Crude extracts from selected endophytic fungi as promising antagonist were followed the method of Kanokmedhakul *et al.* (2006). The fungi were separately cultured in potato dextrose broth at room temperature for one month. The next step was collected the fresh biomass and airdried in the room temperature for dry biomass. The dried fungal biomass of antagonistic fungus were ground with the electrical blender and dissolved with hexane, ethyl acetate, and methanol. The solvents were evaporated *in vacuo* to yield crude hexane, crude ethyl acetate (EtOAc), and crude methanol (MeOH) extracts, respectively. Extraction method was shown in Fig. 3.3. Crude extracts were kept in refrigerator for further experiment (Fig.3.3).

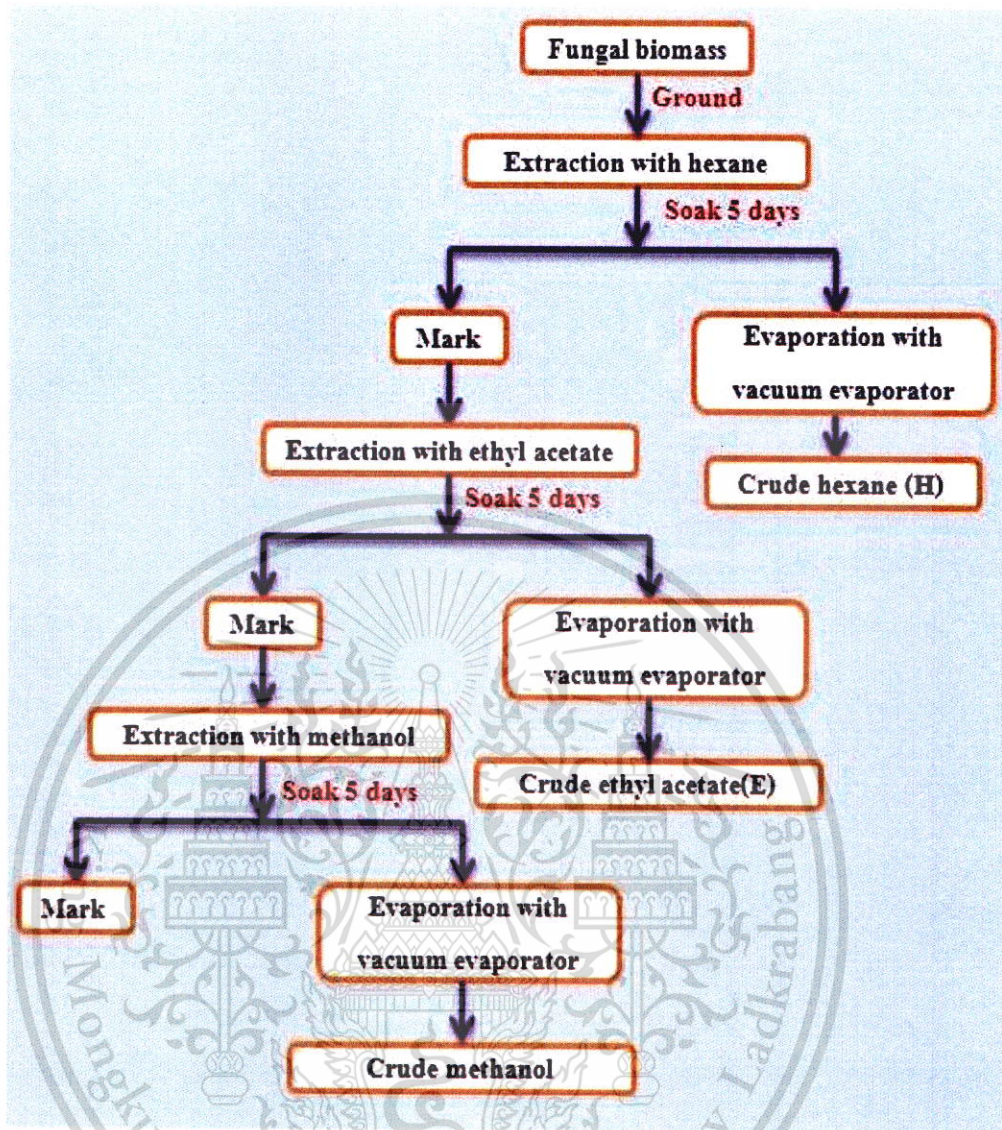


Fig. 3.3. The diagram of crude extracts process

3.4.2 Bioactivities tests of Crude extract against pathogen

The crude extracts were tested for inhibition of the pathogen. The experiment was conducted by using two factors factorial experiment in CRD with four replications. Factor A was represented the crude extracts: A1 = crude hexane extract, A2 = crude ethyl acetate extract and A3 = crude methanol extract. Factor B was represented the different concentrations: B1 = 0 $\mu\text{g/ml}$ (control), B2 = 50 $\mu\text{g/ml}$, B3 = 100 $\mu\text{g/ml}$, B4 = 500 $\mu\text{g/ml}$ and B5 = 1,000 $\mu\text{g/ml}$. Each crude extract was dissolved in 2% dimethyl sulfoxide and added to PDA before autoclaving at 121 °C (15 psi) for 20 minutes. A sterilized 3-mm diameter cork borer was used to transfer agar plugs from the actively growing edge of the pathogen culture. An agar plug was transferred to the center of

5cm diameter Petri dishes of PDA containing crude extract at each concentration and was incubated at room temperature until the mycelium of 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 taken photograph for comparison. Data were collected regarding the number of spore produced by the pathogen and calculated the percentage of spore inhibition. The effective dose (ED_{50}) was calculated using Probit analysis.

3.5 Testing nano products from endophytic antagonists to control orchid

anthracnose *in vivo*

3.5.1 Experimental design

One year old of *Grammatophyllum specinocum* were prepared for the experiment. The experiment was done using RCBD with 4 replications, 5 treatments as follows:

- T1: Un-inoculated anything (Negative control)
- T2: Inoculated with the spore of pathogen 100 μ l at the concentrations 10^6 spores/ml (Positive control)
- T3: Inoculated with pathogen 100 μ l at the concentrations 10^6 spores/ml and apply Nano product from *Chaetomium cochliodes* 10ml/20L of water every 10 days.
- T4: Inoculated with pathogen 100 μ l at the concentrations 10^6 spores/ml and apply Nano product from *Chaetomium cupreum* 10ml/20L of water every 10 days.
- T5: Inoculated with pathogen 100 μ l at the concentrations 10^6 spores/ml and apply with Carbendazem 98% 10ml/20L of water every 10 days.

The abaxial surface of orchid leaves was scratched with a sterile cock borer to make a wound. Then, 100 μ l of spore suspension of *Colletotrichum* sp. was inoculated on the wound in all treatments, except T1. All inoculated orchid plants were covered with water sprayed polyethylene bags for 24 hr.

3.5.2 Formulation of nano products

The crude extract of testing biological activities *in vitro* was used to formulate nano products. The nano products were done using the method of Dar and Soyong (2014). The extract was made into nano-particle by electrospinning. Exactly 2 grams of polylactic acid (PLA) was dissolved in 10mL tetrahydrofuran. The mixture was heated until the polylactic acid was totally melted. The extract was dissolved in a few drops of dimethylsulfoxide and heated to dissolve

completely and then the two mixtures were added together. The resulting mixture was loaded into a syringe and placed into the electrospinning set-up. The tip of the syringe was clipped with the positive pole while the aluminum foil was clipped with the negative pole and serves as the collector. The voltage used was 25 to 30 kilovolts. The product was carefully scraped from the aluminum foil and stored in tightly capped bottles.

3.5.3 Data collection

Disease Severity Index was divided into 9 levels as follows:

1) Healthy, no disease symptoms of the leaves, 2) The length of lesion 1 – 10%, 3) The length of lesion 11 – 20%, 4) The length of lesion 21 – 30%, 5) The length of lesion 31 – 40%, 6) The length of lesion 41 – 50%, 7) The length of lesion 51 – 60%, 8) The length of lesion 61 -70% and 9) The length of lesion more than 71%.

The DSI measured the length of visible lesions on the leaves of orchid, were observed every 10 days for 6 times. The percent disease reduction (%DR) was followed the method of Soyong (2015), was determined using the formula as follows:- $A-B/A \times 100$, where A = score of disease index rating from control treatment inoculated with pathogen and B = score of disease index rating from treatment applied with nano product. Data were statistically analyzed for significant differences using analysis of variance (ANOVA). Comparison among treatment mean was computed using Duncan Multiple Range Test at $P=0.05$ and $P=0.01$.

Chapter IV

Results

4.1 Isolation of endophytic fungi from orchid species

Eleven species of endophytic fungi were isolated from 9 orchid varieties. *Cymbidium dayanum* was most dominant of endophytic fungi which was 7 species and followed by *Grammatophyllum scriptum*, *Dendrobium hercoglossum*, *Dendrobium fimbriaetum*, *Doritis pulcherima*, *Dendrobium friedericksianum*, *Grammatophyllum specinocum*, *Dendrobium crumenatum* and *Dendrobium palpebrae*. *Xylaria* sp. found to be the most wide host range which isolated from 6 orchid species and followed by *Colletotrichum* sp., *Fusarium* sp., *Chaetomium cochliodes*, *Pestalotiopsis* sp., *Phoma* sp., *Curvularia* sp., *Chaetomium cupreum*, *Achaetomium* sp., *Nigrospora* sp. and *Corynascus* sp. (Table 4.1).

Table 4.1. Endophytic fungi which isolated from each orchid species

Orchid varieties	Endophytic fungi
<i>Grammatophyllum scriptum</i>	<i>Chaetomium cochliodes</i> , <i>Chaetomium cupreum</i> , <i>Curvularia</i> sp., <i>Fusarium</i> sp., <i>Pestalotiopsis</i> sp. and <i>Achaetomium</i> sp.
<i>Cymbidium dayanum</i>	<i>Chaetomium cochliodes</i> , <i>Xylaria</i> sp., <i>Colletotrichum</i> sp., <i>Phoma</i> sp., <i>Fusarium</i> sp., <i>Pestalotiopsis</i> sp. and <i>Corynascus</i> sp.
<i>Dendrobium hercoglossum</i>	<i>Chaetomium cochliodes</i> , <i>Xylaria</i> sp., <i>Colletotrichum</i> sp. and <i>Nigrospora</i> sp.
<i>Dendrobium palpebrae</i>	<i>Fusarium</i> sp.,
<i>Dendrobium fimbriaetum</i>	<i>Chaetomium cochliodes</i> , <i>Colletotrichum</i> sp. and <i>Fusarium</i> sp.
<i>Doritis pulcherrima</i>	<i>Xylaria</i> sp., <i>Phoma</i> sp. and <i>Curvularia</i> sp.
<i>Dendrobium crumenatum</i>	<i>Xylaria</i> sp. and <i>Colletotrichum</i> sp.
<i>Dendrobium friedericksianum</i>	<i>Xylaria</i> sp., <i>Fusarium</i> sp. and <i>Pestalotiopsis</i> sp.
<i>Grammatophyllum specinocinum</i>	<i>Xylaria</i> sp., <i>Colletotrichum</i> sp. and <i>Phoma</i> sp.

1. *Chaetomium cochliodes*

Habitat: *Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium hercoglossum* and *Dendrobium fimbriatum*.

The characteristic fungus can be seen in Fig.4.1.

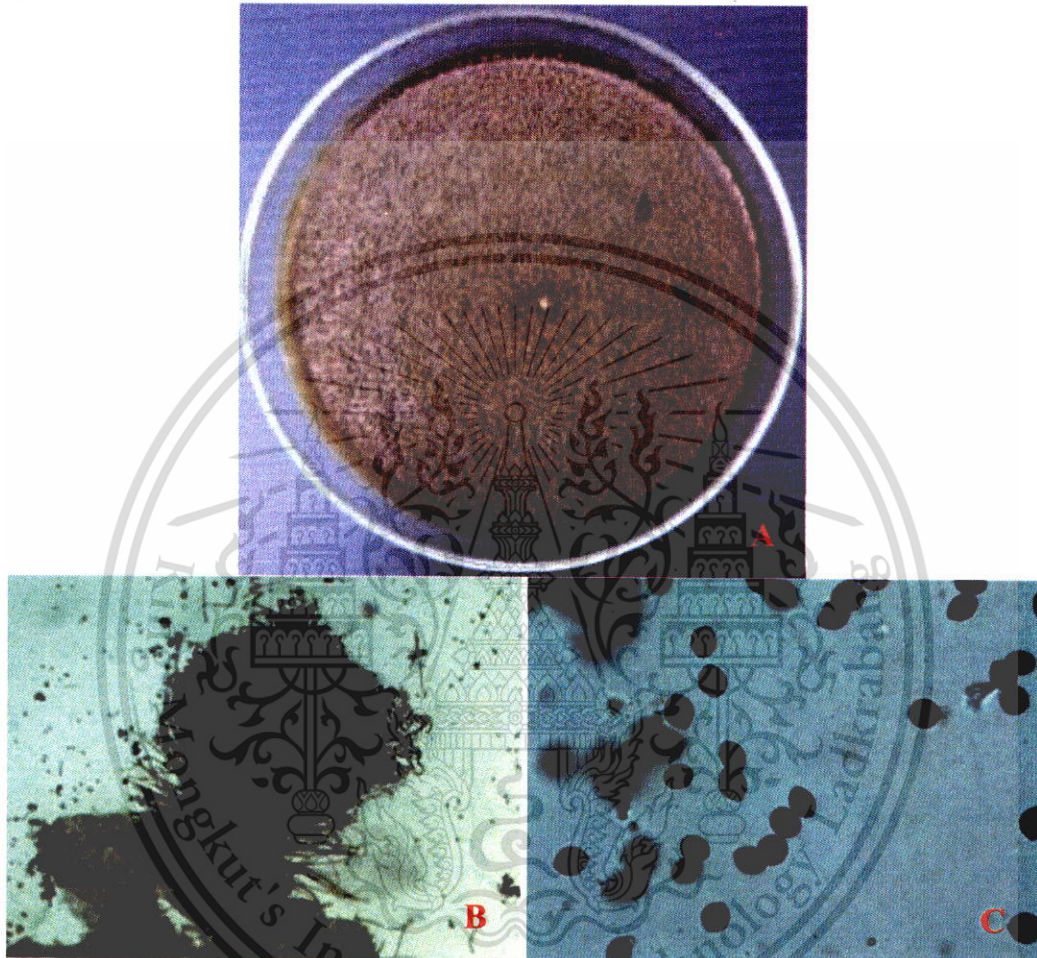


Fig. 4.1. *Chaetomium cochliodes* A: Colony on PDA at 30 days, B: The ascocarp (10X)
C: Ascospores (40X)

2. *Chaetomium cupreum*

Habitat: *Grammatophyllum scriptum*.

The characteristic of fungus can be seen in Fig.4.2.

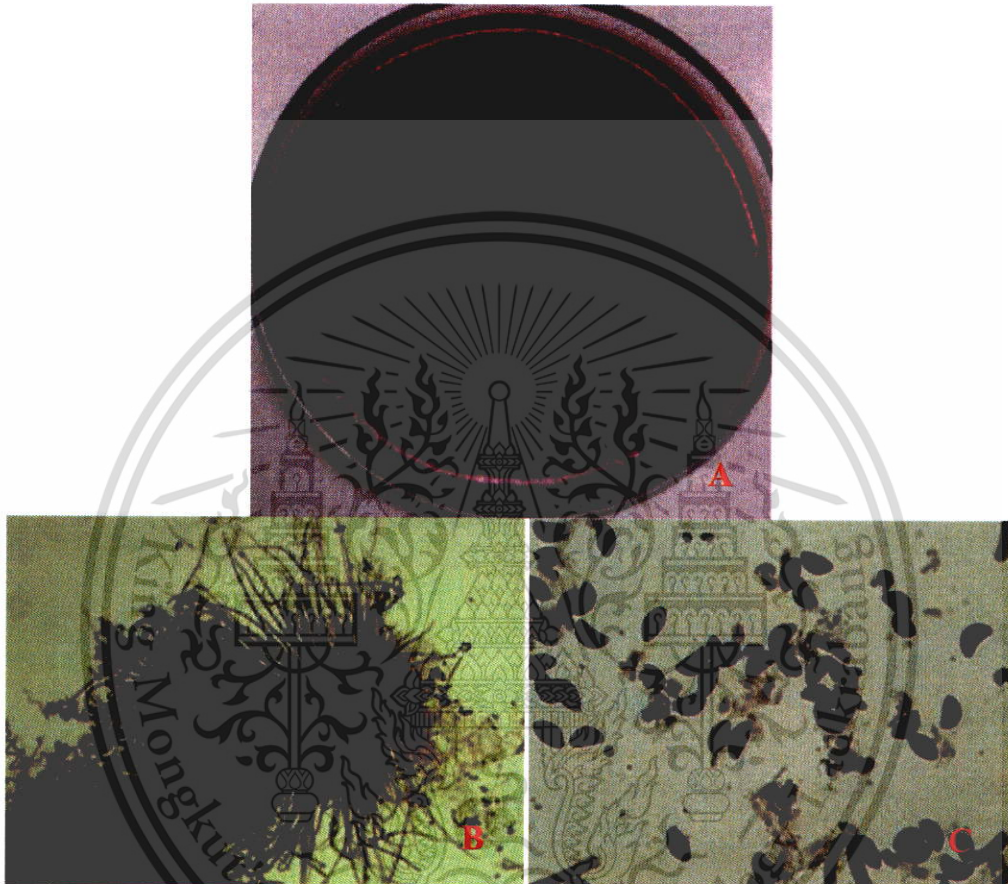


Fig. 4.2. *Chaetomium cupreum* A: Colony on PDA at 30 days B: The ascocarp (10X) and C: Ascospores (40X)

3. *Xylaria* sp.

Habitate: *Cymbidium dayanum*, *Dendrobium hercoglossum*, *Doritis pulcherrima*, *Dendrobium crumenatum*, *Dendrobium friedericksianum* and *Grammatophyllum specinocum*.

The characteristic of fungus can be seen in Fig. 4.3.

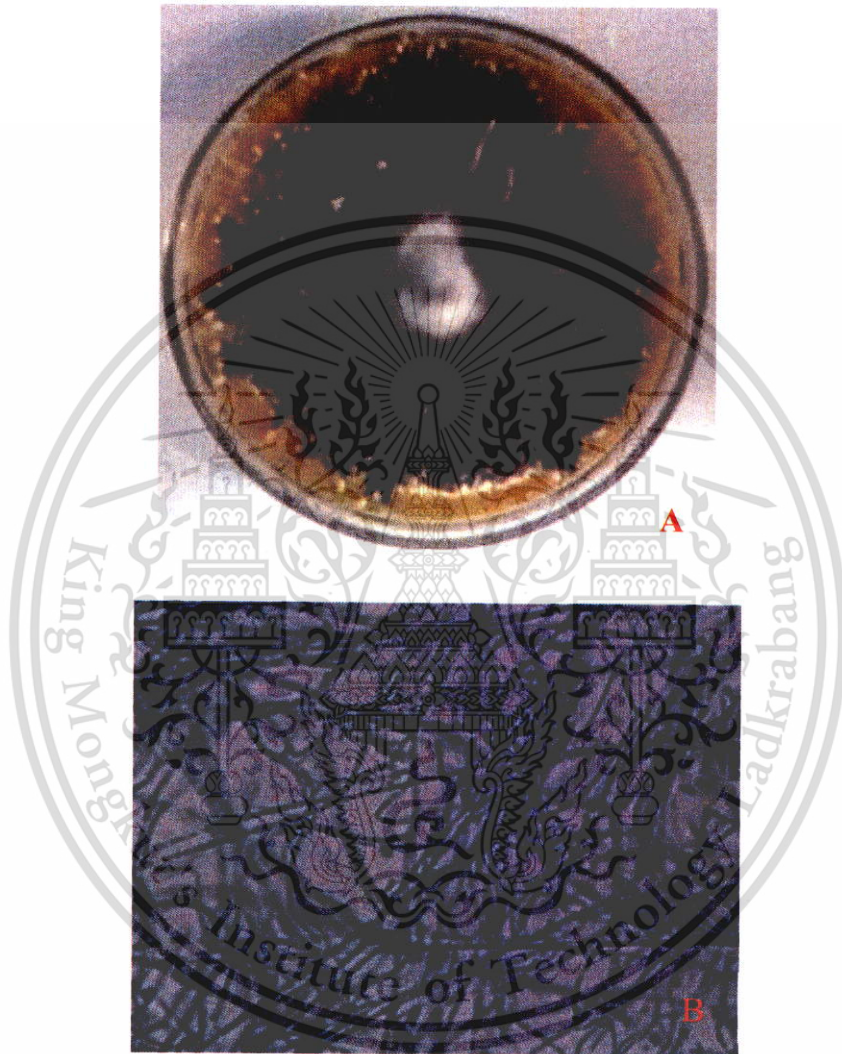


Fig. 4.3. *Xylaria* sp. A: Colony on PDA at 30 days B: Mycelia (40X)

4. *Colletotrichum* sp.

