BIOLOGICAL ACTIVITIES OF ANTAGONISTIC FUNGI TO CONTROL FUSARIUM WILT OF TOMATO

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หัวข้อวิทยานิพนธ์	ฤทธิ์ทางชีวภาพของจุลินทรีย์ต่อต้านในการควบคุมโรคเหี่ยวมะเขือเทศ
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บทคัดย่อ

จากการวิจัยโรคเหี่ยวของมะเขือเทศในพื้นที่ปลูกมะเขือเทศในกรุงเทพ เพชรบูรณ์ ตาก นครราชสีมา บุรีรัมย์ หนองคาย สกลนคร และขอนแก่น พบว่ามีสาเหตุจากเชื้อรา *Fusarium oxysporum* f. sp. *lycopersici* race 2 โดยใช้พื้นฐานการจัดจำแนกลักษณะทางสัณฐานวิทยาและเทคนิคทางชีวโมเลกุล sequencing the internal transcribed spacer (ITS) region ITS1, 5.8S, ITS2, small portion of 18S และ 28S rDNAรวมถึงเทคนิค AFLP จากการวิเคราะห์ความสามารถในการเกิดโรคและ AFLP พบว่า11 isolates เป็นกลุ่มที่ไม่ทำให้เกิดโรค (non-pathogenic or avirulent group) 14 isolatesเป็นกลุ่มที่ทำให้เกิดโรค (as pathogenic group) ซึ่งแบ่งได้เป็น 3 กลุ่มย่อยคือ low virulent (L), moderate virulent (M) และ high virulent (H) Isolate KK2 มีความรุนแรงต่อการเกิดโรคสูงสุดกับมะเขือเทศพันธุ์สีดา จัดจำแนกเป็น *F. oxysporum* f. sp. *lycopersici* race 2 จากผลการทดลองสามารถได้ข้อมูลใหม่เกี่ยวกับ formae specialis ของเชื้อรา *F. oxysproum* f. sp. *lycopersici* ว่าจัดเป็น race 2 ที่สามารถทำให้เกิดโรคเหี่ยวมะเขือเทศได้ทั้งพันธุ์สีดาและพันธุ์เชอรี่

จากการวิจัยจุลินทรีย์ต่อต้านเชื้อรา *Chaetomium brasilense* CB01, *Chaetomium cupreum* CC03, *Chaetomium elatum* ChE01, *Chaetomium lucknowense* CLT01, *Emericella nidulans* EN01 and *Emericella rugulosa* ER01 พบว่าสามารถทำลายยับยั้งเชื้อรา *F. oxysporum* f. sp. *lycopersici* NKSC02ได้ ซึ่งพบว่ากิจกรรมต่อต้านในการยับยั้งการสร้างสปอร์ของเชื้อรา *F. oxysporum* f. sp. *lycopersici* NKSC02 จากการทดสอบสารออกฤทธิ์โดยใช้สารสกัดรวม(crude extracts) และสารบริสุทธิ์ (pure compounds) จากเชื้อจุลินทรีย์ต่อต้าน พบว่าสามารถสร้างสารปฏิชีวนะ เป็นกลไกในการยับยั้งการสร้างสปอร์ของเชื้อรา *F. oxysporum* f. sp. *lycopersici* สารสกัดรวม (crude

extracts) จากเชื้อรา *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 และ*E. rugulosa* ER01 สามารถต่อต้านกิจกรรมของเชื้อรา *F. oxysporum* f. sp. *lycopersici* NKSC02 และสามารถปลดปล่อยสารออกฤทธิ์ในการยับยั้งการเจริญของเชื้อรา *F. oxysporum* f. sp. *lycopersici*

นอกจากนี้สารบริสุทธิ์ Chaetoglobosin-C จากเชื้อรา *Ch. elatum* ChE01 และ*Ch. lucknowense* CLT01 สามารถยับยั้งการสร้างสปอร์ของเชื้อรา *F. oxysporum* f. sp. *lycopersici* NKSC02 ซึ่งมีค่า ED_{50} เท่ากับ 5.94 µg/ml. และสารบริสุทธิ์ tajixanthone จากเชื้อรา *E. rugulosa* ER01 สามารถยับยั้งการสร้างสปอร์ของเชื้อรา *F. oxysporum* f. sp. *lycopersici* NKSC02 ซึ่งมีค่า ED_{50} เท่ากับ 167 µg/ml. นอกจากนี้ Chaetoglobosin C และtajixanthone เป็นสารปฏิชีวนะที่สามารถเข้าทำลายเซลล์ของเชื้อสาเหตุโรคได้

จากการใช้เชื้อก่อโรคของ *F. oxysporum* f. sp. *lycopersici* (1×10^7 spores/ml) ผสมลงในสาร Chaetoglobosin C และ tajixanthone ก่อนปลูกเชื้อลงในมะเขือเทศ พบว่าไม่ทำให้มะเขือเทศเป็นโรคเหี่ยว หลังปลูกเชื้อเป็นเวลา 21 วัน ในขณะที่การปลูกเชื้ออย่างเดียวในในมะเขือเทศ มีอัตราการเกิดโรคเหี่ยวสูงสุด และสารบริสุทธิ์ที่ผสมลงในเชื้อก่อโรคที่ความเข้มข้น 10, 50 และ 100 µg/ml ของ Chaetoglobosin C หรือ tajixanthone มีผลทำให้เซลล์ของเชื้อสาเหตุโรคผิดปกติและไม่สามารถทำให้เกิดโรคเหี่ยวได้ นอกจากนี้ จากการวิจัยยังพบว่ามะเขือเทศพันธุ์สีดา ที่ฉีดพ่นสารสกัด crude EtOAc จากเชื้อรา *E. rugulosa* ที่ 500 และ 1000 µg/ml มีผลทำให้การเกิดโรคเหี่ยวลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับการปลูกเชื้อสาเหตุโรคอย่างเดียว และสามารถแสดงให้เห็นว่าสารสกัด crude EtOAc จากเชื้อรา *E. rugulosa* ที่ 500 และ 1000 µg/ml มีผลต่อการชักนำให้มะเขือเทศสร้างภูมิคุ้มกันโรค

จากการทดสอบยาเชื้อชนิดน้ำมัน และชนิดผงที่ผลิตจากเชื้อรา *Ch. elatum* ChE01, *E. nidulans* EN01 และ *E. rugulosa* ER01 พบว่าสามารถควบคุมโรคเหี่ยวมะเขือเทศพันธุ์สีดาที่มีสาเหตุจากเชื้อรา *F. oxysporum* f. sp. *lycopersici* NKSC02 race 2 ได้ ต้นมะเขือเทศที่ฉีดพ่นยาเชื้อชนิดน้ำมัน และชนิดผงที่ผลิตจากเชื้อรา *Ch. elatum* ChE01, *E. nidulans* EN01 และ*E. rugulosa* ER01 มีอัตราการเกิดโรคเหี่ยวต่ำสุด ซึ่งมีความแตกต่างอย่างมีนัยสำคัญยิ่งทางสถิติเมื่อเปรียบเทียบกับการใช้สารเคมี Prochoraz และการปลูกเชื้อสาเหตุโรคอย่างเดียว นอกจากนี้ยังพบว่าการใช้ยาเชื้อชนิดน้ำมันที่ผลิตจากเชื้อรา *Ch. elatum* ChE01, *E. nidulans* EN01 และ *E. rugulosa* ER01 สามารถเพิ่มการเจริญเติบโตของมะเขือเทศได้ดี เช่นความสูง น้ำหนักสดของต้นและราก ปริมาณและน้ำหนักผลผลิต ดีกว่ายาเชื้อชนิดผง เมื่อเปรียบเทียบกับการใช้สารเคมี Prochoraz และการปลูกเชื้อสาเหตุโรคอย่างเดียว จากการวิจัยสรุปว่าเป็นรายงานครั้งแรกที่สามารถผลิตยาเชื้อชนิดใหม่จากเชื้อรา *Ch. elatum* ChE01, *E. nidulans* EN01 และ *E. rugulosa* ER01 เพื่อใช้ในการควบคุมโรคเหี่ยวมะเขือเทศพันธุ์สีดา จากเชื้อรา *F. oxysporum* f. sp. *lycopersici* race 2 ได้

Thesis	Biological Activities of Antagonistic Fungi to Control Fusarium Wilt of Tomato
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ABSTRACT

The research findings on tomato wilt collected from infested fields in Bangkok, Phetchaboun, Tak, Nakhonratchasima, Burirum, Nongkhai, Sakonnakhon, and Khonkaen provinces resulted to isolate and identify the causal agent as *Fusarium oxysporum* f. sp. *lycopersici* race 2 according to confirmation work on morphological and molecular phylogeny by sequencing the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA and a small portion of the 28S rDNA and AFLP marker.

The pathogenicity test and AFLP analysis revealed that 11 isolates were categorized as non-pathogenic or avirulent group and 14 isolates were categorized as pathogenic group which divided into 3 subgroups of low virulent (L), moderate virulent (M) and high virulent (H). As a result, the isolates of KSoC02, NKRC09, SSoC03 and SSoC04 were shown to be non-pathogenic isolates. Isolate KK2 isolated from Northeast part of Thailand was tested its pathogenicity to cause wilt symptom on tomato Sida var which susceptible to standard tested isolate *F. oxysporum* f. sp. *lycopersici* race 2. This work provided new information on formae specialis of *F. oxysproum* f. sp. *lycopersici* which could classify as race 2 that can cause wilt to different varieties of tomato e.g. Cheery and Sida varieties rather one variety.

The antagonistic fungi of *Chaetomium brasilense* CB01, *Chaetomium cupreum* CC03, *Chaetomium elatum* ChE0, *Chaetomium lucknowense* CLT01,

Emericella nidulans EN01, and *Emericella rugulosa* ER01 were proved to antagonize *F. oxysporum* f. sp. *lycopersici* NKSC02. The antagonism test demonstrated the antagonistic activity of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 to inhibit the conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02. Bioactivities tests of crude extracts and pure compounds from tested antagonistic fungi were proved as a control mechanism. To elucidate the control mechanism involved in the inhibition of *F. oxysporum* f. sp. *lycopersici*, crude extracts of *Ch. brasilense* CB01, *Ch .cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 were confirmed for antifungal activity against of *F. oxysporum* f. sp. *lycopersici* NKSC02. The other control mechanism involved in releasing antibiotic substances to inhibit *F. oxysporum* f. sp. *lycopersici*. All tested crude extracts of *Ch. brasilense* CB01, *Ch.cupreum* CC03, *Ch.elatum* ChE01, *Ch .lucknowense* CLT01, *E. nidulans* EN01 and *E .rugulosa* ER01 were significantly inhibited conidia production of *F. oxysporum* f. sp. *lycopersici*.

It is clearly demonstrated that a pure compound produced by *E. rugulosa* ER01, Chaetoglobosin C, a pure compound produced by *Ch. elatum* ChE01 and *Ch. luckowense* CLT01, inhibited conidia production of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED_{50} value of 5.94 µg/ml. Moreover, tajixanthone, a pure compound produced by *E. rugulosa* ER01, inhibited conidia production of *F. oxysporum* f. sp. *lycopersici* NKSC02 with an ED_{50} value of 167 µg/ml. Chaetoglobosin C and tajixanthone are expressed as a antibiotic substances to destroy the pathogen cells implies antibiosis.

Inocula of *F. oxysporum* f. sp. *lycopersici* (1×10^7 spores/ml) were mixed with chaetoglobosin-C and tajixanthone and inoculated to tomato seedlings caused no symptoms at day 21 while the treatment with pathogen alone showed significantly highest disease severity index. With this, no wilt incidences were appeared at all tested concentration of 10, 50 and 100 µg/ml of either Chaetoglobosin C or tajixanthone. It is stated that chaetoglobosin-C and tajixanthone affected directly to the pathogen inocula implies antibiosis which the occurrences of ruptured cells and abnormal conidia of pathogen.

The research findings indicated that treated tomato seedlings var Sida with crude EtOAc of *E. rugulosa* at 1000 µg/ml gave significant lower DSI from treated with crude EtoAC of *E. rugulosa* at 500 µg/ml when compared to the inoculated

with *F. oxysporum* f. sp. *lycopersici* NKSC02. Disease immunity of Fusarium wilt in Sida variety appeared the highest immunity when treated with crude EtOAc at 1000 µg/ml and followed by treated with crude EtOAc at 500 µg/ml. Microbial extracts expressed to induce immunity in term of microbial elicitors.

Ch. elatum ChE01, *E. nidulans* EN01 and to *E. rugulosa* ER01 were formulated as oil and powder bioformulations gave a good result to control wilt of tomato var Sida caused by *F. oxysporum* f. sp. *lycopersici* NKSC02 race 2. The treated tomatoes showed the lowest wilt incidence in oil and powder bioformulations from *Ch. elatum* ChE01, *E. nidulans* EN01 and to *E. rugulosa* ER01 which significantly differed from Prochoraz and inoculated control. The application of oil bioformulation from *E. rugulosa* could reduce wilt incidence and followed by application of powder bioformulation and Prochoraz whichalso reduced wilt incidence. Based on the result, oil bioformulation from *Ch.elatum* ChE01, *E. nidulans* EN01 and *E. rugulosa* ER01 gave significantly better plant parameters in terms of plant height, plant weight, root weight, number of fruits and fruit weight than powder bioformulation and Prochoraz when compared to the inoculated control with *F. oxysporum* f. sp. *lycopersici*. It is suggested that this new reports of bioformulation of *Ch. elatum* ChE01, *E. nidulans* EN01 and *E. rugulosa* ER01 could be applied to control tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* in the fields.

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Phoutthasone Sibounnavong

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CHAPTER I
INTRODUCTION

1.1. Statement and Significance of the Problems

A tomato (*Lycopersicon esculentum* Mill.) is one of the most widely cultivated, popular and important vegetable crops in the world. There is increasing demand in developed countries for organic tomatoes, as well as heirloom tomatoes, to make up for flavor and texture in commercial tomatoes. Tomato crop is usually attacked by many kinds of diseases such as Fusarium wilt, bacterial wilt, and early blight (Agrios, 1997). Among these diseases, Fusarium wilt is one of the most serious diseases that can cause serious economic losses in many countries. It is caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen. Management of this pathogen is very difficult due to their endophytic growth and persistence in soil. In general, this pathogenic fungus is a limiting factor in the production of many crops and accounts for 10 – 20 percent yield losses annually and can reach as high as 100 per cent (USDA, 2008). It has become one of the most prevalent and damaging diseases wherever tomatoes are grown intensively because the pathogen persists indefinitely in infested soils. The methods used to control vascular wilt are either not very efficient or are difficult to apply. The best way is recommended to control the disease would be selected resistant varieties of tomato (Silva and Bettiol, 2005).

Tomatoes may develop resistance to their race from pathogenic fungus. In addition, there is a report from United State Department of Agriculture in 2008 that the pathogenic fungus is expected to increase when methyl bromide is no longer available. The pathogen has increased and become resistant to chemical fungicides (Silva and Bettiol, 2005). For this reason, alternative methods with emphasis on biological control using fungi or bacteria in controlling the disease have been studied by several researchers to reduce fungicide application and decrease cost of plant production. Recently, there are many reports that some species of fungi can be used as source of biological fungicide to control the diseases (Soytong, 1992a).

At present, the control of most plant diseases is dependent on the use of chemical fungicide because of its effectiveness, reliability, readily available and easy to apply. However, there are several disadvantages of using chemical fungicides.

They are toxic not only to humans but also to other forms of life. The price of chemical fungicide is rapidly increasing which is beyond the reach of ordinary farmers. There are also many reports of environmental pollution due to injudicious application of fungicides. Applications of chemical fungicides affect the environmental condition and can be harmful to the ecosystem. Because of the problems associated with the use of chemicals there is a felt need to search for an alternative method of controlling the diseases which is not only effective and economical but also safe to the environment.

Recently, the use of biological control of plant pathogens has been concerned to the most plant pathologists and many researchers. There are many new species of promising antagonists that can be used to control *Fusarium* wilt of tomatoes. The biocontrol agents and their bioactive compounds extracted from different species of antagonistic fungi were reported to inhibit the growth of many plant pathogenic fungi, including *Fusarium* wilt of tomato (Kanokmedhakul *et al.*, 2006 and 2003, Thongsri and Soytong, 2004, Srinon *et al.*, 2004, Suwannapong and Soytong, 2002 and Sibounnavong, *et al.*, 2009). The bioactive compounds, Trichotoxin A50 extracted from *Trichoderma harzianum* PC01; and Chaetoglobosin C extracted from *Chaetomium globosum.* These compounds have also been reported to elicit resistance or immunity in plants by inducing oxidative burst in plant cells (Soytong, *et al.*, 2001).

1.2. Objectives

The general objective was to evaluate the biological activities of antagonists and its bioactive compounds against *Fusarium oxysporum* f. sp. *lycopesici*.

Specifically, the study was aimed to:

1.2.1. To collect, isolate and identify antagonistic fungi and pathogen by morphological study and molecular phylogeny.

1.2.2. To study the genetic relationship among *F. oxysporum* isolates by pathogenicity test and AFLP marker.

1.2.3. To determine the property of antagonistic fungi for biological control of Fusarium wilt of tomato *in vitro*.

1.2.4. To find out the most effective bioformulations of antagonistic fungi to control Fusarium wilt of tomato in pot experiment

1. 3. Scope of the Study

This research work was covered for collection, isolation and identification of the pathogen from infested soil of tomato's field in Bangkok, Tak and Phetchaboun. Twelve isolates of *F. oxysporum* f. sp. *lycopersici*, which isolated from Burirum, Khonkaen, Nongkhai, Nakhonratchasima and Sakon-nakhon provinces in Thailand were obtained from Assist. Prof. Dr. Chamaiporn Charoenporn (Nakhonratchasima Rachabhat University, Nakhonratchasima, Thailand). All the isolates of the pathogen were to study for genetic variation by using AFLP marker and pathogenicity test. The most effective isolate of *F. oxysporum* f. sp. *lycopersici* was used as causal agent in the further experiment. The biological activities of antagonistic fungi including *Ch. brasilense*, *Ch. cupreum*, *Ch. elatum*, *Ch. lucknowense*, *E. nudulans*, and *E. rugulosa* were studied their ability for controlling of Fusarium wilt of tomato by Bi-culture test and crude extracts of the antagonists bioassay. Two pure compounds namely Chaetogoblosin-C and tajixantone were purified from *Ch. elatum*, *Ch. lucknowense*, and *E. rugolosa* were determined for their fungal metabolite to control the pathogen in the laboratory. Moreover, the antagonistic fungi were evaluated the most effective bioformulations of antagonistic fungi to control Fusarium wilt of tomato in pot experiment.

1.4. Time and Place of the Research Work

The study was conducted at the Biocontrol Research Unit & Mycology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology, King Mongkut's Institute of Technology Ladkrabang, Ladkrabang, (KMITL), Bangkok, Thailand. The study of genetic variation of the pathogen by using AFLP technique was conducted at Department of Plant Pathology, Faculty of Agriculture, Kamphaengsaen campus Kasetsart University. The research work of thesis was started from June 2009 until April 2012.

CHAPTER II
LITERATURE REVIEW

2.1 Characteristics of Tomato Plant

The tomato (*Lycopersicon esculentum*) is native to South America. Genetic evidence shows that the progenitors of tomatoes were herbaceous green plants with small green fruit with a center of diversity in the highlands of Peru. The tomatois aherbaceous, usually sprawling plant in the Solanaceae nightshade family that is typically cultivated for the purpose of harvesting its fruit for human consumption. The fruit of most varieties ripens to a distinctive red color. Tomato plants typically reach to 1–3 meters (3–10 ft) in height, and have a weak, woody stem that often vines over other plants. The leaves are 10–25 centimeters (4–10 in) long, odd pinnate, with 5–9 leaflets on petioles, each leaflet up to 8 centimeters (3 in) long, with a serrated margin; both the stem and leaves are densely glandular-hair. The flowers are 1–2 centimeters (0.4–0.8 in) across, yellow, with five pointed lobes on the corolla; they are borne in a cyme of 3–12 together. It is a perennial, often grown outdoors in temperate climates as an annual (Acquaah, 2002)

2.2. Cultivation

Tomato is one of the most widely cultivated crops in the worldwide. Word tomato production in 2009 was about 150 million tons while tomatoes were produced in the world in 2007 and 2001 were about 125 million and 105 million tons respectively. As it is a relatively short duration crop and gives a high yield, it is economically attractive and the area under cultivation is increasing daily. According to FAOSTAT(2009), China, the largest producer, accounted for about one quarter of the global output, followed by United States and India as shown in Table 2.1.

2.3. Varieties of Tomato

There are more than 4,000 varieties of tomato and are roughly divided into several categories, based mostly on shape, use size (small to large) and color. Botanically, the tomato is a fruit (FAOSTAT, 2009).

Table 2.1. Tomato production in the world in 2009 (in Tons)

Top tomato producers in 2009 (in Tones)	
China	45,365,543
United States	14,141,900
India	11,148,800
Turkey	10,745,600
Egypt	10,000,000
World Total	152,956,115

Food and Agriculture Organization, (FAOSTAT, 2009)
Source: http:// en.wikipedia.org/wiki/Tomato.

However, in 1893, the U.S. Supreme Court declared it a vegetable. Tomato varieties are commonly divided into these categories: cherry sweet tomatoes, usually eaten whole in salads. Plum is a pear shaped, meatier, ideal for tomato products, also called Italian or Roma. Slicing is round or globe-shaped, used mainly for commerce and processed products. Beefsteak is rounding, juicy, used mainly for sandwiches. Other varieties include heirlooms, green, orange and yellow tomatoes. Yellow and orange tomatoes tend to be sweeter than red and green varieties; only red tomatoes, which contain a red pigment, contain lycopene (www. Plants.usda.gov).

2.4. Nutrition of tomato

Tomatoes are now grown and eaten throughout the world. It is used in diverse ways, including raw in salads and processed into ketchup and tomato sauces. They contain a lot of vitamin and lycopene which is the most powerful natural antioxidants (Table 2.2). In some studies, lycopene, especially in cooked tomato has been found to help prevent prostate cancer (http//www.whoods.com). Lycopene has also been shown to protect oxidative damage in many epidemiological and experimental studies. In addition to its antioxidant activity, other metabolic effects of lycopene have also been demonstrated. The richest source of lycopene in the diet is tomato and tomato derived products (Evangelia *et al.*, 2005). Tomato consumption has been associated with decreased risk of breast cancer (Zhang *et al.*, 2009) head and neck cancers (Freedman *et al.*, 2008) and might be strongly protective against

neurodegenerative diseases (Rao and Balachandran, 2002; Fall *et al.*, 1999; Suganuma *et al.*, 2002).

Table 2.2. Nutritional value of red tomato per 100 g

Nutritional value of red tomato per 100g	
Energy	18 Kcal
Carbohydrates	3.9 g
Sugar	2.6 g
Dietary fiber	1.2 g
Fat	0.2 g
Protein	0.9 g
water	94.5 g
Vitamin A	42 µg
Vitamin C	14 mg
Vitamin E	0.54 mg
Vitamin K	237mg
Lycopene	2573 µg

Source: USDA Nutrient Database (2008)

2.5. Fusariumwilt of tomato, causes and symptoms

Fusarium wilt of tomato is caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc) Snyder and Hansen. Economic losses can result in tomato production especially when susceptible varieties of tomatoes are grown under warm climate, sandy soil, low nutrients in nitrogen and phosphorus content but high in potassium and low pH in the areas where tomatoes are grown intensively. *Fusarium* wilt has become one of the most prevalent and damaging diseases because the pathogen is persistent and may indefinitely infest the soils. The symptoms of this disease include strong downward bending of petioles; yellowing, wilting and drying of the lower leaves, often on one side of the plant extensive root necrosis resulting in stunted plant growth and under warm conditions, the plant may die (Agrios, 1997).

In general, *Fusarium* wilt first appears as slight vein clearing on the outer portion of the younger leaves, followed by downward drooping of the older leaves. At

the seedling stage, if the plants are infected by *F. oxysporum,* they may wilt and die soon after the symptom appears. In older plants, vein clearing and leaf epinasty are often followed by stunting, yellowing of the lower leaves, and formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of remaining leaves, and finally death of the entire plant (Agrios, 1997). Browning of the vascular tissue is strong evidence of *Fusarium* wilt. Furthermore, on older plants, symptoms generally become more apparent during the period between blossoming and fruit maturation (Smith *et al.*, 1988). The possible hosts of *Fusarium oxysporum* do not only include tomatoes but also potatoes, sugarcanes, garden beans, cowpeas, prickly pears, cultured zinnias, pansies, Assam rattleboxes, Baba's breaths, and *Musa* sp. like other plant pathogens, *Fusarium oxysporum* has several specialized forms – known as *formae specialis* (f. sp.), that infect a variety of hosts causing various diseases. These include *Fusarium oxysporum* f. sp. *asparagi* (*Fusarium* yellow on asparagus); f. sp. *lycopersici* (wilt on tomato); f. sp. *callistephi* (on China aster); f. sp. *cubense* (Panama disease wilt on banana); f. sp. *dianthi* (wilt on carnation); f. sp. *koae* (on koa); f. sp. *melonis* (on muskmelon); f. sp. *nuvenum* (wilt on watermelon) ; f. sp. *pisi* (on edible pea pod); f. sp. *tracheiphilum* (wilt on *Glycine max*); and f. sp. *zingiberi* (*Fusarium*yellow on ginger) (Jones *et al.,* 1991).

2.6. Biology of the pathogen

In solid culture media such as potato dextrose agar (PDA), the special forms of *F. oxysporum* can have varying appearances. In general, the aerial mycelia first appear white, and then change to a variety of color ranging from violet to dark purple according to the strain (or special form) of *F. oxysporum.* If sporodochia are abundant, the culture may appear cream or orange in color (Smith *et al.,* 1988). *F. oxysporum* produces three types of asexual spores such as microconidia, macroconidia and chlamydospores (Agrios, 1997). Microconidia are one or two celled, and are the type of spore most abundantly and frequently produced by the fungus under all conditions. It is also the type of spore most frequently produced with vessels of infected plants. Macroconidia are three to five celled, gradually pointed and curved toward the ends. These spores are commonly found on the surface of plants killed by this pathogen as well as in sporodochia-like groups. Chlamydospores are round, thick-walled spores, produced either terminally or intercalary on older mycelia

or in macroconidia (Domsch *et al.*, 1993) and Booth (1971) reported that *F. oxysporum* produced fast growing colonies which reach 4.5 – 6.5 cm in diameter in the fourth day at 25° C, aerial mycelia are sparse to abundant and floccose, becoming flat, white or peach, but usually with a purple or tinge of violet. Microconidia are generally abundant and mostly borne on short simple lateral phialide or from sparsely branched conidiophores and never form chains, mostly 3 - 5 septate, ellipsoidal to cylindrical, straight or often curved, 5 - 12 x 2.3 - 3.5 µm. Chlamydospores are terminal or intercalary in hyphae. Conidia are hyaline, smooth – walled or rough end; 5-15 µm. Sclerotial pustules are present in some isolates, pale to green or deep violet.

2.7. Disease cycle

Fusarium oxysporum are abundant and active saprophyte in the soil and organic matter, with some specific forms which are pathogenic to plants. Its saprophytic ability enables it to survive in the soil between crop cycles in infected plant debris (Smith *et al.*, 1988).The fungus can survive either as mycelia, or as any of its three different spore types. Healthy plants can be infected by *F. oxysporum*. If they are grown in soil contaminated with the fungus. The fungus can invade a plant either with its sporangial germ tube or mycelium by invading the roots of the plants. The roots can be infected directly through the root tips, through wounds in the roots or at the formation point of the lateral roots. Once inside the plant, the mycelium grows through the root cortex intercellular. When the mycelium reaches the xylem, it invades the vessels through the xylem pits. At this point, the mycelium remains in the vessels, where it usually advances upwards toward the stem and crown of the plant. As it grows the mycelia branch and produce microconidia, which are carried upward within the vessel by way of the plants sap stream. When the microconidia germinate, the mycelia can penetrate the adjacent xylem vessels through the xylem pits. Due to the growth of the fungus within the plant's vascular tissue, the plant's water supply is greatly affected. This lack of water induces the stomata to close, the leaves wilt, and the plant eventually dies. At the point, the fungus invades the plant's parenchymatous tissue until finally reaching the surface of the dead tissue, it sporulates abundantly. The resulting spores can then be used as new inocula for further spread of the fungus. The fungus invades all tissues; however, if the temperature is low, the infected plants

may produce good yield, in such case the fungus may reach to fruits and penetrate into seeds (Agrios, 1997). The disease cycle is shown in Figure 2.1.

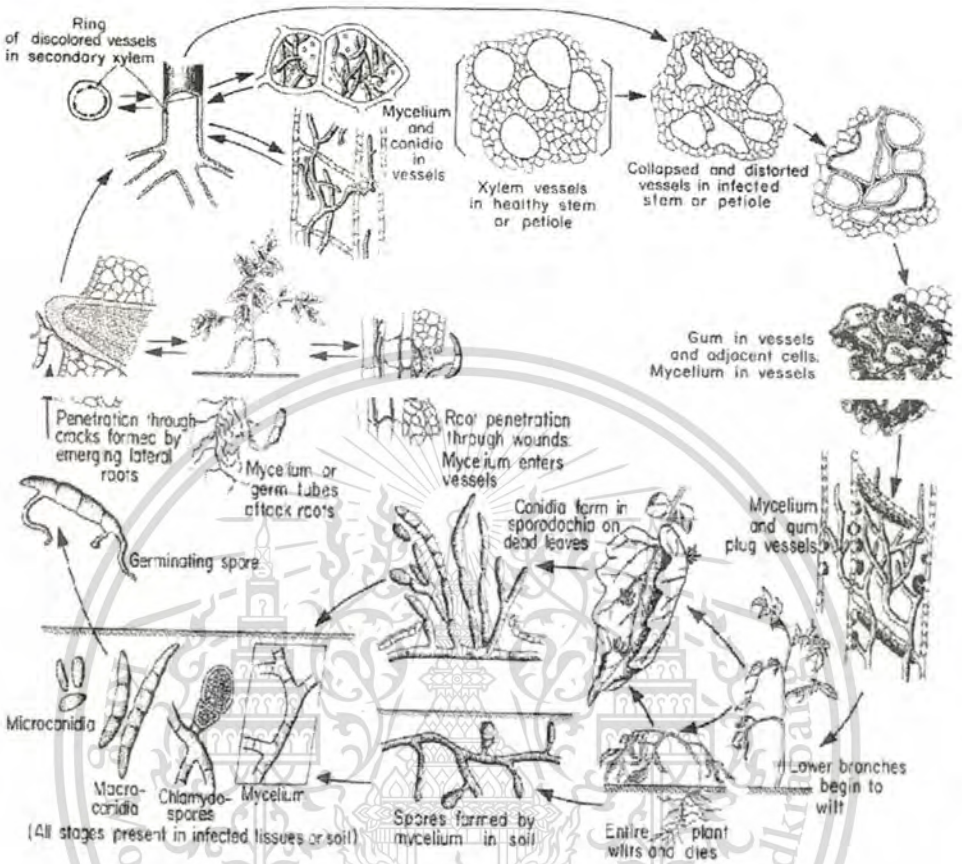


Figure 2.1. Disease and life cycle of *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt (Agrios, 1997).

2.8. *Chaetomium* as antagonistic fungus

Chaetomium is a fungus belonging to Ascomycota of the family Chaetomiaceae which established by Kunze in 1817 (von Arx *et al.*, 1986). *Chaetomium* Kunze is one of the largest genera of saprophytic ascomycetes which comprise more than 300 species worldwide (von Arx *et al.*, 1986; Soytong and Quimio, 1989; Udagawa *et al.*, 1979. *Chaetomium* species are well known as coprophilous, seed and soil fungi (Somrithipol, 2004), and also found in organic compost (Soytong *et al.*, 1990). They degrade cellulose and other organic material and act as antagonist against plant pathogens (Soytong *et al.*, 2001). *Ch. globosum* is reported by several researchers to be a strong cellulose decomposer and express a

very effective antagonist of various soil microorganisms (Aggarwal *et al.*, 2004; Dhingra *et al.*, 1987; Soytong *et al.*, 2001).