Habitats: *Cymbidium dayanum*, *Dendrobium hercoglossum*, *Dendrobium fimbriaetum*, *Dendrobium crumenatum* and *Grammatophyllum specinocum*.

The characteristic of fungus can be seen at Fig.4.4.



Fig. 4.4. *Colletotrichum* sp. A: Colony on PDA at 30 days and B: Conidia (40X)

5. *Nigrospora* sp.

Habitat: *Dendrobium hercoglossum*

The characteristic of fungus can be seen in Fig.4.5.

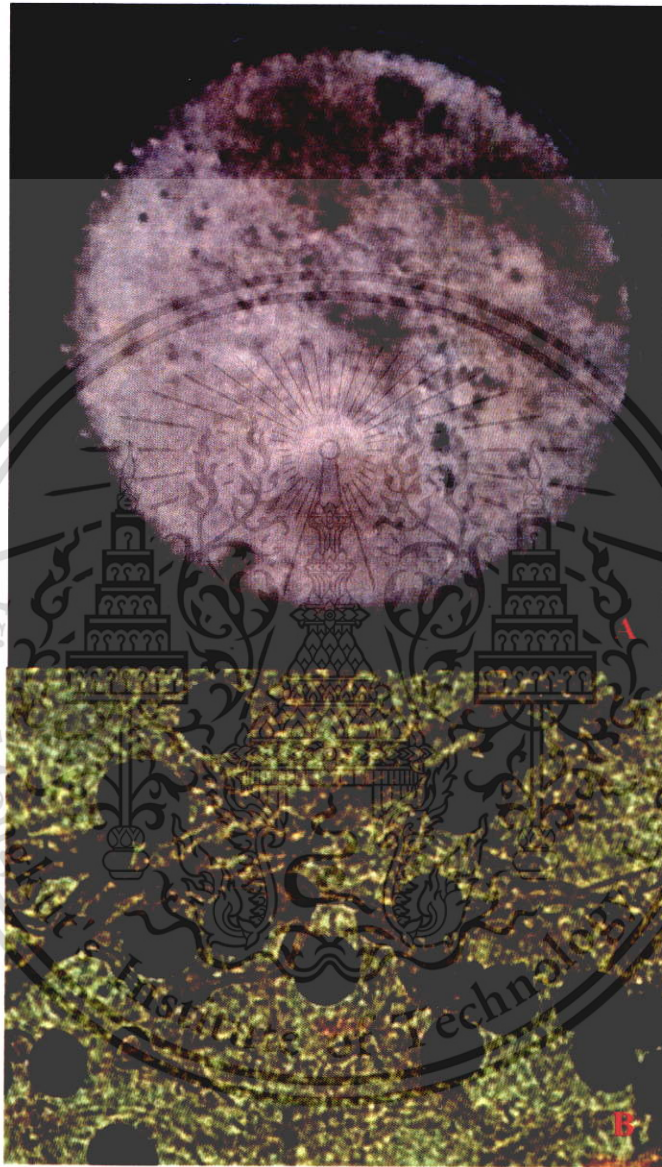


Fig. 4.5. *Nigrospora* sp. A: Colony on PDA at 30 days B: Conidia and conidiophores (40X)

6. *Phoma* sp.

Habitats: *Cymbidium dayanum*, *Doritis pulcherrima* and *Grammatophyllum specinocum*.

The characteristic of fungus can be seen in Fig.4.6.

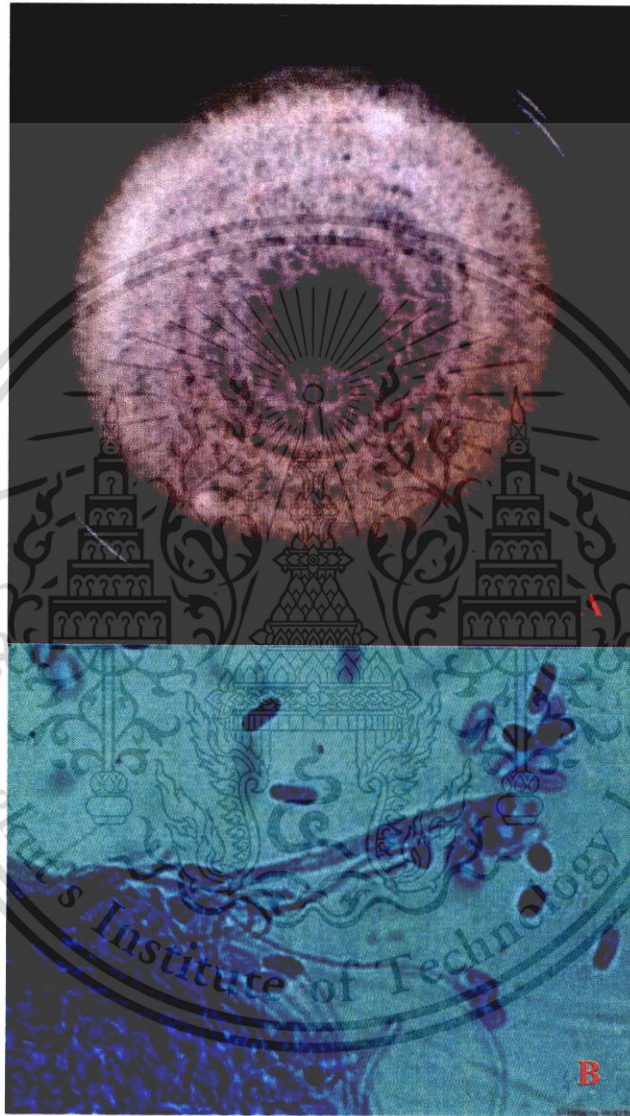


Fig. 4.6. *Phoma* sp. A: Colony on PDA at 10 days and B: Conidia and conidiophores (40X)

7. *Curvularia* sp.

Habitats: *Grammatophyllum scriptum* and *Doritis pulcherrima*.

The characteristic of fungus can be seen at Fig.4.7.

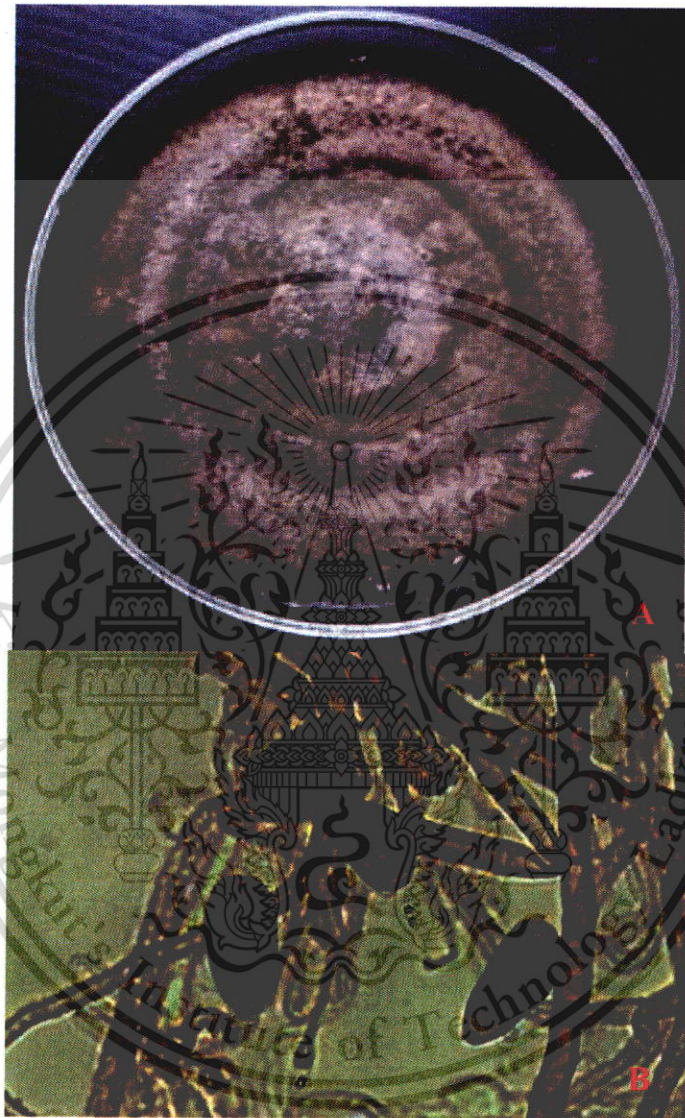


Fig. 4.7. *Curvularia* sp. A: Colony on PDA at 10 days and B: Conidia and Conidiospores (40X)

8. *Fusarium* sp.

Habitat: *Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium palpebrae*, *Dendrobium fimbriatum* and *Dendrobium friedericksianum*.

The characteristic of fungus can be seen in Fig.4.8.



Fig. 4.8. *Fusarium* sp. A: Colony on PDA at 10 days and B: Macroconidia and microconidia (40X)

9. *Pestalotiopsis* sp.

Habitat: *Dendrobium friedericksianum*, *Cymbidium dayanum* and *Grammatophyllum scriptum*.

The characteristic of fungus can be seen in Fig. 4.9

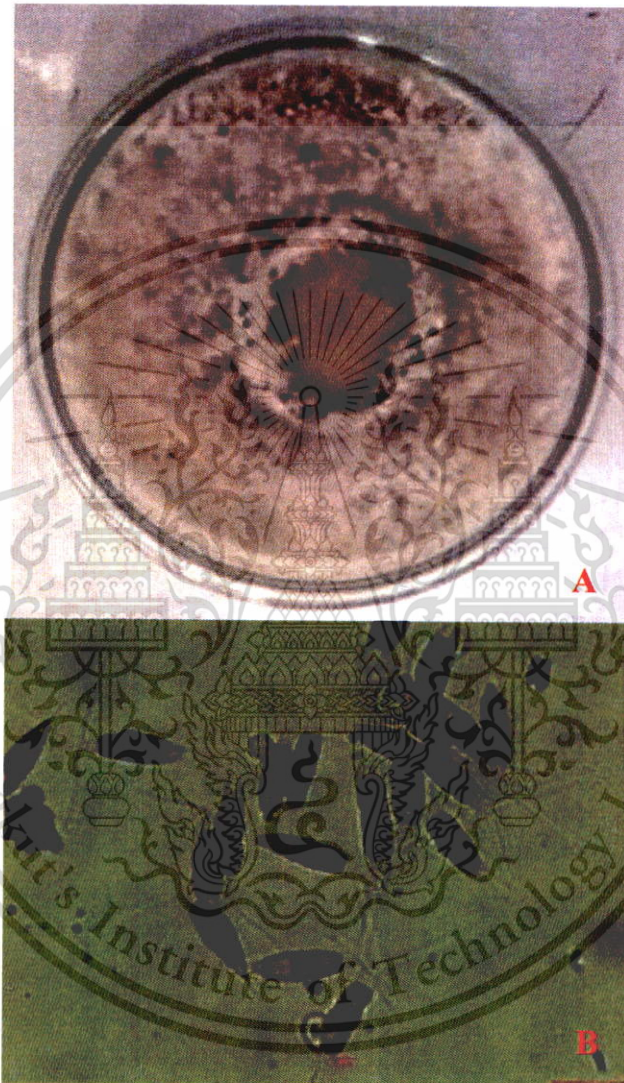


Fig. 4.9 *Pestalotiopsis* sp. A: Colony on PDA at 30 days B: The conidia (40X)

10. *Corynascus* sp.

Habitat: *Cymbidium dayanum*

The characteristic of fungus can be seen in Fig. 4.10.



Fig. 4.10. *Corynascus* sp. A: Colony on PDA 10 days and B: Ascocarp and ascospores (40X)

11. *Achaetomium* sp.

Habitat: *Grammatophyllum scriptum*

The characteristic of fungus can be seen in Fig.4.11.

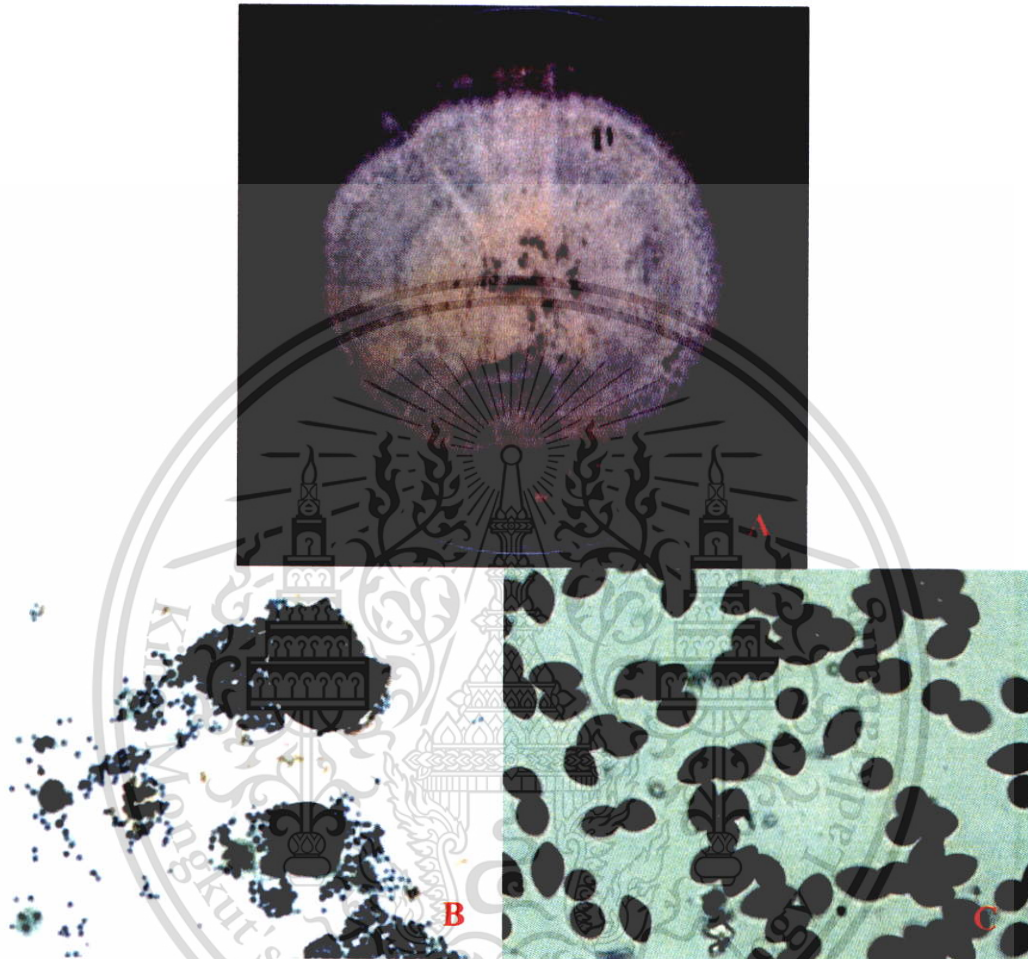


Fig. 4.11. *Achaetomium* sp. A: Colony on PDA at 30 days, B: Ascocarp (10X) and C: Ascospores (40X)

4.2 Isolation of *Colletotrichum* sp. from orchid anthracnose and pathogenicity test

4.2.1 Isolation of *Colletotrichum* sp.

Anthracnose pathogen of *Grammatophyllum specinocum* was isolated and identified as *Colletotrichum* sp. by using transplanting technique. The fungus was subcultured into PDA medium to be pure culture. Characteristic of pathogen was shown in Fig. 4.12.

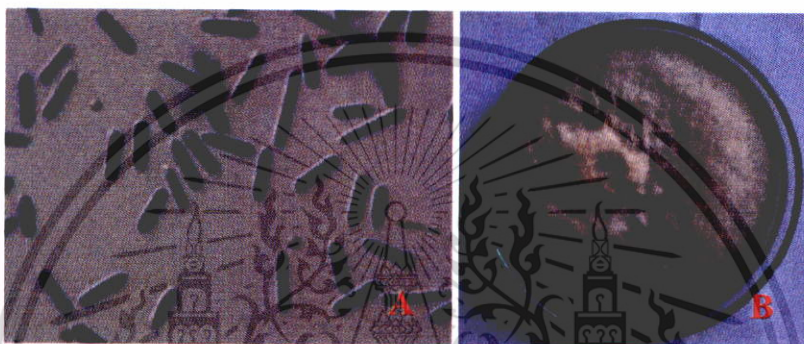


Fig. 4.12. *Colletotrichum* sp. isolated from leaf of *Grammatophyllum specinocum*

A: conidia (40X) and B: Colony on PDA at 10 days

4.2.2 Pathogenicity test

The pathogenicity test was successful done with inoculated into leaf of *Grammatophyllum specinocum*. The inoculated leaf with pathogen showed the symptom of infection with the black rings around the wounds. The leaves without inoculation showed non-infection.

The infected leaf was re-isolated to ensure the fungus and resulted that the fungus was the same with the *Colletotrichum* sp. that inoculated for the first time as shown in Fig. 4.13.

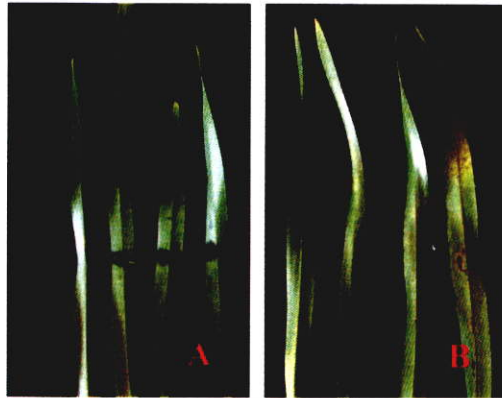


Fig. 4.13. Pathogenicity test of pathogen on leaves of *Grammatophylum specinocum* for 3 days

A: Inoculated with *Colletotrichum* sp. and B: Un-inoculated with *Colletotrichum* sp.

4.3 Bi-culture antagonistic test

Three endophytic fungi, *Achaetomium* sp., *Chaetomium cochliodes* and *Chaetomium cupreum* were tested to inhibit the growth of *Colletotrichum* sp. causing anthracnose in orchids by bi-culture antagonistic test the result showed as follows:

The colony growth inhibition showed that all the antagonists gave significantly different from the control which was 9cm. Colony of *Chaetomium cochliodes* was 5.67cm showed the best ability for inhibition (36.94%) when compared to the control and followed by *Chaetomium cupreum* and *Achaetomium* sp. which was 6.31cm or 29.85% and 6.55cm or 27.21% respectively.

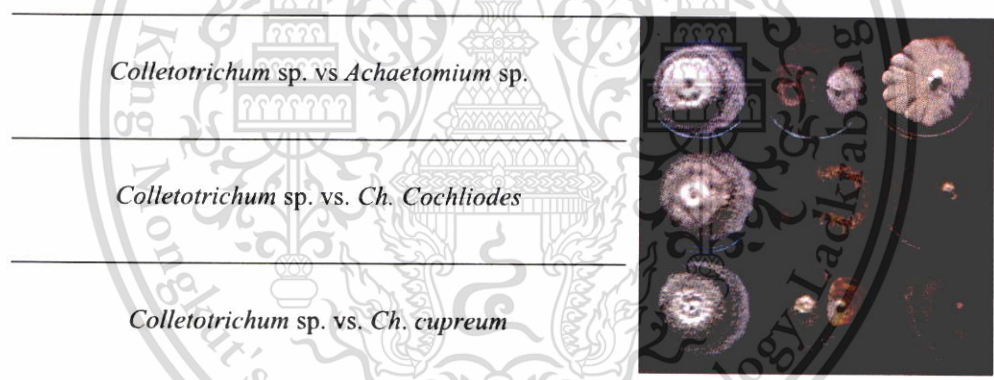
The spore productions showed that all the antagonists gave significantly different when compared to the control which was 55.12×10^6 spores/ml. *Chaetomium cupreum* showed the best ability to inhibit the spore production of pathogen which was 24.25×10^6 spores/ml or 54.39%, and followed by *Chaetomium cochliodes* and *Achaetomium* sp. which were 29.12×10^6 spores/ml and 39.25×10^6 spores/ml respectively and inhibited as 47.2% and 28.81% respectively (Table 4.2 and Fig. 4.14).

Table 4.2. Bi-culture antagonistic test of endophytic fungi against *Colletotrichum* sp.

Antagonists	Colony Growth (cm)	Colony percentages inhibition ²	Number of spore (x10 ⁶ spores/ml)	number of spore Percentages inhibition ²
Control	9.00a ¹	0.00d	55.12a ¹	0.00d
<i>Achaetomium</i> sp.	6.55b	27.21b	39.25b	28.81c
<i>Chaetomium cochliodes</i>	5.67c	36.94a	29.12c	47.2b
<i>Chaetomium cupreum</i>	6.31b	29.85b	24.25c	54.39a
CV%	3.94	10.03	11.16	8.02

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

² Inhibition (%) = $R1-R2/R1 \times 100$ where R1 was colony diameter or number of spores of pathogen in control and R2 was colony diameter or number of spores of pathogen in treated plates.

Fig. 4.14. Bi-culture antagonist test of endophytic fungi against *Colletotrichum* sp.

4.4 Biological activity test *in vitro*

4.4.1 Extraction method

Two most effective species, *Chaetomium cochliodes* and *Chaetomium cupreum*, were chosen to extract for bioactive compounds. Crude extracts were used hexane, ethyl acetate and methanols as solvents. The dry biomass of *Chaetomium cochliodes* 100g gave crude hexane 0.934g, crude ethyl acetate 2.109g and crude methanol 3.219g. The dry biomass of *Chaetomium*

cupreum 100g yielded crude hexane 1.203g, crude ethyl acetate 2.105g and crude methanol 2.804g (Fig. 4.15 and Fig. 4.16).

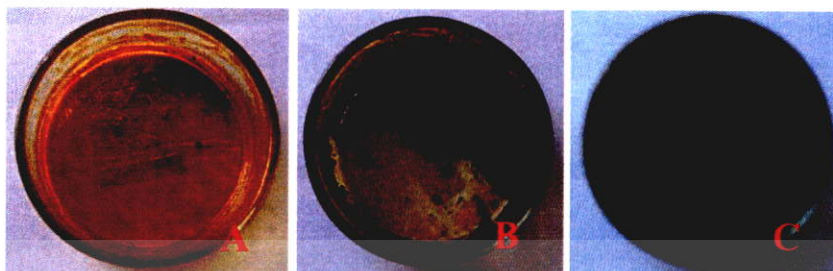


Fig. 4.15. Crude extracts from *Chaetomium cochliodes* A: crude hexane B: crude ethyl acetate and C: crude methanol



Fig. 4.16. Crude extracts from *Chaetomium cupreum* A: crude hexane B: crude ethyl acetate and C: crude methanol

4.4.2 Bioactivities tests of crude extract against *Colletotrichum* sp.

4.4.2.2 Crude extracts from *Chaetomium cochliodes*

Crude extracts from *Chaetomium cochliodes* showed good ability to inhibit the colony growth of *Colletotrichum* sp. Crude hexane at the concentration 10, 50, 100, 500 and 1,000 $\mu\text{g/ml}$ which were 4.18, 4.09, 4.34, 4.11, 3.25 and 3.25cm respectively, when compared to the control (0 $\mu\text{g/ml}$) which was 5cm. The inhibition percentage were 16.5%, 18.25%, 21.25%, 23.5% and 53.75% respectively where the concentration at 1,000 $\mu\text{g/ml}$ was the most inhibition. Crude ethyl acetate at the concentration 10, 50, 100, 500 and 1,000 $\mu\text{g/ml}$ which were 4.39, 4.34, 4.11, 3.25 and 3.23cm respectively, gave significantly different when compared to the control (0 $\mu\text{g/ml}$) which was 5cm. The inhibition percentages were 12.25%, 13.5%, 17.75%, 35% and 35.5% respectively. Crude methanol at the concentration 50, 100, 500 and 1,000 $\mu\text{g/ml}$ which were 4.19, 3.48, 3.45 and 1.99cm respectively, gave significantly different with the control (0 $\mu\text{g/ml}$) which was 5cm. The inhibition percentages were 8.5%, 14.25%, 30.5%, 31% and 60.25% respectively.

The highest inhibition was crude methanol at the concentration 1,000 μ g/ml and crude hexane at the concentration 1,000 μ g/ml which was 60.25% and 53.75% respectively, and followed by crude ethyl acetate at the concentration 1,000 μ g/ml and 500 μ g/ml which was 35.5% and 35% respectively.

All crude extracts from *Chaetomium cochliodes* showed good ability to inhibit the spore production of *Colletotrichum* sp. Crude hexane at the concentration 10, 50, 100, 500 and 1,000 μ g/ml which was 2.96, 2.93, 2.35, 2.18 and 2.05 $\times 10^7$ spores/ml respectively, gave significant different when compared to the control (0 μ g/ml) which was 3.44 $\times 10^7$ spores/ml. The inhibition percentages was 13.75%, 14.75%, 31.29%, 36.6% and 46.12% respectively, where the most inhibition was the concentration 1,000 μ g/ml and followed by 500 μ g/ml and 100 μ g/ml.

Crude ethyl acetate at the concentration 10, 50, 100, 500 and 1,000 μ g/ml which was 2.41, 2.19, 2.07, 1.84 and 1.48 $\times 10^7$ cell/ml respectively, when compared to the control (0 μ g/ml) which was 3.08 $\times 10^7$ cell/ml. The inhibition percentage was 25.5%, 33.15%, 32.8%, 40.05% and 51.77% respectively, where the most inhibition was the concentration 1,000 μ g/ml and followed by 500 μ g/ml and 100 μ g/ml. Crude methanol at the concentration 100, 500 and 1,000 μ g/ml which were 2.21, 1.81 and 1.19 $\times 10^7$ spores/ml respectively, gave significantly different when compared to the control (0 μ g/ml) which was 3.03 $\times 10^7$ cell/ml. The inhibition percentage was 26.76%, 40.05% and 60.87% respectively, where the concentration 1,000 μ g/ml was the most inhibition and followed by the concentrations at 500 μ g/ml and 100 μ g/ml. The ED₅₀ value was 712.0882 μ g/ml (Table 4.3 and 4.4, Fig.4.17, 4.18 and 4.19).

Table 4.3. Crude extracts of *Chaetomium cochliodes* testing for inhibit *Colletotrichum* sp.

Crude extracts	Concentration ($\mu\text{g/ml}$)	Colony diameter (cm) ¹	Growth inhibition (%) ²
Hexane	0	5.00a	0.00f
	10	4.18bcd	16.50de
	50	4.09bcd	18.25de
	100	3.94cde	21.25cd
	500	3.82de	23.50cd
	1,000	2.31g	53.75a
	Ethyl acetate	0	5.00a
10		4.39bc	12.25de
50		4.34bcd	13.50de
100		4.11bcd	17.75de
500		3.25f	35.00b
1,000		3.23f	35.50b
Methanol		0	5.00a
	10	4.58ab	8.50ef
	50	4.19bcd	14.25de
	100	3.48ef	30.5bc
	500	3.45ef	31.00bc
	1,000	1.99g	60.25a
	CV%	6.48	24.37

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

² Inhibition (%) = $\frac{R1-R2}{R1} \times 100$ where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

Table 4.4. Crude extracts of *Chaetomium cochliodes* testing to inhibit spore productions of *Colletotrichum* sp.

Crude extracts	Concentration ($\mu\text{g/ml}$)	Number of spores ($\times 10^7$ spores/ml)	Spore inhibition (%) ²	ED ₅₀ ($\mu\text{g/ml}$)
Hexane	0	3.44a ¹	0.00g	-
	10	2.96b	13.75efg	
	50	2.93b	14.75ef	
	100	2.35cd	31.29cd	
	500	2.18cd	36.60cd	
	1,000	2.05de	46.12bc	
	Ethyl acetate	0	3.08b	
10		2.41c	25.50de	
50		2.19cd	33.15cd	
100		2.07de	32.80cd	
500		1.84e	40.05bcd	
1,000		1.48f	51.77ab	
Methanol		0	3.03b	0.00g
	10	2.97b	2.06fg	
	50	2.93b	3.88fg	
	100	2.21cd	26.76de	
	500	1.81e	40.05bcd	
	1,000	1.19g	60.87a	
	CV%	6.28	27.91	

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

² Inhibition (%) = $\frac{R1-R2}{R1} \times 100$ where R1 was spore number of pathogen in control and R2 was colony spore number in treated plates.

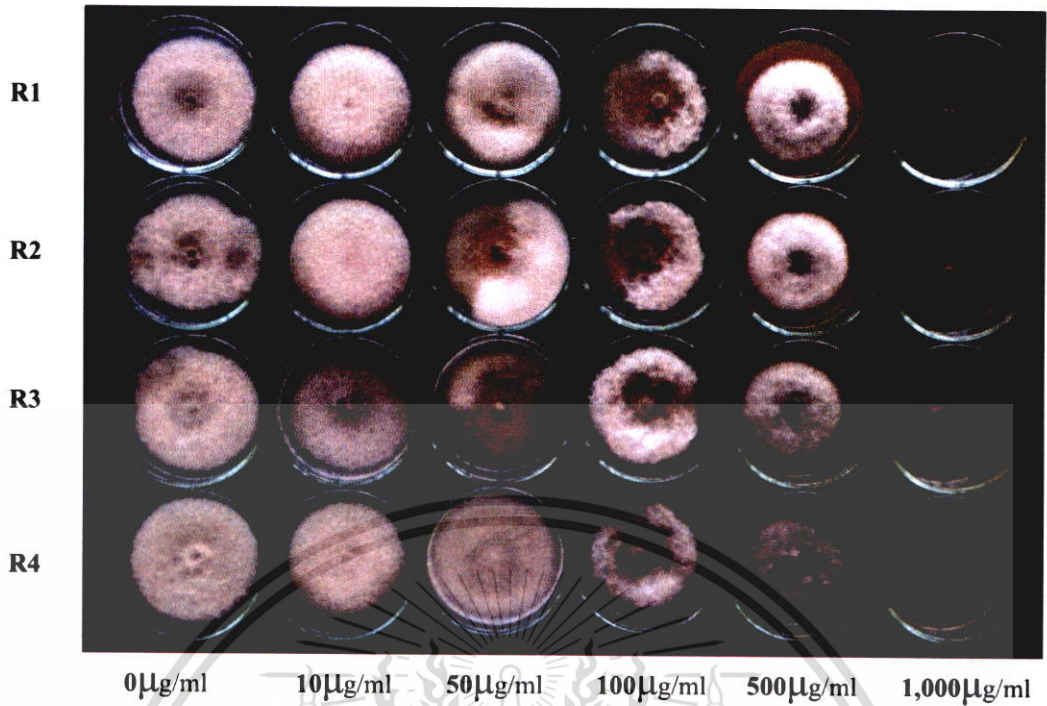


Fig. 4.17. Colony of *Colletotrichum* sp. treated by crude hexane from *Chaetomium cochliodes*

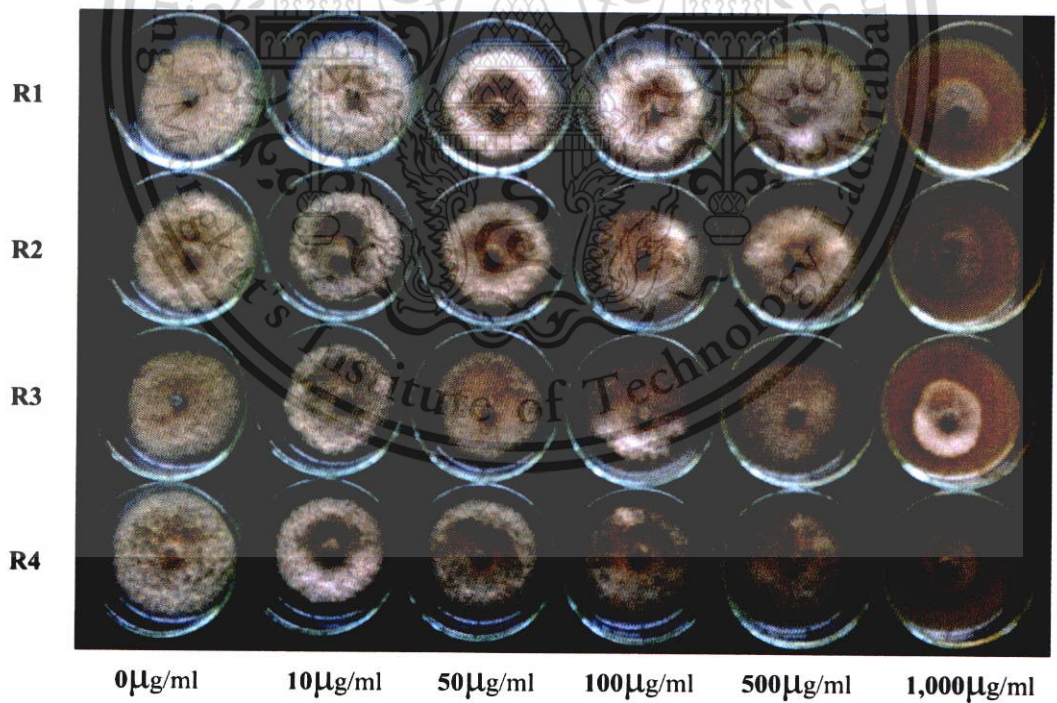


Fig. 4.18. Colony of *Colletotrichum* sp. treated by crude ethyl acetate from *Chaetomium cochliodes*

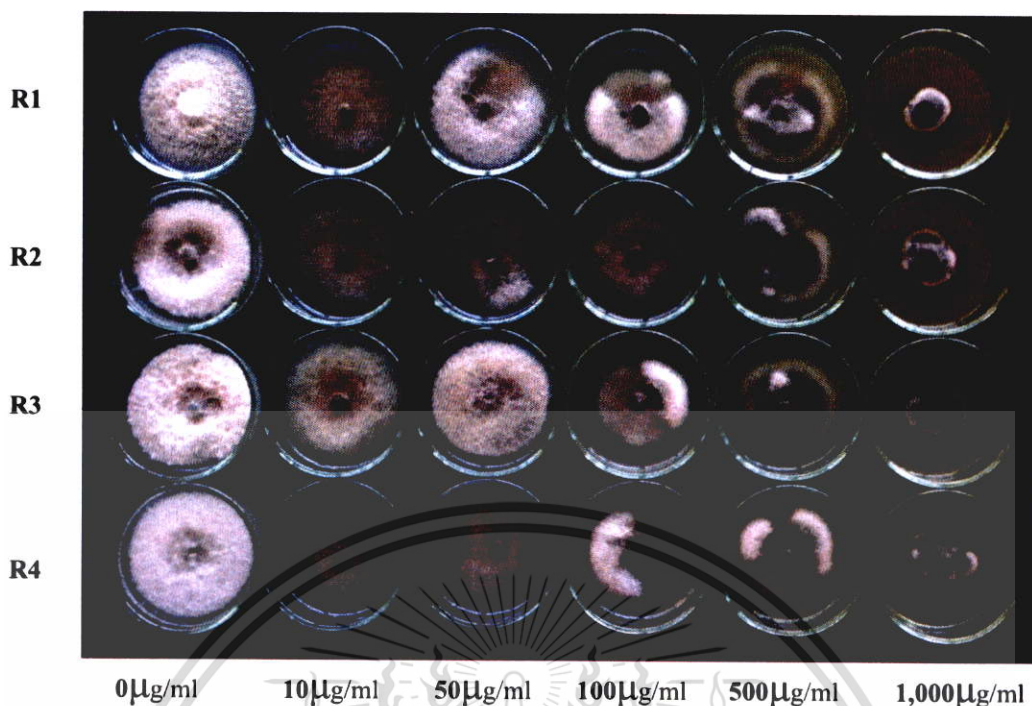


Fig. 4.19. Colony of *Colletotrichum* sp. treated by crude methanol from *Chaetomium cochliodes*

4.3.2.2 Crude extracts from *Chaetomium cupreum*

All the crude extracts from *Chaetomium cupreum* showed the ability to inhibit the growth of *Colletotrichum* sp., both colony and spore production. For colony growth, hexane crude extract at the concentration 10, 50, 100, 500 and 1,000 $\mu\text{g/ml}$ which were 4.21, 4.15, 3.46, 2.85 and 2.73cm respectively, gave significantly different to the control (0 $\mu\text{g/ml}$) which was 5cm. The inhibition percentages at the concentration at 1,000 and 500 $\mu\text{g/ml}$ were the most inhibited which were 40.5% and 38.5% respectively and followed by concentration of 100, 50 and 100 $\mu\text{g/ml}$ which were 31.25%, 16.5% and 15.75% respectively. Ethyl acetate crude extract at the concentration 10, 50, 100, 500 and 1,000 $\mu\text{g/ml}$ which were 4.21, 4.17, 4.08, 3.16 and 1.64cm respectively, gave significantly different when compared to the control (0 $\mu\text{g/ml}$) which were 5cm. The inhibition percentage was the concentration 1,000 $\mu\text{g/ml}$ which was 67.25% and followed by the concentration 500, 100, 50 and 10 $\mu\text{g/ml}$ which were 36.75%, 18%, 15.75% and 15.75% respectively. Methanol crude extract at the concentration 10, 50, 100, 500 and 1,000 $\mu\text{g/ml}$ which were 4.3, 4.04, 3.01, 3.34 and 2.86cm respectively, gave significantly different when compared to the control (0 $\mu\text{g/ml}$) which was 5cm. The most inhibitions were the concentration 1,000, 500 and 100 $\mu\text{g/ml}$ which were 42.75%, 33.1% and 39.75% respectively.

The crude extracts from *Chaetomium cupreum* showed ability to inhibit spore production of *Colletotrichum* sp. Crude hexane at the concentration 10, 50, 100, 500 and 1,000 $\mu\text{g/ml}$ which were 3.88, 3.17, 3.11, 2.37 and 2.05×10^7 spores/ml respectively, gave significantly different when compared to the control (0 $\mu\text{g/ml}$) which was 4.27×10^7 spore/ml. The most inhibition was the concentration 1,000 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ which were 51.35% and 44.45% respectively. The ED_{50} value was 794.8441 $\mu\text{g/ml}$. Crude ethyl acetate at the concentration 10, 50, 100, 500 and 1,000 $\mu\text{g/ml}$ which were 3.38, 3.23, 3.11, 2.31 and 2.05×10^7 spores/ml respectively, gave significantly different with the control (0 $\mu\text{g/ml}$) which was 4.26×10^7 spores/ml. The most inhibition was the concentration 1,000 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ which was 51.27% and 45.55% respectively and followed by the concentration 100, 50 and 10 $\mu\text{g/ml}$ which were 29.34%, 21.83% and 20.54% respectively. The ED_{50} value was 624.2459 $\mu\text{g/ml}$. Crude methanol at the concentration 10, 50, 100, 500 and 1,000 $\mu\text{g/ml}$ which were 3.9, 3.72, 2.64, 2.49 and 1.69×10^7 spores/ml respectively, gave significantly different when compared to the control (0 $\mu\text{g/ml}$) which was 4.07×10^7 spores/ml. The most inhibition was the concentration 1,000 $\mu\text{g/ml}$ which was 58.65%, followed by the concentration 500 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ which were 38.72% and 35.03% respectively. The ED_{50} value was 879.0221 $\mu\text{g/ml}$ (Table 4.5 and 4.6, Fig.4.20, 4.21 and 4.22).

Table 4.5. Crude extracts of *Chaetomium cupreum* testing for inhibit *Colletotrichum* sp.

Crude extracts	Concentration ($\mu\text{g/ml}$)	Colony diameter (cm) ¹	Growth inhibition (%) ²
Hexane	0	5.00a	0.00d
	10	4.21b	15.75c
	50	4.15b	16.50c
	100	3.46c	31.25b
	500	2.85d	38.50b
	1,000	2.73d	40.50b
	Ethyl acetate	0	5.00a
10		4.21b	15.75c
50		4.17b	15.75c
100		4.08b	18.00c
500		3.16cd	36.75b
1,000		1.64e	67.25a
Methanol		0	5.00a
	10	4.30b	13.25c
	50	4.04b	19.25c
	100	3.01cd	39.75b
	500	3.34c	33.10b
	1,000	2.86d	42.75b
	CV%	6.26	25.32

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

² Inhibition (%) = $\frac{R1-R2}{R1} \times 100$ where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

Table 4.6. Crude extracts of *Chaetomium cupreum* testing for inhibit spore production of *Colletotrichum* sp.

Crude extracts	Concentration ($\mu\text{g/ml}$)	Number of spores ($\times 10^7$ spores/ml)	Spore inhibition (%) ²	ED ₅₀ ($\mu\text{g/ml}$)
Hexane	0	4.27a ¹	0.00g	794.8441
	10	3.38bcde	20.96def	
	50	3.17cdef	25.14cde	
	100	3.11def	26.38cd	
	500	2.37gh	44.45ab	
	1,000	2.05gh	51.35ab	
Ethyl acetate	0	4.26a	0.00g	624.2459
	10	3.38bcde	20.53def	
	50	3.33cdef	21.83cde	
	100	3.01def	29.34cde	
	500	2.31gh	45.55ab	
	1,000	2.05gh	51.27ab	
Methanol	0	4.07ab	0.00g	879.0221
	10	3.9abc	4.19fg	
	50	3.72abcd	8.56efg	
	100	2.64egf	35.03bcd	
	500	2.49fg	38.72bc	
	1,000	1.69h	58.65a	
	CV%	11.59	31.31	

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

² Inhibition (%) = $\frac{R1-R2}{R1} \times 100$ where R1 was spore number of pathogen in control and R2 was spore number of pathogen in treated plates.