In Thailand, *Chaetomium* species were screened for their abilities as antagonist in 1989 (Soytong *et al.*, 2001). It has also been reported that some isolate of *Ch. globosum* produce antibiotic substances that can suppress the damping – off of sugar beet cause by *Pythium ultimum* (Di-Pietro *et al.*, 1991). *Ch. cupreum* and *Ch. globosum* have been reported to reduce leaf spot disease of corn cause by *Curvularia lunata*, rice blast cause by *Pyricularia oryzae*, sheath blight of rice cause by *Rhizoctonia oryzae* and tomato wilt cause by *Fusarium oxysporum* f.sp. *lycopersici* (Soytong, 1992). Moreover, *Chaetomium* species are noted for their secondary metabolites content with biological activities. Several types of pure compounds have been investigated from *Chaetomium* species e.g. benzoquinone derivatives (Brewer *et al.*, 1968). A new anthraquinone – chromanone compound named chaetomanone and seven known compounds, ergosterol, ergosterylpalmitate, chrysophanol, chaetoglobosin C, alternariolmonomethyl ether, echinuline and isochaetoglobosin D were found from *Ch. globosum* KMITL-No802 and also reported that chaetomanone and echinulin showed activity towards *Mycobacterium tuberculosis* (Kanokmedhakul *et al.*, 2001). The searches for promising microbial antagonists have been increased tremendously to control diseases. There were numerous reports indicating that *Ch. globosum* could control seedling blight of wheat caused by *Helminthosporium victoriae* (Tveit and Moore, 1954). Spraying the spore suspension of *Ch. globosum* could significantly control apple scab caused by *Venturia inequalis* (Cullen *et al.*, 1984).

Ch. cupreum was also reported to be antagonistic to *Phomopsis sojae* which is a seed-borne pathogen of soybean(Manandhar *et al.*, 1986) and could significantly reduce the growth of seed-borne pathogen of rice e.g. *Curvularia lunata, Drechslera oryzae, Fusarium moniliforme*, and *Pyricularia oryzae* (Soytong, 1992). *Ch. globosum* was reported to significantly suppress tomato wilt in Thailand caused by *Fusarium oxysporum* f. sp. *lycopersici*, and *Pseudomonas solanacearum* (Soytong, 1990 and 1991) while *Ch. cupreum* could control tomato wilt in the fields (Soytong, 1992). The effective strains of *Chaetomium* spp. have been formulated as biological products in the forms of pellet and powder (Soytong, 1993) could effectively control many soil borne plant pathogens (Soytong, 1996). Moreover, the *Chaetomium* product

has a good potential in control of Thielaviopsis Bud Rot of Bottle palm caused by *Hyophorbela genicaulis* (Soytong *et al*, 2005).

The findings of new antagonistic fungi are in progress, *Emericella nidulans* strain (EN01),*Chaetomium elatum* strain (CE01), *Emericella rugulosa* strain (ER01) and *Chaetomium cochlioides* strain CH-VT were reported to be promising antagonistic fungi against plant pathogens in Thailand (personal communication to Dr. Kasem Soytoy). Phonkerd *et al* (2008) reported that *Chaetomium cochlioides* strain Vth01 and CTh 05 can produce new dimericspiro-azaplilones, cochliodones, two new azaphlones, (Figure 2.2) chetoviridines E and F, a new epi-chaetoviridin A that exhibited antimalarial activity against *Plasmodium falciparum* and antituberculosis against *Mycobacterium tuberculosis*, and cytotoxicity against KB, BC1 and NCI-H187 cell lines.

These are promising antagonistic fungi that may possibly produce antibiotics against plant pathogen that implies antibiosis. Moosophon *et al* (2007) reported that chromatographic separation of the crude hexane extract from *E. rugulosa* strain ER, *Ch. elatum* strain CE and *Ch. cochlioides* strain CH-VT from Dr. Kasem Soytong of KMITL, Bangkok, Thailand has led to the isolation of 6 xanthonesshamixanthone (**1**), isoemericellin (**2**), tajixanthone (**3**), tajixanthonemethanoate (**4**), 14-methoxytajixanthone-25-acetate (**5**), and tajixanthone hydrate (**6**). Their structures were identified by spectroscopic method. Removal of solvent from each extract gave crude hexane (8.3g), EtOAc (5.9g), and MeOH extracts (10.9g). The bioactivity evaluation of these isolated compounds is in progress and expressed to be inhibition of some fungal plant pathogens.

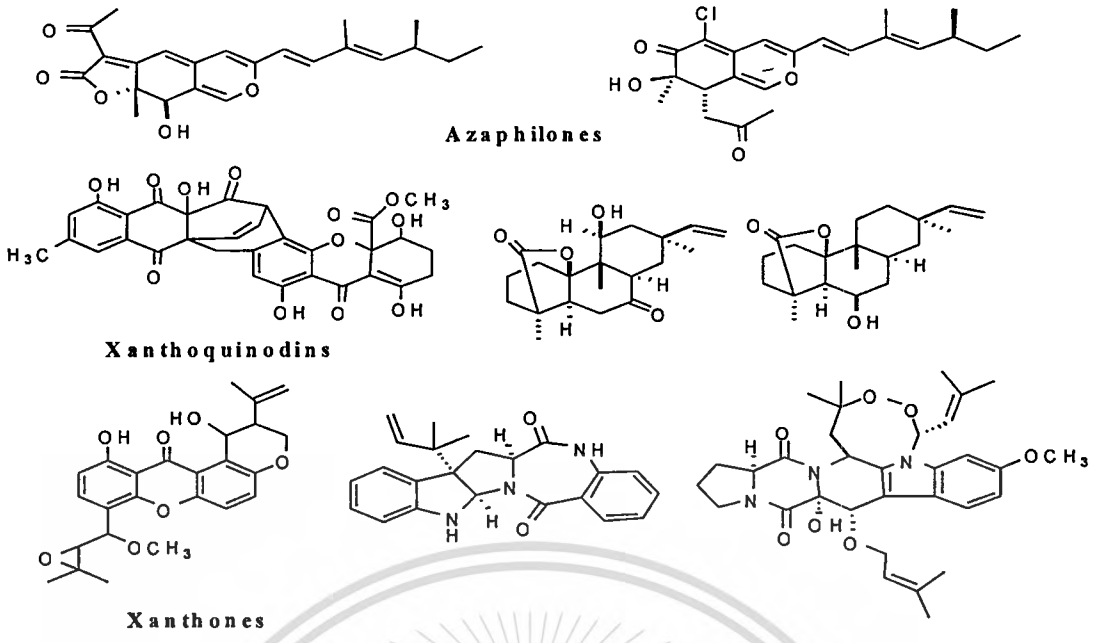


Figure 2.2. Chemical structures of the isolated compounds from *Chaetomium cochlioides* strain Vth01 and CTh 05 (Phonkerd *et al.*, 2008).

In this research finding of *E. nidulans* strain EN, *Ch. elatum* strain CE, *E. rugulosa* strain ER and *Ch. cochlioides* strain CH-VT was firstly reported for those fungi to release some antibiotic substances against human and plant pathogens. Some of these compounds exhibited activity towards *Plasmodium falciparum* (cause of malaria), *Mycobacterium tuberculosis* (TB), *Candida albicans* and cancer cell lines (KB, BC and NCI-H187). Those compounds also showed activity against plant diseases such as *Phytophthora* sp. causing root rot of plants and *Colletotrichum gloeosporioides* causing anthracnose disease (personal communication, Dr. Kasem Soytong). Figure 3 shows the chemical structures of the isolated compounds. Furthermore, *Ch. cochliodes* VTh 01 and *C. cochliodes* CTh 05 were reported to be antagonistic to *F. oxysporum* f.sp. *lycopersici* causing tomato wilt (Phonkerd *et al.*, 2008), Chaetominedione is reported as a new tyrosine kinase inhibitor isolated from the algicolous marine fungus *Chaetomium* sp. (Abdel, 2008).

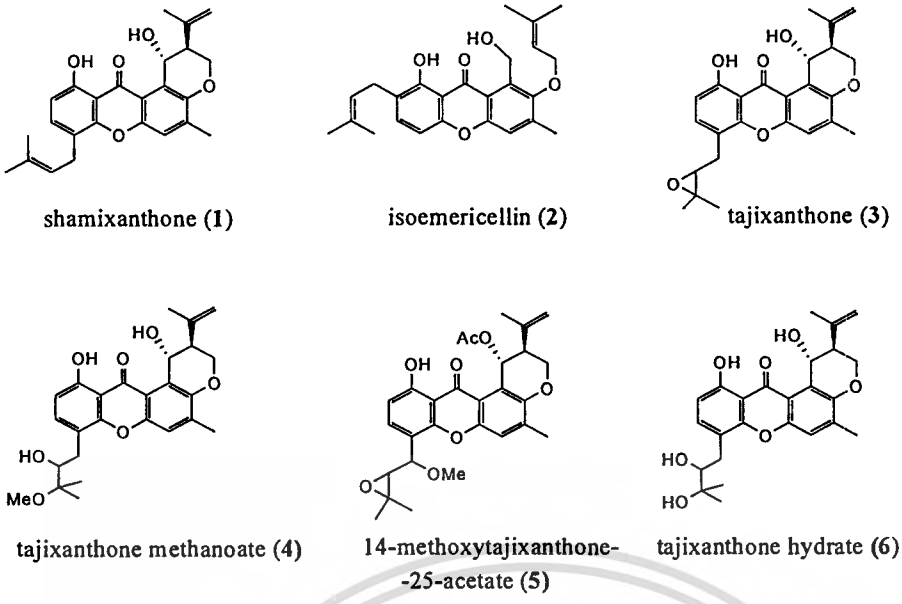


Figure 2.3. Chemical structures of the isolated compounds from *Emericella rugulosa* strain ER01(Moosophon *et al.*, 2007)

The bioactive compounds from antagonistic fungi against plant pathogens have been studied by some researchers which act as one of the control mechanisms that implies antiobiosis. Srinon *et al.* (2004) reported that thebioactive compounds extracted from *Scleroderma citrinum* and *Ch. globosum* CG were tested to inhibit spore production of plant pathogenic fungi e.g. *Colletotrichum gloeosporioides* (citrus anthracnose), *Fusarium oxysporum* f. sp. *lycopersici* (tomato wilt), *Phytophthora parasitica* (root rot of citrus) and *P. palmivora* (root rot of black pepper). Results showed that tested bioactive compounds significantly inhibited sporulation of tested pathogens.

Talubnak *et al.* (2010) reported that the bioactive compound extracted from *Chaetomella* sp. coded as CH/E gave the highest inhibition of *F. oxysporum* f. sp. *lycopersici* causing tomato wilt with the ED_{50} value of 1.09 µg/ml that is significantly different when compared to the non-treated one and followed by the bioactive compounds coded as CH/H (*Chaetomella* sp), CgChi/H, CgChi/M, CgChi/E (*Chaetomium cochliodes*) and Bb/E (*Beauveria bassiana)* with the ED_{50} values of 108, 203, 323, 416 and 817 µg/ml, respectively. They also reported that the bioactive compounds coded Bb/H extracted from *Beauveria bassiana* gave the highest inhibition of *Phytophthora parasitica* causing pummelo root rot with the ED_{50} of 95 µg/ml followed by the bioactive compounds coded as SKP04 (*Emericella rugulosa*),

Bb/M (*Beauveria bassiana*), Bb/E (*Beauveria bassiana*), CgChi/H (*Chaetomium cochlioides*) with the ED_{50} values of 117, 133, 254, 877 and 808 µg/ml, respectively.

2.9. *Emericella* as antagonistic fungus

E. nidulans belongs to Ascomycota. Its colony shows dark green and brown. Colony is growing fast on coconut water dextrose agar (CWDA) medium at room temperature (Sibounnavong *et al.*, 2009). Fruiting structure globose, 125-150 µm surrounded with hullee cells, ascospores purple red, lenticular, smooth walled with two equatorial crests. It can produce anamorph (imperfect stage) namely *Aspergillus nidulans* which produces conidia on phialides, phialide borne in head or vesicle that stand on phialophore and food cell (Domsch *et al*, 1993).

E. nidulans exerted inhibitory activity against *F. oxysporum* f.sp. *lycopersici* in bi-culture test, the conidia and hyphae of the pathogen were deformed, abnormal in shape and showed plugs of protoplast inside cells. Moreover, the crude extract of *E. nidulans* by methanol solvent could inhibit the mycelia growth and spore production of *F. oxysporum* f.sp. *lycopersici* in-vitro (Sibounnavong *et al*, 2009).

The mycofungicide oil and powder formulations of *E. nidulans* gave highly significant to control Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici*. It is also noted that tomato treated with oil and powder formulations of *E. nidulans* gave the highest yield such as fruit weight, number of fruit per plant when compare to the treatment that treated with chemical fungicide (Sibounnavong *et al*, 2010).

2.10. Molecular study

Morphological characterizations of *Fusarium* species which emphasize on microscopic and cultural characteristics are not sufficient to characterize *Fusarium oxysporum* f. sp. *lycopersici* (FOl) from tomato as these characteristics could easily influence by environmental factors. As an alternative molecular methods were used to characterize and to assess genetic variation of FOl from different tomatoes cultivars. Knowledge on the genetic variation is important to determine the genetic relationship between FOl isolates from different tomatoes cultivars. The *Fusarium* species have traditionally been differentiated by their morphological characteristics on selective media (Burgess *et al.*, 1994). However, identification of pathogenic types, or forma

speciales and races of *Fusarium oxysporum* using morphological features is not enough for identification. An inoculation assay using tester plants has been a popular approach of identification of forma speciales. However, this is a time-consuming approach; thus necessitating development of other methods (Woo *et al.*, 1994).

Recently, molecular markers have become popular for identifying species and subspecies in fungi. Some of the techniques that have been reported include amplified fragment length polymorphisms (AFLP) (Vos *et al.*, 1995), random amplified polymorphic DNA (RAPD) (Kalc *et al.*, 1996), and restriction fragment length polymorphisms (RFLP) (Baayen *et al.*, 2000) direct amplification of length polymorphism among others. Differentiation of the *Fusarium* species/subspecies based on comparison of DNA sequences of the ribosomal DNA (rDNA) and internal transcribed spacer (ITS) regions have been reported (Schilling *et al.*, 1996). More recently, Hirano and Arie (2006) have reported differentiation of *Fusarium oxysporum* f. sp *lycopersici* and f .sp *radicislycopersici* by a polymerase chain reaction (PCR)-based method using specific primer sets developed from the knowledge of the partial nucleotide sequences of the *endo (pg1)* and *exo* (*pgx4*) polygalacturonases genes of the fungi. Latiffah *et al.* (2009) stated that Three *Fusarium* species, *F. oxysporum*, *F. proliferatum* and *F. solani* were isolated from the root and stem rot of *Dendrobium* orchid. Their pathogenicity showed that the three *Furarium* species were pathogenic causing root and stem rot on the orchid. Molecular characterization using PCR-RFLP of ITS+5.85 regions showed that the isolates produced similar pattern and UPGMA cluster analysis clearly grouped them were associated with root and stem rot of Dendrobium orchid.

Most of *Fusarium* spp is known as plant pathogenic strain, that cause many diseases such as wilt, root rot and crown rot diseases on a various variety of crops (Nelson *et al.*, 1981). Many researches on *Fusarium* spp. have been focused on studying for plant pathogenic isolates (Mohammadi *et al.*, 2004; Pasquali *et al.*, 2004). However, the nonpathogenic groups represent a significant proportionality of the isolates found and keep most genetic diversity within this species complex (Bao *et al.*, 2002). There is a large deal of genetic relationships between pathogenic and non-pathogenic *F. oxysporum* isolates (Baayen *et al.*, 2000). Skovgaard *et al.* (2002) suggested that particular pathogenic isolates might germinate from non pathogenic strains by mutations affecting a few loci. Some nonpathogenic isolates have been studied to change from pathogenic isolates through loss of virulence (Skovgaard *et*

al., 2002). James *et al.* (2000) reported that some isolates of *Fusarium oxysproum* were highly virulent, whereas others were nonpathogenic fungi. Moreover, both highly virulent isolate and nonpathogenic isolate are not different based on morphological study. Therefore, methods are needed and importance to identify and quantify population of highly virulence of *F. oxysporum* which are molecular techniques that can be used to study. Baayen *et al.* (2000) and Mayak *et al.* (2004) stated that molecular markers have been used to study genetic relationships for pathogenicity in many group of fungi. Using the histone-H3 encoding gene and amplified fragment length polymorphisms (AFLPs) could be used for studying genetic differences between highly virulence, low virulence and nonpathogenic isolates of *F. oxysporum*. These previous results suggested that molecular marker can be used to separate these two phenotypes and compare the phylogenetic relationships of highly virulent *Fusarium* spp. (Donaldson *et al.*, 1995). Amplified fragment length polymorphisms (AFLP) is a powerful technique in molecular marker for studying relationships among isolates of fungi between population and species levels (Cunningham, 1997; Kauserud and Schumacher, 2003; Nelson *et al.*, 1983; Skovgaard *et al.*, 2003; Majer *et al.*, 1998). Moreover, AFLP analysis has been used to investigated genetic variation within and between among different *Fusarium* spp. (Adb-Elsalam *et al.*, 2002; Kiprop *et al.*, 2002; Sivaramakrishnan *et al.*, 2002).

2.11. Biological control

Biological control has become a potential for disease management. A variety of soil microorganisms have demonstrated activity in the control of various soil borne plant pathogens, including Fusarium wilt pathogens. Fusarium-suppressive soils are known to occur in many regions of the world, and suppression has generally been shown to be biological in origin (Alabouvette *et al*, 1998).

Antagonists recovered from Fusarium-suppressive soils, especially nonpathogenic *F. oxysporum,* have been used to reduce Fusarium wilt diseases of several different crops (Larkin *et al.*, 1991). Other biocontrol fungi, such as *Trichoderma* and *Gliocladium* spp have been used to control a variety of fungal pathogens, including *Rhizoctonia, Pythium, Sclerotinia, Sclerotium* and *Fusarium* (Harman, 1991; Lewis *et al.*, 1993 and 1995), and may also be effective against *Fusarium* wilt diseases (Sivan and Chet., 1993 and Zhang *et al*,. 2009).

In addition, several lesser-known groups of biocontrol fungi, including *Laetisaria, Stilbella, Cladorrhinum*, and *Penicillium* spp., have been used to control soil borne pathogens and may have activity against Fusarium diseases. Rhizobacterial strains of *Pseudomonas, Burkholderia,* and *Bacillus* have been used to reduce disease caused by a variety of soilborne pathogens, including *Fusarium* spp. (Decal *et al.*, 2004; Lewis *et al.*, 1995).

Although many different biocontrol strains have showed a potential for some degree of control of Fusarium diseases, strains which can provide the best control of Fusarium wilt of tomato and have potential for effective implementation in commercial agriculture have not yet been identified. It has been suggested that microorganisms isolated from the root or rhizosphere of a specific crop may be better adapted to that crop and may provide better control of diseases than organisms originally isolated from other plant species Such plant associated microorganisms may make better biocontrol agents because they are already closely associated with and adapted to the plant or plant part as well as the particular environmental conditions in which they must function (Cook, 1993).

The screening of such locally adapted strains has yielded improved biocontrol in some cases. The combination of multiple antagonist organisms may provide to improve disease control over the use of single organisms. Multiple organisms may enhance the level and consistency of control by providing multiple mechanisms of action, a more stable rhizosphere community, and effectiveness over a wider range of environmental conditions. In particular, combinations of fungi and bacteria may provide protection at different times or under different conditions, and occupy different or complementary niches. Such combinations may overcome inconsistencies in the performance of individual isolates. Several researchers have observed improved disease control using various combinations of multiple compatible biocontrol organisms (Duffy *et al.*, 1996). Lemanceau and Alabouvette (1991) have demonstrated to enhance biocontrol of Fusarium wilt by combining certain nonpathogenic strains of *F. oxysporum* with fluorescent strains of *Pseudomonas.*

Biological control is another way to avoid the environmental pollution to minimize the intensive use of pesticide (Ibrahim, 2011). *Trichoderma* spp. were commercially applied as biological control agents against fungal pathogen, where *T. harzianum* was found to be effective against the growth of *F. culmorum* (Sharma, 1997) while the dual culture of *T. harzianum* and *T. viride* were most effective in

reducing the mycelia growth of *F. oxysporum* and *Pythium aphanidermatum* (Ark and Thompson, 1959). Basic environmental conditions, such as temperature, moisture, sunlight, and soil physical and chemical characteristics, can greatly affect the physiology of the host plant and subsequent disease development, as well as alter the interactions among plant, pathogen, and biocontrol agent in various ways. This is in addition to potential direct effects on the pathogen and biocontrol organisms and other soil microbes, and all of these effects may influence efficacy of biological control. Other conditions related to the specific pathosystem involved, such as the occurrence of different pathogenic races and variability in disease resistance and susceptibility among host cultivars, also affect the disease response and may influence biological control. There are three known races of *F. oxysporum* f. sp. *lycopersici*, the causal agent of Fusarium wilt of tomato (Alexander *et al.*, 1945, Davis *et al.*, 1988, and Volin and Jone, 1982).

Tomato cultivars with inherent genetic resistance to races 1 and 2 are available and widely used. However, very few cultivars with resistance to race 3 exist, and none are commercially accepted and used. This race is currently causing substantial losses in many tomato-growing areas for biocontrol to be effective, it must control all three races of the pathogen, as well as be capable of controlling disease on many different tomato cultivars with varying levels of disease susceptibility and resistance (Chellemi and Dankers., 1992, Jone *et al.*, 1991 and Mariatt *et al.*, 1996).

On other hand, non pathogenic strain of *Fusarium* spp. have suppressed soil borne disease caused by *Fusarium* spp. under greenhouse and field conditions (Fuchs *et al.*, 1997). Furthermore, a non pathogenic *F. oxysporum* isolate, Fo-B2 has been shown to reduce disease severities of Fusarium wilt of tomato under greenhouse and field conditions (Amemiya, 1996).

Moreover, the efficiency of the biocontrol agent Fo-B2 in suppressing Fusarium wilt of tomato varied under different cultural environments. The ratios of an ED_{50} parameter for Fo-B2 to that of the pathogen CU1 based on their dose-response relationships increased as the environment became less controlled, suggesting that environmentally related efficiency reduction impacted the biocontrol agent more than the pathogen. Recovery of Fo-B2 from seedling hypocotyls and severity of disease indicated that Fo-B2 was most effective when it colonized vascular tissues of the host intensively, and the degree of colonization by the beneficial organism was greatly reduced when soil was not sterile. These results suggest that indigenous populations

of soil microorganisms negatively influenced the efficiency of Fo-B2 inoculations; therefore, early establishment of the fungal antagonist in tomato seedlings prior to out planting may improve the efficacy of this biological control strategy (Shisido *et al*, 2005).

Biological suppression of plant disease has been promoted as a mean to achieve improved. Sustainable crop production systems that are less reliant on chemical input (Adam, 1990). Successful biological control systems commonly employ naturally occurring, antagonistic microorganism that are able to reduce the activities of plant pathogens. Such antagonists (or biological agents) can compete with pathogen for nutrient, inhibit pathogen growth by secreting antibiotics, or reduce pathogen population by through parasitism. In addition some of these microorganisms reduce resistance in host plants, which enhance the plant's ability to defense from pathogen attack. It is essential to determine how biological control may be affected by changing environmental conditions. The influence of varying environmental and cropping conditions including temperature, soil type, light and pathogen isolates, race and cultivar of tomato on biological control of Fusarium wilt of tomato when the temperature regimes ranging from 22-32°C significantly affected disease development and plant physiological parameters (Larkin and Fravel, 1998).

Other conditions related to the specific pathosystem involved, such as the occurrence of different pathogenic races and variability in disease resistance and susceptibility among host cultivars, also affect the disease response and may influence biological control. There are three known races of *F. oxysporum* f. sp. *lycopersici*, the causal agent of Fusarium wilt of tomato (Alexander *et al.*, 1945, Davis *et a.,* 1988; Grattidge and Obrien, 1982; Volin and Jones, 1982).

CHAPTER III
RESEARCH METHODOLOGY

3.1 Collection, Isolation, Identification and pathogenicity test of Fusarium wilt pathogen

Disease samples were collected from infested soil from tomato fields in Bangkok, Pechaboon, and Tak provinces in Thailand. The pathogen was isolated by tissue transplanting and soil plate techniques. Pure cultures of *Fusarium* spp were identified by morphological characteristics under a binocular compound microscope and molecular phylogenic identification. All isolates were maintained on PDA slants and deposited at the Biocontrol Research Unit and Mycology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Twelve isolates of *F. oxysporum*.f. sp. *lycopersici*, which isolated from Burirum, Khonkaen, Nongkhai, Nakhonratchasima and Sakon-nakhon provinces in Thailand were obtained from Assist. Prof. Dr. Chamaiporn Charoenporn (Nakhonratchasima Rachabhat University, Nakhonratchasima, Thailand) as follows:- isolates BRCO3, KK2, KSoC02, NKRC02, NKRC04, NKRC09, NKSC01, NKSC02, NSC01, SRC02, SSoC03, and SSoC04. The morphological identification of the 12 isolates had been confirmed previously by Charoenporn *et al.* (2010) by sequencing the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA and a small portion of the 28S rDNA.

3.1.1. Morphological Identification

All the isolates of *Fusarium* spp. were cultured on PDA at the centre of the Petri dishes (9cm diameter) and incubated in the room temperature approximately (27-30°C) and studied under compound microscope for morphological characteristics. The characteristics were recorded are growth rate of colony every 48 hours (mm), hyphal characteristics, shape and size of spore, types of spores and other structures that needed for morphological identification were also recorded, measured, and taken the photo under compound microscope and comparison with the isolates were offered by Assist. Prof. Dr. Chamaiporn Charoenporn as the standard for identification.

3.1.2 Molecular Identification

The molecular study is extremely important for confirmation the species. All isolates were studied for confirmation of species, relationship between isolate to variety of tomatoes and collected sources by techniques of AFLP, according to the method of Mohmed *et al.* (2003).

3.1.3 Genetic relationship among isolates of *F. oxysporum* f. sp. *lycopersici* by using AFLP marker

3.1.3.1. DNA extraction

50 mg of grounded fungal biomass was extracted with 0.5 ml of extraction buffer (50mM Tris-HCl, 850mM NaCl, 100 mM EDTA, and 1% SDS) and incubated at 65°C for 30 min then added with Phenol ($^1/_2$ vol) and Chloroform:IAA (24:1) ($^1/_2$ vol). After centrifugation at 13000 rmp for 10, the DNA on the upper aqueous phase was precipitated by additional 1vol of Chloroform:IAA (24:1). After centrifugation at 13000 rpm for 10 min, the DNA molecules were added with 2 vol of absolute ethanol and incubated at -20°Cfor 1 hour. After centrifugation at 13000 rpm) for 10 min, the DNA molecules were washed by 70% ethanol and centrifugation at 13000 rpm for 10 min in two times. The end product of DNA molecules were dissolved in 100 µl of TE (10mM Tris HCl 8.0, 1mM EDTA). The DNA concentration was measured using on 1% agarose gel electrophoresis.

3.1.3.2. Fingerprinting analysis using AFLP marker

The AFLP reactions were performed as described by Vos *et al.*(1995) and Mohmed *et al.* (2003) with the following modifications: Genomic DNA (500 ng.) was digested with a combination of restriction enzymes *Eco* RI (50 Units) and *Tru* 9I (*Mse* I) (10 uints) in a mix of 10x ligase buffer ATP, 0.5 M NaCl and BSA. The sample was incubated at 37° C for 1 hour. The digested DNA fragments were ligated to their respective adapter pair of both enzymes in a reaction of T4 DNA ligase (1u) and T4 DNA ligase buffer with ATP (1x) and incubated at 37° C for 3 hours.

After the restriction-ligation products were diluted 10 fold with TE buffer (10 mM tris, 0.1 mM EDTA,pH 8.0). The first amplifications were carried out with 1 selective nucleotide at 3' end of each primer in volume of 25 µl of PCR buffer containing PCR buffer (1x), dNTP (0.2mM), each primer (E+A/M+G, E+G/M+A, E+C/M+G and E+G/M+C) 5pmole, $MgCl_2$ (2.5mM), Taq polymerase (0.5u). This preamplification was carried out in a thermal cycler programmed for 20 cycles of 30 sec at 94°C, 60 sec at 56 °C, 60 sec at 72 °C and hold 16 °C for 15 min. The selective amplifications were performed using selected combinations of primers with two or three selective nucleotides (Table 1). All seventeen primers combinations were screened to investigate the most suitable primers. They were carried out in volumes of 20 µl of PCR buffer containing 5 µl diluted pre-amplified DNA, PCR buffer (1x), dNTP (0.2mM), each primer 5 pmole , MgCl2 (2.5mM) and Taq polymerase (1u). The PCR amplifications were performed with an initial denaturation at 94°C for 30 sec followed by 12 cycles of 94°C for 30sec, annealing at 65 °C each cycle was reduced by 1°C for 30 sec and extension step at 72 °C for 60sec. in each of the following 10 cycles, the annealing temperature was reduced by 1 °C. The next 30 PCR cycles continued of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 60 sec.

For gel analysis, the amplification reaction products were mixed with 10 µl of formamide dye (98% formamide, 10 mM EDTA pH 8.0, 0.3% bromo phenol blue and 0.3% xylene cyanol) and heat at 95 °C for 3 min and quickly cooled on ice. Each sample (2 µl) was examined on a 5% polyacrylamide gel plus 7M urea on a Model S2 sequencing gel electrophoresis apparatus. Electrophoresis was performed at constant power 50 W for 3 hr. After electrophoresis, the gel plate was removed, fixed in 10% acetic acid for 30 min, and washed in distilled water 3 times for 2 min. The gels plate were stained in silver solution (1 g of silver nitrate and 1.5 ml of 37% formaldehyde per liter) for 30 min and rinsed with distilled water. After staining, the gels were developed in a cool developer solution (30 g of sodium carbonate, 1.5 ml of 37% formaldehyde and 0.01 g of sodium thiosulfate) until the bands appeared. The staining was stopped by adding 10% acetic acid (fixed solution) for 1-2 min, rinsed with distilled water for 2 min and dried under fume hood overnight.

3.1.3.3. Data analysis

The fingerprint patterns were scored for polymorphic bands as binary data by 1 (present) or 0 (absent). The binary data was analyzed with the computer program NTSYS pc version 2.02 (Rohlf, 1993). An unweighted pair group arithmetic mean method (UPGMA) cluster analysis was performed using the DICE's similarity coefficient. Dendrogram was generated with the tree option (TREE) and a cophenetic value distance matrix was derived from dendrogram with a COPH program in NTSYSpc. The cophenetic value distance matrix was compared for level of correlation with the original matrix with the MXCOMP NTSYS program. Bootstrap values were calculated with 1000 replications by Winboot program (Yap and Nelson, 1996).

The total numbers of polymorphic bands were recorded in the GenAlex6 format. A principle coordinate plot based on genetic distances between all pairs of AFLP genotypes was generated in GenAlex6 and was used to generate a two-dimensional principal coordinate analysis was based on the population of AFLP genotypes in the PCA plot. Neighbor joining tree based Nei's (1978) genetic distance was generated using UPGMA modified from neighbor procedure of PHYLIP version 3.5.

3.1.4. Pathogenicity tests

All *F. oxysporum* isolates were tested for pathogenicity to tomato seedlings var Sida and Cherry using Koch's postulates to confirm pathogenic isolates. All isolates were sub-cultured and multiplied on PDA and incubated for 7–10 days at room temperature approximately (30–32°C). The inoculum of pathogen was adjusted to1×10^7 spores/ml before inoculating to 20–day–old tomato seedlings var. Sida. The roots of tomato seedlings were washed under running sterilized water and cut at five points on the root tips before dipping the roots into a 20 ml spore suspension for 15 min. A control was performed by dipping seedling roots into sterile distilled water. The seedlings were then potted in sterilized soil. After 10 days, symptoms of disease were recorded using the Disease Severity Index (DSI) and rated according to Sibounnavong *et al.* (2009, 2010) as follows: 1 = no symptoms, 2 = 1–20% of leaves yellow and wilted, 3 = 21–40% of leaves yellow and wilted, 4 = 41–60 % leaves

yellow and wilted, 5 = 61–80% of leaves yellow and wilted, and 6 = 81–100 % of leaves yellow and wilted. The experiment was conducted using a completely randomized design (CRD) with six replications of each treatment. The experiment was repeated twice. Virulence was categorized according to the DSI, following the method used by Charoenporn *et al.* (2010) as follows:- non-pathogenic (DSI =1), low virulence (DSI $\leq$ 3.50), moderate virulence (DSI > 3.50 – 4.50), and highly virulence (DSI > 4.50). The most virulent isolate was selected for further experiments (Fig. 3.1 and 3.2).