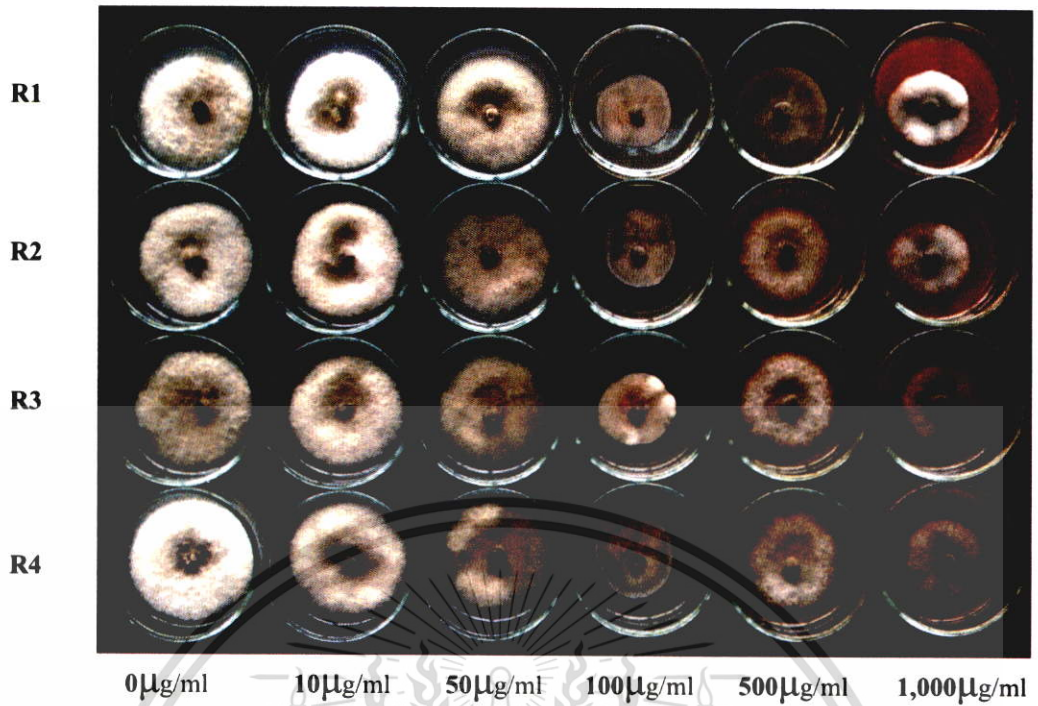


Fig. 4.20. Colony of *Colletotrichum* sp. treated hexane from *Chaetomium cupreum*

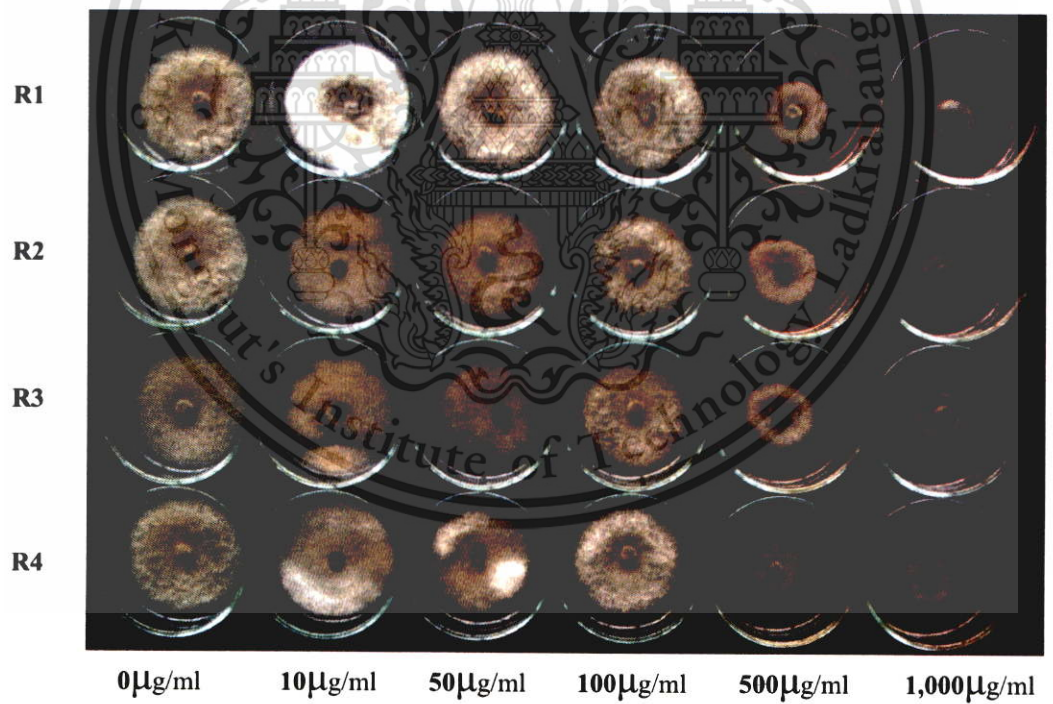


Fig. 4.21. Colony of *Colletotrichum* sp. treated ethyl acetate from *Chaetomium cupreum*

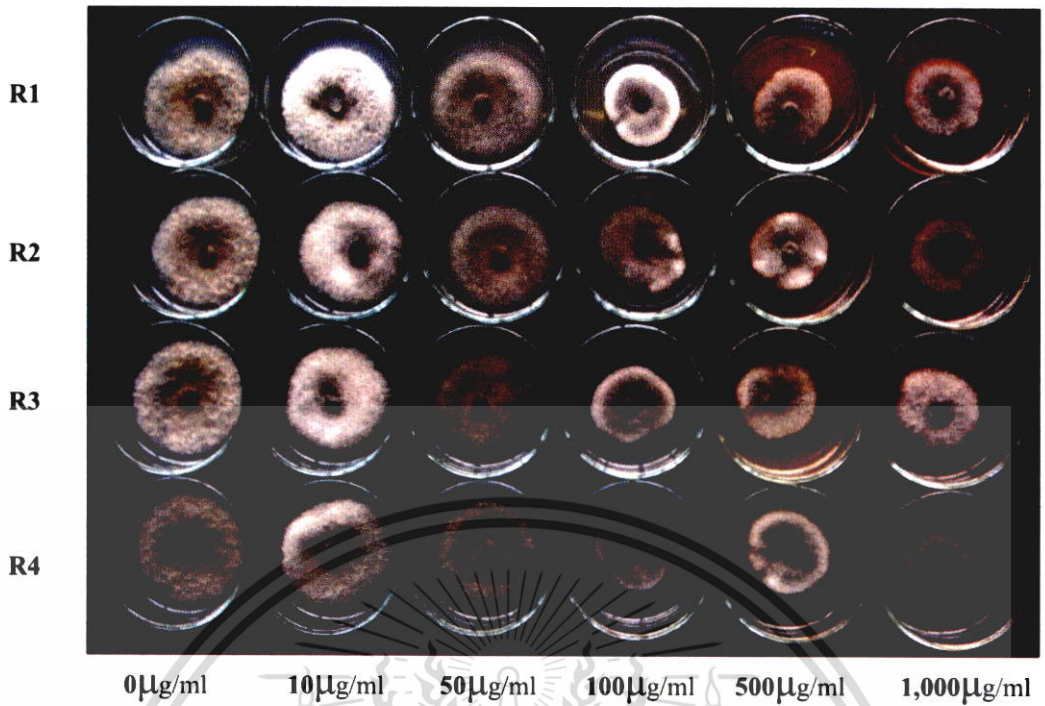


Fig. 4.22. Colony of *Colletotrichum* sp. treated by methanol from *Chaetomium cupreum*

4.5 Testing nano products from effective endophytic antagonist to control orchid anthracnose *in vivo*

Nano products from two endophytic fungi, *Chaetomium cochliodes* and *Chaetomium cupreum* were done with the method of Dar and Soyong. (2014) (Fig. 4.23).

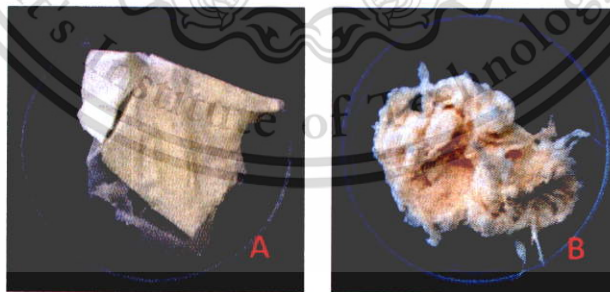


Fig. 4.23. Nano particles A: *Chaetomium cochliodes* and B: *Chaetomium cupreum*

Result showed that application of nano-products from *Chaetomium cochliodes* and *Chaetomium cupreum* and carbendazim 98% gave non-significantly differed when compared to the inoculated with *Colletotrichum* sp. causing leaf anthracnose of *Grammatophyllum specinocum* at 10 days of inoculation (Table 4.7, Fig. 4.24).

After 20 days of inoculation, it showed that carbendazim treatment was significantly lower disease severity index (2.75) than nano-product of *Chaetomium cochliodes* (3.00) and *Chaetomium cupreum* (3.5) treatment when compared with inoculated with pathogen treatment. Disease severity index (DSI) in nano-products of *Chaetomium cochliodes* and *Chaetomium cupreum* (4.25) and carbendazim treatment (3.75) were significantly lower DSI than the inoculated one (6.00) at 30 days after inoculation.

DSI at 40 days found that nano-products of *Chaetomium cochliodes* and *Chaetomium cupreum* (5.75) were significantly lower than the inoculated one but carbendazim treatment was significantly lower DSI (4.50) than all treatments.

DSI at 50 days, it was found that DSI of carbendazim treatment (3.25) significantly lower than nano-product of *Chaetomium cochliodes* and *Chaetomium cupreum* (5.75) when compared to the inoculated with pathogen (9.00).

Result showed clearly significant different in DSI at 60 days which carbendazim treatment gave the best result to control leaf anthracnose of *Grammatophyllum specinocum* (DSI = 2.75) and followed by nano-product of *Chaetomium cochliodes* (DSI = 5.50) and nano-product of *Chaetomium cupreum* (DSI = 5.50) respectively when compared to the inoculated with pathogen (DSI = 9.00) (Table 4.7).

It revealed that all treatments of nano-products and carbendazim reduced anthracnose disease of *Grammatophyllum specinocum*. Anthracnose reduction was significantly higher in carbendazim (37.38%) which higher than nano-product of *Chaetomium cochliodes* (28.22%) and nano-product of *Chaetomium cupreum* (27.98%) at 30 days. Disease reduction at 40 days revealed that all treatments were not significantly differed. Disease reduction at 50 days showed

that carbendazim treatment was significantly higher reduced disease (58.33%) than nano-product of *Chaetomium cochliodes* (33.33%) and nano-product of *Chaetomium cupreum* (36.11%) respectively.

Disease reduction at 60 days were clearly demonstrated that carbendazim treatment was significantly highest disease reduction (69.45%) and followed by nano-products of *Chaetomium cochliodes* and nano-product of *Chaetomium cupreum* (38.89%) respectively (Table 4.8).

Table 4.7: Disease Severity Index of anthracnose on the leaves of *Grammatophyllum specinocum*

Treatments	DIS ¹					
	10 days	20 days	30 days	40 days	50 days	60 days
T1 (non inoculated)	1.00b ²	1.00b	1.00c	1.00c	1.00d	1.00cd
T2 (Inoculated pathogen)	2.00a	3.50a	6.00a	7.50a	9.00a	9.00a
T3 (Nano-product of <i>Ch. cochliodes</i>)	2.00a	3.00a	4.25b	5.75ab	6.00b	5.50b
T4 (Nano-product of <i>Ch. cupreum</i>)	2.00a	3.50a	4.25b	5.75ab	5.75b	5.50b
T5 (Carbendazim 98%)	1.75a	2.75c	3.75b	4.50b	3.25c	2.75c
C.V. %	13.99	18.25	18.21	23.55	14.95	21.59

¹Disease Severity Index (DSI): 1 =Healthy, no disease symptoms of the leaves, 2 =The length of lesion 1 – 10%, 3 = The length of lesion 11 – 20%, 4 = The length of lesion 21 – 30%, 5 = The length of lesion 31 – 40%, 6 = The length of lesion 41 – 50%, 7 = The length of lesion 51 – 60%, 8 = The length of lesion 61 -70% and 9 = The length of lesion more than 71%

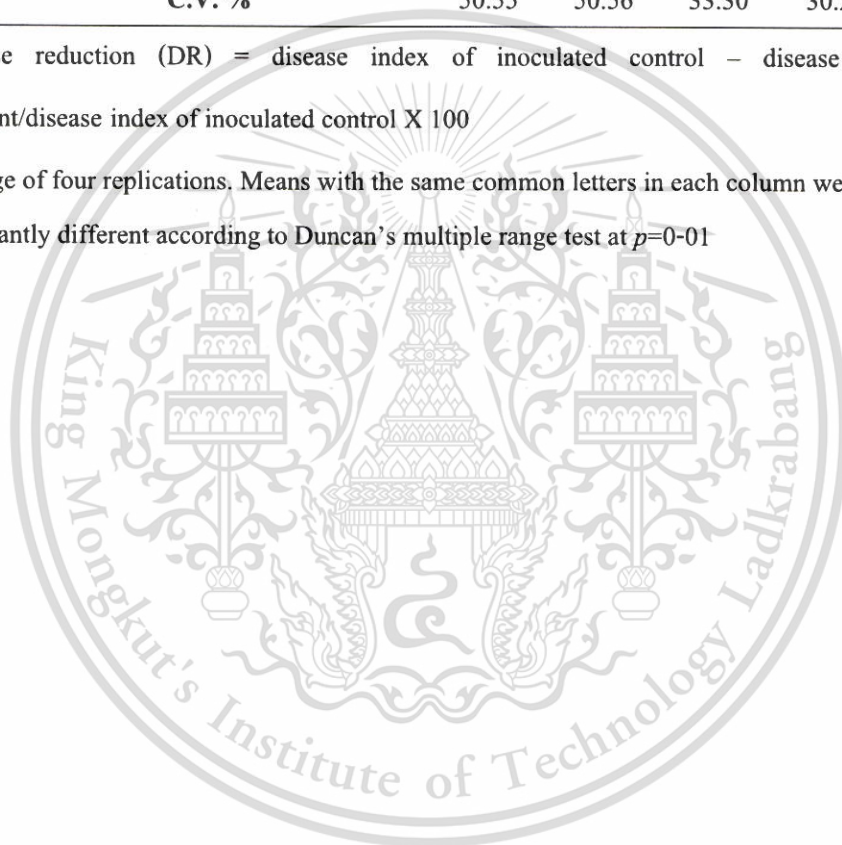
²Average of four replications. Means with the same common letters in each column were not significantly different according to Duncan's multiple range test at $p=0-01$

Table 4.8 Disease reduction after treatments

Treatments	DR ¹ (%)			
	30 days	40 days	50 days	60 days
Nano-product of <i>Ch. cochliodes</i>	28.22a ²	22.99a	33.33b	38.89b
Nano-product of <i>Ch. cupreum</i>	27.98a	23.36a	36.11ab	38.89b
Carbendazim 98%	37.38a	37.70a	58.33a	69.45a
C.V. %	50.55	50.56	33.30	30.29

¹Disease reduction (DR) = $\frac{\text{disease index of inoculated control} - \text{disease index of treatment}}{\text{disease index of inoculated control}} \times 100$

²Average of four replications. Means with the same common letters in each column were not significantly different according to Duncan's multiple range test at $p=0.01$



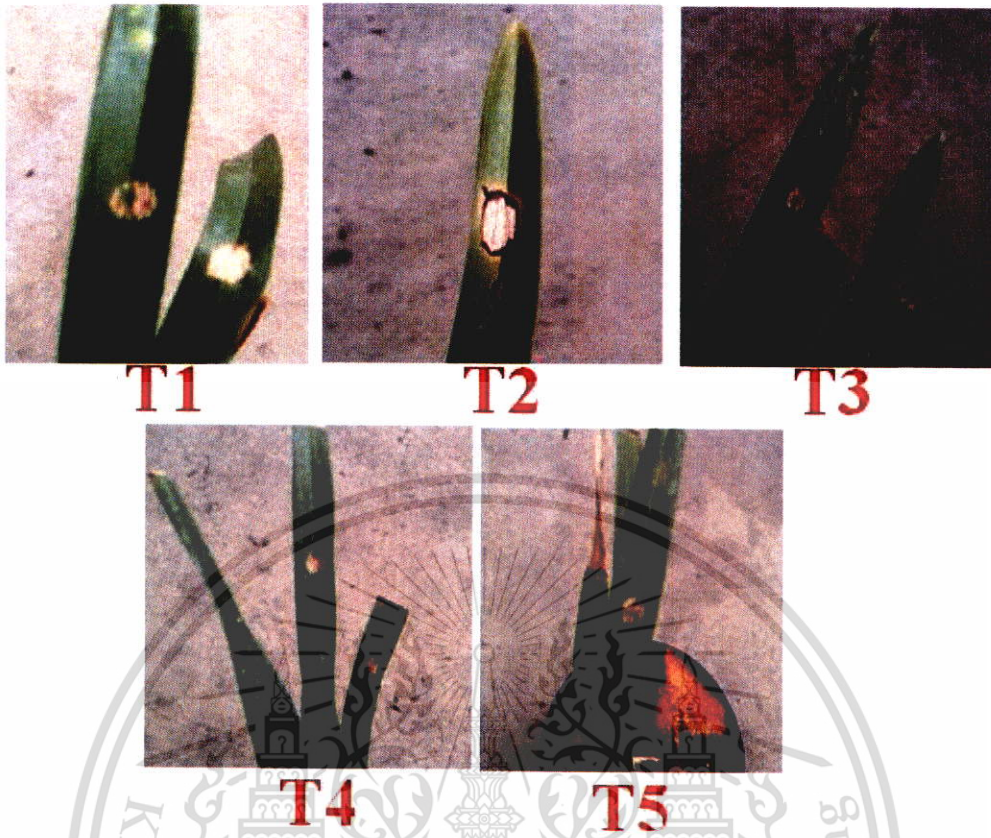


Fig. 4.24. Anthracnose lesions on the leaves of *Grammatophyllum specinocum* on 10th day after inoculated the pathogen

T1 Negative control (non inoculated)

T2 Positive control (inoculated pathogen)

T3 Pathogen + nano product of *Chaetomium cochliodes*

T4 Pathogen + nano product of *Chaetomium cupreum*

T5 Carbendazim 98%

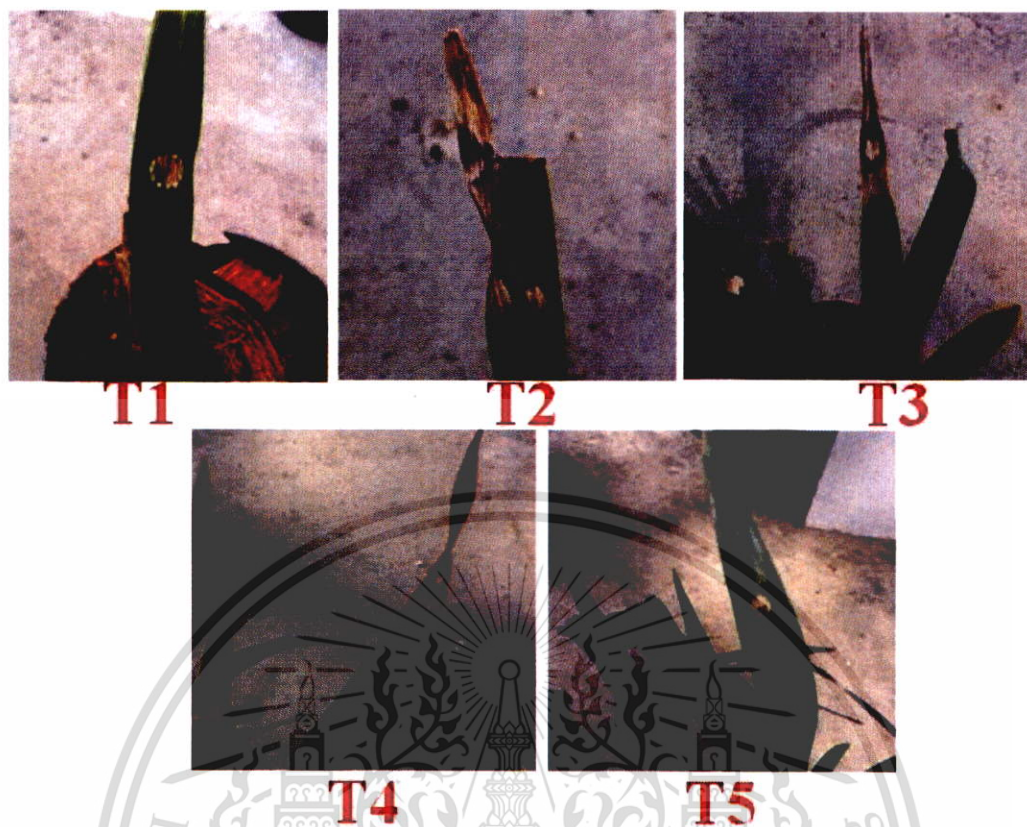


Fig. 4.25. Anthracnose lesions on the leaves of *Grammatophyllum specinocum* on 20th day after inoculated the pathogen

T1 Negative control (non inoculated)

T2 Positive control (inoculated pathogen)

T3 Pathogen + nano product of *Chaetomium cochliodes*

T4 Pathogen + nano product of *Chaetomium cupreum*

T5 Carbendazim 98%

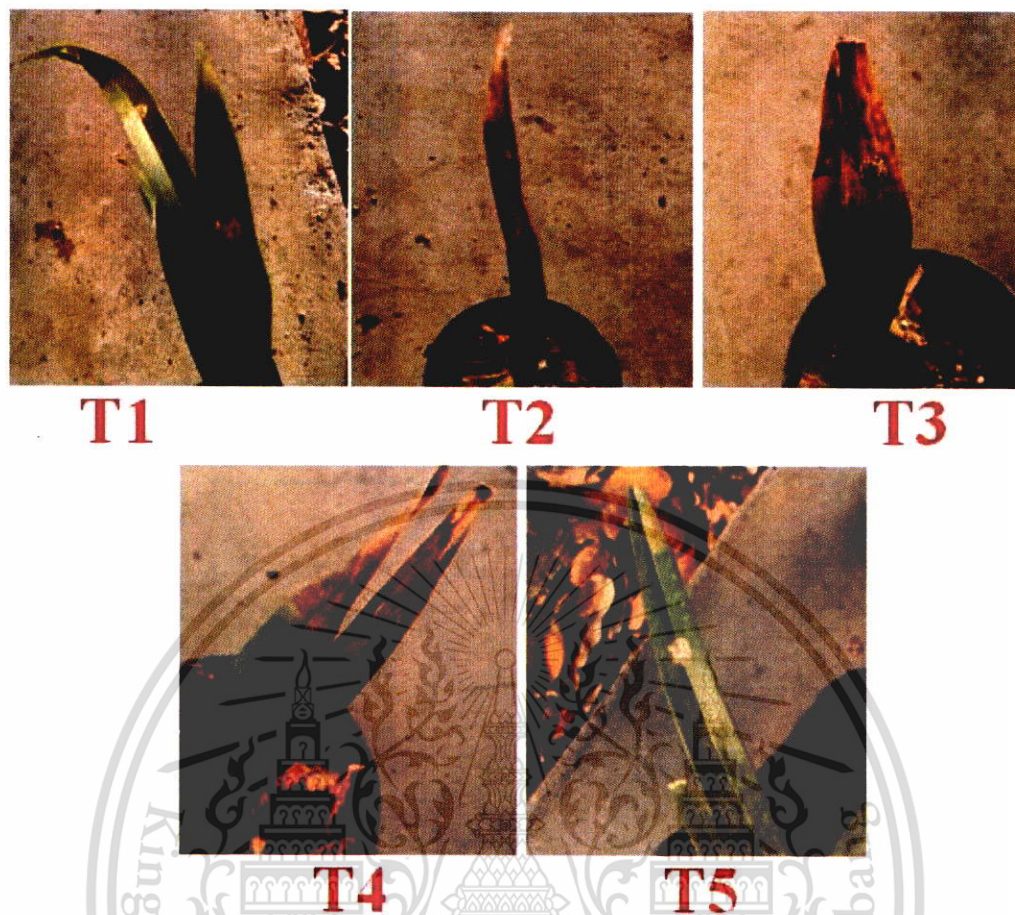


Fig. 4.26. Anthracnose lesions on the leaves of *Grammatophyllum specinocum* on 30th day after inoculated the pathogen

T1 Negative control (non inoculated)

T2 Positive control (inoculated pathogen)

T3 Pathogen + nano product of *Chaetomium cochliodes*

T4 Pathogen + nano product of *Chaetomium cupreum*

T5 Carbendazim 98%

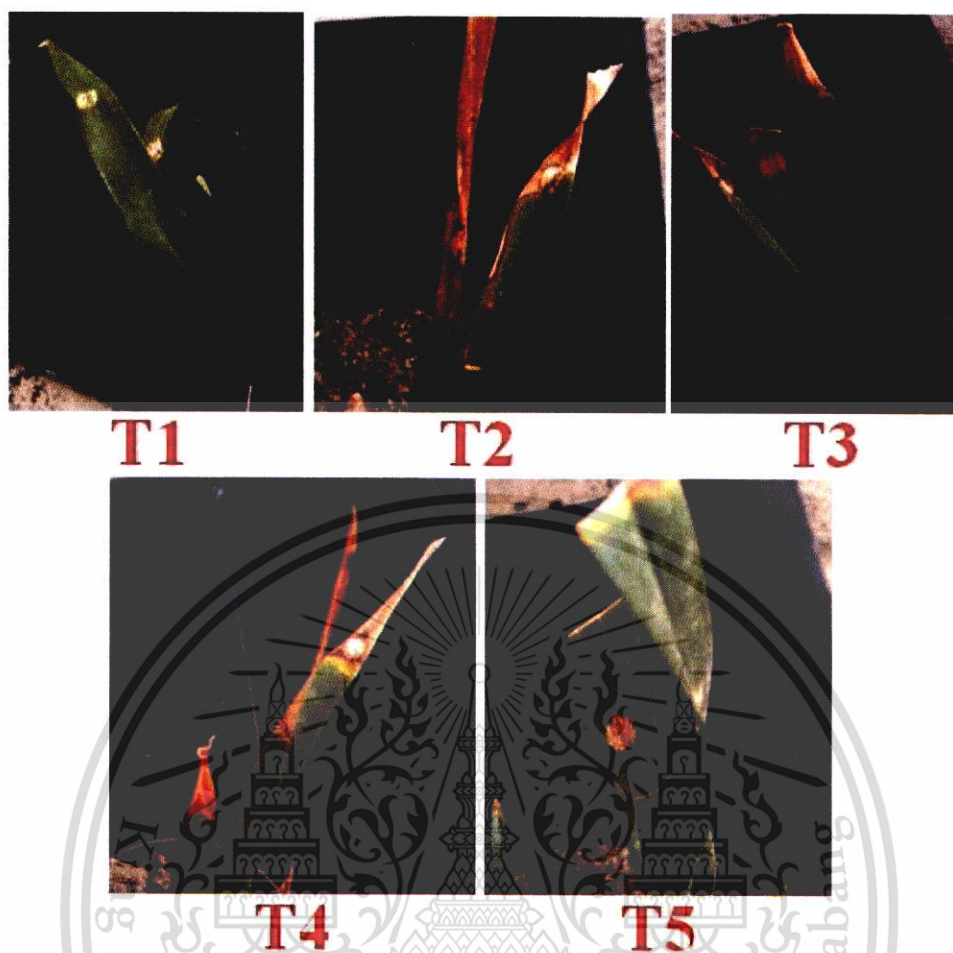


Fig. 4.27. Anthracnose lesions on the leaves of *Grammatophyllum specinocum* on 40th day after inoculated the pathogen

T1 Negative control (non inoculated)

T2 Positive control (inoculated pathogen)

T3 Pathogen + nano product of *Chaetomium cochliodes*

T4 Pathogen + nano product of *Chaetomium cupreum*

T5 Carbendazim 98%

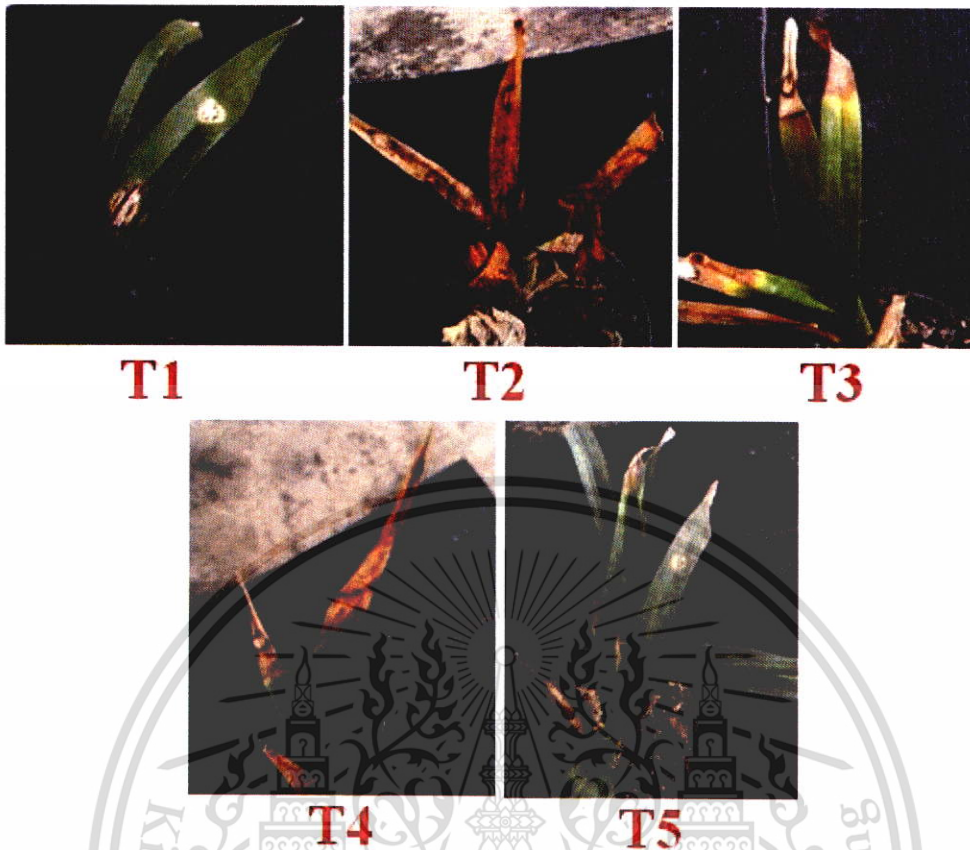


Fig. 4.28. Anthracnose lesions on the leaves of *Grammatophyllum specinocum* on 50th day after inoculated the pathogen

T1 Negative control (non inoculated)

T2 Positive control (inoculated pathogen)

T3 Pathogen + nano product of *Chaetomium cochliodes*

T4 Pathogen + nano product of *Chaetomium cupreum*

T5 Carbendazim 98%

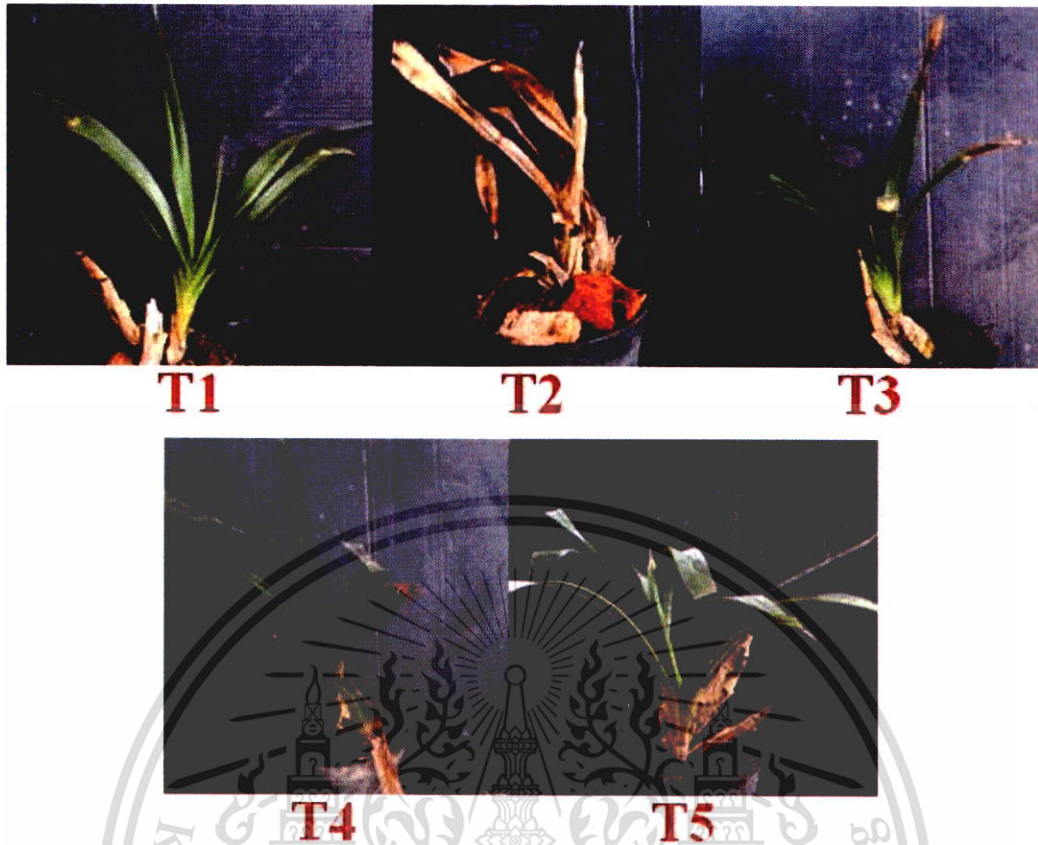


Fig. 4.29. Anthracnose lesions on the leaves of *Grammatophyllum specinocum* on 60th day after inoculated the pathogen

T1 Negative control (non inoculated)

T2 Positive control (inoculated pathogen)

T3 Pathogen + nano product of *Chaetomium cochliodes*

T4 Pathogen + nano product of *Chaetomium cupreum*

T5 Carbendazim 98%

Chapter V

Discussion

Eleven species of endophytic fungi were isolated from 9 orchid species. *Cymbidium dayanum* was most dominant of endophytic fungi which was 7 species and followed by *Gammatophyllum scriptum*, *Dendrobium hercoglossum*, *Dendrobium fimbriaetum*, *Doritis pulcherima*, *Dendrobium friedericksianum*, *Grammatophyllum specinocum*, *Dendrobium crumenatum* and *Dendrobium palpebrae*. *Xylaria* sp. was the most wide host range and followed by *Colletotrichum* sp., *Fusarium* sp., *Chaetomium cochliodes*, *Pestalotiopsis* sp., *Phoma* sp., *Curvularia* sp., *Chaetomium cupreum*, *Achaetomium* sp., *Nigrospora* sp. and *Corynascus* sp.

Xylaria sp. found to be endophytic fungus which isolated from other orchid species. Chen *et al.* (2013) isolated this species from 7 species of *Dendrobium* in China, it also isolated from *Bulbophyllum neilgherrense* and *Pholidota pallida* (Sawmya *et al.* 2013). *Colletotrichum* sp. was the fungus which mostly isolated from orchid. Behera *et al.* (2013) isolated this species from 3 orchid species, *Acampe praemorsa*, *Cymbidium aloifolium* and *Vanda testacea* at India. Previous study showed that the endophytic fungus, *Colletotrichum gloeosporioides* was isolated from *Justicia gendarussa*, a medicinal plant, had ability to produce taxin that was a potent anticancer drug used widely in the treatment of a variety of cancers (Gangadevi, and Muthumary, 2008). *Fusarium* sp. was a fungus that mostly isolated from orchids. Sufaati *et al.* (2016) isolated *Fusarium solani* from 3 orchid species, *Phaius tankervilleae*, *Dendrobium lancifolium* and *Calanthe triplicate* in Indonesia. *Fusarium redolens* isolated from Himalayan yew can produce taxol, the anticancer drug (Garyali, 2013). Sogra and Raj.(2014) isolated *Fusarium oxysporum* from leaf of *Nothapodytes foetida*, ethyl acetate extract from this fungus show ability to inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. *Chaetomium cochliodes* was the common fungus that can isolate from soil, but in this study it was isolated as an endophytic fungus. It was not the strange because other study was indicated that this species was endophytic fungi. Gange *et al.* (2012) isolated this species from *Cirsium arvense*. Li *et al.* (2006) indicated that epipolythiodioxopi-perazines and chaetocochin was distilled from the ethyl acetate extract of the solid-state fermented rice culture of the fungus *Chaetomium cochliodes*. Their structures were elucidated on the basis of spectroscopic analysis. Compounds 1, 3, and 4 exhibited significant cytotoxicity in vitro against cancer cell lines Bre-04,

Lu-04, and N-04. *Pestalotiopsis* sp. is known as a pathogen that caused many diseases in plants, but it also an endophytic fungus familiar with orchid. Tempesta *et al.* (2011) isolated this fungus from leaves of *C. mrcranthum* and *O. adendrobium* and found 29 strains of *Pestalotiopsis* sp. Wei *et al.* (2007) isolated this species that associated with plants of Podocarpaceae, Theaceae and Taxaceae in southern China also obtained 24 species. Kumaran *et al.* (2010) isolated *Pestalotiopsis versicolor* and *Pestalotiopsis neglecta* as endophytic fungi from Japanese Yew tree, *Taxus cuspidate*, and indicated that they can produce taxol concentration induces increased cell death in cancer. *Phoma* sp. was endophytic fungi that can isolate from many plants. Wang *et al.* (2012) isolated *phoma* sp. from chinese medicinal plant, *Arisaema erubescens*, and showed that the bioactive metabolites from this species can inhibited the growth of four plant pathogenic fungi (*Fusarium oxysporium*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, and *Magnaporthe oryzae*). This species also isolated from roots of cucumber in Korea and showed the ability to produce Gibberellins and Indoleacetic Acid and Promotes host-plant growth during stress (Waqas *et al.*, 2012). Zakaria *et al.* (2010) isolated *Curvularia* sp. species from *Oryza sativa*. Aharwal *et al.* (2014) also isolated this fungus from *Calotropis procera* (Linn.) R.Br., a widely used medicinal plant in India. It is noticed that *Chaetomium cupreum* as a fungus that have ability to inhibit many pathogens. This species was isolated as endophytic fungus from *Macleaya cordata* and have ability to antifungal activity against *Rhizoctonia solani*, *Botrytis cinerea*, *Pythium ultimum*, and antitumor activities against HL-60 and A549. It is toxic to *Artemia salina* (Bi *et al.* 2010). *Nigrospora* sp. isolated from deep sea water at Arabian Sea and The secondary metabolites produced from this organism showed potent antimicrobial and anticancer activities with immediate application to cosmetics and pharmaceutical industries. (Arumugam *et al.* 2015).

Three endophytic fungi, *Achaetomium* sp., *Chaetomium cochliodes* and *Chaetomium cupreum* were tested to inhibit the growth of *Colletotrichum* sp. causing anthracnose in orchids by bi-culture antagonistic test. The colony growth inhibition showed that all the antagonists gave significantly different from the control which was 9cm. *Chaetomium cochliodes* was 5.67cm showed the best ability for inhibition (36.94%) when compared to the control and followed by *Chaetomium curpreum* and *Achaetomium* sp. which was 6.31cm or 29.85% and 6.55cm or 27.21% respectively. The spore productions showed that all the antagonists gave significantly different when compared to the control 55.12x10⁶ spores/ml. *Chaetomium cupreum* showed the best ability to inhibit the spore production of pathogen which was 24.25x10⁶ spores/ml or 54.39%,

and followed by *Chaetomium cochliodes* and *Achaetomium* sp. which were 29.12×10^6 spores/ml and 39.25×10^6 spores/ml respectively and inhibited as 47.2% and 28.81% respectively. As result showed that all endophytic fungi had ability to inhibit growth of pathogen both colony and spore productions. Nguyen *et al.* (2014) reported that *Chaetomium cochliodes* showed abilities to inhibit growth of *Fusarium roseum* caused leaf anthracnose of coffee. This species also showed abilities to inhibit growth of *Drechslera oryzae* causing leaf spot of rice (*Oryza sativa*) (Samawit *et al.* 2012). There are some previous studies about *Chaetomium cupreum* for biocontrol. Nguyen *et al.* (2014) indicated that this species had abilities to inhibit the growth of *Pestalotia* sp. caused leaf spot in tea by bi-culture tested and it had abilities to control *Fusarium oxysporum* causing tomato wilt (Sibounnavong *et al.* 2012).