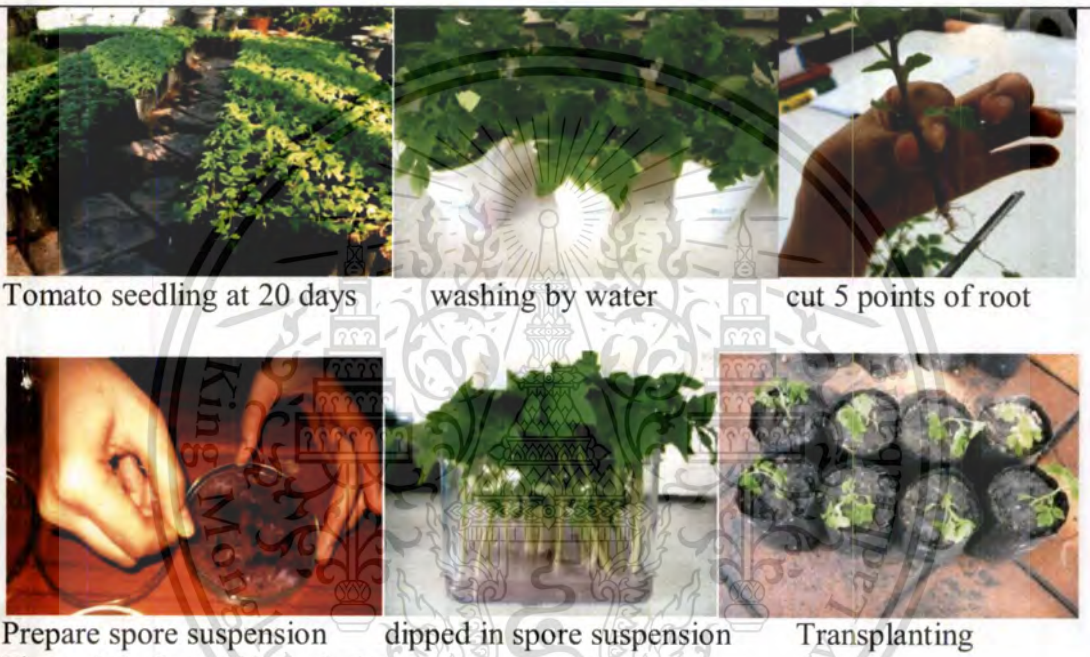


Figure 3.1. Step of inoculation

Figure 3.2. Disease severity index

Where level 1: healthy plant, 2: symptoms on leaves are wilt and yellow 1-20%, 3: Symptoms on leaves are wilt and yellow 21-40%, 4: Symptom on leaves are wilt and yellow 41-60%, 5: Symptom on leaves are wilt and yellow 61-80%and 6: Symptom on leaves are wilt and yellow 81-100%.

3.2. Study on Antagonistic isolates

The promising antagonistic fungi were offered from Assoc. Prof. Dr. Kasem Soytong. Six isolates of antagonists were studied as follows:- *Chaetomium brasilense* CB01, *Chaetomium cupreum* CC03, *Chaetomium elatum* ChE01, *Chaetomium lucknowense* CLT01, *Emericella nidulans* EN01 and *Emericella rugulosa* ER01. Those isolates were cultured on potato dextrose agar and periodically observed under compound binocular microscope to study morphological characters.

3.3. Antagonism tests

3.3.1 Bi-culture antagonistic tests

Antagonistic fungi namely *Chaetomium brasilense* CB01, *Chaetomium cupreum* CC03, *Chaetomium elatum* ChE01, *Chaetomium lucknowense* CLT01, *Emericella nidulans* EN01 and *Emericella rugulosa* ER01. These isolates were tested to determine their ability to antagonize the *F. oxysporum* f. sp. *lycopersici* isolate identified as the most virulent in the pathogenicity test. The test was conducted using the methods of Soytong (1992); Sibounnavong *et al.* (2009), and Charoenporn *et al.* (2010). 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 fungi and used to inoculate 9-cm diameter PDA plates: an agar plug of the pathogen was placed on one side of the plate opposite an agar plug of an antagonistic fungus. Plates inoculated with a single plug of an antagonistic fungus or of the pathogen acted as the controls. The plates were incubated at room temperature for 30 days. The experiment was performed using a completely randomized design (CRD) with four replications. Data were collected regarding colony diameter (cm) and the number of conidia produced by the pathogen. A haemacytometer was used to count the number of conidia. Percentage inhibition of pathogen colony growth and of conidia production was calculated using the following formula: - % inhibition = A-B/A × 100; where A = colony diameter or number of conidia produced by the pathogen on the control plate and B = colony diameter or number of conidia produced by the pathogen when inoculated opposite

an antagonistic fungus. Analysis of variance was statistically analyzed and treatment means were compared using Duncan's Multiple Range Test (DMRT) at $p = 0.05$ and 0.01. The experiment was repeated twice.

3.3.2 Bioactivities tests of crude extracts and pure compounds from antagonistic fungi

3.3.2.1 Extraction method

Crude extracts from each antagonistic fungus were obtained from the method used by Kanokmedhakul *et al.* (2006), Moosophon *et al.* (2009), and Thohinung *et al.* (2010). The fungi were cultivated in potato dextrose broth at room temperature for 30 days. The dried fungal biomass of each antagonistic fungus was ground and sequentially extracted with hexane, ethyl acetate, and methanol. The solvents were then evaporated *in vacuo* to yield crude hexane, crude ethyl acetate (EtOAc), and crude methanol (MeOH) extracts, respectively. The extracts were separated and purified using chromatographic methods to obtain the compounds. The structures of these compounds were identified by spectroscopic methods, IR, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR (COSY, HMQC, HMBC, and NOESY).

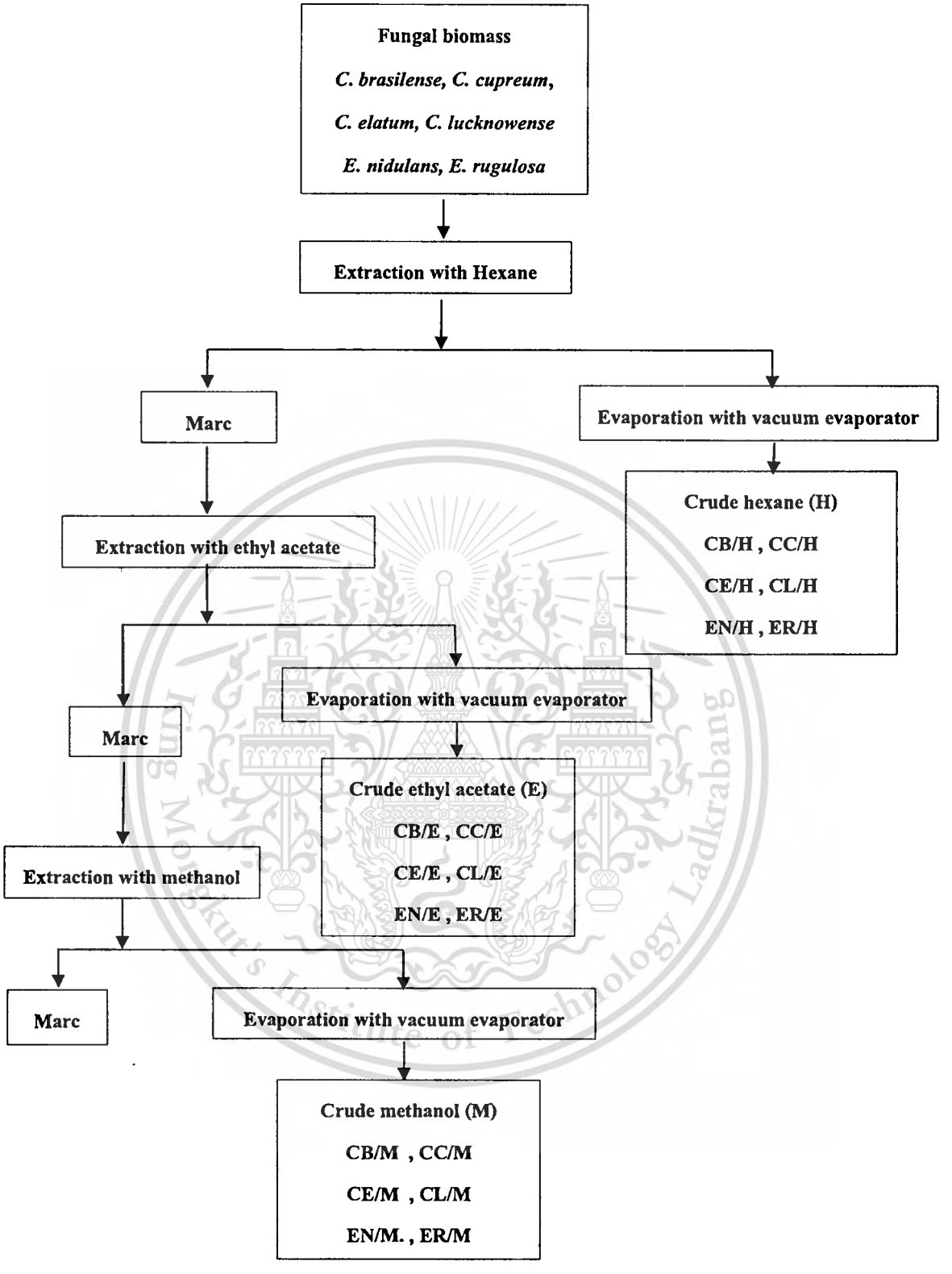
The crude extraction method was followed the method of Kanokmedhakul *et al.* (2006) and Suwannapong (2004). The mycelium disc of promising antagonistic fungi on PDA was taken sterilized cork borer with 3mm diameter from the edge of growing colony and transferred into PDB (Potato Dextrose Broth) then incubated in static state at room temperature for 30 days. The fungal mycelium mat was removed from PDB by cheesecloth filtration and air dried at room temperature approximately 27-30°C. The fresh and dry weight of mycelia mats were weighed and recorded. The dried mycelia mats of each promising antagonistic fungus were separately ground with electrical blender and dissolved with hexane (H) at 1:1 (vol/vol) for extraction and incubated at room temperature by shaking for one day.

The fungal extracting was separated out of the marc by filtration through filter paper (Whatman No.4). The marc was further extracted with ethyl acetate (EtOAc) and finally with methanol (MeOH) using the same procedure as extraction by hexane. The solvents extracted from hexane, ethyl acetate and methanol were separately extracted by using a rotary vacuum evaporator. Each crude extract was weighed and

kept in small bottle then kept in the refrigerator at 4°C until testing for experiment. Extraction method is shown in flow chart.

3.3.2.2 Bioactivities tests of crude extract against *Fusarium oxysporum* f. sp. *lycopersici*

The crude extracts were tested for inhibition of the most virulent isolate of *F. oxysporum* f. sp. *lycopersici*. The experiment was conducted by using a factorial experiment in CRD with four replications. Factor A represented the different solvents: A1 = crude hexane, A2 = crude ethyl acetate and A3 = crude methanol. Factor B represented the different concentrations: B1 = 0 µg/ml (control), B2 = 50 µg/ml, B3 = 100 µg/ml, B4 = 500 µg/ml and B5 = 1,000 µg/ml. Each crude extract was dissolved in dimethyl sulfoxide and added to PDA before autoclaving at 121°C (15 psi) for 30 minutes. To perform the assay, a sterilized 3-mm diameter cork borer was used to remove agar plugs from the actively growing edge of the pathogen culture. An agar plug was transferred to the center of 5 cm diameter Petri dishes of PDA containing crude extract at each concentration and incubated at room temperature until the pathogen on the control plates had grown over the plate. Data were collected regarding the number of conidia produced by the pathogen and used to



Flow chart of crude extraction of fungal biomass method

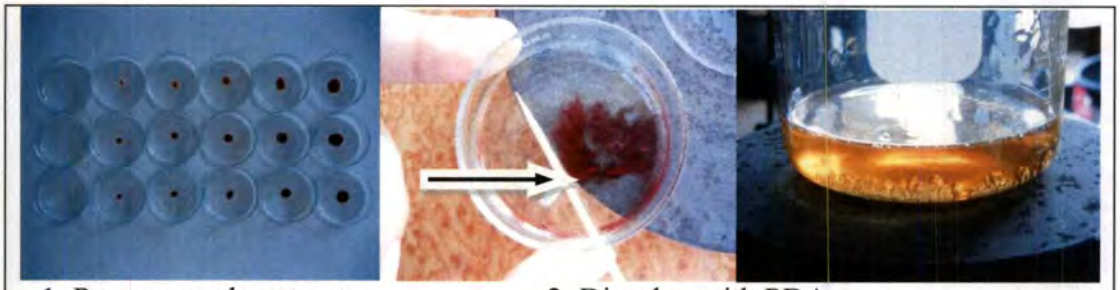
calculate as the percentage of conidia inhibition. The effective dose (ED_{50}) was calculated using Probit analysis. The experiment was repeated twice.

3.3.2.3. Pure compound bioassay against *Fusarium oxysporum* f. sp *lycopersici*

Pure compounds of chaetoglobosin-C from *Ch. elatum* and *Ch. lucknowense* and tajixanthone from *E. rugulosa* were offered from Assoc. Prof. Dr. Somdej Kanokmedhakul, Khon Kaen University, Thailand. It was separately tested for their antifungal activity against *F. oxysporum* f. sp. *lycopersici*. A sterilized 3-mm diameter cork borer was used to remove agar plugs from the actively growing edge of the pathogen culture. An agar plug was transferred to the center of 5-cm diameter Petri dishes of PDA containing either pure compounds of Chaetoglobosin–C or tajixanthone at each concentration and incubated at room temperature until the pathogen on the control plates grown over the plate. The experiment was performed using a CRD with four replications. Treatments comprised four different concentrations: 0, 10, 50 and 100 μg/ml. (Fig.3.3). The experiment was repeated twice. Data were collected regarding the number of conidia produced by the pathogen and calculated for percentage conidial inhibition. The ED_{50} was calculated using Probit analysis.

3.3.2.4. Effect of fungal metabolites to *Fusarium oxysporum* f. sp. *lycopersici* and its pathogenicity loss

The roots of 20– day– old tomato seedlings var. Sida were washed under running sterilized water and cut at five points on the root tips before dipping the roots into each treatment a 20 ml spore suspension of 1×10^7 spores/ml mixed with different concentration of pure compounds for 15 min. The experiment was conducted by using a factorial experiment in CRD with four replications. Factor A represented the pure compounds: A1 = tajixanthone, and A2 = Chaetoglobosin C. Factor B represented the different concentrations: B1 = 0 (control), B2 = 10, B3 = 50, and B4 = 100 μg/ml. A control was performed by dipping seedling roots into sterile distilled water. The seedlings were then planted in pots which contained sterilized soil. The experiment was repeated twice. Disease incidence was recorded using the Disease Severity Index used previously in the pathogenicity test.



1. Prepare crude extract 2. Dissolve with PDA

3. Sterilezed by autoclaving for 30 minutes 4. Poured into sterilezed plates

5. transferred agar plug of pathogen onto PDA mixed with crude extract and incubated at room temperature for 5-7 days

Figure 3.3. Technique for biological activities test

3.3.2.5. Effect of antagonistic crude extract for disease immunity of wilt incidence in tomato Sida variety

The experiment was conducted by using a CRD with four replications. Treatments were conducted as follows: T1= inoculated with *F. oxysporum* f. sp. *lycopersici*. T2= inoculated with the mixture of *F. oxysporum* f. sp. *lycopersici* and antagonistic crude extract 500 µg/ml., T3= inoculated with the mixture of *F. oxysporum* f. sp. *lycopersici* and antagonistic crude extract 1,000 µg/ml., and T4= non-inoculated with *F. oxysporum* f. sp. *lycopersici* which served as control. The roots of 20– day– old tomato seedlings var. Sida were washed under running sterilized

water and cut at five points on the root tips before dipping the roots into each treatment. A 20 ml spore suspension of 1×10^7 spores/ml mixed with different concentrations of crude extract for 15 min. The seedlings were then planted in pots which contained sterilized soil. The experiment was repeated twice. DSI was scored as previous experiment and disease immunity (%) was computed as follows:- DSI in control – DSI in treatment/ DSI in control X 100.

3.4. Evaluation of Bioformulations to control Fusarium wilt of tomato *in vivo*

The selected effective antagonists were screened to formulate and evaluate its efficacy to control Fusarium wilt of tomato var Sida in pot experiment. The bioformulations were separately performed as powder and oil bioformulations. The most effective antagonists were cultured on PDA until grown full plate, and then the spore were removed from the plates and used in each formulated fungicide which modified from the work of Soytong *et al.* (2001).

Soil preparation was prepared as soil mixture, sand : compost at the ratio of 8:2:2 (vol/vol/vol) before autoclaving at121°C or 15 lbs/inch^2 for 2 hours then put into pot for each treatment before planting the inoculated tomato seedlings. Sida variety was used in this experiment.

The virulence isolates of *F. oxysporum* f. sp. *lycopersici* was cultured on PDA for 7 days before spore suspension were made. Pathogen inocula were prepared at proper rate of concentration. Tomato seedlings at 20 days were used in the experiment. All tested tomato seedlings were inoculated with the most virulent isolate of *F. oxysporum* f. sp. *lycopersici*. The root tip of tomato were cleaned by running off water then the roots were cut for 2 cm at 5 points and dipped in the pathogen inoculum suspension for 10 min before transplanting into sterilized soil in pot experiment. After transplanting the tomato seedlings were treated every 10 days for each treatment until harvesting. The experiments were done using Randomized Block Design (RCBD) with four replications. Treatments were performed as follows:- T1=non-inoculated control, T2= inoculated with *F. oxysporum* f. sp. *lycopersici* (control), T3=powder formulation at the rate of 20 g/20 L of water by spraying every 10 days, T4=oil formulation at the rate of 20 ml/20 L of water by spraying every 10 days, T5=culture filtrate of effective antagonist at 20 ml/plants at every 10 days, and

T6=chemical fungicide (Prochoraz) 20 g/20 L of water. The experiment was repeated two times.

Data were collected as plant height (cm), plant weight (g), number of fruits/plant, weight of fruit (g) was collected. Disease severity index (DSI) was observed and rated every 30 days after inoculation based on a diseased rating scale. Disease index was scored by the method of Sibounnavong *et al* (2010).

The percent disease reduction (% DR) was calculated using the formula as follows: - A-B/A X 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 bioformulation.

Data were analyzed for significant differences using analysis of variance (ANOVA). Comparison among treatment mean using Duncan Multiple Range Test at P=0.05 and P=0.01.

CHAPTER IV
RESULTS

4.1. Collection, Isolation, Identification and pathogenicity test of Fusarium wilt pathogen

4.1.1 Sample collection and isolation of *Fusarium* spp.

Disease samples were collected from infested tomatoes in the fields in Bangkok, Phetchaboun, Tak, Nakhonratchasima, Burirum, Nongkhai, Sakonnakhon, and Khon kaen provinces. Samples were taken from rhizosphere soil and infected tomato plant which showed yellow and wilt. The pathogen was isolated by transferring surface-sterilized plant tissue to a potato dextrose agar (PDA) and soil plate technique on GANA medium following the methods use by Agrios (1997). Result showed that two isolates were isolated from Bangkok including BKRF01, BKRS01 isolates, 7 isolates PBRS101, PBRS102, PBRS103, PBRS104, PBRS201, PBRS202, PBRS203 were isolated from Phetchaboun, 4 isolates MSRS01, MSRS02, TRS01, and TRS02 from Tak . Twelve isolates of *F. oxysporum* f. sp. *lycopersici*, which isolated from Burirum, Khonkaen, Nongkhai, Nakhonratchasima and Sakon-nakhon provinces in Thailand were obtained from Assist. Prof. Dr. Chamaiporn Charoenporn (Nakhonratchasima Rachabhat University, Nakhonratchasima, Thailand) as follows:- BRCO3, KK2, KSoC02, NKSC01, NKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03, and SSoC04 isolates. The morphological identification of the 12 isolates had been confirmed previously by Charoenporn *et al.* (2010) by sequencing the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA and a small portion of the 28S rDNA. Pure cultures of *F. oxysporum* f. sp. *lycopersici* were identified by morphological characteristics under a binocular compound microscope. All the isolates of *Fusarium oxysporum* were maintained on PDA slants and deposited at the Biocontrol Research Unit and Mycology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand (Table 4.1). All the isolates were studied for morphological characters such as colony, conidiophores, macroconidia and microconidia on potato dextrose agar (PDA) which incubated at room

temperature (28-32˚C). Morphological characters of all isolates were identified under microscope and measured for their sizes.

Table 4.1 Isolates of *Fusarium oxysporum* f. sp. *lycopersici* from collection sites.

Sources	**Isolates***	**Samples**	**species**
Bangkok	BKRF01	root	*F. oxysporum*
	BKRS01	root	*F. oxysporum*
Phetchabun	PBRS101	fruit	*F. oxysporum*
	PBRS102	fruit	*F. oxysporum*
	PBRS103	fruit	*F. oxysporum*
	PBRS104	fruit	*F. oxysporum*
	PBRS201	root	*F. oxysporum*
	PBRS202	stem	*F. oxysporum*
	PBSS203	stem	*F. oxysporum*
Tak	MSRS01	stem	*F. oxysporum*
	MSRS02	stem	*F. oxysporum*
	TRS01	stem	*F. oxysporum*
	TRS02	stem	*F. oxysporum*
Burirum	BRC03	stem	*F. oxysporum*
KhonKaen	KK2	stem	*F. oxysporum*
	KSoC02	stem	*F. oxysporum*
Nongkhai	NKSC01	stem	*F. oxysporum*
	NKSC02	stem	*F. oxysporum*
	NKRC02	root	*F. oxysporum*
	NKRC04	root	*F. oxysporum*
	NKRC09	root	*F. oxysporum*
Nakhonratchasima	NSC01	rhizosphere soil	*F. oxysporum*
Sakon Nakhon	SRC02	soil	*F. oxysporum*
	SSoC03	rhizosphere soil	*F. oxysporum*
	SSoC04	rhizosphere soil	*F. oxysporum*

* 1st alphabet means provinces:- BK = Bangkok, P = Pathumthani, N = Nakhon Ratchasima, B = Burirum, S = Sakon Nakhon, K = Khon Kaen, NK = Nong Khai and M = Mukdahan 2 nd alphabet means samples:- S = stem, R = root, F = fruit, So = rhizosphere soil, 3rd alphabet means name of collector:- C = Chamaiporn, 4th alphabet means number of isolates.

4.1.2 Morphological Identification

1. Description of *Fusarium oxysporum* isolates BKRF01

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia are shaped in fusiform, slightly curved, 3-5 septate, 15.6-31.4 x 2.4-4.5 µm. Microconidia are abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.8 x 1.7-3.3 µm. Chlamydospores appear terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.7 x 2.4-3.5 µm (Fig 4.1).

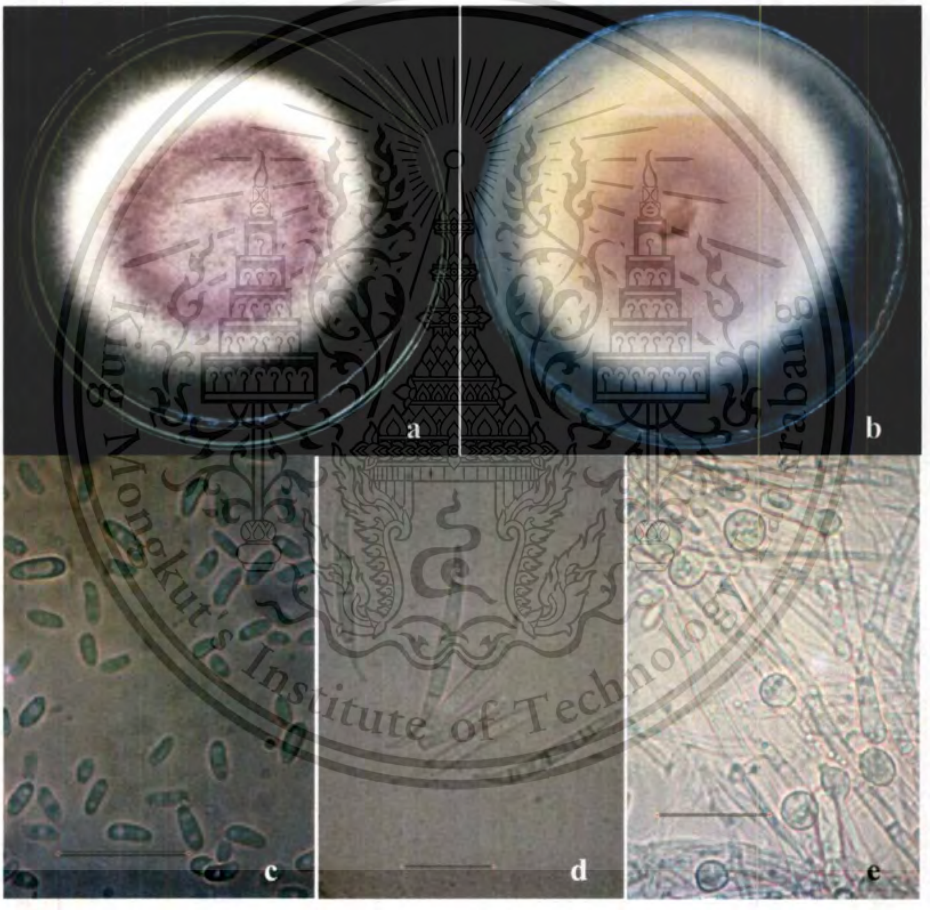


Figure 4.1 Morphological character of *Fusarium oxysporum* BKRF01. a = front surface of colony on PDA, b = back surface of colon , c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

2. Description of *Fusarium oxysporum* isolate KSoC02

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge.Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.4-32.4 x 2.4-4.4 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.4 x 1.6-3.4 µm. Chlamydospores appear terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.8 x 2.4-3.4 µm.(Fig.4.2)

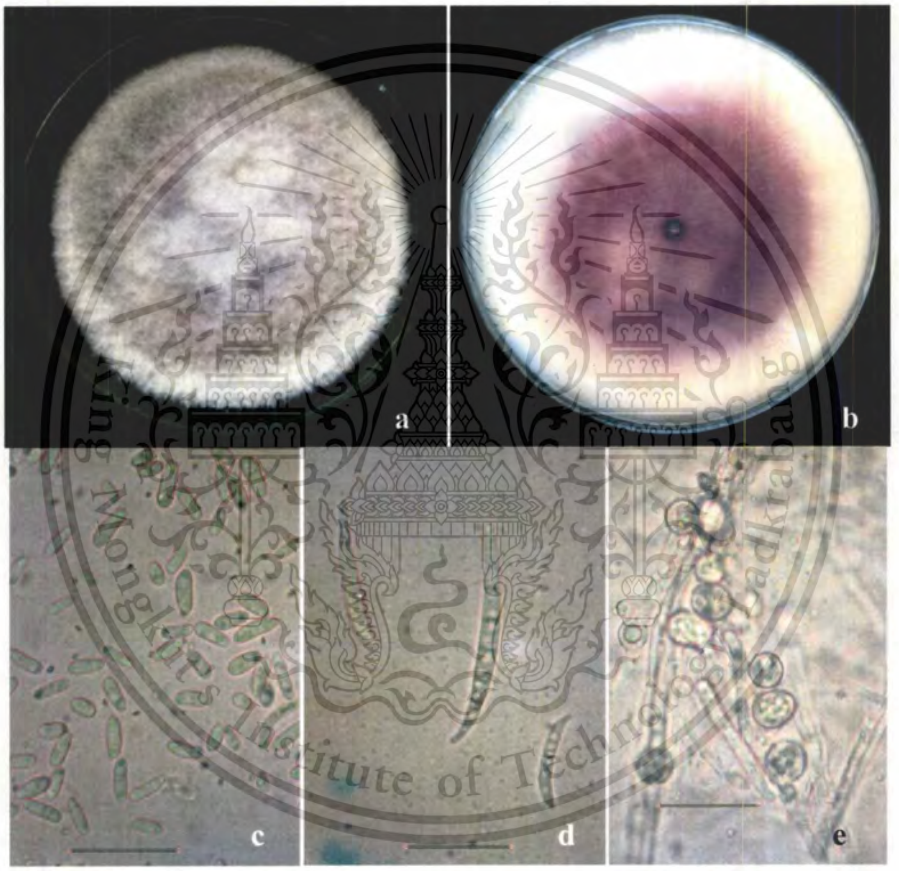


Figure 4.2 Morphological character of *Fusarium oxysporum* KSoC02. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

3. Description of *Fusarium oxysporum* isolate SSoC04

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.5-32.6 x 2.4-4.3 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.2 µm. Chlamydospores: terminal or intercalary in hyphae, smooth or roughen wall, 2.3-3.6 x 2.4-3.5 µm.(Fig. 4.3)

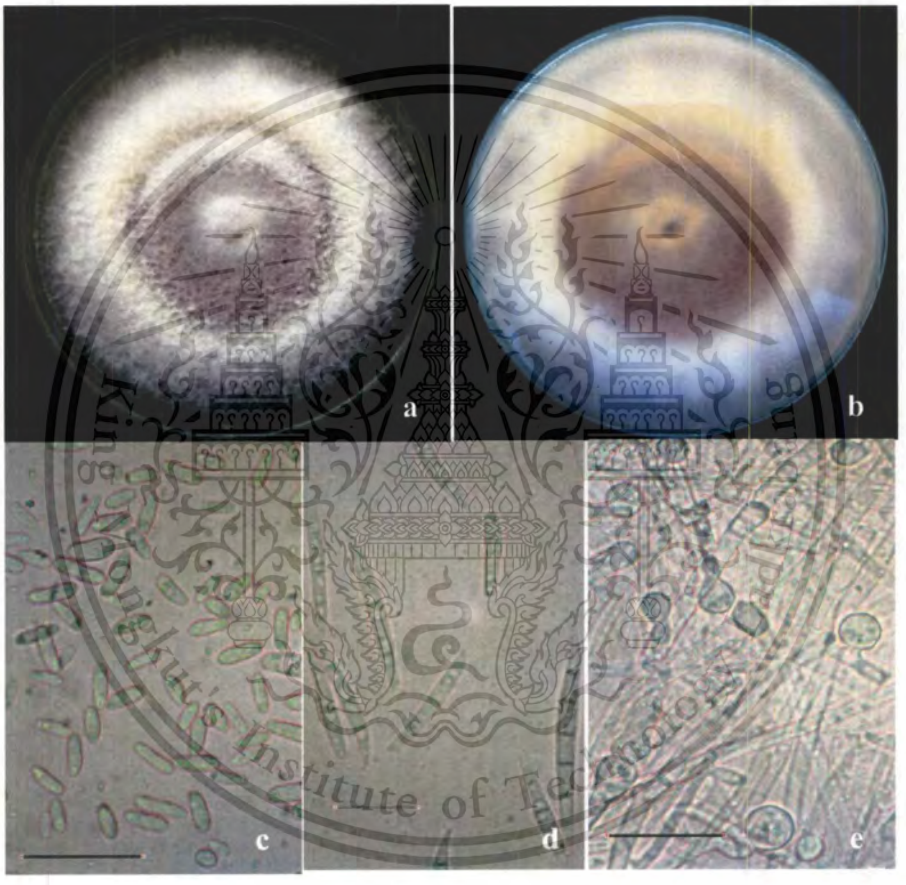


Figure 4.3 Morphological character of *Fusarium oxysporum* SSoC04. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

4 Description of *Fusarium oxysporum* isolate SRC02

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.7-33.4 x 2.5-4.5 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.3 x 1.6-3.5 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.8 x 2.5-3.5 µm. (Fig 4.4).

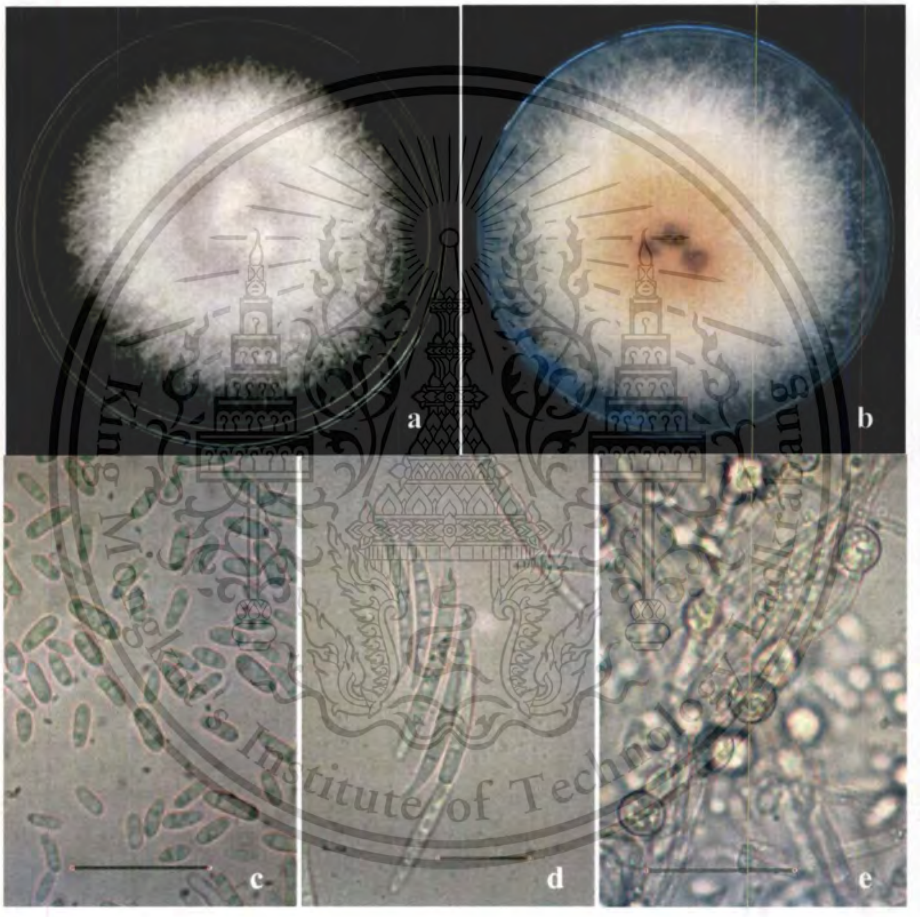


Figure 4.4. Morphological character of *Fusarium oxysporum* SRC02. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

5. Description of *Fusarium oxysporum* isolate BKRS01

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 14.7-33.4 x 2.6-4.5 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-8.5 x 1.5-3.5 µm. Chlamydospores: terminal or intercalary in hyphae, smooth or roughen wall, 2.5-3.8 x 2.4-3.6 µm. (Fig. 4.5).