Crude extract from *Chaetomium cochliodes* showed good ability to inhibit the colony growth of *Colletotrichum* sp. This study similar to Samawit *et al.* (2012) was indicated that crude hexane; crude ethyl acetate and crude methanol inhibited the spore production of *Drechslera oryzae* which caused leaf spot of rice which was 86.11%, 85.84% and 74.92% respectively. The crude extract of *Chaetomium cochliodes* can inhibit *Colletotrichum* sp. confirmed to Nguyen *et al.* (2014) who reported that crude extract of hexane, ethyl acetate and methanol at the concentration 1,000 μ g/ml can inhibit the spore productions of *Fusarium roseum* which causing wilt of coffee which were 60.87%, 78.16% and 74.57% respectively. Soyong (2014) reported that crude hexane, ethyl acetate and methanol from *Chaetomium cochliodes* at the concentrations 50 μ g/ml can inhibited the spore productions of *Drechslera oryzae* causing brown leaf spot in rice which were 54.99%, 63.14% and 48.96% respectively.

All the crude extracts from *Chaetomium cupreum* showed the ability to inhibit the growth of *Colletotrichum* sp. both colony growth and spore production. This study similar to Samawit *et al.* (2012) who indicated that crude hexane; crude ethyl acetate and crude methanol from *Chaetomium cupreum* inhibited the spore production of *Drechslera oryzae* which caused leaf spot of rice which was 76.30%, 72.23% and 71.40% respectively. Sibounnavong *et al.* (2012) reported that 3 crude extract, hexane, ethyl acetate and methanol, of *Chaetomium cupreum* showed abilities to inhibit spore production of *Fusarium oxysporum* F.sp. *lycopersici* which was 91.92%, 93.75% and 97.37% respectively.

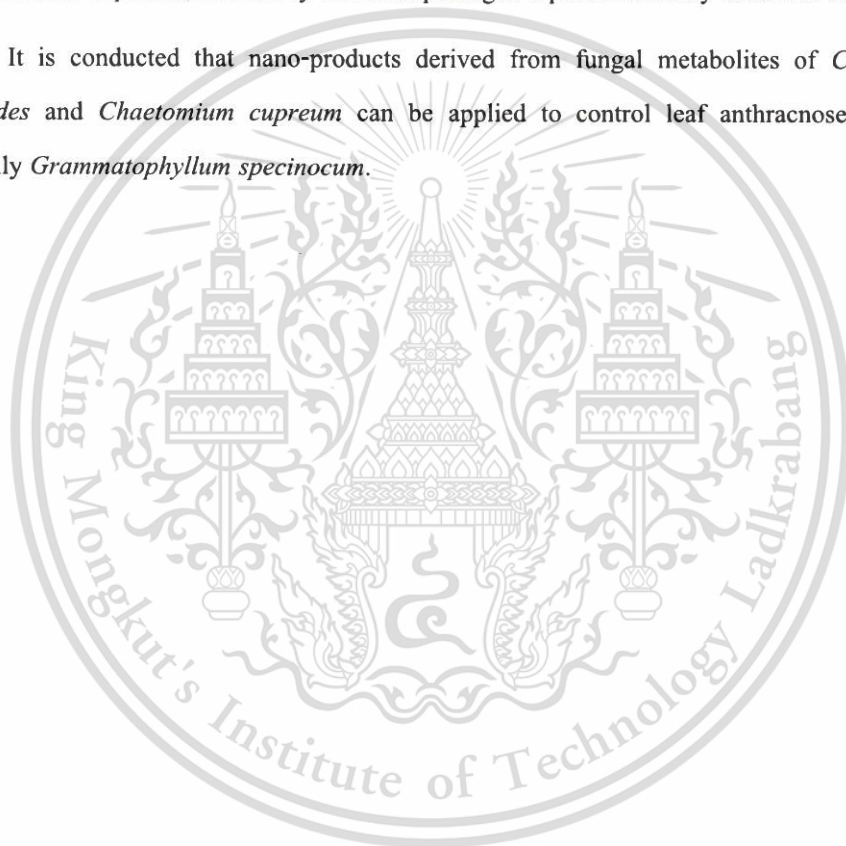
The research finding found that two effective endophytic fungi from orchid, *Chaetomium cupreum* and *Chaetomium cochliodes* inhibited *Colletotrichum* sp. causing anthracnose of *Grammatophyllum specinocum* which similar to Noiaium and Soyong (2000) reported that *Chaetomium cupreum* can be inhibited *Colletotrichum gloeosporiodes* causing anthracnose of mango.

Crude methanol from *Chaetomium cochliodes* expressed antifungal activity against *Colletotrichum* sp. causing leaf anthracnose of orchid which the ED₅₀ value of 712µg/ml. Crude hexane, crude ethyl acetate and crude methanol from *Chaetomium cupreum* exhibit antifungal against leaf anthracnose of orchid at ED₅₀ values of 794, 624 and 879µg/ml, respectively. Similar results and also reported by Soyong *et al.* (2001).

Application of nano-products formulated from crude extracts of *Chaetomium cochliodes* and *Chaetomium cupreum* to control leaf anthracnose of orchid were investigated and give a good result. Nano-products from *Chaetomium* sp. could reduce anthracnose disease of *Grammatophyllum specinocum* as compared to carbendazim chemical fungicide. As result, Dar and Soyong. (2014) reported that nano-particles from *Chaetomium* sp. could give a good control of *Fusarium oxysporum* f. sp. *lycopersici* and *Colletotrichum capsici* causing chilli anthracnose. This is because of these endophytic fungi may produced antibiotic substance as Waksman and Bugie. (1944) found that *Chaetomium cochliodes* produced a biotic substance active principally against gram-positive bacteria. Three new epipolythiodioxo-piperazines, chaetocochins A (1), B (2), and C (3), along with dethio-tetra (methylthio) chetomin (4) and chetomin (5) which were isolated from the ethyl acetate extract of the solid-state fermented rice culture of the fungus *Chaetomium cochliodes*. Their structures were also elucidated on the basis of spectroscopic analysis. Compounds 1, 3, and 4 exhibited significant cytotoxicity in vitro against cancer cell lines Bre-04, Lu-04, and N-04 (Li *et al.* 2006). Meiler and Taylor. (1971) reported that cochliodinol, an antibiotic substance from *Chaetomium cochliodes* against *Fusarium oxysporum*. The result showed that cochliodinol at 15µg/ml reduced the respiration rate of microspores of *Fusarium oxysporum*, with glucose or succinate as substrate, by about 70%, and also inhibited their germination. The inhibitory effect was partially reversed by the addition of magnesium ions to the medium. *Chaetomium cupreum* also had many antibiotic substances used as metabolite against pathogens. Kanokmedhakul *et al.* (2006) reported that 3 new azaphilones name rotiorionals A-C (1-3), two new stereoisomers, (-) rotiorin (4) and epi-siochromophilone II (5) and

a known compound, rubrorotiorin (6), were isolated from the fungus *Chaetomium cupreum*. Structures were established on the basis of spectroscopic evidence. Compounds 1, 3, 4 and 6 exhibited antifungal activity against *Candida albicans*. Panthama *et al.* (2014) reported that 2 new angular types of azaphilones, isochromophinal and ochrephilonol isolated from *Chaetomium cupreum* showed moderated cytotoxicity against KB and NCI-H187 cell lines. Mao *et al.* (2010) indicated that oosporein from *Chaetomium cupreum* had antifungal activity against *Rhizoctonia solani*, *Botrytis cinerea*, *Pythium ultimum*, and antitumor activities against HL-60 and A549. It is toxic to *Artemia salina*. As all reference showed that 2 endophytic fungi, *Chaetomium cochliodes* and *Chaetomium cupreum*, had ability to inhibit pathogen it produced many antibiotic substances.

It is conducted that nano-products derived from fungal metabolites of *Chaetomium cochliodes* and *Chaetomium cupreum* can be applied to control leaf anthracnose of orchid especially *Grammatophyllum specinocum*.



Chapter VI

Conclusion

Eleven endophytic fungi was isolated from 9 orchid varieties, *Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium hercoglossum*, *Dendrobium palpebrae*, *Dendrobium fimbriatum*, *Doritis pulcherrima*, *Dendrobium crumenatum*, *Dendrobium friedericksianum* and *Grammatophyllum specinocum*. They were identified as *Chaetomium cochliodes*, *Chaetomium cupreum*, *Xylaria* sp., *Colletotrichum* sp., *Fusarium* sp., *Curvularia* sp., *Achaetomium* sp., *Phoma* sp., *Nigrospora* sp., *Pestalotiopsis* sp., and *Corynascus* sp.

Colletotrichum sp. is found to be caused anthracnose in orchids was isolated from leaf of *Grammatophyllum specinocum*.

Bi-culture test showed that all antagonists showed ability to inhibit both colony growth and spore productions of *Colletotrichum* sp. significantly different when compared to control. Colony inhibition showed that *Chaetomium cochliodes* was the most inhibition and followed by *Chaetomium cupreum* and *Achaetomium* sp. Spores inhibition showed that *Chaetomium cupreum* gave the best spore inhibition of pathogen and followed by *Chaetomium cochliodes* and *Achaetomium* sp.

Two species of endophytic fungi, *Chaetomium cochliodes* and *Chaetomium cupreum* showed best results to inhibit *Colletotrichum* sp. causing leaf anthracnose of orchid. Crude extract from *Chaetomium cochliodes* showed ability to inhibit *Colletotrichum* sp. when compared to control.

The research finding was reported that two effective endophytic fungi from orchids could release antifungal metabolites to inhibit *Colletotrichum* sp. causing leaf anthracnose of orchid (*Grammatophyllum specinocum*). The developed antifungal metabolites to be nano-products can be controlled leaf anthracnose of orchid.

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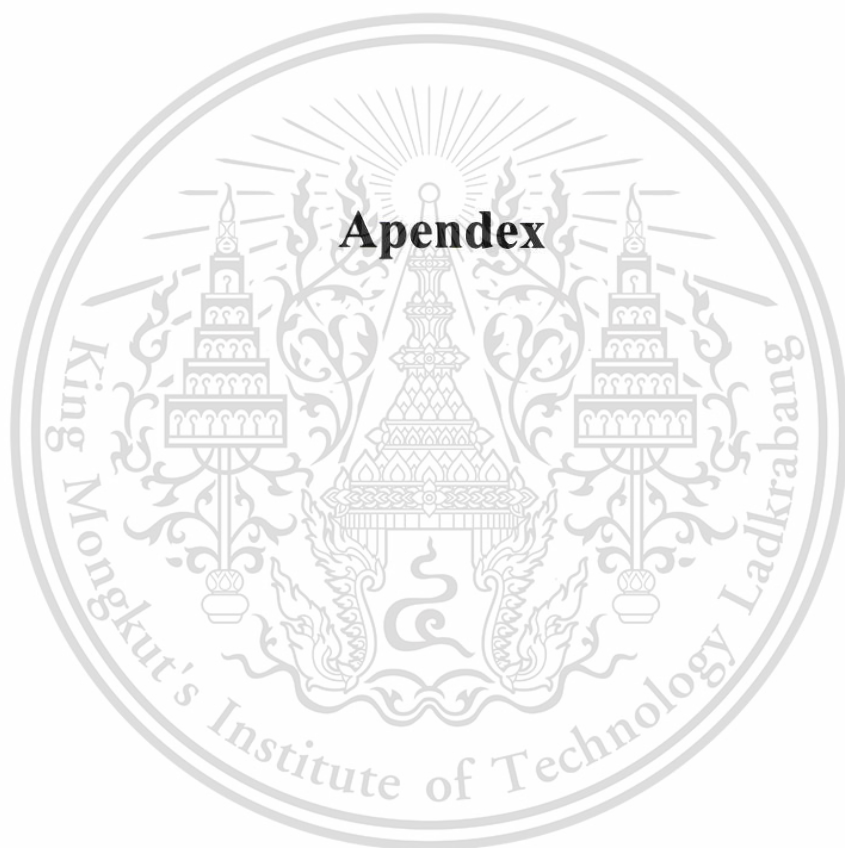
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Published Paper

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Vannak, S., Sarayut P. and Soyong K. 2015. "Antifungal activities of endophytic fungi isolated from orchids against *Colletotrichum* sp. caused anthracnose in orchids". **Journal of Agricultural Technology**. 11(8):1949-1961.



Isolation of endophytic fungi from some orchid varieties

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Sour V., Phonpho S and Soyong K. (2015) Isolation of endophytic fungi from some orchid varieties. Journal of Agricultural Technology 11(5):1243-1254

Endophytic fungi from orchids in Chantaburi province was studied. Endophytic fungi were isolated from 9 orchid varieties in greenhouse condition. Leaves and roots of *Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium hercoglossum*, *Dendrobium palpebrae*, *Torenia fournieri*, *Doritis pulcherrima*, *Dendrobium crumenatum*, *Dendrobium friedericksianum* and *Grammatophyllum specinocum* were collected. All samples were collected then brought to laboratory. Isolation was done by surface sterilization, removed the outer surface and got inner tissues then place on water agar and transferred culture to potato dextrose agar to be pure culture. The morphological identification based on colony and agar color, liquid drops, colony shapes, the growth rate and spores characteristic Twelve species are identified as *Chaetomium cochliodes*, *Chaetomium cupreum*, *Xylaria* sp., *Colletotrichum* sp., *Nigrospora* sp., *Phoma* sp., *Curvularia* sp., *Fusarium* sp., *Pestalotiopsis* sp., *Corynascus* sp. and *Cladosporium*-like fungi.

Keywords: Endophytic fungi, Orchids

Introduction

Plants are always colonized with other microorganisms. Endophytic fungi are one kind of microorganism that live within healthy plant tissue causing no harm to the host plant. The meaning of endophytic fungi is so broad. Modern usage of the term endophytic fungi in mycology refers to those fungi which live within leaves stems roots and other part of apparently healthy host plants and can not be seen in visually signs of infection (Stone J.K. *et al.*, 2004). Dreyfuss and Chapela (1994) estimated that there may be at least one million species of endophytic fungi alone. It means that almost all plant species are usually infected with endophytic fungi.

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Nowaday endophytic fungi take attention from many scientists as estimated that such species may useful as sources of anticancer, antidiabetic, insecticidal and immunosuppressive compounds (Strobel, G. and Daisy, B., 2003). Endophytic fungi from medicinal plants can be used for the development of drugs. For example *Fusarium oxysporum* isolated from *Catharanthus roseus* plant can produce Vinblastine and Vincristine, excellent anti-cancer drugs (Kumar A. *et al*, 2013).

Orchids are monocotyledonous plants and taxonomically belong to Orchidales and Orchidaceae. There are 15000 to 25000 of orchid species distributed all around the world, mostly can found in tropical environment. (Thamasiri K., 2005). Fungal endophytes have now been investigated in a large number of orchid species from around the world (Perkins *et al*. 1995). The fungi involved with orchids are almost all members of the phylum Basidiomycota group. The results may vary with the species of orchid.

Materials and methods

Plant sample collection

Roots and leaves of 9 orchid varieties (*Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium hercoglossum*, *Dendrobium palpebrae*, *Torenia fournieri*, *Doritis pulcherrima*, *Dendrobium crumenatum*, *Dendrobium friedricksianum* and *Grammatophyllum specinocum*). Sample were collected in greenhouse from Rajamangala University Technology Tawan-ok Chantaburi Campus, Chantaburi province, Thailand on 1st December 2014.

All samples were placed in sterile polythene bags and brought to laboratory in an icebox and stored at 4.5 °C before isolating endophytic fungi within 48 hours.

Isolation and identification of endophytic fungi

The surface sterilization was done by procedure used by Blumenstein (2010) with some modifications. Symptomless roots of each plant species were cut into 2–3 mm sections were rinsed in a sequence distilled water for 1 min, 75% ethanol for 30s, 3% NaClO for 1 min, and 75% ethanol for 30s and then rinsed in sterile distilled water three times. Each root was placed on water agar (WA). The isolates were subcultured on potato dextrose agar for morphological identification and conidial production.

The endophytic fungi were identified initially according to their colony, mycelium and spore characteristics according to the discription of Barnett and Hunter (1998) and Von Arx (1978).

Results and Discussion

Many isolates can't identify the species because it doesn't produce spores. Twelve species belonging to Ascomycota were isolated from 9 orchid varieties. They are as follow:

1. *Chaetomium cochliodes*

Habitat: *Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium hercoglossum* and *Torenia fournieri*.

The colonies are quit slowly growing, for cultured on PDA medium in room temperature for 7 days have 7 cm in diameter, cottony and white in color initially and then become soft-green. *C. cochliodes* has distinctive small brown 'lemon' or 'football' shaped ascospores. (Arx, J.A. von *et al.* 1986).

This species also isolated as endophytic fungi from *Cirsium arvense*, (Gange AC. Et al., 2012). Li Gy et al. 2006 indicated that epipolythiodioxopiperazines and chaetocochin were isolated from the ethyl acetate extract of the solid-state fermented rice culture of the fungus *Chaetomium cochliodes*. Their structures were elucidated on the basis of spectroscopic analysis. Compounds 1, 3, and 4 exhibited significant cytotoxicity in vitro against cancer cell lines Bre-04, Lu-04, and N-04.



Fig. 1: *Chaetomium cochliodes* A: The colony that culture on PDA 30 days, B The spores under microscops 10X C The ascospores under microscops 40X

2. *Chaetomium cupreum*

Habitat: *Grammatophyllum scriptum*.

The growth was quit slow, cultured on PDA medium for 7 days the growth have 4.4 cm in diameter. The hypheae are red and it produces the red color around mycelia.

Septate hyphae, perithecia, asci and ascospores are visualized. Perithecia are large, red color, fragile, globose to flask shaped and have filamentous, hair-like, brown to black appendages (setae) on their surface. Perithecia have ostioles (small rounded openings) and contain asci and ascospores inside (De Hoog *et al.*, 2000).

This species was isolated as endophytic fungus from *Macleaya cordata* and have ability to antifungal activity against *Rhizoctonia solani*, *Botrytis cinerea*, *Pytium ultimum*, and antitumor activities against HL-60 and A549. It is toxic to *Artemia salina* (Bi Z.M. *et al.*, 2010).

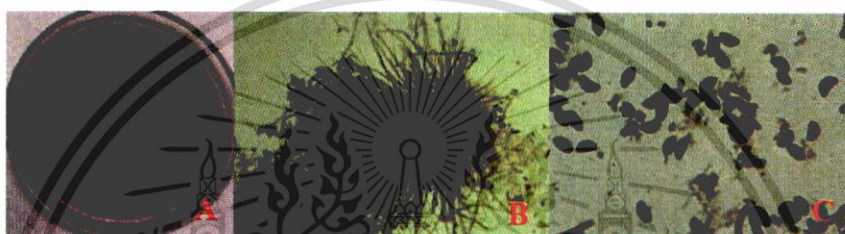


Fig. 2: *Chaetomium cupreum* A: The colony cultured on PDA 30 days B: The spores under microscops 10X and C: The ascospores under microscops 40X

3. *Xylaria* sp.

Habitate: *Cymbidium dayanum*, *Dendrobium hercoglossum*, *Doritis pulcherrima*, *Dendrobium crumenatum*, *Dendrobium friedericksianum* and *Grammatophyllum specinocum*.

The fungi have white mycelia; the growth was quit slowl, for cultured on PDA in room temperature for 7 days have 5.8 cm in diameter. They produce the stroma on 20th day but didn't produce ascus and ascospore.

Xylaria sp. was the common species that can isolated from other orchid varieties. Previous research also found that this species are most colonized in 7 *Dendrobium* sp. in China (Chen J. *et al.* 2013). Sawmya K. *et al.* (2013) also isolated *Xylaria* sp. from *Bulbophyllum neilgherrense* and *Pholidota pallida*.

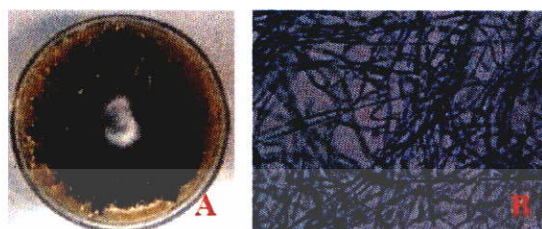


Fig. 3: *Xylaria* sp. A: Colony culture on PDA 30 days B: Mycelia of xylaria sp. under microscops 40X

4. *Colletotrichum* sp.

Habitats: *Cymbidium dayanum*, *Dendrobium hercoglossum*, *Torenia fournieri*, *Dendrobium crumenatum* and *Grammatophyllum specinocum*.

The growth on PDA medium 7 days have 8.5-9 cm, the mycelia initially white-grey and then become black-brown. The conidia have no color with rods shape (Busarakham K.,2001).