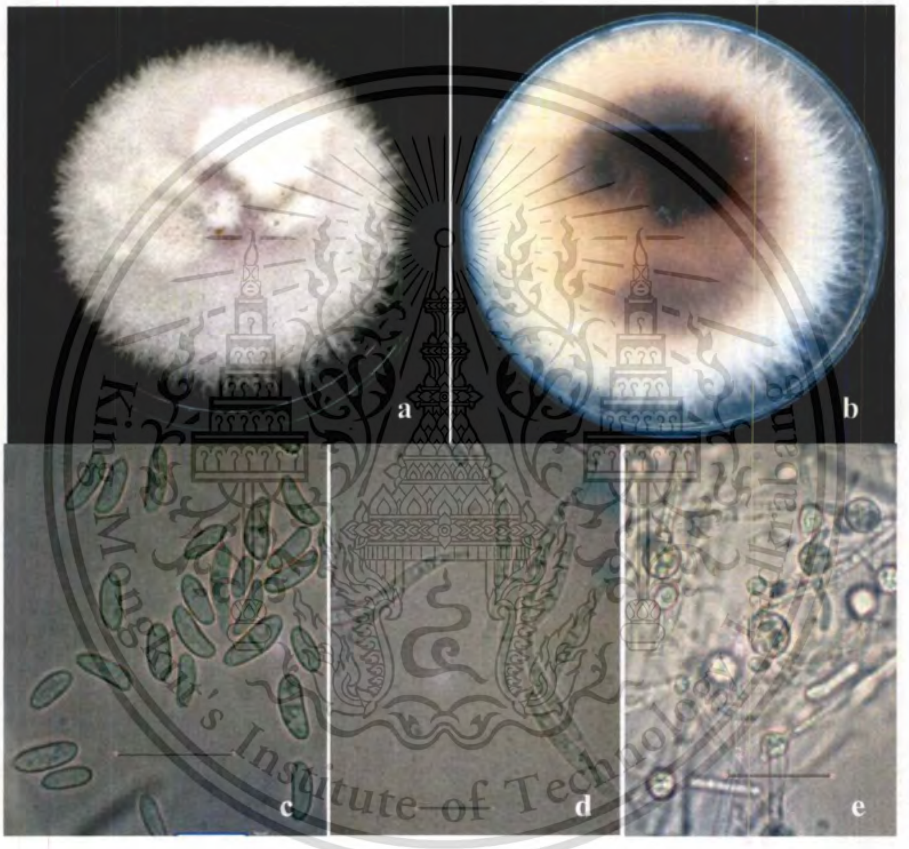


Figure 4.5 Morphological character of *Fusarium oxysporum* BKRS01. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

6. Description of *Fusarium oxysporum* isolate BRC03

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 13.2-34.4 x 2.5-3.8 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, straight, 4.1-9.3 x 1.5-3.0 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.2-3.9-2.0-3.5 µm.(Fig.4.6).

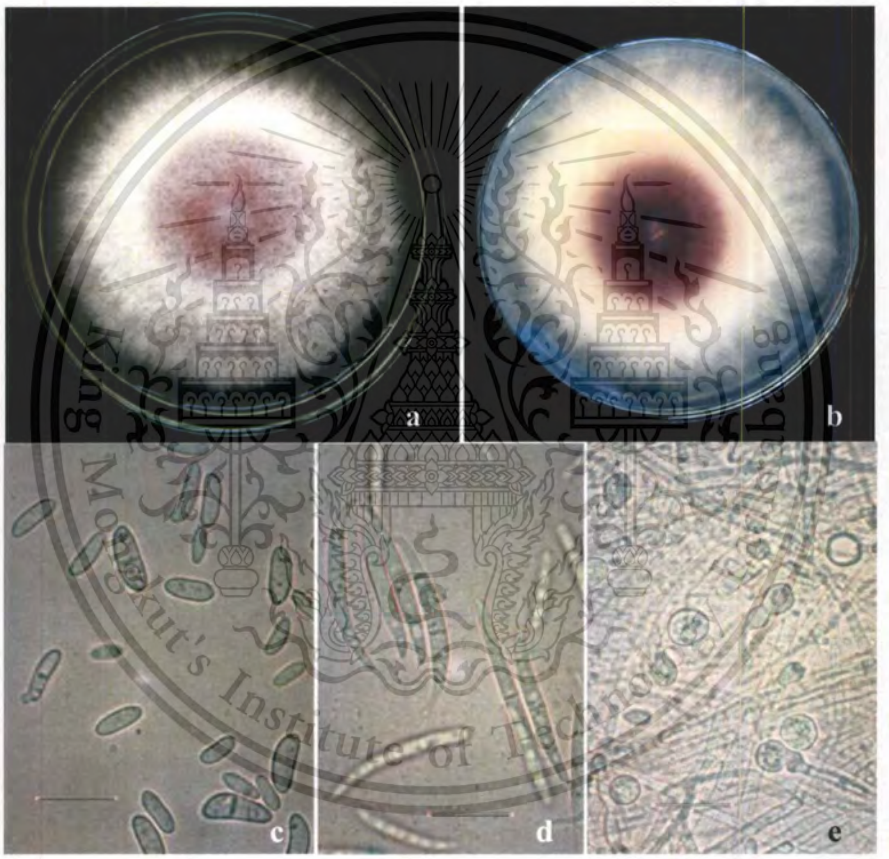


Figure 4.6. Morphological character of *Fusarium oxysporum* BRC03. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

7. Description of *Fusarium oxysporum* isolate SSoC03

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PSA. Aerial mycelium sparse to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.7-32.4 x 2.4-4.3 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.2 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.7 x 2.4-3.5 µm. (Fig.4.7).

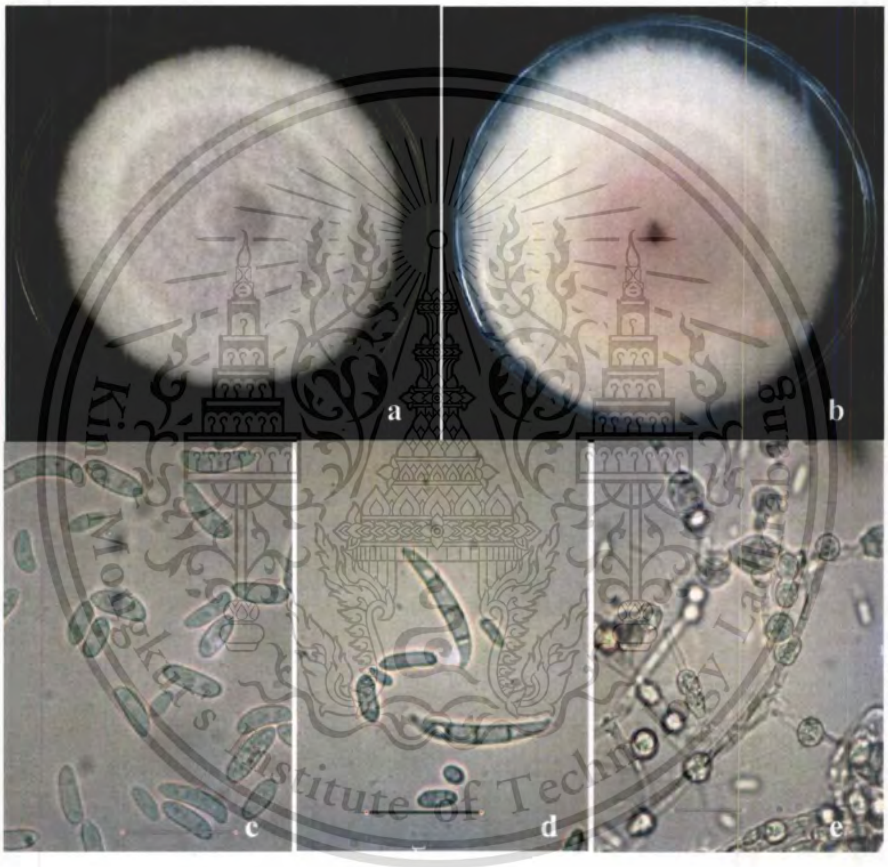


Figure 4.7. Morphological character of *Fusarium oxysporum* SSoC03. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

8. Description *Fusarium oxysporum* isolate NKRC09

Colony is fast-growing, reaching 9 cm diameter in 7-9 days on PDA at 28-32° C. Aerial mycelium sparse to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 22.7-33.1 x 2.2-3.9 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, straight, 7.0-9.9 x 2.0-3.0 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.3-3.8 x 2.0-3.6 µm. (Fig.4.8).

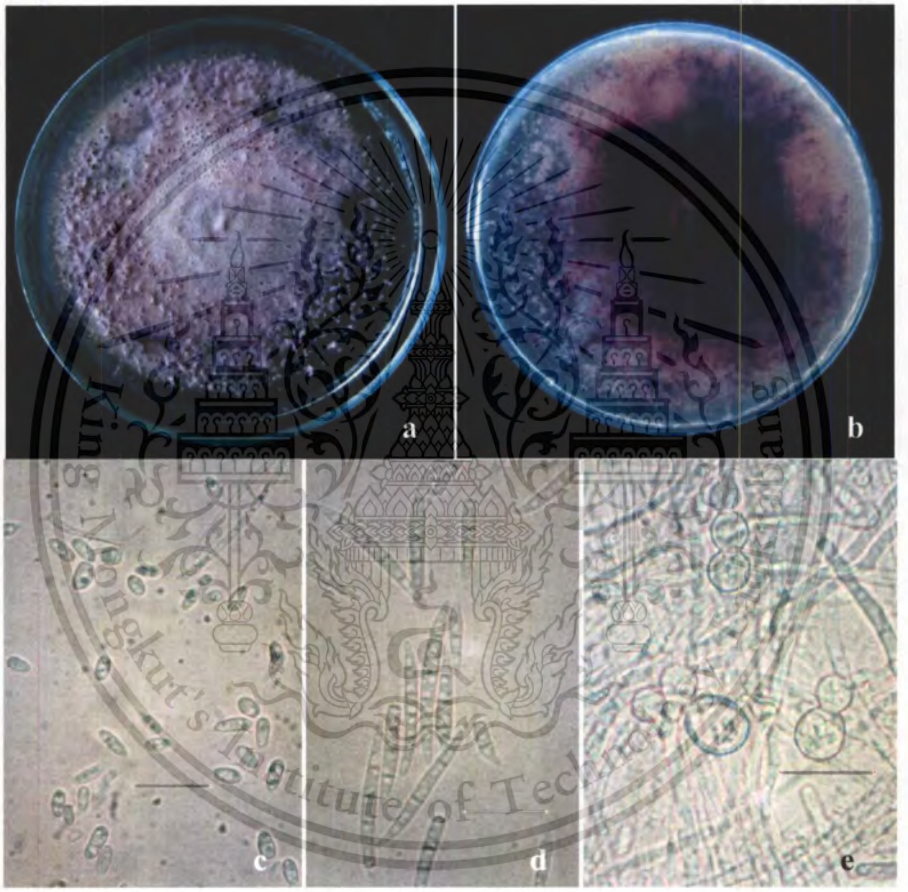


Figure 4.8 Morphological character of *Fusarium oxysporum* NKRC09. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

9. Description of *Fusarium oxysporum* isolate NKRC02

Colony is fast-growing, reaching 9 cm diameter in 7-9 days on PDA at 28-32° C. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 26.5-42.1 x 3.5-5.0 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, straight, 10.1-18.1 x 3.1-4.8 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.9 x 1.9-3.2 µm. (Fig.4.9).

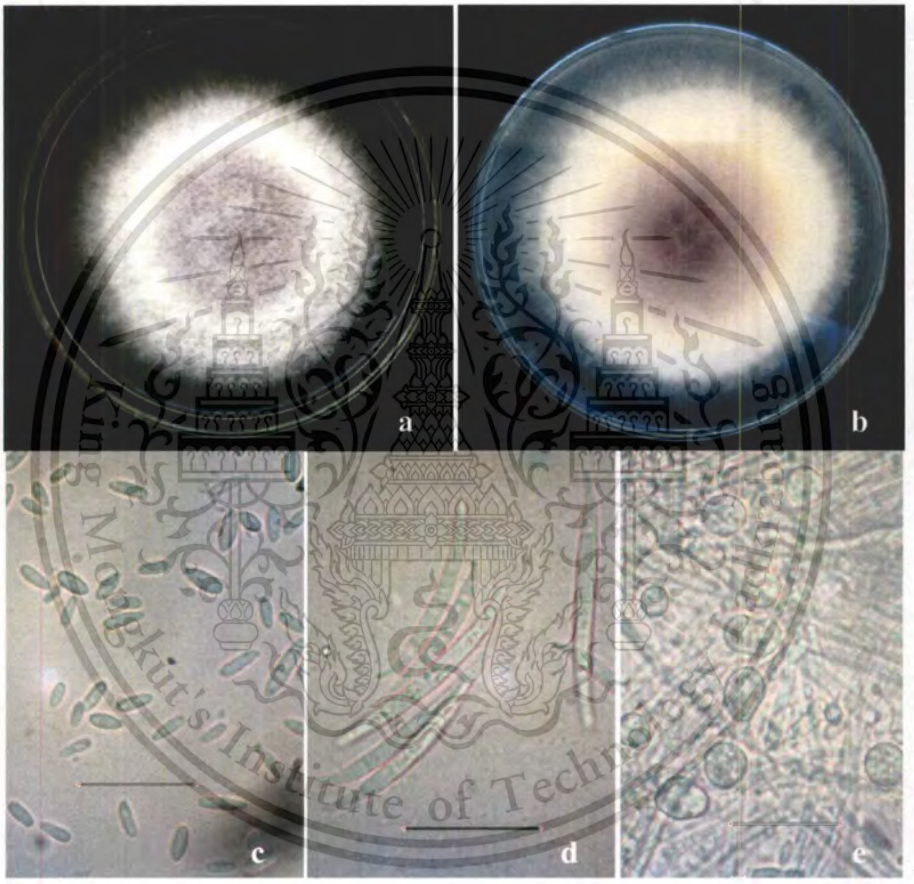


Figure 4.9 Morphological character of *Fusarium oxysporum* NKRC02. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

10. Description of *Fusarium oxysporum* isolate NKRC04

Colony is fast-growing, reaching 9 cm diameter in 7-9 days on PDA at 28-32° C. Aerial mycelium is sparsed to abundant and becoming felted, white with violet tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.3-26.1 x 2.6-3.9 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, straight, 3.4-10.3 x 1.7-3.2 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.6-3.6 x 1.7-3.1 µm. (Fig.10).

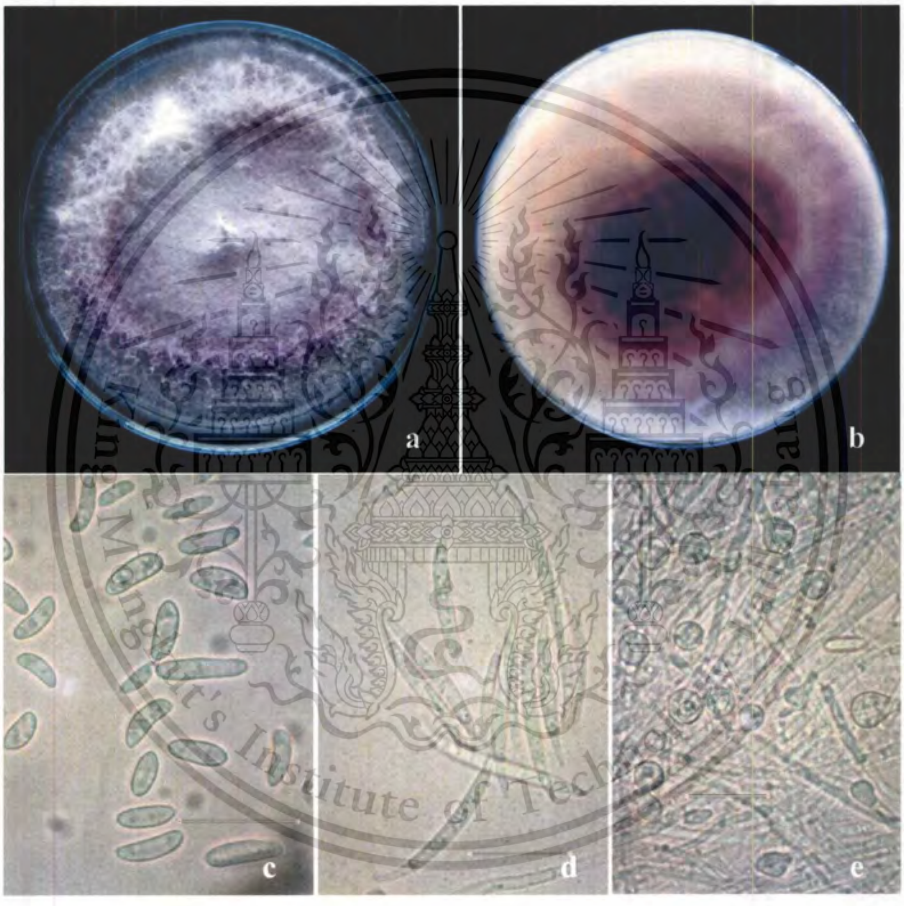


Figure 4.10 Morphological character of *Fusarium oxysporum* NKRC04. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

11. Description of *Fusarium oxysporum* isolate NSC01

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved3-5 septate, 15.5-25.6 x 2.4-3.8 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, straight, and 4.1-10.7 4.1 M 10.7-2.7 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.3-4.5 x 2.0-4.1 µm. (Fig.4.11).

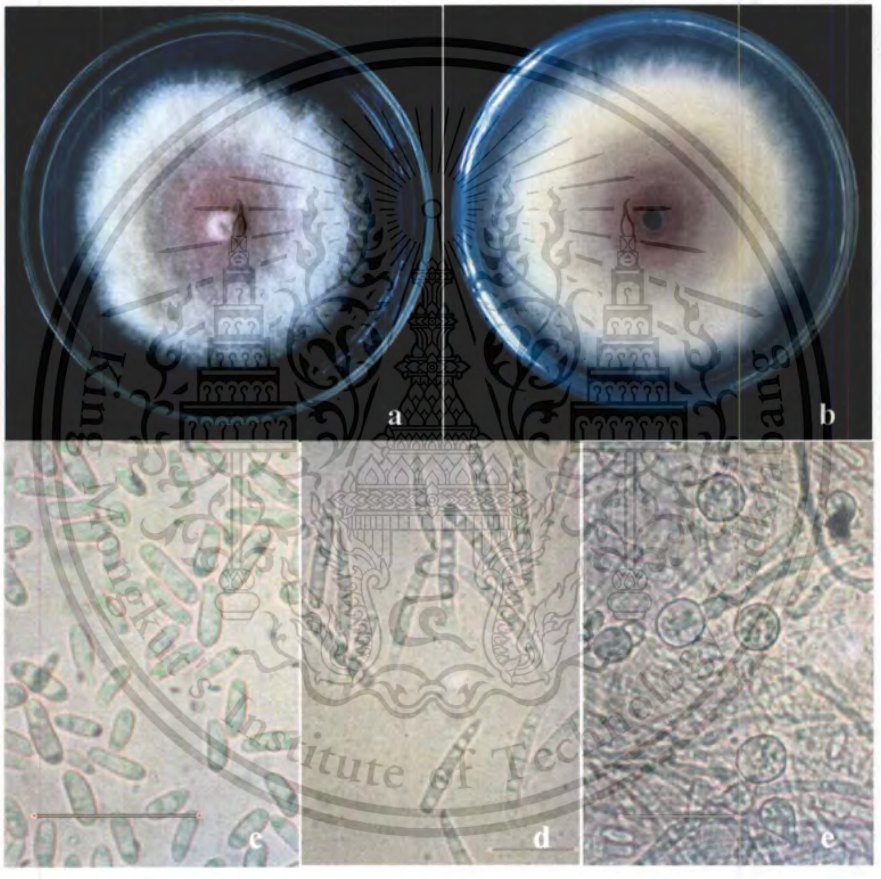


Figure 4.11 Morphological character of *Fusarium oxysporum* NSC01. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

12. Description of *Fusarium oxysporum* isolate MSRS01

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 16.5-33.4 x 2.5-4.4 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.7 x 1.6-3.5 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.9 x 2.5-3.5 µm. (Fig.4.12).

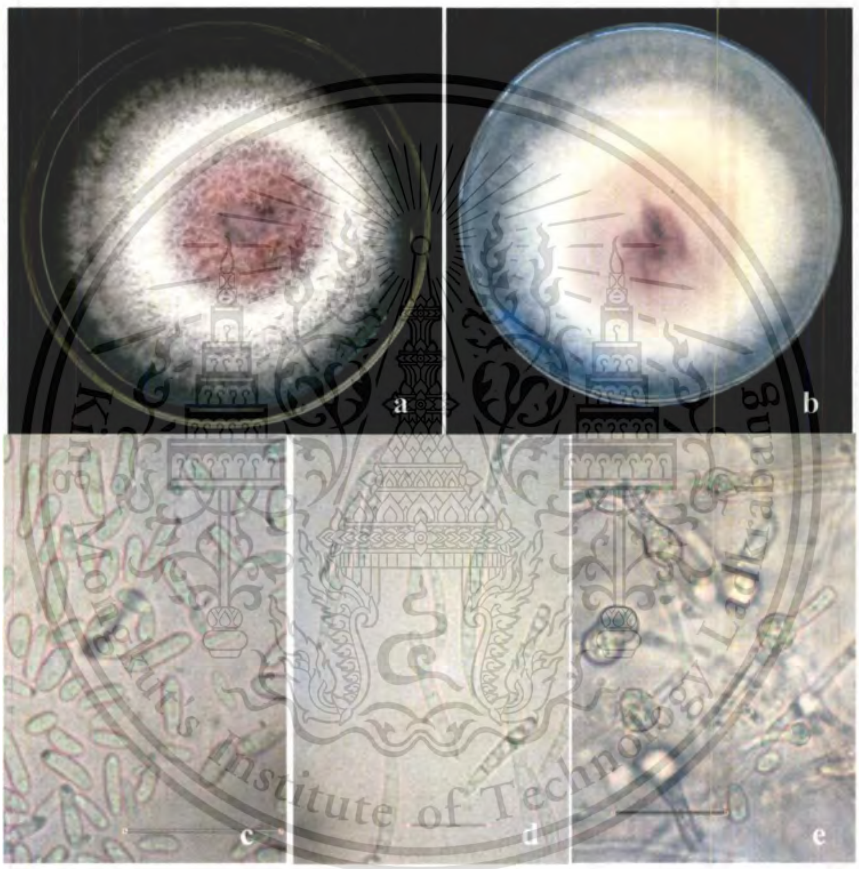


Figure 4.12 Morphological character of *Fusarium oxysporum* MSRS01. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

13. Description of *Fusarium oxysporum* isolate MSRS02

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 14.9-33.4 x 2.5-4.6 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.2 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.5-3.7 x 2.5-3.7 µm.(Fig.4.13).

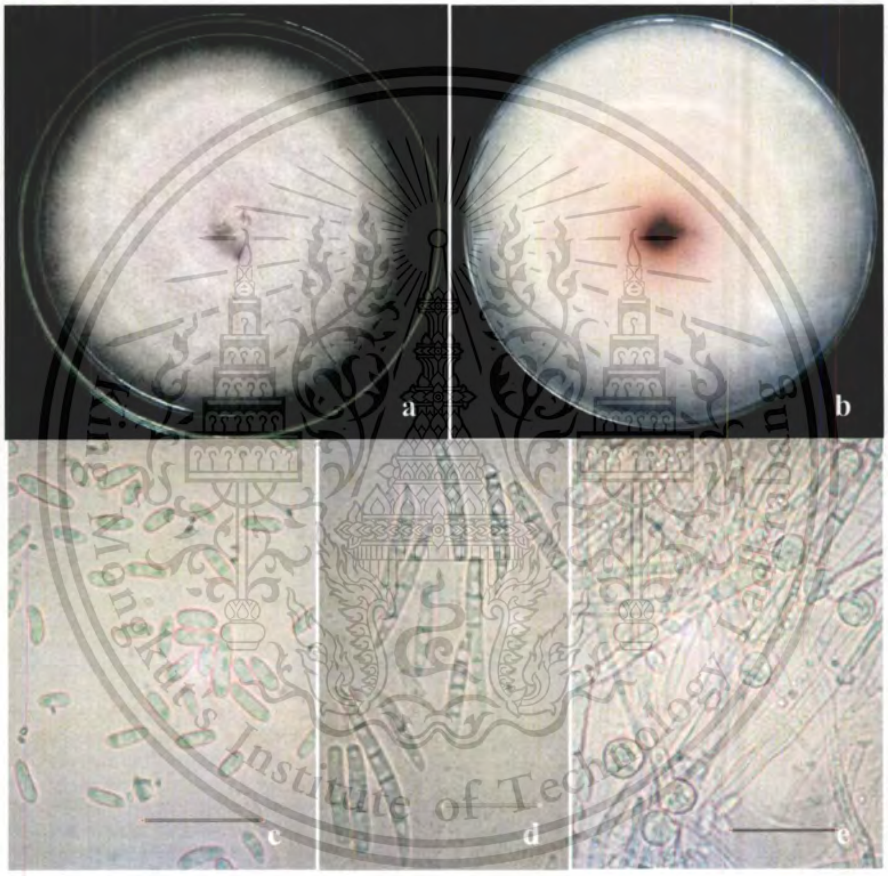


Figure 4.13 Morphological character of *Fusarium oxysporum* MSRS02. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

14. Description of *Fusarium oxysporum* isolate PBRS102

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.4-33.4 x 2.5-4.6 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.3-7.8 x 1.6-3.1 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.5-3.8 x 2.4-3.5 µm.(Fig.4.14).

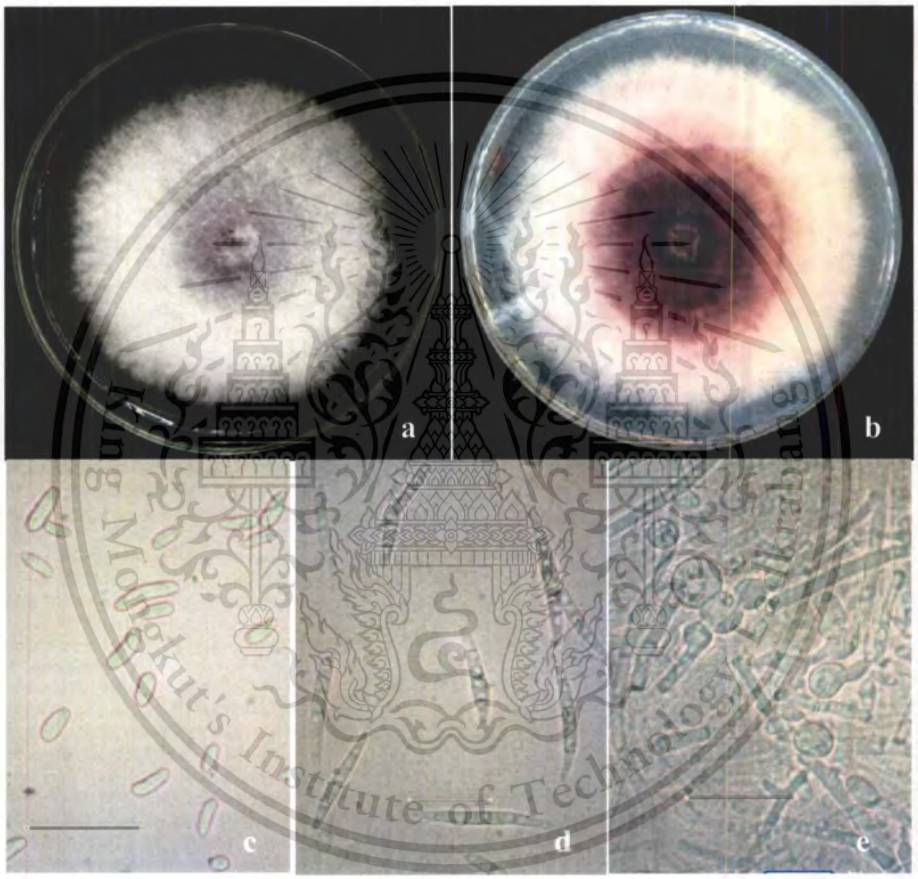


Figure 4.14 Morphological character of *Fusarium oxysporum* PBRS102. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

15. Description of *Fusarium oxysporum* isolate PBRS203

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.7-33.4 x 2.5-4.3 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.4 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.6 x 2.4-3.7 µm.(Fig.4.15).

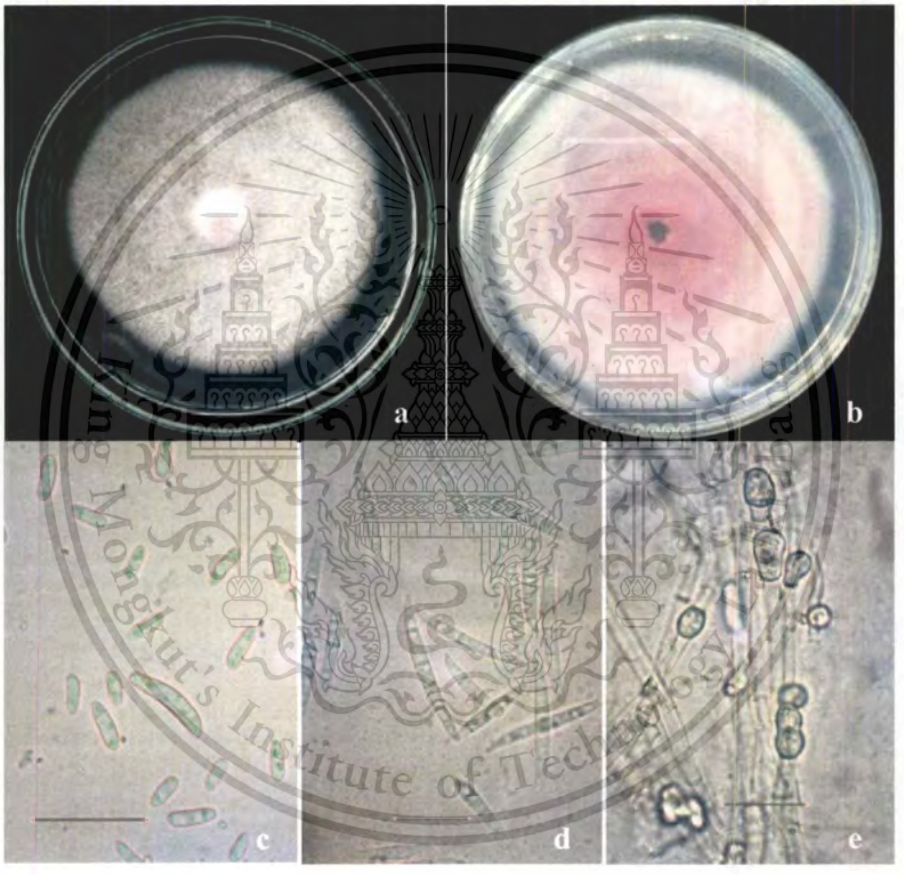


Figure 4.15 Morphological character of *Fusarium oxysporum* PBRS203 a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

16. Description of *Fusarium oxysporum* isolate TRS01

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 16.7-32.6 x 2.5-4.4 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.2 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.7 x 2.4-3.5 µm.(Fig.4.16).

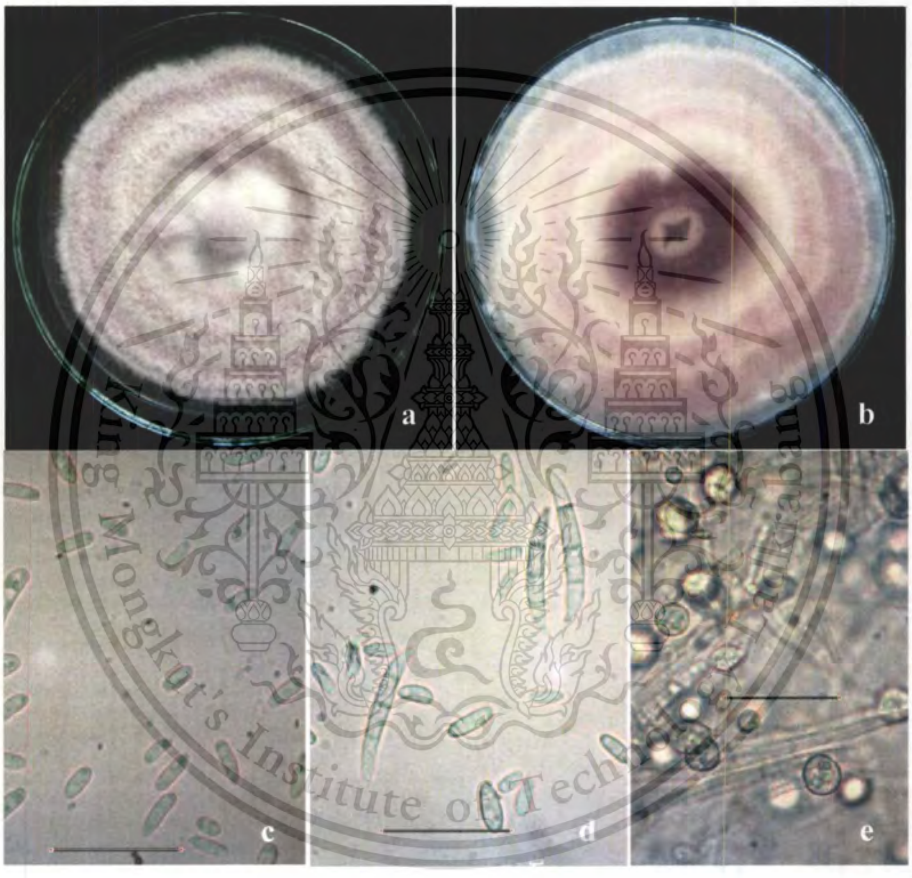


Figure 4.16 Morphological character of *Fusarium oxysporum* TRS01. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

17. Description of *Fusarium oxysporum* isolate PBRS201

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.7-32.5 x 2.4-4.8 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.2 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.6 x 2.4-3.5 µm. (Fig.4.17).

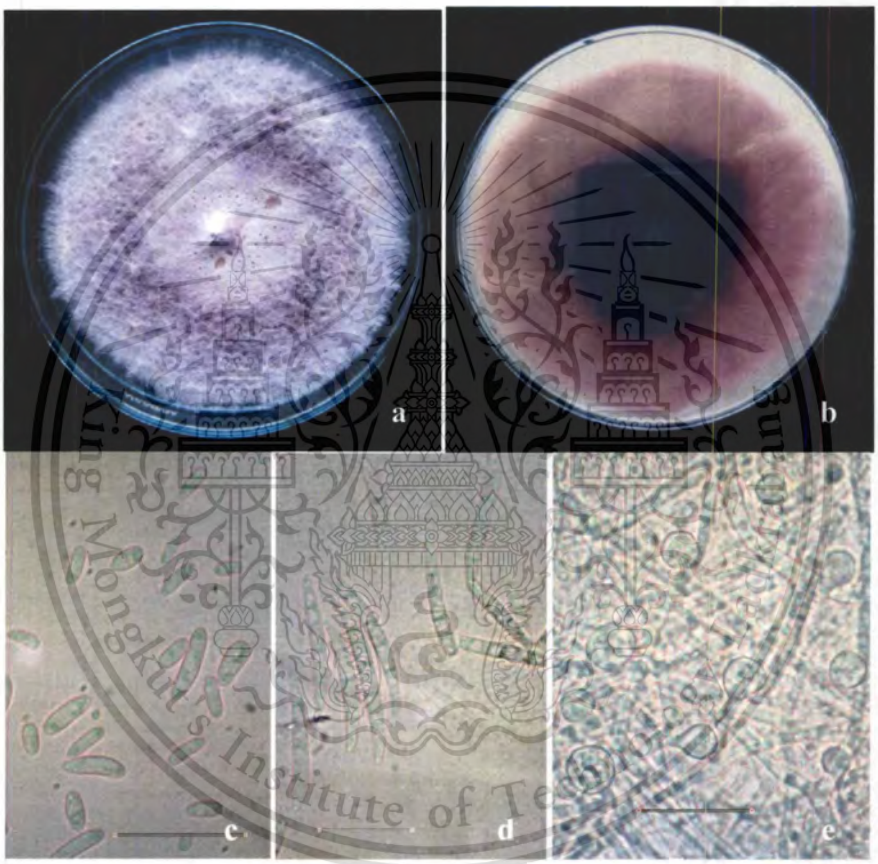


Figure 4.17 Morphological character of *Fusarium oxysporum* PBRS201. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

18. Description of *Fusarium oxysporum* isolate PBRS103

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.7-32.5 x 2.5-4.3 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.3 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.7 x 2.5-3.5 µm.(Fig.4.18).

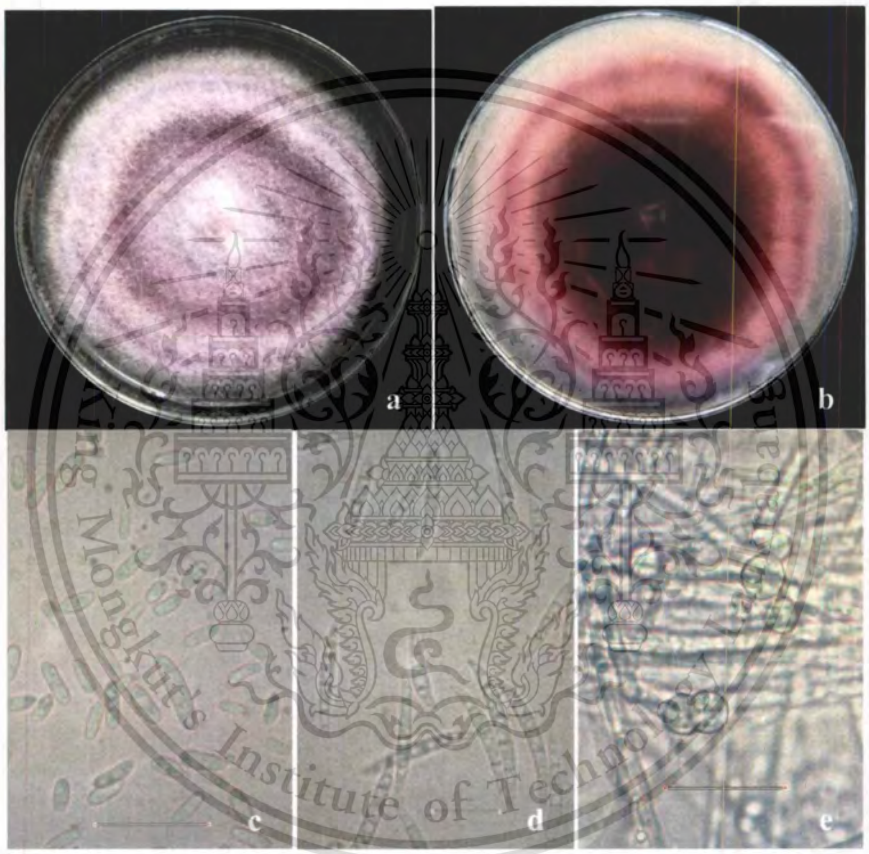


Figure 4.18 Morphological character of *Fusarium oxysporum* PBRS103. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

19. Description of *Fusarium oxysporum* isolate PBRS101

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.7-33.4 x 2.4-4.7 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.7-3.2 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.5 x 2.4-3.5 µm. (Fig.19).

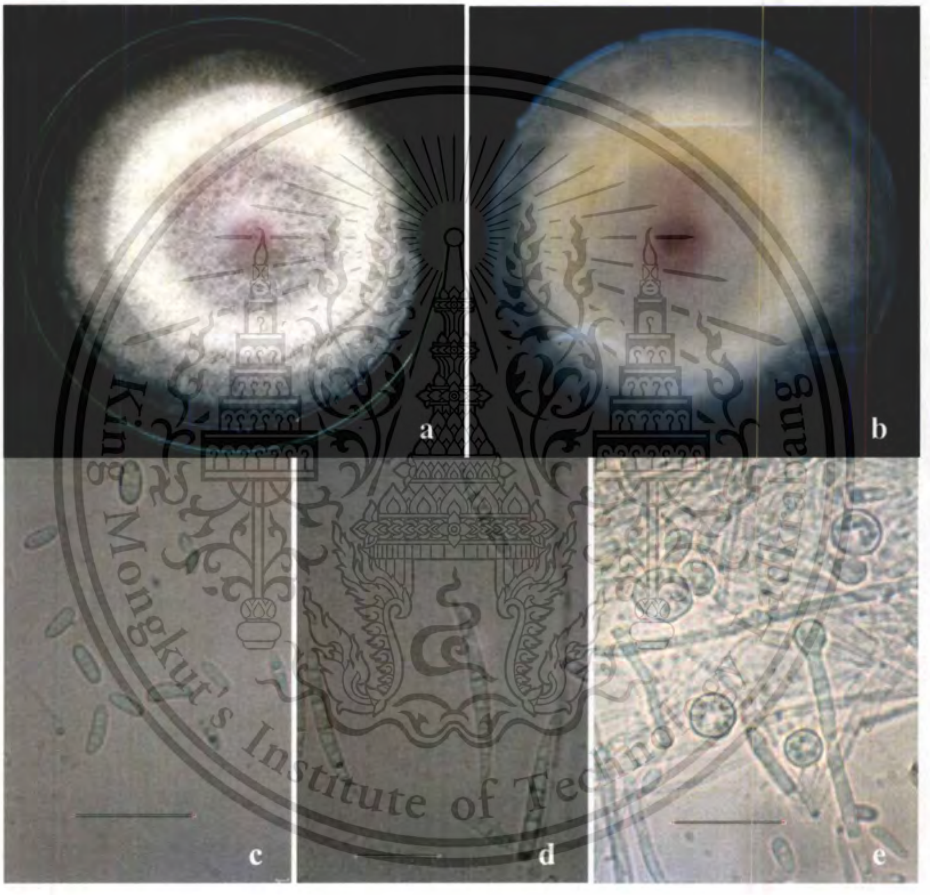


Figure 4.19 Morphological character of *Fusarium oxysporum* PBRS101. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

20. Description of *Fusarium oxysporum* isolate PBRS104

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.7-33.4 x 2.4-4.5 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.4 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.5-3.7 x 2.4-3.7 µm.(Fig.4.20).

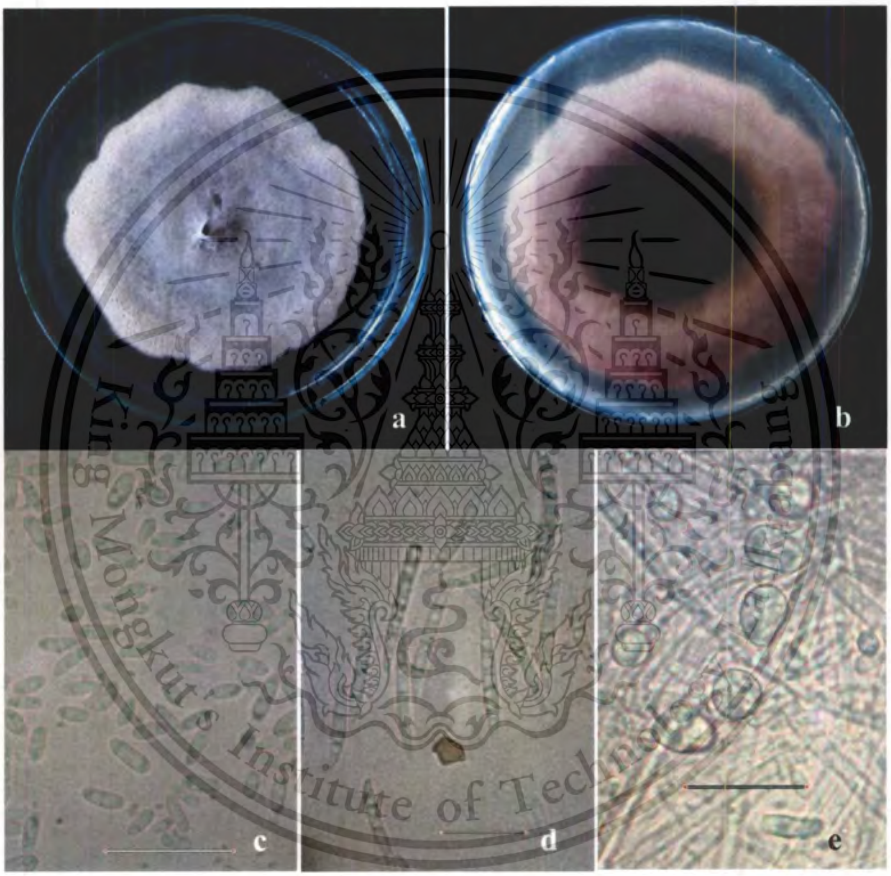


Figure 4.20 Morphological character of *Fusarium oxysporum* PBRS104. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

21. Description of *Fusarium oxysporum* isolate TRS02

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 14.7-32.5 x 2.5-4.3 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.8 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.7 x 2.4-3.5 µm.(Fig.4.21).

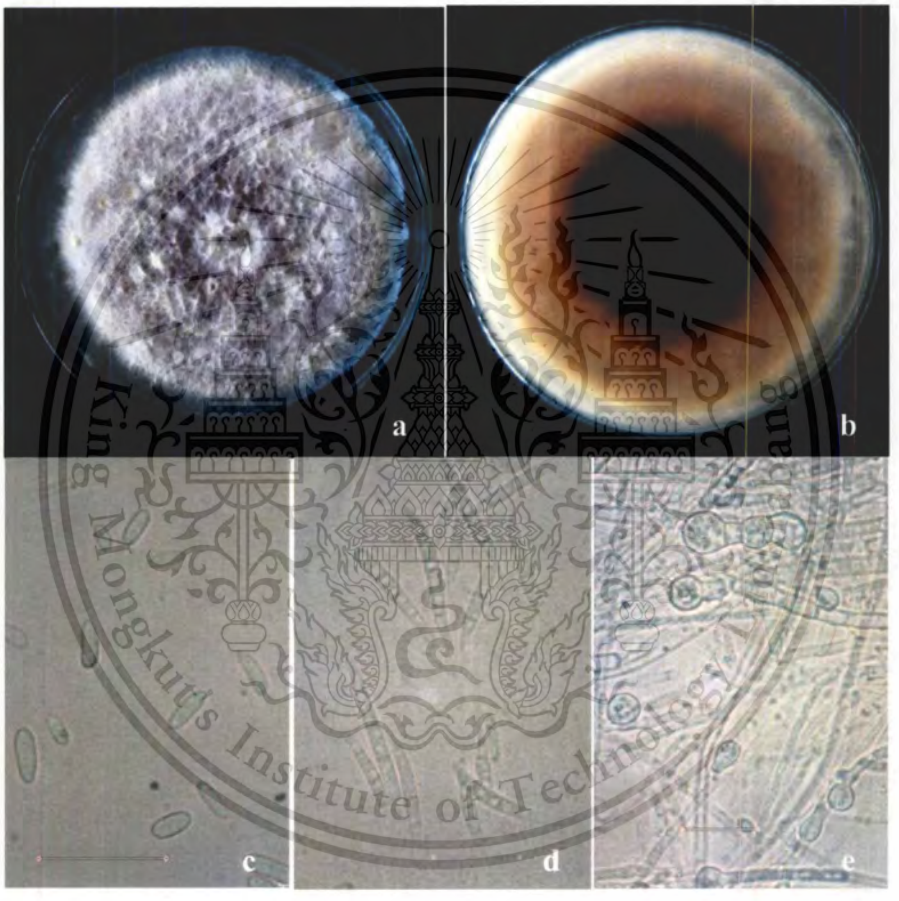


Figure 4.21 Morphological character of *Fusarium oxysporum* TRS02. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

22. Description of *Fusarium oxysporum* isolate PBRS202

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 15.4-32.7 x 2.4-4.3 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, 4.0-7.5 x 1.6-3.5 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.4 x 2.4-3.5 µm.(Fig.4.22).

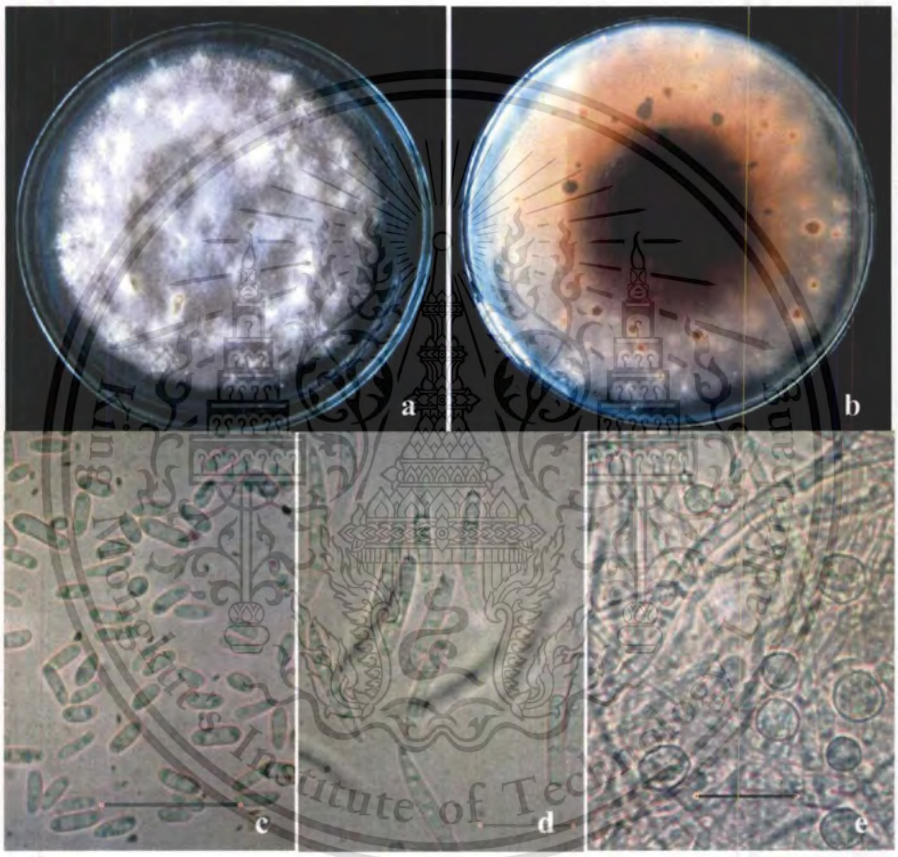


Figure 4.22 Morphological character of *Fusarium oxysporum* PBRS202. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

23. Description of *Fusarium oxysporum* isolate KK2

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 14.1-27.4 x 2.1-3.7 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, straight, 4.2-9.6 x 1.3-3.7 µm Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.2-4.9-1.9-3.1 µm.(Fig.4.23).

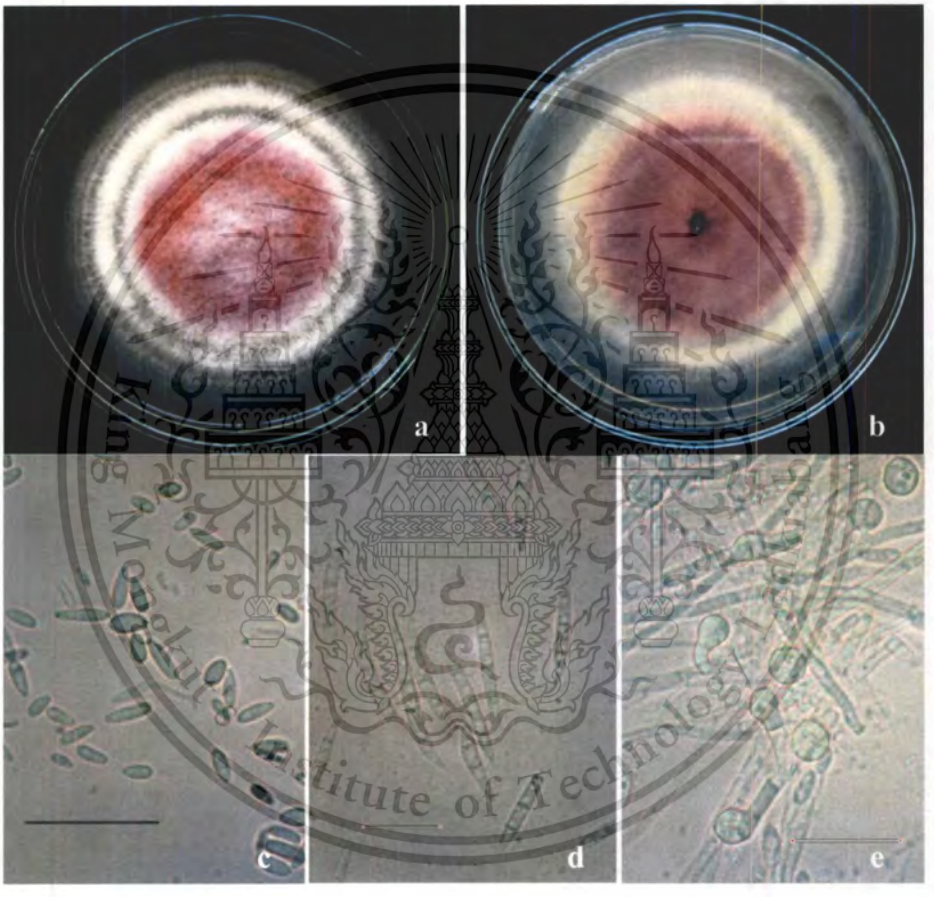


Figure 4.23 Morphological character of *Fusarium oxysporum* KK2. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

24. Description of *Fusarium oxysporum* isolate NKSC01

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 16.0-32.8 x 2.2-3.5 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, straight, 3.5-6.0 x 1.5-2.9 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.6-3.7 x 2.2-3.4 µm.(Fig.4.24).

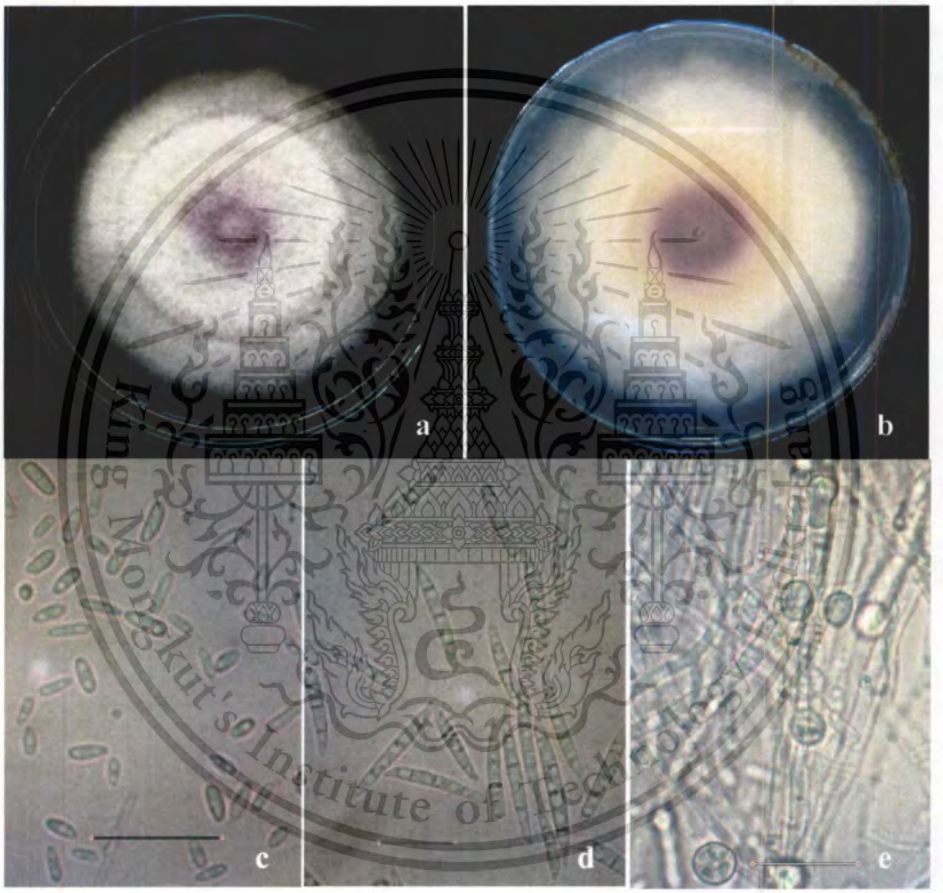


Figure 4.24 Morphological character of *Fusarium oxysporum* NKSC01. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

25. Description of *Fusarium oxysporum* isolate NKSC02

Colony is fast-growing, reaching 9 cm diameter in 7-9 days at 28-32° C on PDA. Aerial mycelium is sparsed to abundant and becoming felted, white with purple tinge. Conidiophores are shorted, formed singly and branched. Macroconidia shaped in fusiform, slightly curved, 3-5 septate, 21.3-31.2 x 2.2-3.6 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, straight, 5.6-11.7 x 1.4-2.4 µm. Chlamydospores terminal or intercalary in hyphae, smooth or roughen wall, 2.4-3.5 x 2.1-3.3 µm.(Fig.4.25).

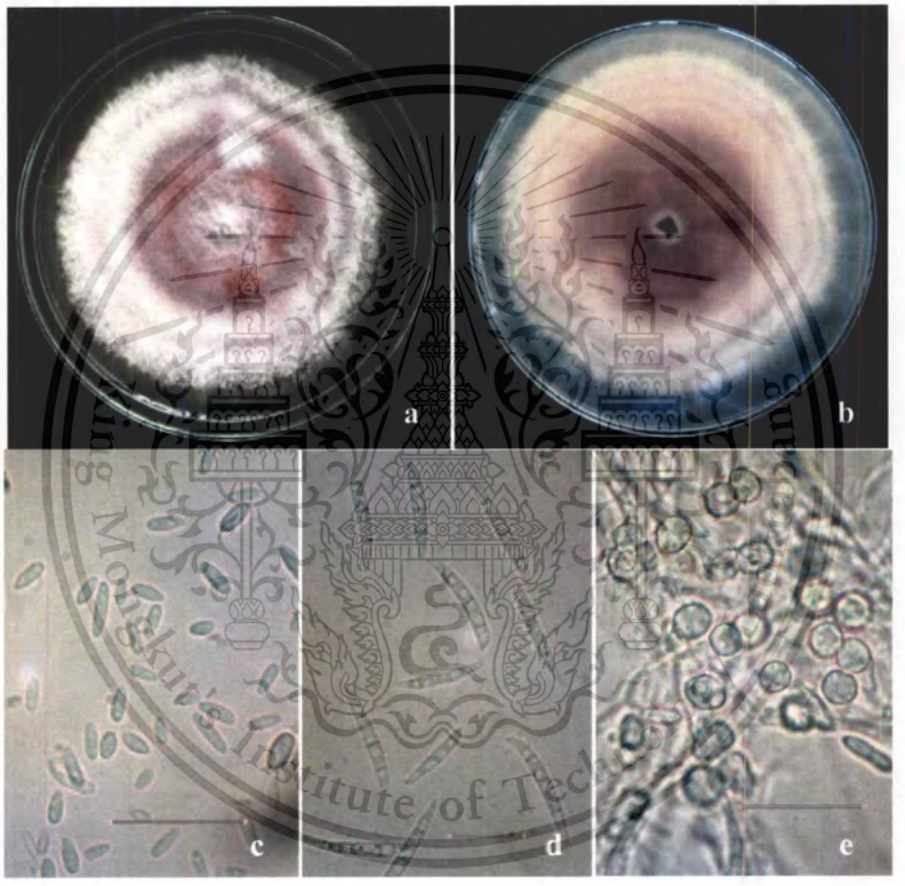


Figure 4.25 Morphological character of *Fusarium oxysporum* NKSC02. a = front surface of colony on PDA, b = back surface of colony, c = microconidia, d = macroconidia, e = chlamydospores, scale bar = 10 µm.

4.1.3. Pathogenicity Tests

Twenty-five isolates of *F. oxysporum* f. sp. *lycopersici* (Table 4.1) were proved for pathogenicity with two varieties of tomatoes, Sida and Cherry varieties. Tomato seedlings at 21 day-old were tested by using Koch's postulates method.

4.1.3.1. Pathogenicity test of *F. oxysproum* f. sp. *lycopersici* with Sida variety

The results showed that 18 isolates from Bangkok, Phetchabun, Tak, Burirum and Sakornnakorn categorized in low virulence group which DSI ranged from 1.25 to 2.37 which including isloates (BKRF01, BKRS01, PBRS101, PBRS102, PBRS103, PBRS104, PBRS201, PBRS202, PBRS203, MSRS01, MSRS02, TRS01, TRS02, TRS03, BRC03, SRC02, SSoC03, and SSoC04), while isolate of KK2 from Khonkaen was grouped into moderated virulence, and there were two isolates were high virulence NKSC01 and NKSC02 which showed disease severity index at 4.55 and 5.25 respectively. Four isolates from Nakhonratchasima were appeared non-pathogenic fungi (NKRC02, NKRC04, NKRC09 and NSC01) Two isolates of NKSC01 and NKSC02 from Nongkhai were high virulence as shown in (Table 4.2, Fig. 4.26 – 4.29). Therefore, the highly virulent isolate NKSC02 was chosen for further studies.

4.1.3.2. Pathogenicity test of *F. oxysproum* f. sp. *lycopersici* with Cherry variety

Result showed that 11 isolates of BKRS01, BKRF01, BRC03, KSoC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03 and SSoC04 from Bangkok, Burirum, Nakhonratchasima and Sakornnakorn were non-pathogenic or avirulent group (DSI = 1). The pathogenic isolates were showed 11 isolates of PBRS101, PBRS102,PBRS103, PBRS104, PBRS201, PBRS202, PBRS203, MSRS01, MSRS02, TRS01 and TRS02 from Phetchabun and Tak were low virulent (L), one isolate of KK2 was moderate virulent (M) and two isolates of NKSC01 ,NKSC02 were high virulent (H) as shown in Table 4.3 and Fig 4.30, 4.31, 4.32, 4.33, and 4.34. It revealed that NKSC01 and NKSC02 from Nongkhai were high virulence to both tomato varieties of Sida and Cherry.

Table 4.2. Isolates of *Fusarium* spp. and their pathogenicity group in tomato var. Sida at 21 days

Provinces	**Isolates**	**DSI[2]**	**Pathogenicity group[4]**
Bangkok	BKRF01	1.87cde	Low virulence
	BKRS01	1.25de	Low virulence
Phetchabun	PBRS101	1.87cde	Low virulence
	PBRS102	1.87cde	Low virulence
	PBRS103	1.87cde	Low virulence
	PBRS104	1.87cde	Low virulence
	PBRS201	1.87cde	Low virulence
	PBRS202	1.87cde	Low virulence
	PBSS203	2.12cd	Low virulence
Tak	MSRS01	1.87cde	Low virulence
	MSRS02	1.87cde	Low virulence
	TRS01	2.37c	Low virulence
	TRS02	2.37c	Low virulence
Burirum	BRC03	1.25de	Low virulence
KhonKaen	KK2	4.25b	Moderate virulence
	KSoC02	1.25de	Low virulence
Nongkhai	**NKSC01**	4.55ab	High virulence
	NKSC02	5.25a	High virulence
	NKRC02	1e	Non-pathogenic
	NKRC04	1e	Non-pathogenic
	NKRC09	1e	Non-pathogenic
Nakhonratchasima	NSC01	1e	Non-pathogenic
SakonNakhon	SRC02	1.25de	Low virulence
	SSoC03	1.87cde	Low virulence
	SSoC04	1.87cde	Low virulence

DSI[1] Disease severity index of pathogenicity test Cherry var., DSI[2] Disease severity index of pathogenicity test Sida var.