Previous study from India showed that the endophytic fungus, *Colletotrichum gloeosporiodes* was isolated from *Justicia gendarussa*, a medicinal plant have ability to produce Taxol that is a potent anticancer drug used widely in the treatment of a variety of cancers (Gangadevi V. and Muthumary J., 2008).

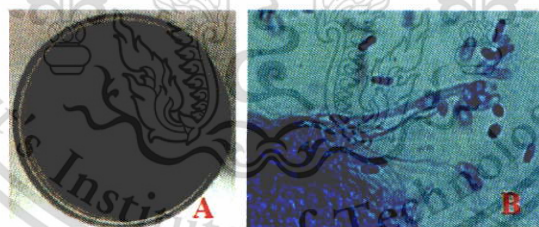


Fig. 4: *Colletotrichum* sp. A: The colony culture on PDA 30 days and B: The spores under microscope 40X

5. *Nigrospora* sp.

Habitat: *Dendrobium hercoglossum*

Nigrospora grows quit fast and produces woolly colonies on potato dextrose agar (PDA) at room temperature, on 7th days has 9 cm in diameter. Color of the colony is white initially and then becomes gray with black areas and turns to black eventually from both front and reverse. Spores (conidia) are produced singly on swollen urn-shaped conidiophores and are egg-shaped to

flattened-spherical, black, and often have an equatorial colourless line or germ slit.

This species also can isolated from deep sea environment at Arabian Sea and The secondary metabolites produced from this organism showed potent antimicrobial and anticancer activities with immediate application to cosmetics and pharmaceutical industries. (Arumugam GK, *et al*, 2015)

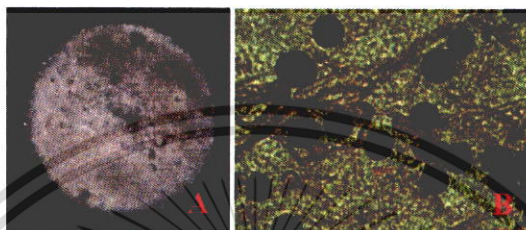


Fig. 5: *Nigrospora* sp. **A:** The colony culture on PDA 30 days **B:** The hyphae and spores under microscope 40X

6. *Phoma* sp.

Habitats: *Cymbidium dayanum*, *Doritis pulcherrima* and *Grammatophyllum specinocum*.

The hyphae were growing rapidly, for 2 days that cultured on PDA medium have 6.5 cm in diameter. The hyphae first growth have white color and than become black-brown. Spores are colorless and unicellular. The pycnidia are black and depressed in the tissues of the host.

Phoma sp. are endophytic fungi that can isolated from many plants. Wang LW *et al.* (2012) isolated *phoma* sp. from chinese medicinal plant *Arisaema erubescens* and showed that the bioactive metabolites from this species can inhibited the growth of four plant pathogenic fungi (*Fusarium oxysporium*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, and *Magnaporthe oryzae*). This species also isolated from roots of cucumber in Korea and showed that have ability to produce Gibberellins and Indoleacetic Acid and Promotes Host-Plant Growth during Stress (Waqas M. *et al.*, 2012).

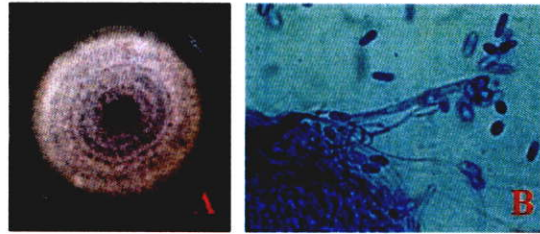


Fig. 6: *Phoma* sp1. A: The colony culture on PDA 30 days and B: The spores and mycelia under microscope 40X

7. *Curvularia* sp.

Habitats: *Grammatophyllum scriptum* and *Doritis pulcherrima*.

The growth was quit fast for cultured on PDA medium for 2 days have 5.2cm in diameter. From the front, the color of the colony is white to pinkish gray initially and turns to black as the colony matures. From the reverse, it is dark brown to black. Conidia are pale brown, with three or more transverse septa (phragmoconidia) and are formed apically through a pore (poroconidia) in a sympodially elongating geniculate conidiophore similar to *Drechslera*. Conidia are cylindrical or slightly curved, with one of the central cells being larger and darker. Germination is bipolar and some species may have a prominent hilum (McGinnis, M.R. 1980).

Zakaria L. *et al.* (2010) isolated this species from *Oryza sativa*. Aharwal R.P. *et al.* (2014) also isolated this fungus from *Calotropis procera* (Linn.) R.Br. a widely used medicinal plant in India.



Fig. 7: *Curvularia* sp. A: The colony culture on PDA 30 days and B: The spores under microscope 40X

8. *Fusarium* sp.

Habitat: *Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium palpebrae*, *Torenia fournieri* and *Dendrobium friedericksianum*.

The hyphae are white. The growth was quit fast, for cultured on PDA in room temperature, for 4 days have 4.9 cm in diameter. The conidia are fusiform, slightly curved, pointed at the tip. *Fusarium* is a filamentous fungus widely distributed on plants and in the soil. It is found in normal mycoflora of commodities, such as rice, bean, soybean, and other crops.

Previous study showed that *Fusarium redolens*, isolated from Himalayan yew can produce taxol, the anticancer drug (Garyali S, 2013). Sogra F.M. and Raj M.B.(2014) isolated *Fusarium oxysporum* from leaf of *Nothapodytes foetida*, ethyl acetate extract from this fungus show ability to inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*.

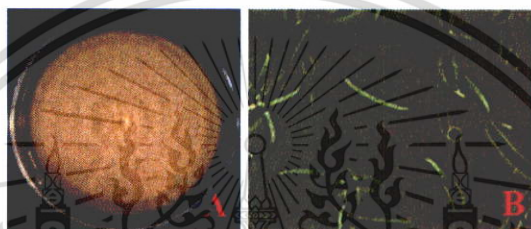


Fig. 8: *Fusarium* sp. A: The colony culture on PDA 30 days and B: The spores under microscope 40X

9. *Pestalotiopsis* sp.

Habitat: *Dendrobium friedericksianum*, *Cymbidium dayanum* and *Grammatophyllum scriptum*.

The mycelia are white in color. This species have quit slow of growing. For growing on PDA in room temperature for 7 days have 5.5cm in diameter. Conidiophores (annellides) produced within compact fruiting structures (aecervuli or pycnidia). Spores (conidia) 4 to 5 celled, with the two or three central cells dark brown, and with two or more apical appendages or hairs.

Pestalotiopsis sp. is the common endophytic fungi that can isolate from orchids and other plants. They also isolated from other orchid varieties from other country such as Tempesta S. *et al.* (2011) isolated endophytic fungi from leaves of *C. mrcranthum* and *O. adendrobium* also found 29 strains of *Pestalotiopsis* sp. Wei J.G *et al.* (2007) isolated this species that associated with plants of Podocarpaceae, Theaceae and Taxaceae in southern China also obtained 24 species. Kumaran RS *et al.* (2010) isolated *Pestalotiopsis* ver-

sicolor and *Pestalotiopsis neglecta* as endophytic fungi from Japanese Yew tree, *Taxus cuspidata* and indicated that they can produce taxol concentration induces increased cell death in cancer.

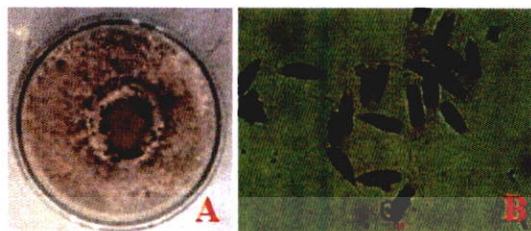


Fig. 9: *Pestalotiopsis* sp. A: The colony culture on PDA 30 days B: The conidia under microscopes 40X

10. *Corynascus* sp.

Habitat: *Cymbidium dayanum*

The fungus have quit fast in growth. Only 3 days the mycelia have 9.0 cm in diameter. It has black-white mycelia, produce lots of black spores and inside spores there are lots of little ascospores.



Fig. 10: *Corynascus* sp. A: The colony culture on PDA 30 days and B: The spores and ascospores under microscopes 40X

11. *Cladosporium*-like fungi

Habitat: *Grammatophyllum scriptum*, *Cymbidium dayanum*, *Dendrobium palpebrae*, *Doritis pulcherrima* and *Dendrobium crumenatum*

The initial growth the mycelia have white color and then become black. The growth was quit fast. On 6th day of culture on PDA have 9 cm in diameter. The mycelia have branches and the lots of small spore.

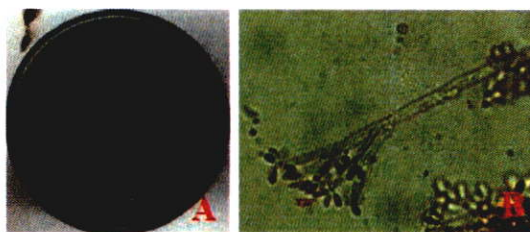


Fig. 11: *Cladosporium*-like fungi A: The colony culture on PDA 30 days and B: The spores and mycelia under microscops 40X

12. *Achaetomium* sp.

Habitat: *Grammatophyllum scriptum*

The mycelia growth look like the yellow sheets and this fungus growth quit slow, cultured on PDA medium in room temperature for 7 days have 5.5 cm in diameter. The spores are large with yellow in color. Inside the spores have many ascospores.



Fig. 12: *Achaetomium* sp. A: The colony culture on PDA 30 days B: The spores under microscops 10X and C: The ascospores under microscops 40X

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Antifungal activities of endophytic fungi isolated from orchids against *Colletotrichum gloeosporioides* caused anthracnose in orchids

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Vannak S., Sarayut P. and Soyong K. (2015). Antifungal activities of endophytic fungi isolated from orchids against *Colletotrichum* sp. caused anthracnose in orchids. Journal of Agricultural Technology 11(8):1949-1961.

Crude extracts from endophytic fungi, *Daldiniaescholtzii*, *Chaetomium cochliodes* and *Chaetomium cupreum*, were isolated from orchids showed ability to inhibit the mycelia growth and spore production of *Colletotrichum gloeosporioides* caused anthracnose in orchids. Crude hexane, ethyl acetate and methanol from *Daldiniaescholtzii* at the concentrations 1,000 µg/ml showed the ability to inhibit mycelia growth of *Colletotrichum* sp. which was 75.5%, 61.75% and 41.75% respectively, inhibited spore productions which were 65.5%, 69.45% and 33.09% respectively which the ED₅₀ values as 220, 104 and 2971 µg/ml, respectively. Crude extract from *Chaetomium cochliodes* at the concentrations 1,000 µg/ml can inhibit the mycelia growth of *Colletotrichum* sp. which was 53.75%, 35.5% and 60.25% respectively, inhibited spore production of 46.12%, 51.77% and 60.87% respectively and the ED₅₀ values as 1754, 1879 and 712 µg/ml, respectively. Crude extract from *Chaetomium cupreum* also showed ability to inhibit *Colletotrichum* sp., at the concentrations 1,000 µg/ml that inhibited the growth of mycelia as 40.5%, 67.25% and 37.75%, inhibited spore productions as 51.35%, 51.27% and 58.65%, respectively and the ED₅₀ value 794, 624 and 879 µg/ml, respectively.

Keywords: endophytic fungi, orchid, *Daldiniaescholtzii*, *Chaetomium cochliodes*, *Chaetomium cupreum*, *Colletotrichum* sp.

Introduction

Resently, there are many works that studying about endophytic fungi from many plants, both fungi and bacteria. Endophytic microorganisms are those that colonize the healthy plant internal tissue (Stone, *et al.*, 2004). The meaning of term "endophyte" is as broad as its literal definition and spectrum of potential hosts and inhabitants. Endophytes are used for both bacteria and fungi (Schulz, *et al.*, 2006). The endophytic fungi have three major roles as saprophyte on dead or senescing tissue, Mutualism that can protect the host

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plant from pathogen or can promote plant growth and probably expressed as latent pathogens and avirulent pathogens at early stages of infection. Recently endophytic fungi have attracted the attention from many scientists in the world as estimated that such species may be useful as sources of anticancer, antidiabetic, insecticidal and immune-suppressive compounds (Strobel and Daisy, 2003). Orchids are among the plant groups that have aroused most widespread interest among scientists and horticulturists, both for their study and use. They have been subjected to particularly high commercial demand over the past 40 years for the beauty of their structure and their vividly colored flowers, steeped in symbolism and mystery. Some countries have declared orchid species as their national flowers (Clemente, 2009). The orchid cultivation has many problems specially, fungal diseases. Among these, anthracnose caused by *Colletotrichum gloeosporioides* is a most destructive disease and known to cause great losses to the orchid growers in terms of both quality and quantity (Duff and Daly, 2002). *C. gloeosporioides* can attack leaves, petioles and blooms during periods of prolonged leaf moisture and high humidity (Bailey, *et al.*, 1992). The ability to cause latent or quiescent infections has grouped *Colletotrichum* among the most important post-harvest pathogens (Freeman, 1998). *C. gloeosporioides*, known as one of the world's most important pathogens, is a species complex comprising morphologically indistinguishable but genetically isolated species and has been reported on broad range of hosts (Cai, 2009).

The objectives of research findings were to test the isolated endophytic fungi, *Chaetomium cochliodes*, *Chaetomium cupreum* and *Daldiniaescholtzii* from orchid varieties against *C. gloeosporioides* causing anthracnose in orchids.

Materials and methods

Isolates of endophytic fungi

The endophytic fungi were isolated and reported as previous study (Sour, *et al.*, 2015).

Isolation of pathogen

The infected leaf anthracnose in orchid was isolated by using transplanting technique (Agrios, 2005). The leaf was cut in to small pieces, washed by distilled water for one time, put in 5 % ethanol for 3 minutes, pour in 0.5% sodium hypochlorite solution 2 minutes and washed in distilled water thrice, dried with autoclaved tissue paper, then transferred to water agar (WA) and

incubated at room temperature until mycelia emerge. The mycelia were transferred to Potato Dextrose Agar (PDA) for pure culture isolation.

Pathogenicity test

The healthy leaf of *Dendrobium speciosum* were used to test for pathogenicity. The experiment was performed as Completely Randomized Design (CRD) with four replications by detached leaf method. Treatments were set up as inoculated leaves and non-inoculated leaves which served as the controls. The leaf samples were washed by distilled water for one time, dried with autoclaved tissue paper. The pathogen was cultured on PDA and collected as spore suspension at concentration of 1×10^6 spores/ml, then dropped onto wounded leaves. The controls were dropped only sterilized distilled water. All treated leaves were put into plastic box as moist chamber and incubated at room temperature for gathering data of disease level.

Extraction method

Crude extracts from endophytic fungi act as the promising antagonists were done which followed the method of Kanokmedhakul *et al.* (2006). The fungi were separately cultured in potato dextrose broth at room temperature for one month. The dried fungal biomass of antagonistic fungus was ground with the electrical blender and dissolved with hexane for 7 days before running in vacuo to yield crude hexane extract. The marc was then continued to dissolved in ethyl acetate (EtOAc) as the same manner to get crude ethyl acetate and crude methanol extract, respectively. Crude extracts were kept in refrigerator until use.

Bioactive tests of crude extracts against pathogen

The experiment was conducted by using two factors factorial experiment in CRD with four replications. Factor A represented the crude extracts: A1 = crude hexane extract, A2 = crude ethyl acetate extract and A3 = crude methanol extract. Factor B represented the different concentrations: B1 = 0 µg/ml (control), B2 = 10 µg/ml, B3 = 50 µg/ml, B4 = 100 µg/ml, B5 = 500 µg/ml and B6 = 1,000 µg/ml. Each crude extract was dissolved in 2% dimethyl sulfoxide and added to PDA before autoclaving at 121°C (15 psi) for 20 minutes. A sterilized 3-mm diameter cork borer was used to cut at the colony peripheral to get culture agar plug, then transferred to the center of 5 cm diameter Petri dishes of PDA containing each crude extract at each concentration and incubated at room temperature until the pathogen on the control plates growing full plate. The

pathogen cells in each treatment were observed under compound microscope and taken photograph for comparison. Data were collected regarding the mycelia growth and number of spore produced by the pathogen and calculated the percentage of conidia inhibition. Data were subjected analysis of variance (ANOVA) 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.

Results

Isolation of pathogen

Colletotrichum gloeosporioides was isolated from leave of orchid, *Grammatophyllum specinocum*. The detail characteristic were observed under compound microcope to see conidiophores and conidia, then measured (data not shown) as seen in Fig.1.



Fig.1. *Colletotrichum gloeosporioides*. Left = conidiophores and conidia; right = pure culture on PDA

Pathogenicity test

The pathogenicity test was successful done by inoculated the spore suspension to wounded leaves of *Grammatophyllum specinocum*. The inoculated pattern showed clearly symptom and no symptom showing in the uninoculated controls as seen in Fig.2.

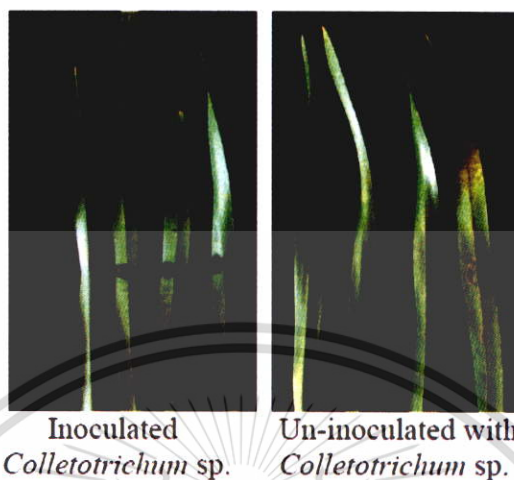


Fig.2. Show the infection and un-infection of pathogen on leaves of *Grammatophyllum speciosum*

Bioactive tests of crude extracts against pathogen

Hexane crude extract from *Dalmaniaescholtzii* at the concentrations 500 and 1,000 μ g/ml gave significantly different in colony diameters of *Colletotrichum* sp. which were 1.9 and 1.22 cm, respectively when compared to the control (0 μ g/ml) 5cm. Ethyl acetate crude extract at the concentration of 100, 500 and 1,000 μ g/ml which were 3.98, 3.33 and 2.01cm respectively gave significantly different when compared with the control (0 μ g/ml). Methanol crude extract at the concentrations 50, 100, 500 and 1,000 μ g/ml also gave significantly different which were 3.91, 4.46, 3.35 and 2.63cm respectively when compared to the control (0 μ g/ml).

Hexane crude extract from *Chaetomium cochliodes* at all the concentrations of 10, 50, 100, 500 and 1,000 μ g/ml showed colony diameters of 4.18, 4.09, 3.94, 3.82 and 2.31cm respectively which gave significantly different when compared to the control (0 μ g/ml). Ethyl acetate crude extract all the concentrations of 10, 50, 100, 500 and 1,000 μ g/ml showing colony diameters of 4.39, 4.34, 4.11, 3.25 and 3.23cm, respectively which gave significantly different when compared to the control (0 μ g/ml). Methanol crude extract at the concentrations 50, 100, 500 and 1,000 μ g/ml, which were 4.19, 3.48, 3.45 and 1.99cm, respectively gave significantly different when compared to the control (0 μ g/ml) 5cm.

Hexane crude extract from *Chaetomium cupreum* at all the concentrations of 10, 50, 100, 500 and 1,000 μ g/ml showing colony diameters of 4.21, 4.15, 3.46, 2.85 and 2.73cm, respectively which significantly differed to the control (0 μ g/ml). Ethyl acetate crude extract at all the concentrations of 10, 50, 100,

500 and 1,000µg/ml, showing colony diameters of 4.21, 4.17, 4.08, 3.16 and 1.64cm, respectively which significant differed when compared to the control (0µg/ml). Methanol crude extract at all the concentrations of 10, 50,100, 500 and 1,000µg/mlshowing colony diameters of 4.3, 4.04, 3.01, 3.34 and 2.86cm, respectively which significantly differed when compared to the control (0µg/ml) as seen in Table 1.

The highest inhibition to tested pathogen showed that crude hexane extract at 1,000 µg/ml of from *Daldiniaescholtzii*was 75.5%, followed by crude hexane extract at the concentrations 500µg/ml which was 62.25% and crude ethyl acetate at the concentrations 1,000µg/ml which was 61.75%.

Crude extracts from *Chaetomiumcochliodes*inhibited mycelium growth of tested pathogen at different level of inhibition. The highest inhibition was shown in crude methanol extract at the concentration 1,000µg/ml which was 60.25%, and followed by crude hexane extraxctat the concentration 1,000µg/ml which was 53.75% and crude ethyl acetate extract at the concentrations of 1,000µg/ml which was 35.5%.

Table 1: Effect of crude extracts from antagonistic fungi on mycelia growth of *Colletotrichum* sp.