Table 4.3. Isolates of *Fusarium* spp. and their pathogenicity group in tomato var. Cherry at 21 days

Provinces	**Isolates**	**DSI**[1]	**Pathogenic or non-pathogenic isolates**
Bangkok	BKRS01	1.00 e[2]	Non-pathogenic
	BKRF01	1.00 e	Non-pathogenic
Phetchabun	PBRS101	2.00 d	Low virulence
	PBRS102	2.00 d	Low virulence
	PBRS103	2.00 d	Low virulence
	PBRS104	2.00 d	Low virulence
	PBRS201	2.00 d	Low virulence
	PBRS202	2.00 d	Low virulence
	PBRS203	2.00 d	Low virulence
Tak	MSRS01	2.00 d	Low virulence
	MSRS02	2.00 d	Low virulence
	TRS01	2.00 d	Low virulence
	TRS02	2.00 d	Low virulence
Burirum	BRC03	1.00 e	Non-pathogenic
KhonKaen	KK2	4.25 c	Moderate virulence
	KSoC02	1.00 e	Non-pathogenic
Nongkhai	**NKSC01**	4.75 b	High virulence
	NKSC02	5.50 a	High virulence
	NKRC02	1.00 e	Non-pathogenic
	NKRC04	1.00 e	Non-pathogenic
	NKRC09	1.00 e	Non-pathogenic
Nakhonratchasima	NSC01	1.00 e	Non-pathogenic
SakonNakhon	SRC02	1.00 e	Non-pathogenic
	SSoC03	1.00 e	Non-pathogenic
	SSoC04	1.00 e	Non-pathogenic

[1]DSI = Disease severity index:- avirulence (DSI =1), low virulence (DSI $\leq$ 3.50), moderate virulence (DSI > 3.50 – 4.50), and high virulence (DSI > 4.50).

2 Average of two repeated experiments from eight replications. Means followed by a common letter were significantly different by DMRT at P=0.01.

Figure 4.26 Disease severity index of tomato seedlings var. Sida after inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02 at 21 days in low virulent group.

Figure 4.27 Disease severity index of tomato seedlings var. Sida after inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02 at 21 days in moderate virulent group.

Figure 4. 28 Disease severity index of tomato seedlings var. Sida after inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02 at 21 days in high virulent group.

Figure 4.29. Disease severity index of tomato seedlings var. Sida after inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02 at 21 days in non-pathogenic isolates.

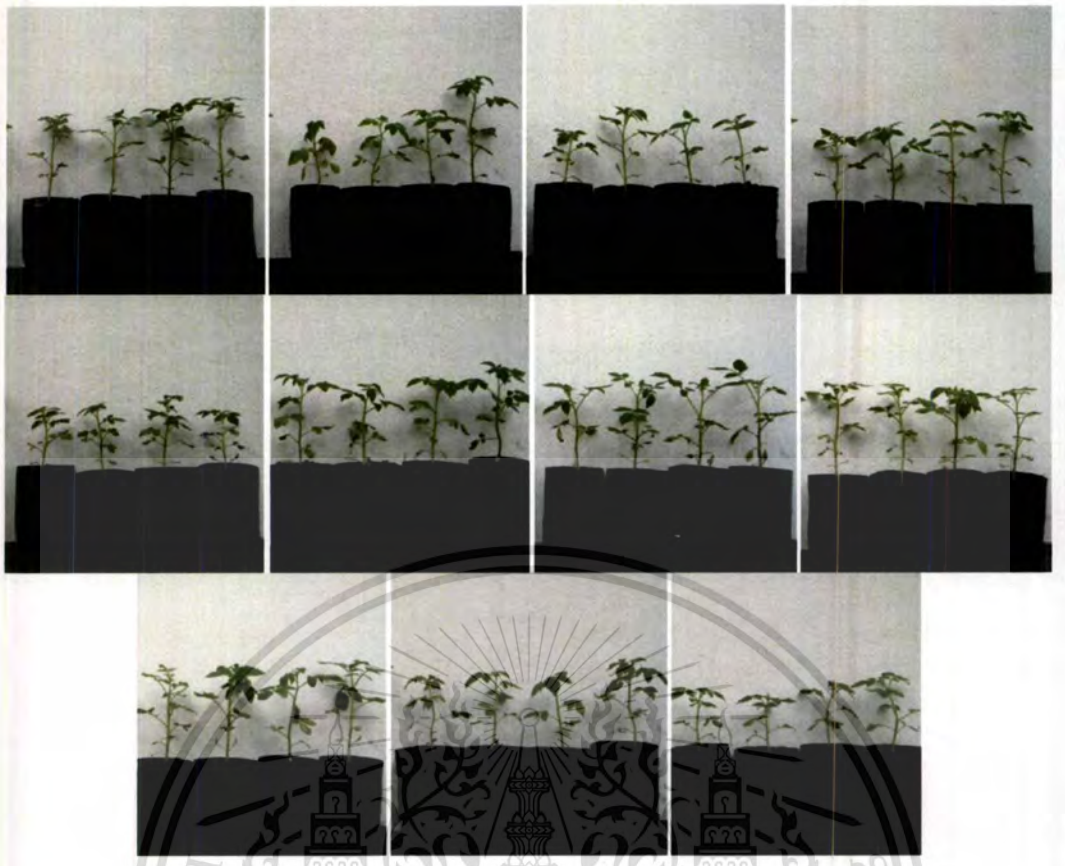


Figure 4.30. Disease severity index of tomato seedlings var. Cherry after inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02 at 21 days in low virulent group.

Figure 4.31 Disease severity index of tomato seedlings var. Cherry after inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02 at 21 days in moderate virulent group.

Figure 4. 32 Disease severity index of tomato seedlings var. Cherry after inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02 at 21 days in high virulent group.

Figure 4.33. Disease severity index of tomato seedlings var. Cherry after inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02 at 21 days in non-pathogenic group.

Figure 4. 34. Control treatment

4.1.4. Genetic relationship among *Fusarium oxysporum* f. sp. *lycopersici* isolates using AFLP markers

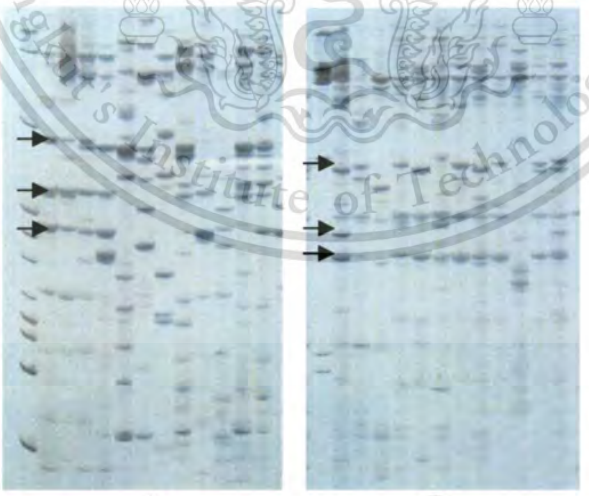
4.1.4.1. DNA fingerprint analysis using AFLP marker

Seventeen primers combination were screened on five isolates for investigating suitable primers combination use for further study. Result showed that there were only three primers combination including EcoRI+G/MseI+ACG, EcoRI+G/MseI+CAC, EcoRI+ACG/MseI+G gave highly number of polymorphic bands when compared with others primer combination which resolved 22, 22.4 and 20.5 polymorphic bands, respectively as shown in (Figure 4.35 and Table 4.4). The three primers were chosen for further screening on 25 isolates of *F. oxysporum* f. sp. *lycopersici*. A total 81 polymorphic bands were amplified using primers combination with EcoRI (E)+3 and MseI (M+1) and EcoRI (E)+1 and MseI (M+3) at the 3' end of the primers on 25 isolates of *F. oxysporum* f. sp. *lycopersici*. The polymorphic bands were analyzed using NTSYS program (Figure 4.36). Cluster analysis divided all the isolates into two major groups at 30% Dice' coefficient similarity. Group 1 described as non-pathogenic isolate group (avirulence) which consisted of KSoC02, BKRF01, SSoC04, SRC02, BKRS01, BRC03, SSoC03, NKRC02, NKRC04, NKRC09, and NSC01. Group 2 described as pathogenic isolate group which divided into 3 subgroups as follows:- subgroup 1 was low virulent isolates of MSRS01, MSRS02, PBRS102, PBRS203, TRS01, PBRS201, PBRS103, PBRS101, PBRS 104, TRS02 and PBRS202; subgroup 2 was moderate virulent isolates of KK2, and subgroup 3 was high virulent isolates of NKSC01 and NKSC02. A UPGMA tree was resulting from AFLP cluster analysis showed 85.4% bootstrap value of Isolates NKSC01 and NKSC02 which high virulent isolates were causing wilt disease of tomato var. Cherry. Among the pathogenic isolates that grouped into low virulence (L), AFLP cluster analysis showed over 60% of bootstrap. Moreover, 99.5% of bootstrap value for non-pathogenic or avirulent group (Figure 4.36). Thus, there was very clearly demonstrated the relationship between degree of virulence and their genetic relationship by AFLP marker and pathogenicity test. Moreover, It is also clearly shown that the phenetic dendrogram generated by UPGMA on genotypes in 8 populations as pop1: Khonkaen province, pop2:Bangkok province, pop3: Sakornnakorn province, pop4:Burirum province, pop5: Nongkhai province, pop6: Nakhonratchasima province, pop7: Tak province and pop8:Phetchabun province.

With this, a principal coordinate analysis (PCA) grouped all of the *Fusarium* spp. isolates into eight major clusters. It observed that pop 1: Khonkaen and pop 5: Nongkai is located in the Northeast of Thailand where majority of planted areas of tomatoes in which these geographical areas were found more moderate and high virulent isolates (Figure 4.37).

Table 4.4. Total number of polymorphic bands of screening primer pairs

EcoRI primer	MseI primer	No. of bands
A	GTA	17
G	ACG	22
G	AAC	12.6
G	AGC	18.8
C	GTA	17
G	CTA	11.2
G	CGC	12.2
G	CAC	22.4
AG	GT	8.2
AC	GT	11.8
ACG	G	20.5
ACT	G	15.8
AGC	G	17
GCG	C	15.2
GTC	C	16
CGC	G	8
CTG	G	11.8



A **B**

Figure 4.35. DNA fingerprint of *Fusarium oxysporum* f. sp. *lycopersici* by AFLP markers using E+ACG/M+G (A) and E+G/M+CAC (B) primers. The polymorphic bands shown by arrows and lane 1 are 100 bp plus DNA Ladder (Fermentas).

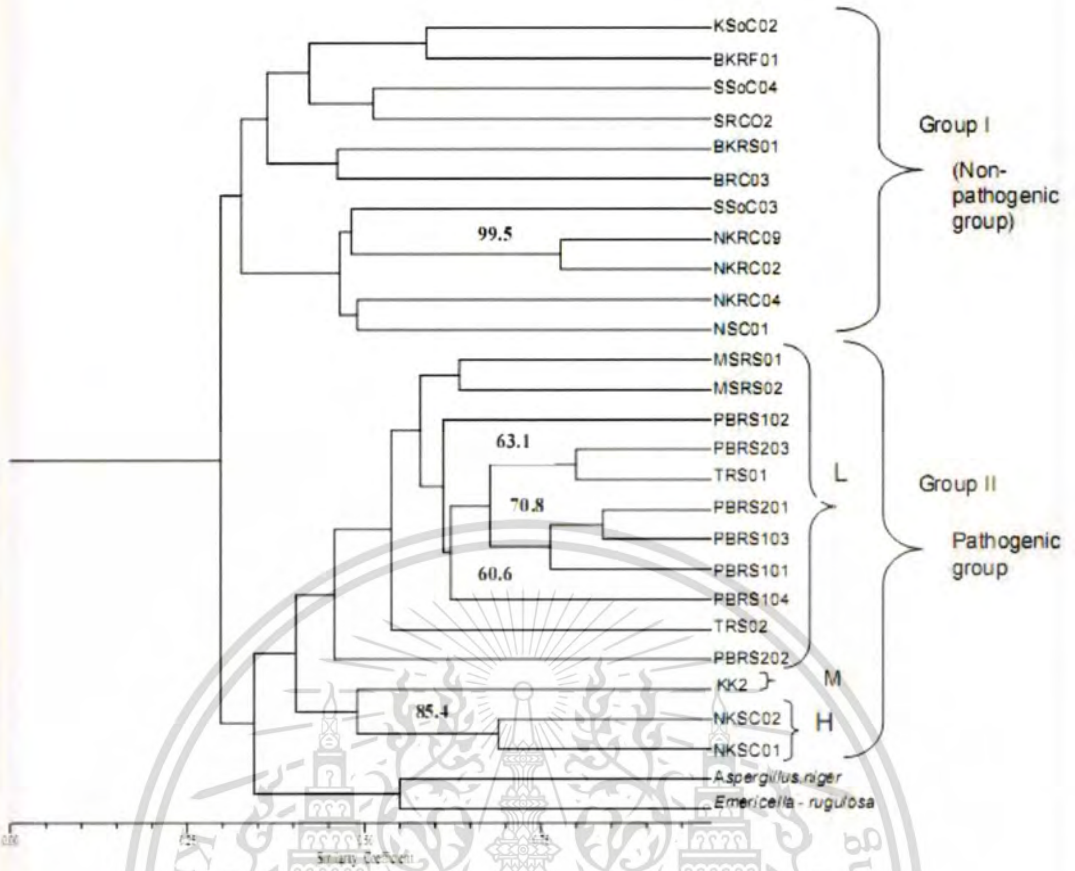
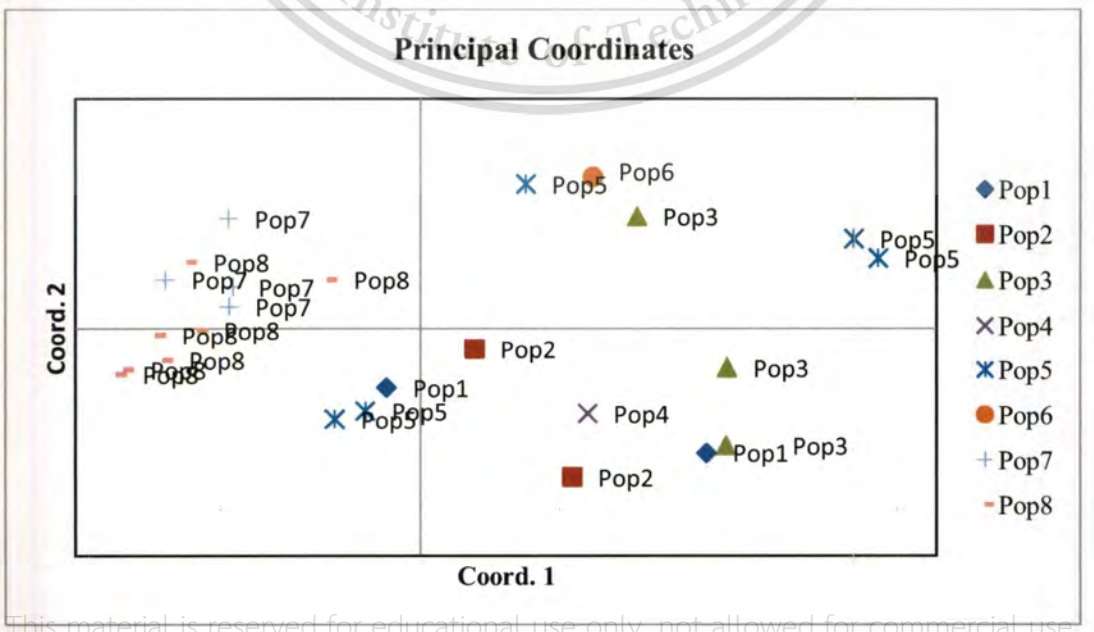


Figure 4.36 Phenetic dendrogram of *Fusarium oxysporum* f. sp. *lycopersici* isolates based on the binary matrix of polymorphic bands, using the UPGMA algorithm and Dice's similarity coefficient (NTSYS program). Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branch.



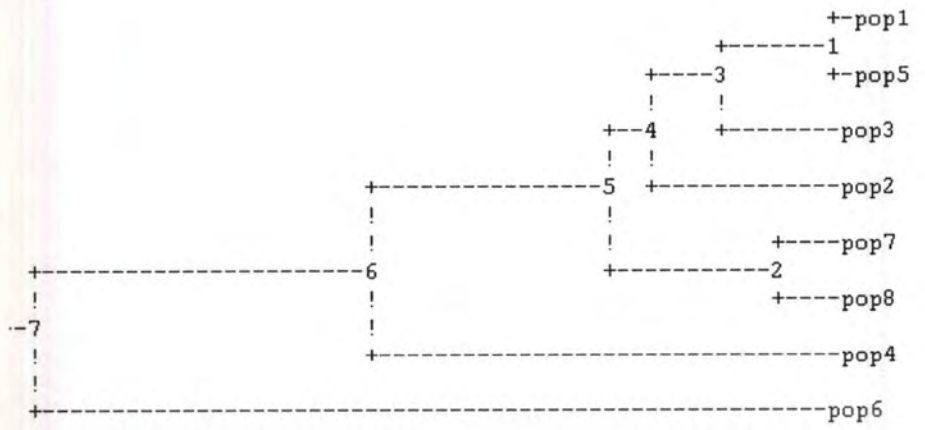


Figure 4.37. A, Principle coordinate analysis plot of AFLP genotypes of 25 isolates of *Fusarium oxysporum* f. sp. *lycopersici* from Thailand based on genetic Nei's (1978) genetic distance between genotypes. B, Dendrogram based Nei's genetic distance using UPGMA modified from neighbor procedure of PHYLIP version 3.5 based on AFLP genotypes in 8 populations, pop1: Khonkaen; pop2: Bangkok; pop 3: Sakon nakhon; pop4: Burirum; pop5: Nongkai; pop6 Nakhonratchasima; pop7: Tak and pop8: Phetchabun.

4.2 Antagonistic fungi

4.2.1. *Chaetomium brasilense* Batista & Pontual

Colonies are moderately growing on PDA, reaching a diameter of 5-6 cm in 10 days, with floccose aerial mycelium, becoming dark due to pigmented. Mycelium is grey or nearly white, reverse black when old. Ascomata are superficial or spherical, ostiolate, 80-105.9 x 121.0-200 µm, with a dark wall of angular cell. Ascomatal hairs are flexuous, undulate or spiraly coiled, septate, dark brown, verrucose or warty. Asci fasciculate, cylindrical, short stalk, with 8 ascospores. Ascospores ovate, bilaterally flattened, dark brown when mature, 5-6 x 6-7 x 7-8.5 µm, with a germ pore at the attenuated end. (Figure 4.38).

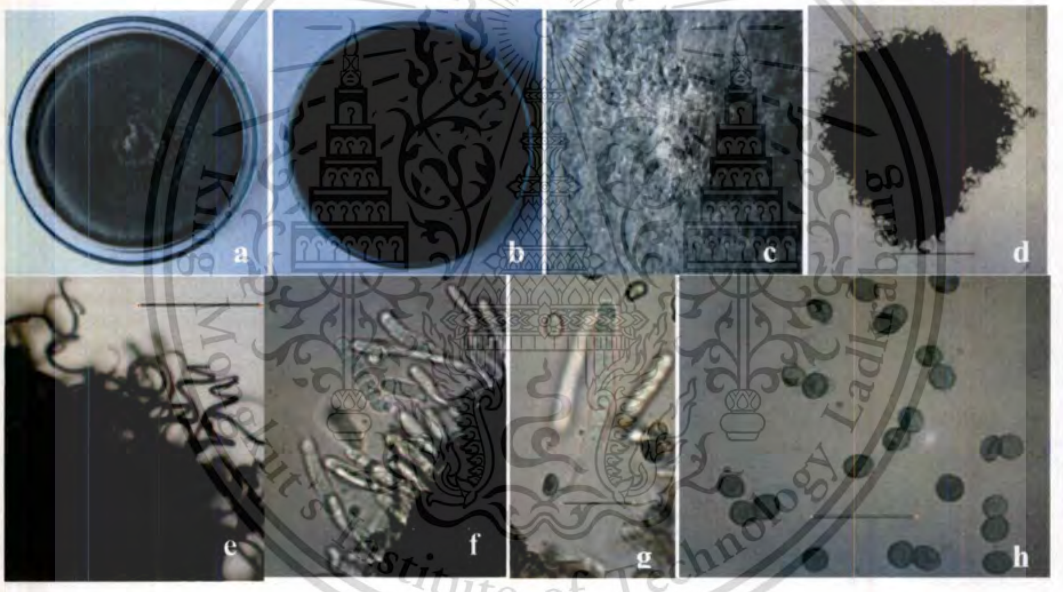


Figure 4.38. Morphological characteristic of *Chaetomium brasilense* at 10 days old culture on PDA. a. surface colony, b. back surface colony, c. mycelial mass, d. ascomata, e. ascomatal hairs, f. young asci, h. 8 ascospores in an ascus, i. ascospores. Bar. d= 100 µm, e,f,g,h=10µm.

4.2.2. *Chaetomium cupreum* Ames

Colonies usually are red due to pigment exudates. Ascomata are red, maturing within 10-14 days, ovate in shape, 79.7-142.7 x 94.7-151.5 µm. Ascomatal hairs arcuate, apically circinate or coiled, septate. Asci are clavate in shape with 8 ascospores per ascus. Ascospores are reniform, 4.7-6.7 x 6.7-10 µm, with a single apical germ pore. (Figure 4. 39).

Figure 4.39. Morphological characteristic of *Chaetomium cupreum* at 10 days old culture on PDA. a. surface colony, b. back surface colony, c. mycelial mass, d. ascomata, e. ascomatal hairs, f. young asci, h.ascospores. Bar. d= 100 µm, e,f,g=10µm.

4.2.3. *Chaetomium elatum* Kunze

Colonies on PDA with a daily growth rate of 5-6 mm, sparse aerial mycelia, white, exudates yellowish green color; ascomata maturing within 21 days, olivaceous or grey-green in reflected light, superficial, spherical to ovate, ostiolate, 182-410 µm with a brown wall of textura intricate on surface; ascomatal hairs numerous, long, flexuous, dichotomously or branched, mostly in the upper part, dark brown, septate, verrucose, 4-5 µm broad near the base; asci clavate to fusuform, stalked, 8-spored, evanescent, 32-45 x 15-19 µm; ascospores limoniform, bilaterally flattened, brown when mature, thick-walled, 12-15 x 9-12 x 8-10 µm, with an apical germ pore. (Figure 4.40).

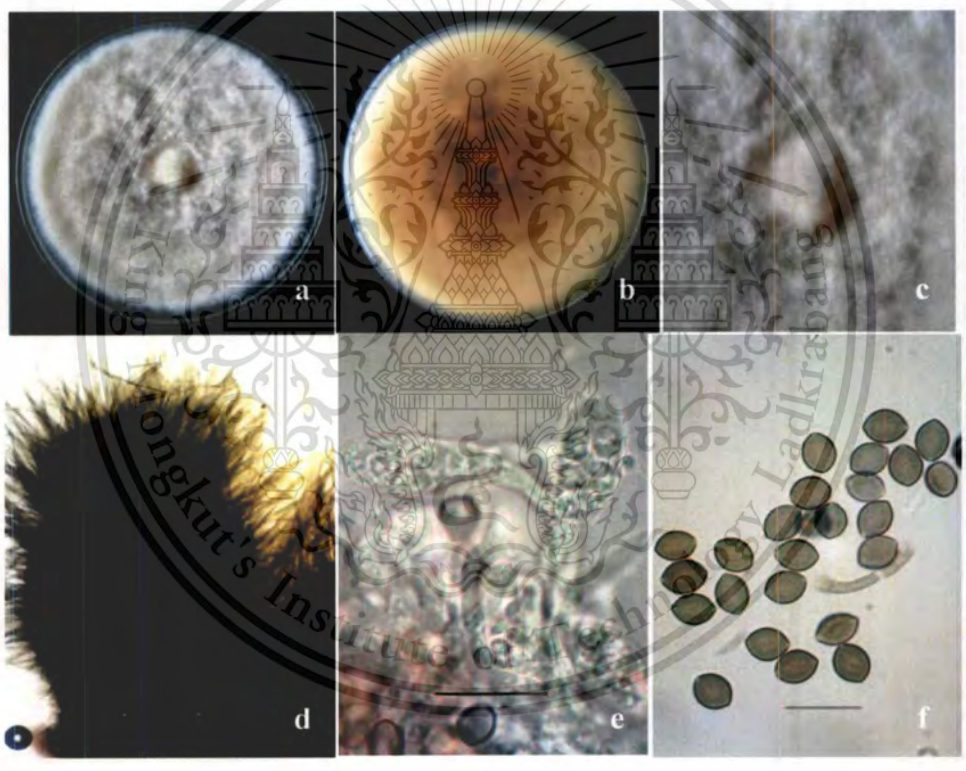


Figure 4.40. Morphological characteristic of *Chaetomium elatum* at 10 days old culture on PDA. a. surface colony, b. back surface colony, c. mycelial mass, d. ascomata, e. young asci, f.ascospores. Bar. d= 100 µm, e,f,=10µm.

4.2.4. *Chaetomium lucknowense* Rai & Tewari

Colonies grow on PDA full plate (9 cm diameter) in 10-12 days at room temperature (28-32°C), olive green to brown or grey when mature. Ascomata are grey green, superficial, spherical or ovate shaped, 178.5-205.5 µm. Ascomatal hairs are numerous, flexous. Asci shaped in clavate with short stalks, 8 ascospores, 24.5-27.5 x 12.2-13.6 µm. Ascospores shaped in ovate, olivaceous brown, 6.5-8.5 x 5.5-6.8 µm, with a germ pore (as seen in Figure 4.49). (Figure 4.41)

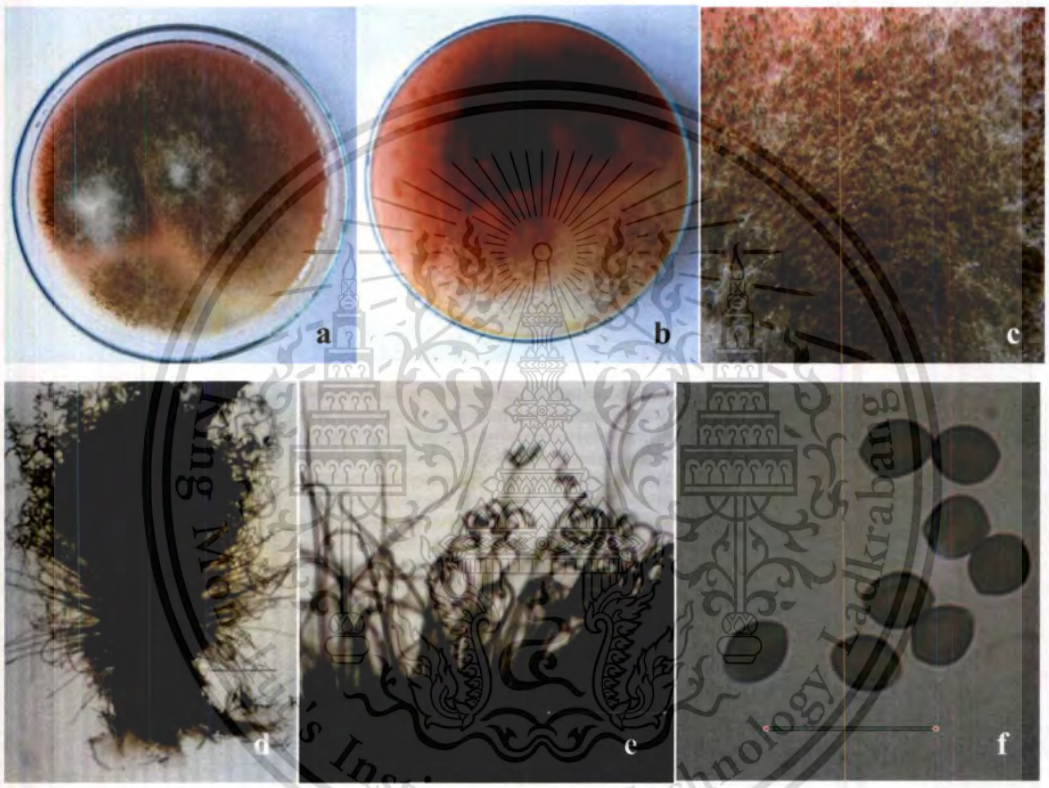


Figure 4.41 Morphological characteristic of *Chaetomium lucknowense* at 10 days old culture on PDA. a. surface colony, b. back surface colony, c. mycelial mass, d. ascomata, e. young asci, f.ascospores. Bar. d= 100 µm, e,f,=10µm.

4.2.5. *Emericella nidulans* (Eidam) Vuill.

Anamorph: *Aspergillus nidulans* (Eidam) Winter

Conidia diameters on PDA 5-6 cm in two weeks at room temperature (28-30°C), green from conidia becoming brownish from ascomata. Ascomata (Cleistothecia) are abundant, dull yellow globose, 130.0-345.0 µm in diameter, surrounded by hülle cells. Hülle cells ellipsoidal to globose, 9.5-20.0 µm in diameter. Asci are globose to subglobose, 8-spored. Ascospores are red to purple, smooth, 2.8-4.5 × 3.5-5.8 µm, with two narrow longitudinal flanges, with entire margins (Figure 4.42).

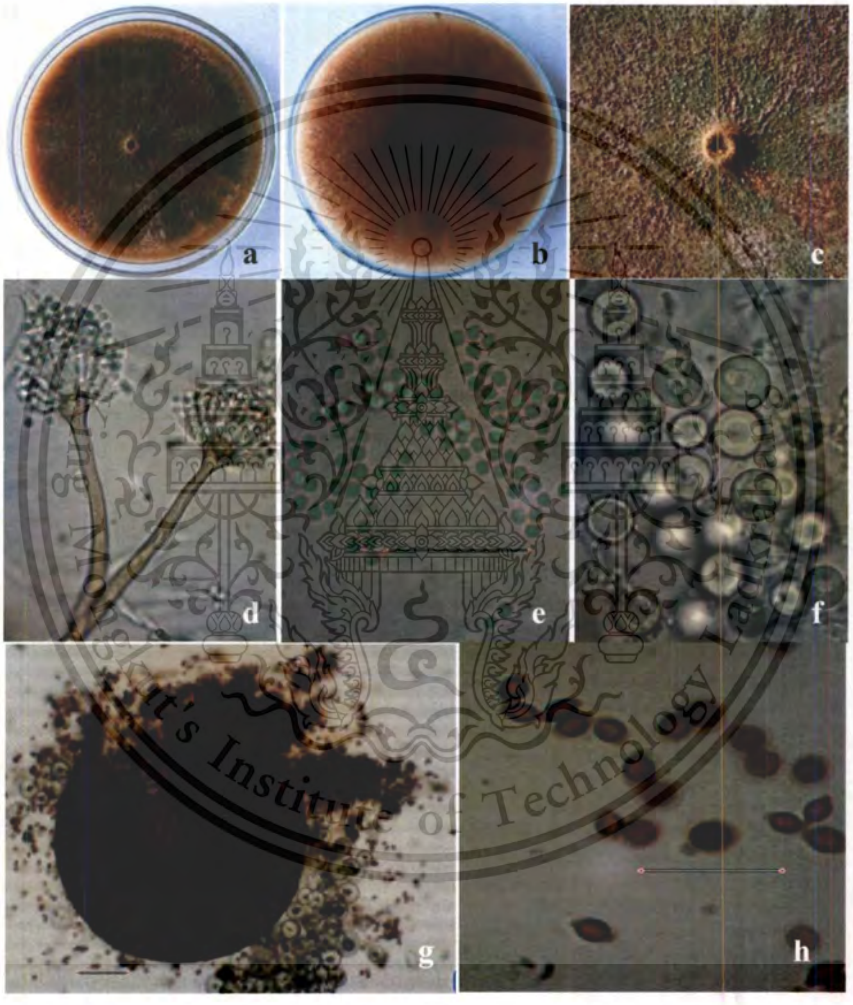


Figure 4.42. Morphological characteristic of *Emericella nidulans* at 10 days old culture on PDA. a. surface colony, b. back surface colony, c. mycelial mass, d. thalli in imperfect stage, e. conidia, f. hulle cells, g. cheistothecium or ascomatum, h. ascospores in perfect stage

4.2.6. *Emericella rugulosa* (Thom & Raper) C.R. Benjamin

Anamorph: *Aspergilus rugulovalvus*, formerly *A. rugulosus* Thom & Raper

Colonies diameter on PDA 5-6.0 cm in two weeks at room temperature, green to dark green colors from conidia. Conidia are globose, rugulose, 3-4 µm in diameter. Conidiolphores smooth-walled, pale brownish, 50-80 µm, conidial heads columnar. Colonies become brownish from ascomata. Ascomata (Cleistothecium) are abundant, dull yellow globose, 250.0-410.0 µm in diameter, surrounded by dark brown, globose hülle cells. Hülle cells are ellipsoidal to globose, 9.5-35 µm in diameter. Asci are globose to subglobose, 8-spored. Ascospores are purple-red lenticular, rugulose, with two sinuate equatorial crests, 2.5-4.2 × 3.2-4.5 µm (Figure 4.43).

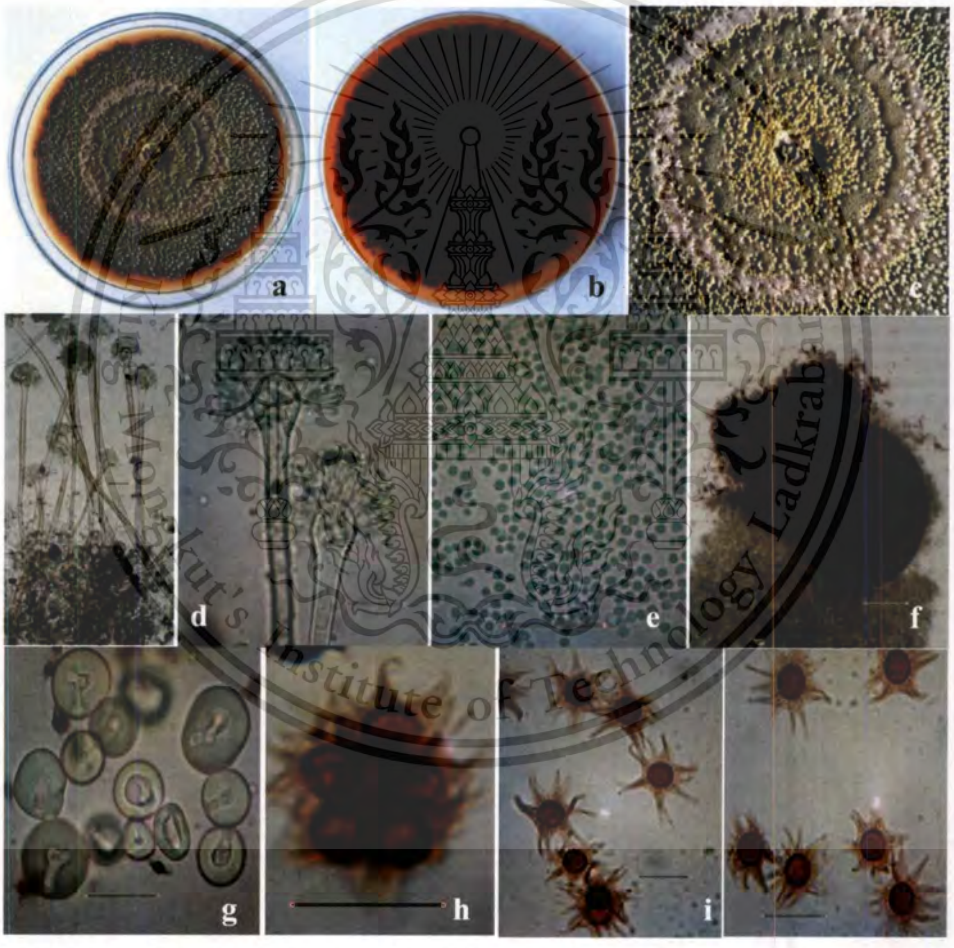


Figure 4.43. Morphological characteristic of *Emericella rugulosa* at 10 days old culture on PDA. a. surface colony, b. back surface colony, c. mycelial mass, d. thalli in imperfect stage, e. conidia, f. cheistothecium or ascomatum, g. hulle cells, h. ascospores in an ascus, i. ascospores.

4.3 Antogonism Tests

4.3.1 Bi-culture antagonistic tests

The antagonistic fungi including *Chaetomium brasilense* CB01, *Chaetomium cupreum* CC03, *Chaetomium elatum* ChE01, *Chaetomium lucknowense* CLT01, *Emericella nidulans* EN01 and *Emericella rugulosa* ER01 were tested for their abilities to inhibit the growth of *F. oxysporum* f. sp. *lycopersici* NKSC02. Results showed that *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 significantly inhibited colony growth of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 6.06, 5.56, 2.25, 5.45, 5.85 and 5.68 cm, respectively when compared to the control plate (Table 4.5 and Fig 4.45). *Ch. elatum* ChE01 gave significantly better inhibition of colony growth at 74. 99 % than *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 which the percent inhibition of colony growth of 32.66, 38.19, 39.43, 35.13 and 36.80 %, respectively (Fig 4.46). It is interesting that *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01 and *E. nidulans* EN01 gave least number of pathogen conidia and not significantly different in number of pathogen conidia in bi-culture plates which were 1.53 $x10^7$, 0.91 $x10^7$, 1.49 $x10^7$and 1.39 $x10^7$, respectively and followed by *Ch. lucknowense* CLT01 and *E. nidulans* EN01 which the number of pathogen conidia in bi-culture plates were 3.19 $x10^7$ and 2.48 $x10^7$, respectively. With this, it also showed that *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01 and *E. nidulans* EN01 were not significantly inhibited the pathogen conidia in bi-culture plates which were 77.5, 86.51, 77.72 and 79.37 %, respectively while *Ch. lucknowense* CLT01 and *E. nidulans* EN01 inhibited the pathogen conidia of 53.18 and 63.27 %, respectively (Table 4.5 and Fig 4.46, 4.47 and 4.48).

Table 4.5. Colony diameter, number of conidia, percent inhibition of colony and conidia of *F. oxysproum* f. sp. *lycopersici* in Bi-culture test at 30 days

Treatments	Colony diameter (cm)	Number of conidia($x10^7$)	% inhibition of colony growth	% inhibition of conidia
Control	9.00a[1]	6.90a		
CB01 vs Fol[2]	6.06b	1.53c	32.63b	77.51a
CC03 vs Fol	5.56b	0.91c	38.19b	86.51a
ChE01 vs Fol	2.25c	1.49c	74.99a	77.72a
CLT01 vs Fol	5.45b	3.19b	39.43b	53.18b
EN01 vs Fol	5.83b	1.39c	35.13b	79.37a
ER01 vs Fol	5.68b	2.48b	36.80b	63.27b
CV (%)	5.06	16	8.07	7.88

[1] Average of four replications, Means followed by the same letters were not significantly different by DMRT at P=0.01, [2] *F. oxysproum* f. sp. *lycopersici*.

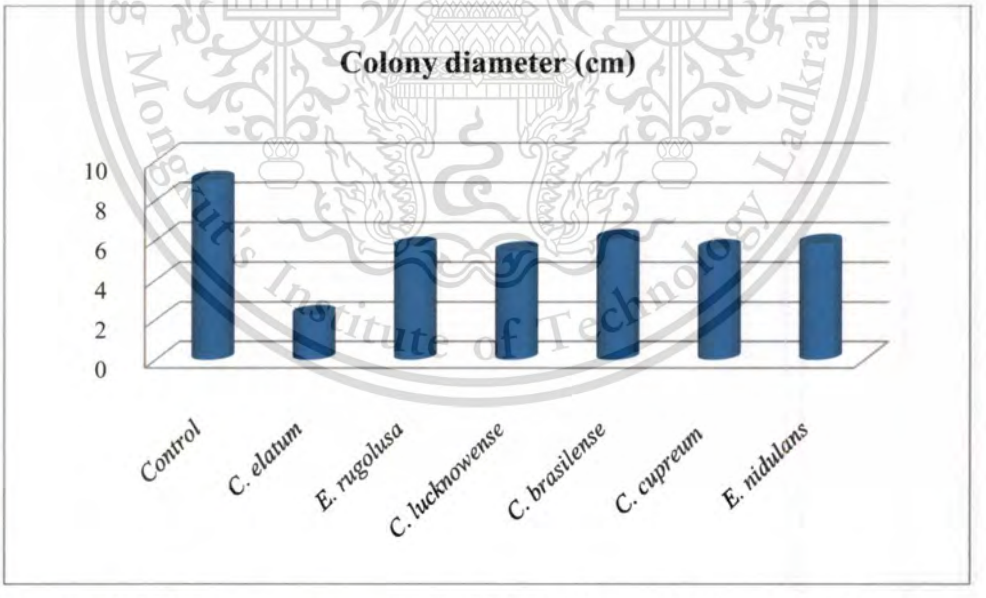


Figure 4.44 Colony diameter (cm) of the pathogen in Bi-culture plate.

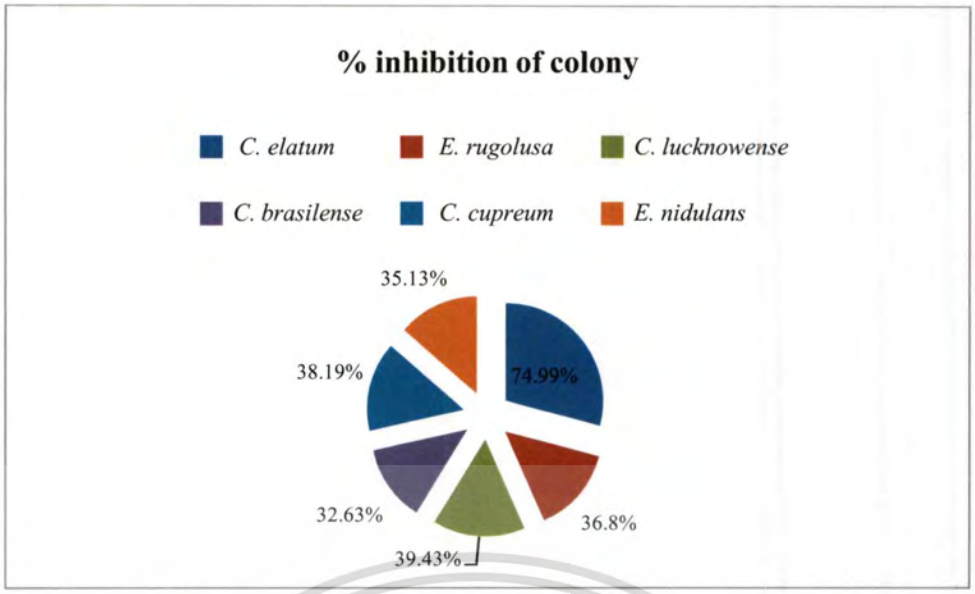


Figure 4.45 Percent inhibition of conidia of the pathogen in Bi-culture plates.

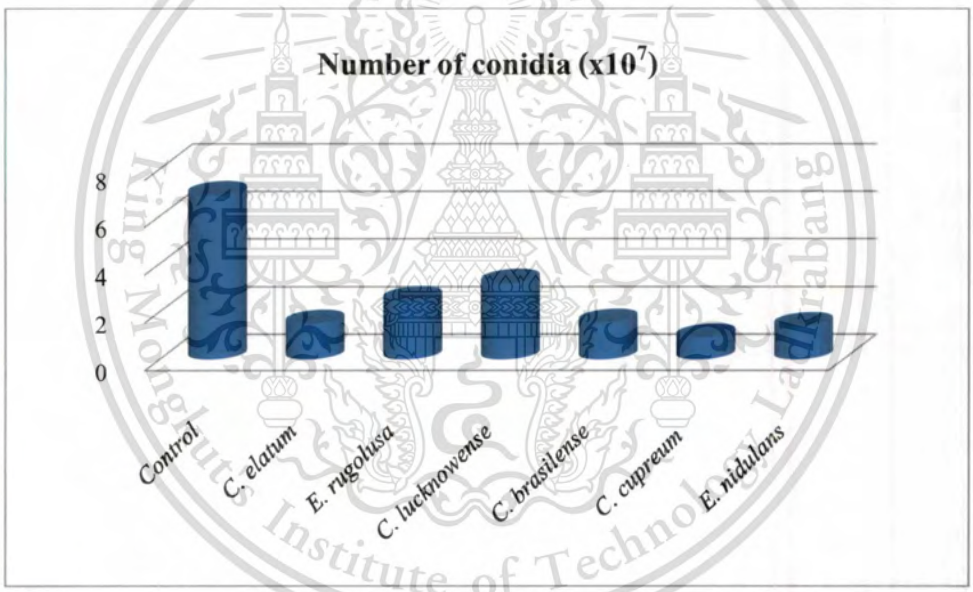


Figure 4.46 Number of conidia of the pathogen in Bi-culture plates.

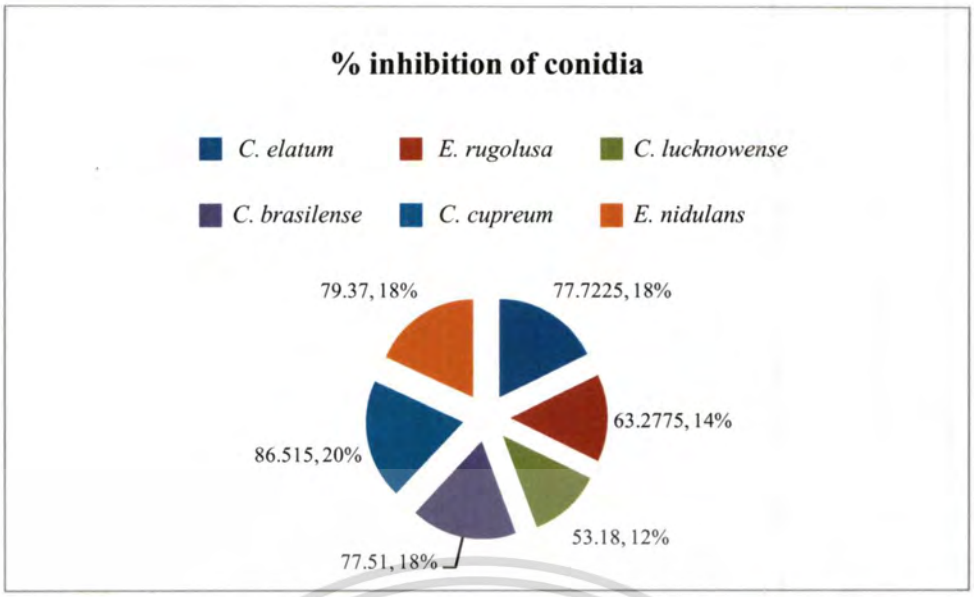


Figure 4.47 Percent inhibition of conidia of the pathogen in Bi-culture plates.

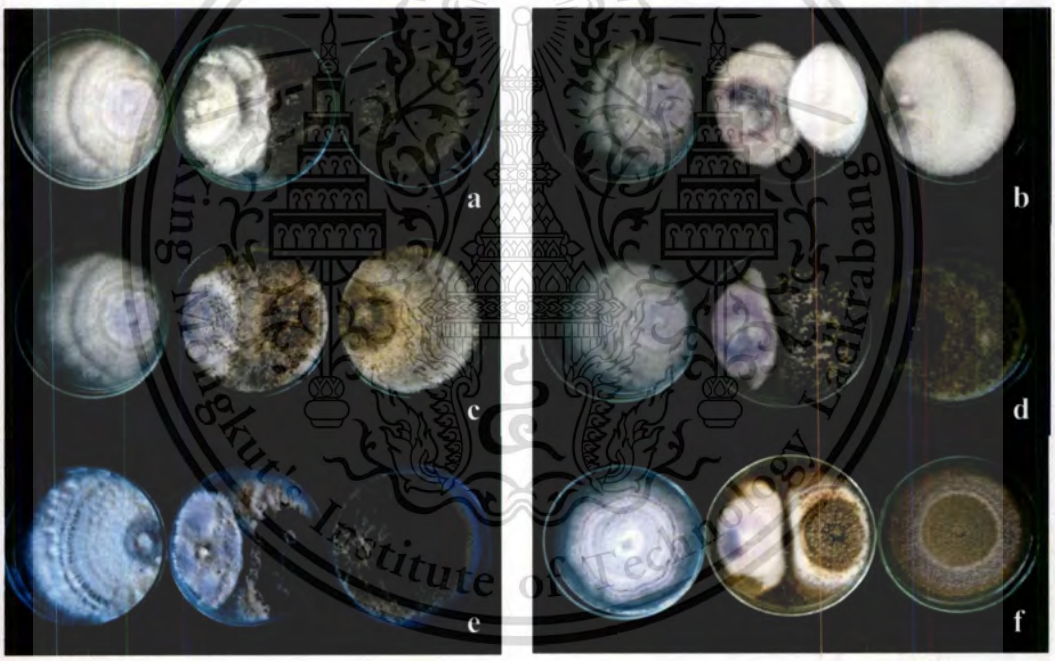


Figure 4.48 Bi-culture test of antagonistic fungi and pathogen. a. *C. brasilense* vs. *F. oxysporum* NKSC02, b. *Ch. cupreum* vs. *F .oxysporum* NKSC02, c. *Ch. elatum* vs. *F. oxysporum* NKSC02, d. *Ch. lucknowense* vs. *F. oxysporum* NKSC02, e. *E. nidulans* vs. *F. oxysporum* NKSC02, f. *E. rugulosa* vs. *F. oxysporum* NKSC02.

4.3.2. Bioactivities tests of crude extracts and pure compounds from antagonistic fungi

4.3.2.1 Extraction results

The antagonistic fungi; *Chaetomium brasilense* CB01, *Chaetomium cupreum* CC03, *Chaetomium elatum* ChE01, *Chaetomium lucknowense* CLT01, *Emericella nidulans* EN01 and *Emericella rugulosa* ER01 were cultured in PDB (7.5 L each). After 30 days, fungal biomass were removed from PDB and dried at air temperature over night (Fig 4.49).

Figure 4.49 Fungal biomasses of antagonistic fungi were used for crude extractions. A. *Ch. brasilense*, B. *Ch. cupreum*, C. *Ch. elatum*, D. *Ch. lucknowense*, E. *E. nidulans*, and F. *E. rugulosa*

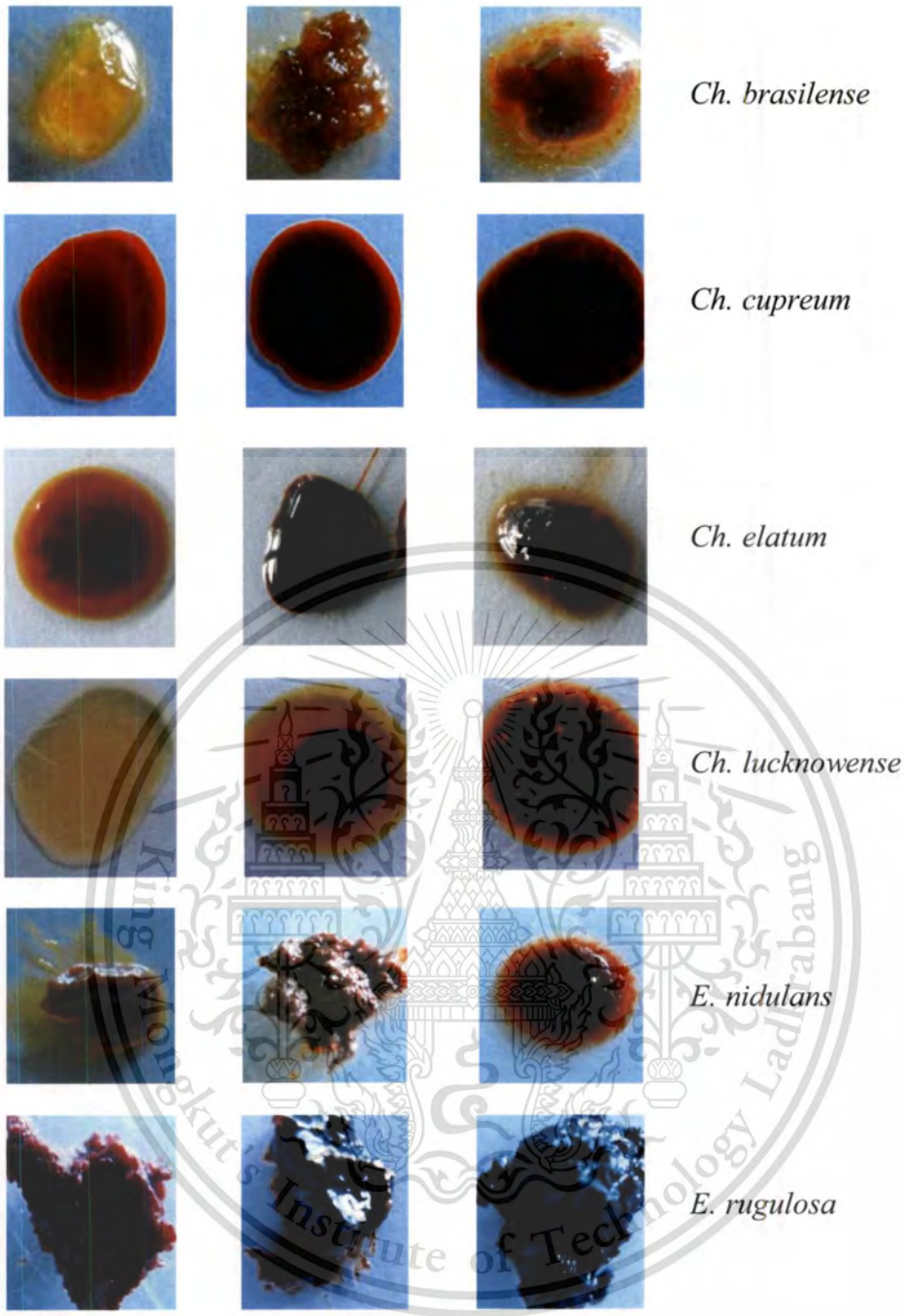


Figure 4.50 Characteristics of crude extracts from antagonistic fungi

Dried weight fungal biomasses of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01were weighted as 70, 70, 40, 50, 50 and 70 g, respectively. *Ch. brasilense* CB01 yielded crude hexane 2.5 g (3.57%), crude EtOAc 6.5 g (9.28%) and 6.76 g (9.65 %). *Ch. cupreum* CC03 yielded crude hexane 3g (4.28%), crude EtOAc 5.5 g(7.85 %) and

6.5 g(8.85 %) of crude MeOH. *Ch. elatum* ChE01 yielded crude hexane 0.44g (1.1%), crude EtOAc 2.5 g(6.25%) and 3.2 g (8%) of crude MeOH. *Ch. lucknowense* CLT01 yielded crude hexane 1.09 g (2.18 %), crude EtOAc 2.86 g (5.72%) and 4.67 g (9.34 %) of crude MeOH. *E. nidulans* EN01 yielded crude hexane 0.5g (1.25 %), crude EtOAc 0.88 g (2.2%) and 2.25 g (5.60 %) of crude MeOH and *E. rugulosa* ER01 yielded crude hexane 1.55 g (2.21 %), crude EtOAc3.45 g (4.92%) and 4.43 g (6.32 %) of crude MeOH (Table4.6 , Fig 4.51).

Table 4. 6 Yields of fungal biomass and crude extracts of antagonistic fungi

Antagonists	Dried weight (g)	Crude hexane (g)	% yield	Crude EtOAc (g)	% yield	Crude MeOH (g)	% yield
Ch. brasilense	70	2.5	3.57	6.5	9.28	6.76	9.65
Ch. cupreum	70	3	4.28	5.5	7.85	6.2	8.85
Ch. elatum	40	0.44	1.1	2.5	6.25	3.2	8
Ch.lucknowense	50	1.09	2.18	2.86	5.72	4.67	9.34
E. nidulans	40	0.5	1.25	0.88	2.2	2.25	5.60
E. rugulosa	70	1.55	2.21	3.45	4.92	4.43	6.32

4.3.2.2 Bioactivities tests of crude extract against *Fusarium oxysporum* f sp *lycopersici*

Crude extracts of antagonistic fungi namely *Chaetomium brasilense* CB01, *Chaetomium cupreum* CC03, *Chaetomium elatum* ChE01, *Chaetomium lucknowense* CLT01, *Emericella nidulans* EN01 and *Emericella rugulosa* ER01 at different concentrations of 0, 10, 50, 100, 500, and 1,000 g/ml were tested for inhibition of *F. oxysporum* f. sp. *lycopersici* NKSC02 which obtained from previous experiment.

Hexane crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 3.67, 3.19, 2.67, 2.37 and 1.94 cm, respectively when compared to the control (0 µg/ml) of 5 cm. EtOAc crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml

gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 3.05, 2.92, 2.64, 2.27 and 2.22 cm, respectively when compared to the control (0 µg/ml) of 5 cm. MeOH crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 3.67, 3.50, 2.97, 2.77 and 2.22 cm, respectively when compared to the control (0 µg/ml) of 5 cm. (Table 4.7 and Fig 4.52).

Hexane crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 5.00, 4.87, 4.47, 4.45 and 4.12 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. EtOAc crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 5.00, 3.92, 3.67, 3.54 and 3.40 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. MeOH crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 4.47, 4.12, 3.74, 3.54 and 3.25 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. (Table 4.7 and Fig 4.53).

Hexane crude extract from *Ch. elatum* ChE01at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 4.12, 4.02, 3.92, 3.27 and 3.12 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. EtOAc crude extract from *Ch. elatum* ChE01at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 3.80, 3.35, 3.19, 2.55 and 2.44 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. MeOH crude extract from *Ch. elatum* ChE01at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 4.75, 4.12, 4.04, 3.90 and 3.80 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. (Table 4.7 and Fig 4.54).

Hexane crude extract from *Ch. lucknowense* CLT01the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 4.90, 4.80, 4.09, 3.92 and 3.85 cm,

respectively when compared to the control (0 µg/ml) of 5.00 cm. EtOAc crude extract from *Ch. lucknowense* CLT01the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 4.87, 4.77, 4.52, 4.22 and 3.80 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. MeOH crude extract from *Ch. lucknowense* CLT01at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 4.90, 4.72, 4.69, 3.87 and 3.57 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. (Table 4.7 and Fig 4.55).

Hexane crude extract from *E. nidulans* EN01 the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 5.00, 4.85, 4.50, 4.32 and 4.09 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. EtOAc crude extract from *E. nidulans* EN01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 5.00, 3.92, 3.72, 3.47 and 3.47 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. MeOH crude extract from *E. nidulans* EN01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 5.00, 3.90, 3.40, 3.09 and 2.60 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm.(Table 4.7 and Fig 4.56).

Hexane crude extract from *E. rugulosa* ER01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 5.00, 5.00, 5.00, 4.80 and 4.29 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. EtOAc crude extract from *E. rugulosa* ER01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 5.00, 4.97, 4.87, 4.70 and 3.67 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. MeOH crude extract from *E. rugulosa* ER01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 5.00, 5.00, 4.95, 4.90 and 4.62 cm, respectively when compared to the control (0 µg/ml) of 5.00 cm. (Table 4.7 and Fig 4.57).

It revealed that crude extract at 1000 µg/ml from hexane of *Ch. brasilense* CB01 gave significantly better inhibited the colony growth of *Fusarium oxysporum* f.sp. *lycopersici* as 61 % better than crude extracts from EtOAc and MeOH which were 55.50 and 55.50 % (Table 4.8).

Crude extract at 1000 µg/ml from MeOH of *Ch. cupreum* CC03gave significantly better inhibited the colony growth of *Fusarium oxysporum* f.sp. *lycopersici* as 35 % better than crude extracts from EtOAc and hexane which were 35.0 and 17.5 %, respectively. Table 4.8.

Crude extract at 1000 µg/ml from EtOAc of *Ch. elatum* ChE01 gave significantly better inhibited the colony growth of *Fusarium oxysporum* f.sp. *lycopersici* as 51.0 % better than crude extracts from hexane and MeOH which were 37.5 and 24.0 %, respectively. Crude extract at 1000 µg/ml from MeOH of *Ch. lucknowense* CLT01 gave significantly better inhibited the colony growth of *Fusarium oxysporum* f.sp. *lycopersici* as 28.5 % better than crude extracts from EtOAc and hexane which were 24.0 and 23.0 %, respectively. Crude extract at 1000 µg/ml from MeOH of *E. nidulans* EN01 gave significantly better inhibited the colony growth of *Fusarium oxysporum* f. sp. *lycopersici* as 48.0 % better than crude extracts from EtOAc and hexane which were 30.5 and 18.0 %, respectively. Crude extract at 1000 µg/ml from EtOAc of *E. rugulosa* ER01gave significantly better inhibited the colony growth of *Fusarium oxysporum* f. sp. *lycopersici* as 26.5 % better than crude extracts from hexane and MeOH which were 14.0 and 7.5 %, respectively (Table 4.8).

Hexane crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 21.5 $x10^7$, 15.93 $x10^7$, 14.0 $x10^7$ and 2.16 $x10^7$, respectively.when compared to the control (0 µg/ml) of 35.78 $x10^7$ EtOAc crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02which were 26.71 $x10^7$, 19.61 $x10^7$, 11.48 $x10^7$, 5.35 $x10^7$ and 4.40 $x10^7$cm, respectively when compared to the control (0 µg/ml) of 36.24 $x10^7$ cm. MeOH crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 11.83 $x10^7$, 9.84 $x10^7$, 8.52 $x10^7$, 4.28 $x10^7$ and 1.07 $x10^7$, respectively when compared to the control (0 µg/ml) of 35.72 $x10^7$ (Table 4.9).

Hexane crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02which were 15.93 $x10^7$, 8.64 $x10^7$, 6.82 $x10^7$, 5.94 $x10^7$and 3.18 $x10^7$, respectively when compared to the control (0 µg/ml) of 39.50 $x10^7$. EtOAc crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 14.43 $x10^7$, 8.87 $x10^7$, 7.68 $x10^7$, 4.48 $x10^7$and 2.40 $x10^7$, respectively when compared to the control (0 µg/ml) of 38.47 $x10^7$. MeOH crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02which were 13.93 $x10^7$, 8.43 $x10^7$, 6.16 $x10^7$, 2.86 $x10^7$and 1.07 $x10^7$, respectively when compared to the control (0 µg/ml) of 41.00 $x10^7$ (Table 4.9).

Hexane crude extract from *Ch. elatum* ChE01at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 13.79 $x10^7$, 11.83 $x10^7$, 8.75 $x10^7$, 7.76 $x10^7$and 5.90 $x10^7$, respectively when compared to the control (0 µg/ml) of 37.81 $x10^7$. EtOAc crude extract from *Ch. elatum* ChE01at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 13.07 $x10^7$, 10.82 $x10^7$, 4.64 $x10^7$, 2.89 $x10^7$ and 1.65 $x10^7$, respectively when compared to the control (0 µg/ml) of 37.68 $x10^7$. MeOH crude extract from *Ch. elatum* ChE01at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 29.03 $x10^7$, 23.00 $x10^7$, 12.84 $x10^7$, 9.06 $x10^7$and 7.54 $x10^7$, respectively when compared to the control (0 µg/ml) of 38.62 $x10^7$ (Table 4.9).

Hexane crude extract from *Ch. lucknowense* CLT01at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 39.50 $x10^7$, 37.62 $x10^7$, 34.12 $x10^7$, 30.43 $x10^7$and 15.93 $x10^7$, respectively when compared to the control (0 µg/ml) of 43.31 $x10^7$. EtOAc crude extract from *Ch. lucknowense* CLT01the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 38.50 $x10^7$, 36.62 $x10^7$, 33.05 $x10^7$, 22.50 $x10^7$and 12.00 $x10^7$, respectively when compared to the control (0 µg/ml)

of 43.81 x10^7. MeOH crude extract from *Ch. lucknowense* CLT01at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02which were 29.93 x10^7, 26.93 x10^7, 14.43 x10^7, 10.50 x$10^7$and 3.81, respectively when compared to the control (0 µg/ml) of 42.75 x10^7 (Table 4.9).

Hexane crude extract from *E. nidulans* EN01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 14.07 x10^7, 12.78 x10^7, 11.91 x10^7, 10.11 x10^7 and 5.82 x10^7, respectively when compared to the control (0 µg/ml) of 14.67 x10^7. EtOAc crude extract from *E. nidulans* EN01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 12.31 x10^7, 11.11 x10^7, 10.11 x10^7, 7.13 x10^7 and 5.33 x10^7, respectively when compared to the control (0 µg/ml) of 15.00 x10^7. MeOH crude extract from *E. nidulans* EN01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 11.81 x10^7, 10.08 x10^7, 5.70 x10^7, 3.77 x10^7 and 2.61 x10^7, respectively when compared to the control (0 µg/ml) of 14.10 x10^7 (Table 4.9).

Hexane crude extract from *E. rugulosa* ER01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 16.67 x10^7, 15.43 x10^7, 12.33 x10^7, 7.92 x10^7 and 4.22 x10^7 , respectively when compared to the control (0 µg/ml) of 17.62 x10^7. EtOAc crude extract from *E. rugulosa* ER01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 15.61 x10^7, 14.47 x10^7, 8.66 x10^7, 4.50 x10^7 and 2.15 x10^7, respectively when compared to the control (0 µg/ml) of 17.25 x10^7. MeOH crude extract from *E. rugulosa* ER01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in conidia production by *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 16.37 x10^7, 15.44 x10^7, 14.33 x10^7, 13.05 x10^7 and 7.54 x10^7, respectively when compared to the control (0 µg/ml) of 17.50 x10^7 as shown in Table 4.9.