Crude extract	Mycelia growth (cm) of <i>Colletotrichum</i> sp. At each concentration (µg/ml)					
	0	10	50	100	500	1,000
<i>Daldiniaescholtzii</i>						
Hexane	5a ¹	4.75ab	4.45abc	4.5abc	1.9fg	1.22g
EtOAc	5a	4.7abc	4.23abc	3.98bcd	3.33de	2.01f
MeOH	5a	4.21abc	3.91cd	4.46abc	3.35de	2.63ef
<i>Chaetomiumcochliodes</i>						
Hexane	5a	4.18bcd	4.09bcd	3.94cde	3.82de	2.31g
EtOAc	5a	4.39bc	4.34bcd	4.11bcd	3.25f	3.23f
MeOH	5a	4.58ab	4.19bcd	3.48ef	3.45ef	1.99g
<i>Chaetomiumcupreum</i>						
Hexane	5a	4.21b	4.15b	3.46c	2.85d	2.73d
EtOAc	5a	4.21b	4.17b	4.08b	3.16cd	1.64e
MeOH	5a	4.3b	4.04b	3.01cd	3.34c	2.86d

¹Average of four replications. Means followed by the same letters in each column were not significantly different by DMRT at P=0.01.

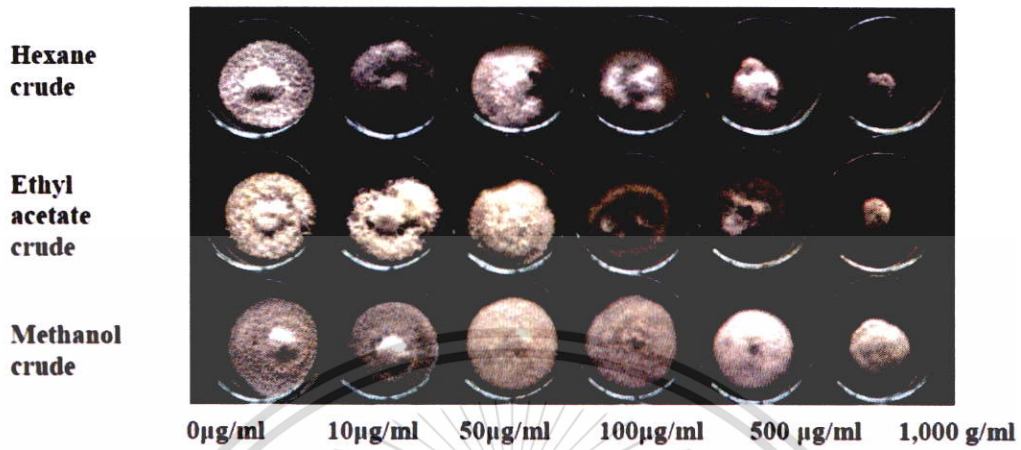


Fig.3. Crude extract test of *Daldiniaescholtzii* against *Colletotrichum* sp.

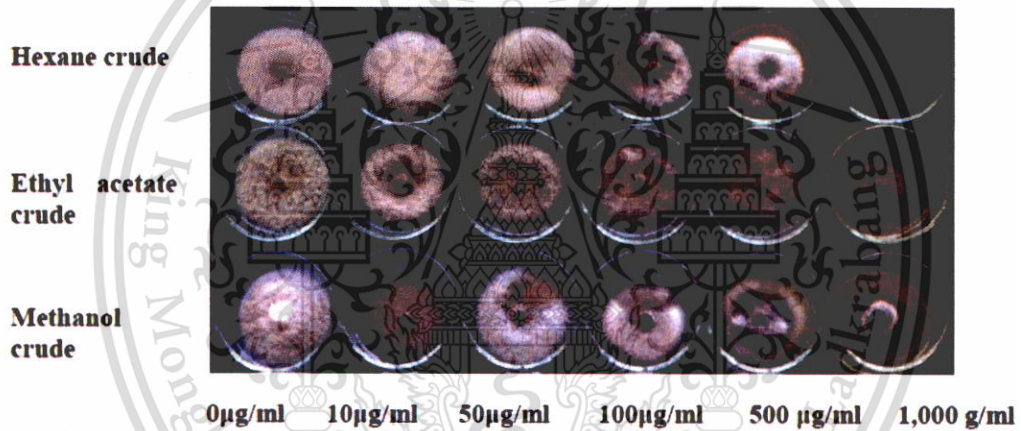


Fig.4. Crude extract test of *Chaetomiumcochliodes* against *Colletotrichum* sp.

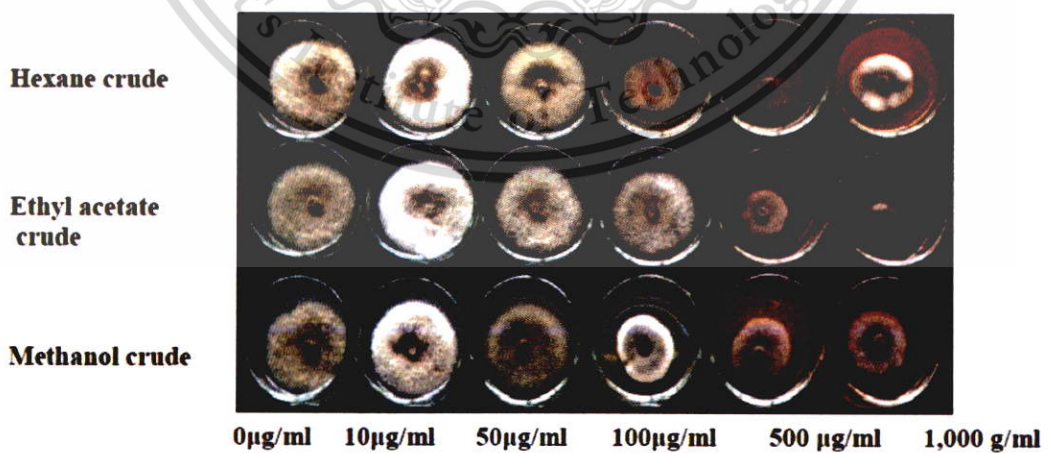


Fig.5. Crude extract test of *Chaetomiumcupreum* against *Colletotrichum* sp.

Crude extracts from *Chaetomiumcupreum* inhibited mycelium growth of the tested pathogen at different levels. The highest inhibition to tested pathogen was crude ethyl acetate extract at the concentration 1,000 μ g/ml which was 67.25 μ g/ml, and followed by crude methanol extract at the concentration 1,000 μ g/ml which was 42.75%, and crude hexane extract at the concentrations 1,000 μ g/ml which was 40.4% as seen in Table 2.

Table 2: The percentages effected of crude extracts from antagonistic fungi on mycelia growth of *Colletotrichum* sp.

Crude extracts	Percentage Colony inhibition of <i>Colletotrichum</i> sp.				
	10	50	100	500	1,000
<i>Daldiniaeschscholtzii</i>					
Hexane	5e ¹	11e	10e	62.25ab	75.5a
EtOAc	6e	15.75de	20.5de	33.35cd	61.75ab
MeOH	15.75de	21.75de	9.25e	33.1cd	47.5bc
<i>Chaetomiumcochliodes</i>					
Hexane	16.5de	18.25de	21.25cd	23.5bcd	53.75a
EtOAc	12.25de	13.5de	17.75de	35b	35.5b
MeOH	8.5e	14.25de	30.5bc	31bc	60.25a
<i>Chaetomiumcupreum</i>					
Hexane	15.75d	16.5d	31.25bc	38.5b	40.5b
EtOAc	15.75d	15.75d	18cd	36.75	67.25a
MeOH	13.25d	19.25cd	39.75b	33.1b	42.75b

¹Average of four replications. Means followed by the same letters in each antagonist were not significantly different by DMRT at P=0.01.

Hexane crude extract of *Daldiniaeschscholtzii* at the concentrations 50, 100, 500 and 1,000 μ g/ml significantly inhibited spore production which were 2.74x10⁷, 2.67x10⁷, 1.46x10⁷ and 1.24x10⁷ spores/ml, respectively when compared to the control (3.6x10⁷ spore/ml). Ethyl acetate crude extract at all the concentrations of 10, 50, 100, 500 and 1,000 μ g/ml inhibited spore production which were 2.65x10⁷, 2.59x10⁷, 2.45x10⁷, 1.8x10⁷ and 1.15x10⁷ spores/ml respectively, when compared to the control (3.78x10⁷ spores/ml). Methanol crude extract at the concentrations of 50, 100, 500 and 1,000 μ g/ml inhibited spore production which were 2.89x10⁷, 2.59x10⁷, 1.8x10⁷ and 1.65x10⁷ spores/ml which significantly differed from the controls (3.76x10⁷ spores/ml). Crude extract of methanol, hexane and ethyl acetate inhibited spore production showed the ED₅₀ values of 2971, 220 and 104 μ g/ml, respectively.

Hexane crude extract of *Chaetomiumcochliodes* at all the concentrations of 10, 50, 100, 500 and 1,000 μ g/ml inhibited spore production which were

2.96×10^7 , 2.93×10^7 , 2.35×10^7 , 2.18×10^7 and 2.05×10^7 spores/ml, respectively which significantly differed when compared to the control (3.44×10^7 spores/ml). Ethyl acetate crude extract at all the concentrations 10, 50, 100, 500 and $1,000 \mu\text{g/ml}$ inhibited spore production which were 2.41×10^7 , 2.19×10^7 , 2.07×10^7 , 1.84×10^7 and 1.48×10^7 spores/ml, respectively which significantly differed when compared to the control (3.08×10^7 spores/ml). Methanol crude extract at the concentrations 100, 500 and $1,000 \mu\text{g/ml}$ inhibited spore production which were 2.21×10^7 , 1.81×10^7 and 1.19×10^7 spores/ml, respectively which significantly differed to the control (3.03×10^7 spores/ml). Crude extract of ethyl acetate, hexane and methanol inhibited spore production of tested pathogen which the ED_{50} values of 1879, 1754 and $712 \mu\text{g/ml}$, respectively.

Hexane crude extract of *Chaetomium cupreum* at all concentrations of 10, 50, 100, 500 and $1,000 \mu\text{g/ml}$ inhibited spore production which were 3.38×10^7 , 3.17×10^7 , 3.11×10^7 , 2.37×10^7 and 2.05×10^7 spores/ml, respectively which significantly differed when compared to the control (4.27×10^7). Ethyl acetate crude extract at the concentrations of 10, 50, 100, 500 and $1,000 \mu\text{g/ml}$ inhibited spore production which were 3.38×10^7 , 3.23×10^7 , 3.11×10^7 , 2.31×10^7 and 2.05×10^7 spores/ml respectively gave significantly different when compared to the control ($0 \mu\text{g/ml}$) 4.26×10^7 spores/ml. Methanol crude extract at the concentration of 100, 500 and $1,000 \mu\text{g/ml}$ inhibited spore production which were 2.64×10^7 , 2.49×10^7 and 1.69×10^7 spores/ml that significantly differed when compared to the control (4.07×10^7 spores/ml). The ED_{50} values of crude methanol, hexane and ethyl acetate inhibit spore production of tested pathogen were 879, 794 and $624 \mu\text{g/ml}$, respectively as seen in Table 3.

Table 3: Effect of crude extracts from antagonistic fungi on spore number

Crude extract	Spore numbers ($\times 10^7$) of <i>Colletotrichum sp.</i> at each concentration (spores/ml)						ED ₅₀
	0	10	50	100	500	1,000	
<i>Daldiniaeschscholtzii</i>							
Hexane	3.6a ¹	3.38ab	2.74c	2.67c	1.46e	1.24e	220.9214
EtOAc	3.78ab	2.65c	2.59c	2.45cd	1.35e	1.15e	104.7066
MeOH	3.76a	3.17ab	2.89b	2.59c	1.8c	1.65d	2971.033
<i>Chaetomium cochlioides</i>							
Hexane	3.44a	2.96b	2.93b	2.35cd	2.18cd	2.05de	1754.045
EtOAc	3.08b	2.41c	2.19cd	2.07de	1.84e	1.48f	1879.879
MeOH	3.03b	2.97b	2.93b	2.21cd	1.81e	1.19g	712.0882
<i>Chaetomium cupreum</i>							
Hexane	4.27a	3.38bcde	3.17cdef	3.11def	2.37gh	2.05gh	794.8441
EtOAc	4.26a	3.38bcde	3.23cdef	3.11def	2.31gh	2.05gh	624.2459
MeOH	4.07ab	3.9abc	3.72abcd	2.64egf	2.49fg	1.69h	879.0221

¹Average of four replications. Means followed by the same letters in each antagonist were not significantly different by DMRT at P=0.01.

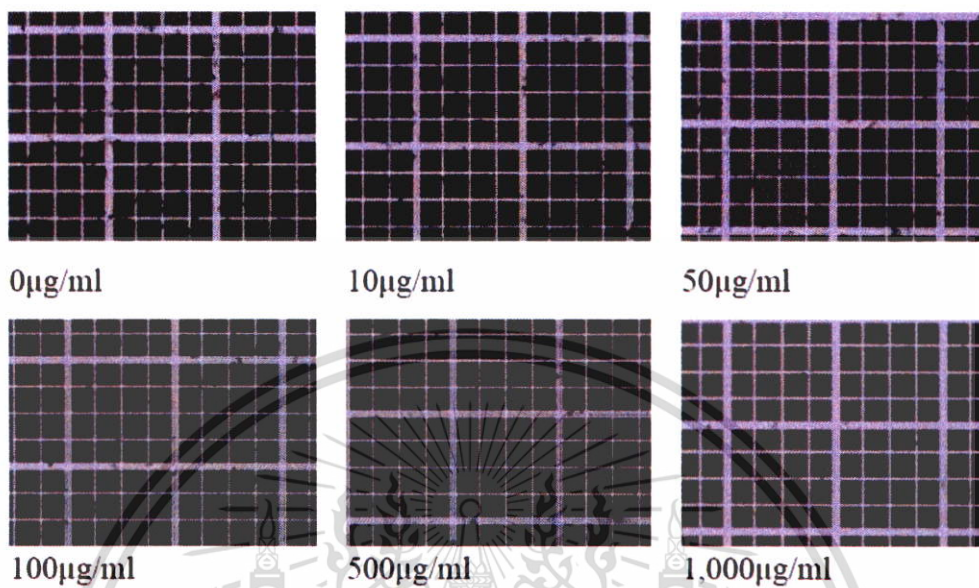


Fig.6. Spore production of *Colletotrichum* sp. at different concentrations

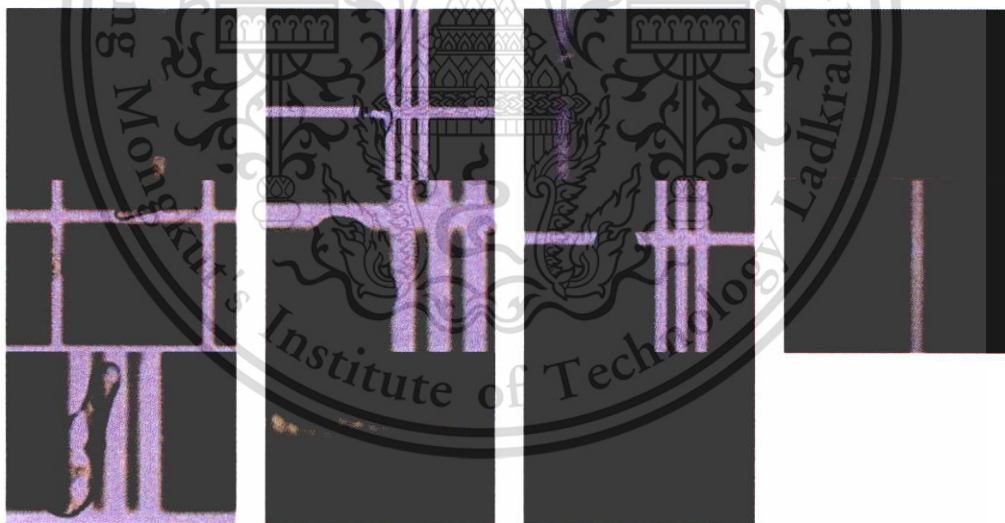


Fig.7. Abnormal spores of *Colletotrichum* sp. at

The spore production of *Colletotrichum gloeosporioides* was significantly inhibited by metabolites from *Daldiniaescholtzii*. The highest spore inhibition was crude ethyl acetate at the concentrations 1,000 µg/ml (69.45%), followed by crude hexane at concentration 1,000 µg/ml which was 65.5%, crude ethyl acetate which was 64.34% and crude hexane at the concentration of 500 µg/ml was 59.32%.

Crude extracts from *Chaetomium cochliodes* significantly inhibited spore production of *Colletotrichum gloeosporioides*. The highest spore inhibition was crude methanol at the concentrations 1,000 µg/ml (60.87%), followed by crude ethyl acetate at the concentrations 1,000 µg/ml (51.77%) and crude hexane at the concentration 1,000 µg/ml (46.12%). The lowest percentages were crude methanol at the lowest concentrations 10 and 50 µg/ml.

Crude extracts from *Chaetomium cupreum* also showed inhibition of spore production (*Colletotrichum gloeosporioides*). The highest spore inhibition was crude methanol extract at the concentration 1,000 µg/ml (58.65%), followed by crude hexane and ethyl acetate extracts at concentration of 1,000 µg/ml which were 51.35% and 51.27%, respectively.

Table 4: Effect of crude extracts from antagonistic fungi on spore inhibition of *Colletotrichum gloeosporioides*.

Crude extract of	Percentages of spore inhibition of <i>Colletotrichum sp.</i>				
	10	50	100	500	1,000
<i>Daldiniaescholtzii</i>					
Hexane	6.15g ¹	23.68defg	25.83def	59.32ab	65.5a
EtOAc	30cde	31.48cde	34.98cd	64.34ab	69.45a
MeOH	10.42fg	14.03efg	31.12cde	33.09cd	34.24bc
<i>Chaetomium cochliodes</i>					
Hexane	13.75ef	14.75ef	31.29cd	36.6bcd	46.12abc
EtOAc	25.5de	33.15cd	32.8cd	40.05bcd	51.77ab
MeOH	2.06f	3.88f	26.76de	40.05bcd	60.87a
<i>Chaetomium cupreum</i>					
Hexane	20.96def	25.14cde	26.38cde	44.45abc	51.35ab
EtOAc	20.53def	23.59de	25.97cde	45.55ab	51.27ab
MeOH	4.19f	8.56ef	35.03bcd	38.72bcd	58.65a

¹Average of four replications. Means followed by the same letters in each antagonist were not significantly different by DMRT at P=0.01.

Discussions

As the results, It is showed that all crude extracts from *Daldiniaescholtzii*, *Chaetomium cochliodes* and *Chaetomium cupreum* can

inhibit the colony growth and spore production of *Colletotrichum gloeosporioides* causing anthracnose of orchid. The crude extracts from *Daldiniaescholtzii* expressed its ability to inhibit the colony growth and spore production of tested pathogen. There was no previous studied about crude extracts or fungal metabolites from the species against *Colletotrichum gloeosporioides* causing anthracnose of orchid. So, this is reported to be the first time. But *Daldiniaescholtzii* was reported to express a feature of many immunosuppressive substance (Zhang, et al., 2008) who discovered Dalesconol A and B polyketides with showing immunosuppressive activity which initially isolated from *Daldiniaescholtzii* and two years later, they discovered daeschol A, dalesconol C, 2, 16-dihydroxyl-benzo fluoranthene and dalmanol A which were isolated from *D. escholtzii* (Zhang, et al., 2011). Moreover, helicascotide C, a new lactone with fungistatic activity against *Cladosporium cucumerinum* was isolated together with helicascotide A from an Indonesian marine algal-associated *D. escholtzii* strain was reported by Tarman et al. (2012).

The crude extracts of *Chaetomium cochliodes* can inhibit *Colletotrichum* sp. causing tea anthracnose was confirmed by Nguyen Van Thiep et al. (2014) who stated that crude extract from hexane, ethyl acetate and methanol at the concentration 1,000 µg/ml can also inhibit the spore productions of *Fusarium roseum* causing wilt of coffee which were 60.87%, 78.16% and 74.57% respectively. Soyong (2014) reported that crude hexane, ethyl acetate and methanol from *Chaetomium cochliodes* at the concentrations 50 µg/ml can inhibit the spore productions of *Drechslera oryzae* causing brown leaf spot in rice which were 54.99%, 63.14% and 48.96% respectively.

Crude extracts from *Chaetomium cupreum* also showed ability to control *Collectotrichum* sp. Tathan, et al. (2012) reported that crude extract from this fungus can inhibit the growth of *Drechslera oryzae* causing rice leaf spot. Hung Phung Manh et al. (2014) also reported *Chaetomium cupreum* was actively inhibited spore production of *Pythium aphanidermatum* causing root rot of in pomelo.

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