It revealed that crude extract at 1000 µg/ml from MeOH of *Ch. brasilense* CB01 gave significantly better inhibited the conidia production by *Fusarium oxysporum* f. sp. *lycopersici* as 96.98 % and ED_{50} value of 2.99 better than crude

extracts from Hexane and EtOAc which were 93.95, 87.85 % while ED_{50} value of 29.87 and 38.99 reapectively.

Crude extract at 1000 µg/ml from MeOH of *Ch. cupreum* CC03gave significantly better inhibited the conidia production by *Fusarium oxysporum* f. sp. *lycopersici* as 97.37 % and ED_{50} value of 2.65 better than crude extracts from hexane and EtOAc which were 93.75 and 91.92 %, while ED_{50} value of 2.33 and 2.38 respectively. Crude extract at 1000 µg/ml from EtOAc of *Ch. elatum* ChE01gave significantly better inhibited the conidia production by *Fusarium oxysporum* f. sp. *lycopersici* as 95.10 % and ED_{50} value of 3.39 better than crude extracts from hexane and MeOH which were 83.85 and 80.44 % and ED_{50} value of 0.65 and 63.42 respectively. Crude extract at 1000 µg/ml from MeOH of *Ch. lucknowense* CLT01 gave significantly better inhibited the conidia production by *Fusarium oxysporum* f. sp. *lycopersici* as 91.07 % and ED_{50} value of 53 better than crude extracts from EtOAc and hexane which were 72.59 and 63.68 % and ED_{50} value of 921 and 393 respectively. Crude extract at 1000 µg/ml from MeOH of *E. nidulans* EN01 gave significantly better inhibited the conidia production by *Fusarium oxysporum* f. sp. *lycopersici* as 81.44 % and ED_{50} value of 112 better than crude extracts from EtOAc and hexane which were 64.40 and 60.28 % and ED_{50} value of 915 and 379 respectively. Crude extract at 1000 µg/ml from EtOAc of *E. rugulosa* ER01gave significantly better inhibited the conidia production by *Fusarium oxysporum* f. sp. *lycopersici* as 87.55 % and ED_{50} value of 138 better than crude extracts from hexane and MeOH which were 76.00 and 55.41 % and ED_{50} value of 313 and 1372 respectively as shown in Table 4.10.

Table 4.7. Effect of crude extracts from antagonistic fungi on mycelia growth of *Fusarium oxysporum* f. sp. *lycopersici* NKSC02

Crude extracts	Colony diameter (cm) of *Fusarium oxysporum* f.sp. *lycopersici* concentration (µg/ml))					
	0	10	50	100	500	1000
Ch. brasilense						
Hexane	5a[1]	3.67b	3.19c	2.67f	2.37g	1.94h
EtOAc	5a	3.05cd	2.92de	2.64f	2.27g	2.22g
MeOH	5a	3.67b	3.50b	2.97de	2.77ef	2.22g
Ch. cupreum						
Hexane	5a	5.00a	4.87a	4.47b	4.45b	4.12b
EtOAc	5a	5.00a	3.92d	3.67ef	3.54f	3.40g
MeOH	5a	4.47b	4.12c	3.74e	3.54f	3.25 h
Ch. elatum						
Hexane	5a	4.12b	4.02bc	3.92bc	3.27d	3.12d
EtOAc	5a	3.80c	3.35d	3.19d	2.55e	2.44e
MeOH	5a	4.75a	4.12b	4.04bc	3.90bc	3.80c
Ch. lucknowense						
Hexane	5a	4.90ab	4.80bcd	4.09g	3.92h	3.85hi
EtOAc	5a	4.87bc	4.77cd	4.52e	4.22f	3.80i
MeOH	5a	4.90ab	4.72d	4.69d	3.87hi	3.57j
E. nidulans						
Hexane	5a	5.00a	4.85a	4.50b	4.32c	4.09d
EtOAc	5a	5.00a	3.92e	3.72f	3.47g	3.47g
MeOH	5a	5.00a	3.90e	3.40g	3.09h	2.60i
E. rugulosa						
Hexane	5a	5.00a	5.00a	5.00a	4.80abc	4.29d
EtOAc	5a	5.00a	4.97a	4.87ab	4.70bc	3.67e
MeOH	5a	5.00a	5.00a	4.95a	4.90ab	4.62c

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

Table 4.8. Effect of crude extracts from antagonistic fungi for percentage of colony inhibition growth of *Fusarium oxysporum* f. sp. *lycopersici* NKSC02.

Crude extracts	Colony inhibition of *Fusarium oxysporum* f.sp. *lycopersici* (%)				
	10	50	100	500	1000
Ch. brasilense					
Hexane	26.5g[1]	36.0f	46.5c	52.5b	61.0a
EtOAc	39.0ef	46.0cd	47.0c	54.5b	55.5b
MeOH	26.5 g	30.0 g	40.5 def	44.5 cde	55.5b
Ch. cupreum					
Hexane	0.0h	2.5h	10.5g	11.0g	17.5f
EtOAc	0.0h	21.5 e	26.5 cd	29.0 bc	32.0 ab
MeOH	10.5 g	17.5 f	25.0 d	31.0 b	35.0 a
Ch. elatum					
Hexane	7.5d	19.5cd	21.5cd	34.5b	37.5b
EtOAc	24.0c	33.0b	37.0b	49.0a	51.0a
MeOH	5.0e	17.5d	19.0cd	22.0cd	24.0c
Ch. lucknowense					
Hexane	2.0 g	4.0 fg	18.0 c	21.5 b	23.0 b
EtOAc	2.5 g	4.5 fg	9.5 e	15.5 d	24.0 b
MeOH	2.0 g	5.5 f	6.0 f	22.5 b	28.5 a
E. nidulans					
Hexane	0.0	3.0g	10.0 f	13.5 f	18.0 e
EtOAc	0.0	21.5 de	25.5 d	30.0 c	30.5 c
MeOH	0.0	22.0 de	32.0 c	38.0 b	48.0 a
E. rugulosa					
Hexane	0.0e	0.0e	0.0e	4.0 cde	14.0 b
EtOAc	0.0e	0.5 e	2.5 de	6.0cd	26.5a
MeOH	0.0e	0.0e	1.0e	2.0de	7.5 c

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

Table 4.9. Effect of crude extracts from antagonistic fungi against conidia production of *Fusarium oxysporum* f. sp. *lycopersici* NKSC02.

Crude extracts	Number of conidia(x10^7) of *Fusarium oxysporum* f.sp. *lycopersici* concentration (µg/ml)					
	0	10	50	100	500	1000
Ch. brasilense						
Hexane	35.78a[1]	21.51c	15.93d	14.50d	4.10gh	2.16hi
EtOAc	36.24a	26.71b	19.61c	11.48e	5.35g	4.40gh
MeOH	35.72a	11.83e	9.84ef	8.52f	4.28gh	1.07i
Ch. cupreum						
Hexane	39.50b	15.93d	8.64fg	6.82hi	5.94i	3.18k
EtOAc	38.47c	14.43e	8.87f	7.68gh	4.48j	2.40k
MeOH	41.00a	13.93e	8.43fg	6.16i	2.86k	1.07 l
Ch. elatum						
Hexane	37.81a	13.79d	11.83ef	8.75gh	7.76h	5.90i
EtOAc	37.68a	13.07d	10.82f	4.64j	2.89k	1.65l
MeOH	38.62a	29.03b	23.00c	12.84de	9.06g	7.54h
Ch. lucknowense						
Hexane	43.31a	39.5 b	37.62cd	34.12e	30.43f	15.93i
EtOAc	43.81a	38.5 bc	36.62d	33.05e	22.5h	12.00k
MeOH	42.75a	29.93f	26.93g	14.43j	10.50 l	3.81m
E. nidulans						
Hexane	14.67ab	14.07b	12.78c	11.91d	10.11f	5.82h
EtOAc	15.00 a	12.31cd	11.11e	10.11f	7.13g	5.33h
MeOH	14.10ab	11.81d	10.08f	5.70h	3.77i	2.61j
E. rugulosa						
Hexane	17.62a	16.67abc	15.43def	12.33g	7.92h	4.22i
EtOAc	17.25ab	15.61cde	14.47ef	8.66h	4.50i	2.15j
MeOH	17.50 ab	16.37bcd	15.44def	14.33f	13.05g	7.54h

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

Table 4.10 Effect of crude extracts from antagonistic fungi for percentage of conidia inhibition of *Fusarium oxysporum* f. sp. *lycopersici* NKSC02.

Crude extracts	Conidia inhibition of *Fusarium oxysporum* f.sp. *lycopersici* (%)					
	10	**50**	**100**	**500**	**1000**	**ED_{50}**
Ch. brasilense						
Hexane	39.83k[1]	55.44i	59.47h	88.53c	93.95b	29.87
EtOAc	33.17l	45.88j	68.29g	85.22d	87.85c	38.99
MeOH	66.87g	72.38f	76.09e	87.98c	96.98a	2.99
Ch. cupreum						
Hexane	59.64j	78.23g	82.71de	84.38de	91.92b	2.33
EtOAc	62.46i	76.92g	82.17ef	88.34c	93.75b	2.38
MeOH	65.99h	79.41fg	85.49d	93.13b	97.37a	2.65
Ch. elatum						
Hexane	63.46j	68.67gh	76.35f	78.87ef	83.85d	0.65
EtOAc	65.30ij	71.26g	87.65c	92.29b	95.61a	3.39
MeOH	24.76l	40.43k	66.69hi	76.52f	80.44e	63.42
Ch. lucknowense						
Hexane	8.78j	13.10hi	21.14g	29.70f	63.18c	921
EtOAc	12.10ij	16.39h	24.55g	48.62d	72.59b	393
MeOH	29.98f	36.97e	66.20c	75.39b	91.07a	53
E. nidulans						
Hexane	4.03i	12.88h	18.80g	31.10ef	60.28c	915
EtOAc	17.90gh	25.88f	32.55e	52.40d	64.40c	379
MeOH	16.16gh	28.39ef	59.46c	73.17b	81.44a	112
E. rugulosa						
Hexane	5.12h	12.18efg	29.86d	55.06c	76.00b	313
EtOAc	9.43fgh	16.03ef	49.75c	73.87b	87.55a	138
MeOH	6.43gh	11.60efgh	17.89e	25.26d	55.41c	1372

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

4.3.2.3 Pure compound bioassay against *Fusarium oxysporum* f. sp. *lycopersici*

Chaetoglobosin C was isolated from *Ch. elatum* ChE01 and *Ch. luckowense* CLT01 and tajixanthone isolated from *E. rugulosa* ER01 which elucidated using chromatographic methods to obtain these compounds (Figure 4.57). The structures were identified by spectroscopic methods, IR, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR (COSY, HMQC, HMBC, and NOESY). Chaetoglobosin-C, a pure compound produced by *Ch. elatum* ChE01 and *Ch. luckowense* CLT01, inhibited conidia production of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED_{50} value of 5.94 µg/ml. Moreover, tajixanthone, a pure compound produced by *E. rugulosa* ER01, inhibited conidia production of *F. oxysporum* f. sp. *lycopersici* NKSC02 with an ED_{50} value of 167 µg/ml (Table 4.11).

4.3.2.4 Effect of fungal metabolites to *Fusarium oxysporum* f. sp. *lycopersici* and its pathogenicity loss

Inoculum of *F. oxysporum* f. sp. *lycopersici* (1×10^7 spores/ml) treated with pure compounds of chaetoglobosin-C and tajixanthone inoculating to tomato seedlings caused no symptoms at day 21 while the treatment with pathogen alone showed significantly highest disease severity index as shown in (Table 4.12). No disease incidences were appeared at all tested concentration of 10, 50 and 100 µg/ml of either chaetoglobosin-C or tajixanthone which significantly differed from the control. It revealed that the antibiotic substances of chaetoglobosin-C and tajixanthone affected directly to the pathogen conidial inocula which implies antibiosis mechanism of control. Moreover, the occurrences of ruptured cells and abnormal conidia thereafter mixing with each pure compound of chaetoglobosin-C and tajixanthone were observed under the microscope (Fig. 4.58).

4.3.2.5 Effect of antagonistic crude extract for disease immunity of wilt incidence in tomato var Sida

Crude EtoAC of *E. rugulosa* was selected to test for disease immunity. Result found that treated tomato seedlings var Sida with crude EtoAC of *E. rugulosa*

at 1000 µg/ml gave significant lower DSI from treated with crude EtoAC of *E. rugulosa* at 500 µg/ml when compared to the inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02.

Disease immunity to Fusarium wilt in Sida varieiy showed the highest immunity of 80.95 % when treated with crude EtoAC at 1000 µg/ml and followed by treated with crude EtoAC at 500 µg/ml which showed immunity of 30.09 %. As shown in (Table 4.13).

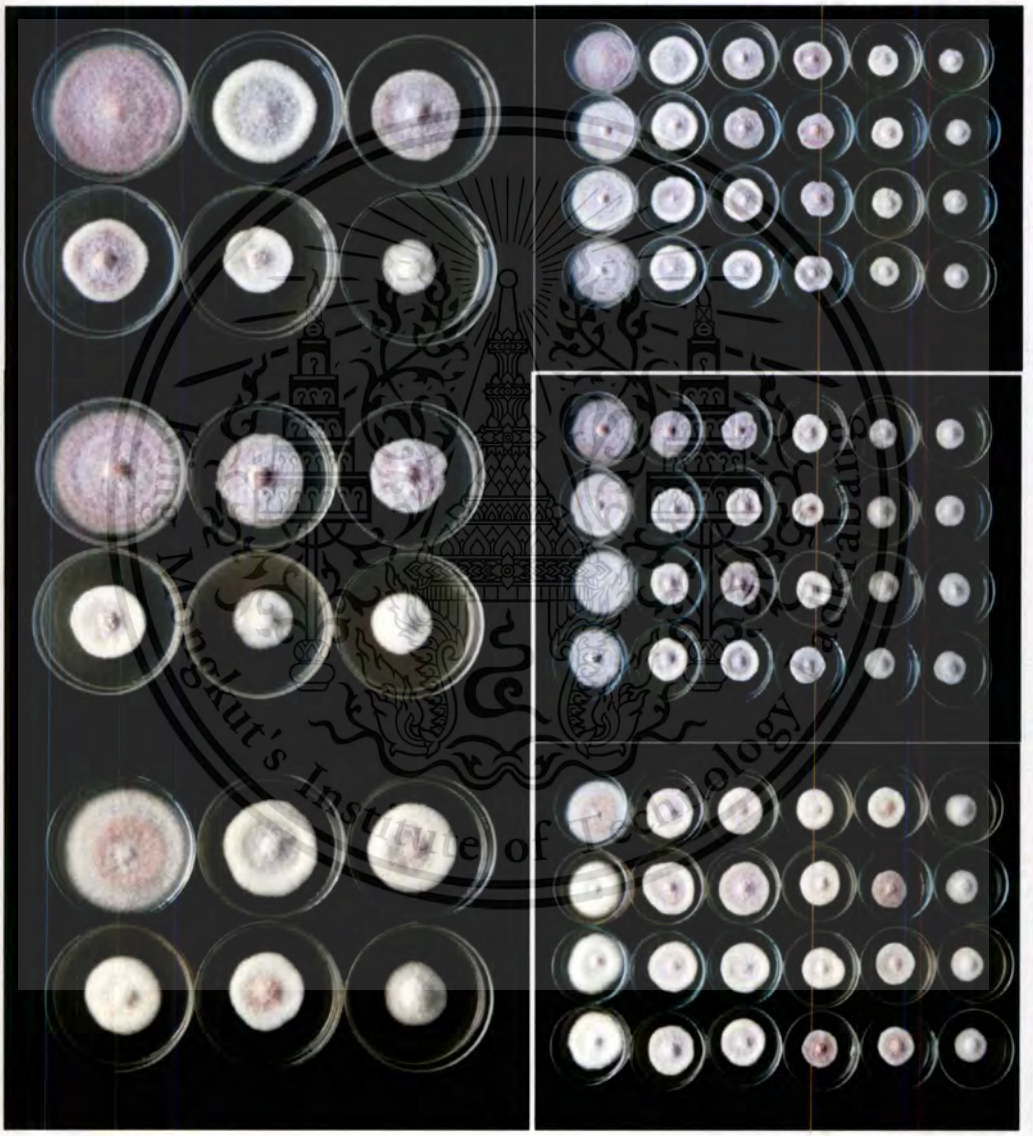
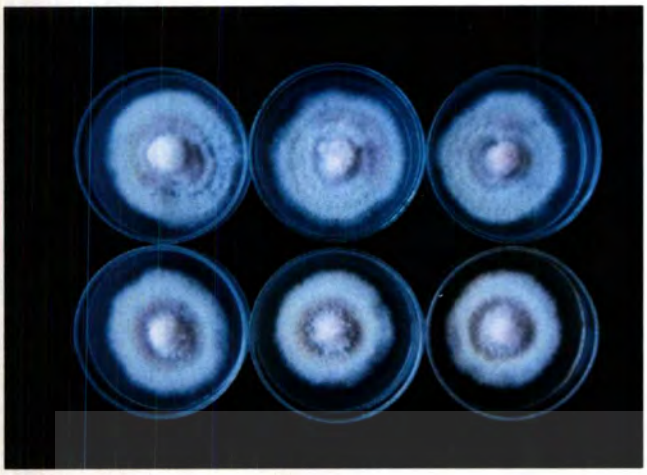
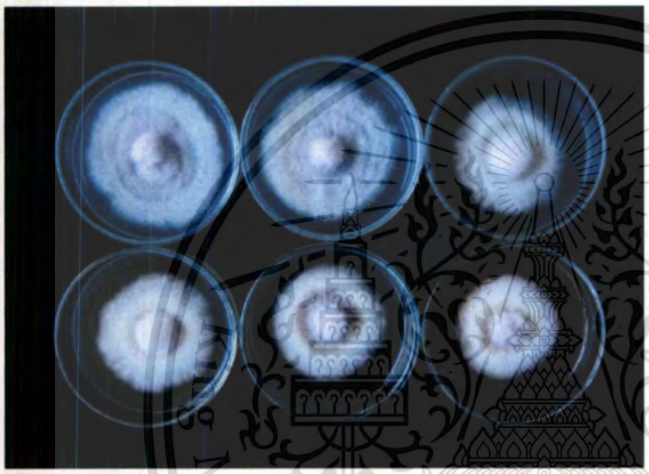


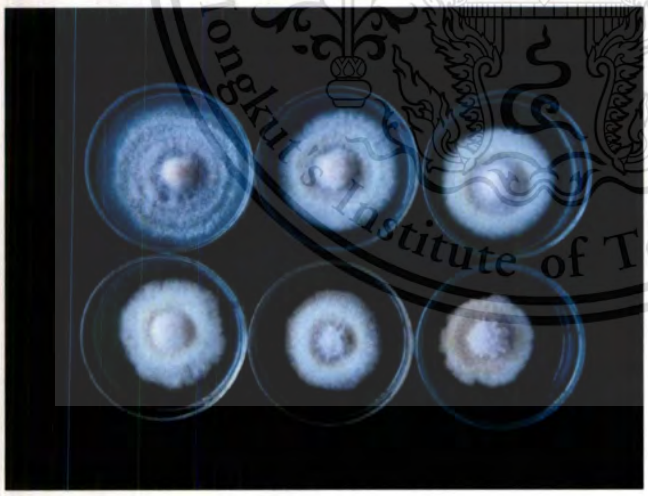
Figure 4.51 Five-day old colony of *F. oxysporum* f. sp. *lycopersici* NKSC02 from testing crude extracts of *Ch. brasilense* ; above row from left to right at 0, 10, and 50 µg/ml concentrations; below row from left to right at 100, 500, and 1000 µg/ml concentrations.



Crude hexane



Crude ethyl acetate



Crude methanol

Figure 4.52 Five-day old colony of *F. oxysporum* f. sp. *lycopersici* NKSC02 from testing crude extracts of *Ch. cupreum* ; above row from left to right at 0, 10, and 50 µg/ml concentrations; below row from left to right at 100, 500, and 1000 µg/ml concentrations

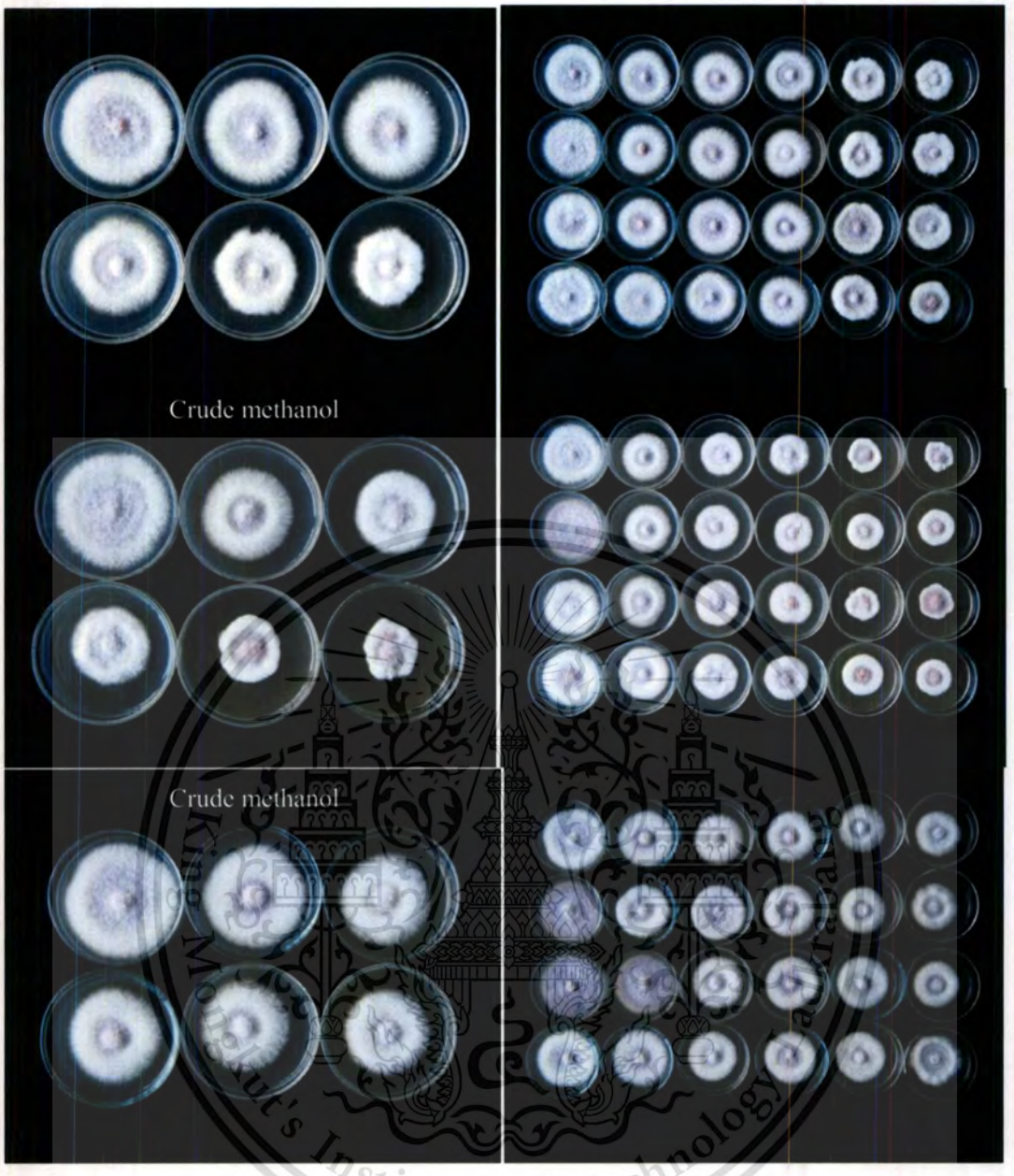


Figure 4.53 Five-day old colony of *F. oxysporum* f. sp. *lycopersici* NKSC02 from testing crude extracts of *Ch. elatum* ; above row from left to right at 0, 10, and 50 µg/ml concentrations; below row from left to right at 100, 500, and 1000 µg/ml concentrations

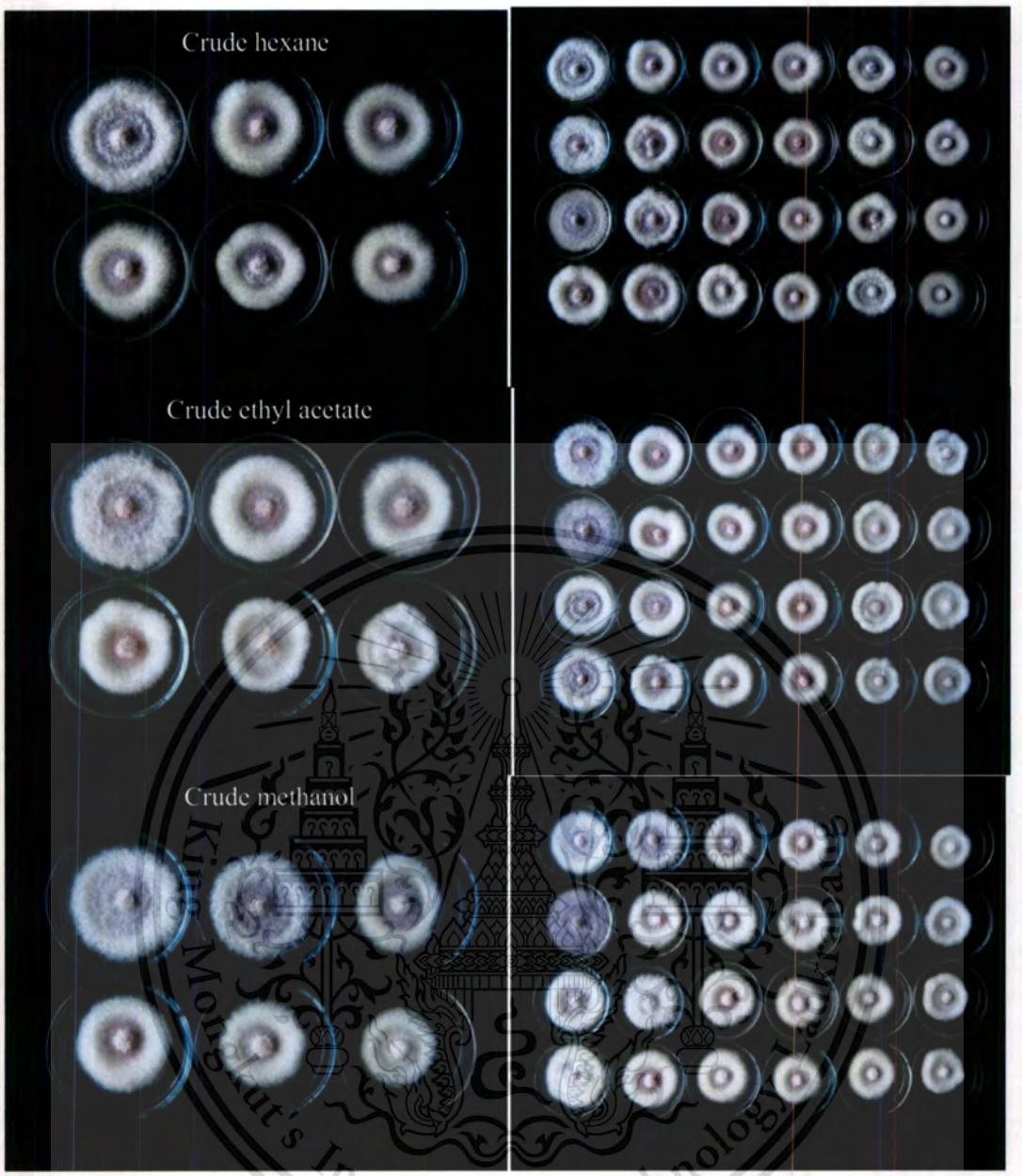


Figure 4.54 Five-day old colony of *F. oxysporum* f. sp. *lycopersici* NKSC02 from testing crude extracts of *Ch. lucknowense* ; above row from left to right at 0, 10, and 50 µg/ml concentrations; below row from left to right at 100, 500, and 1000 µg/ml concentrations

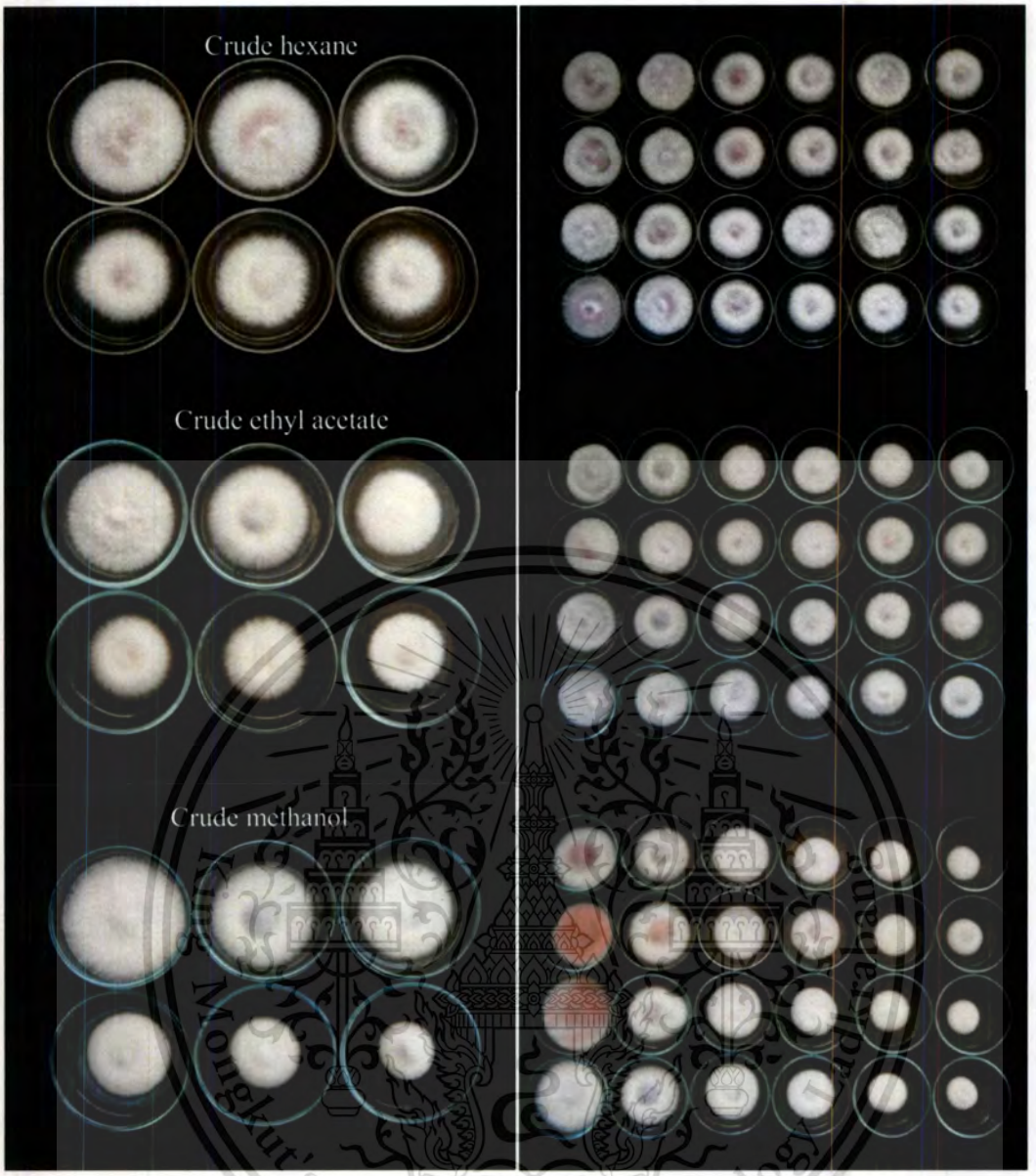
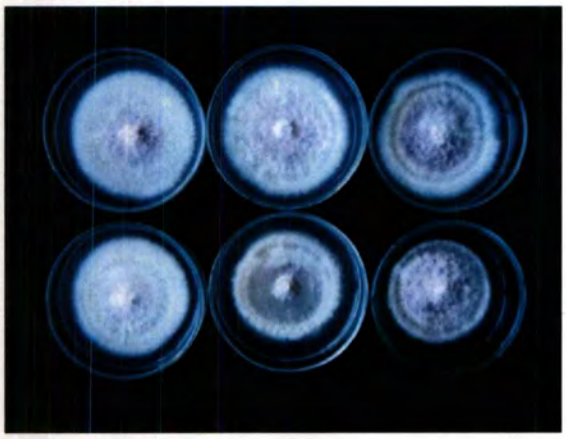
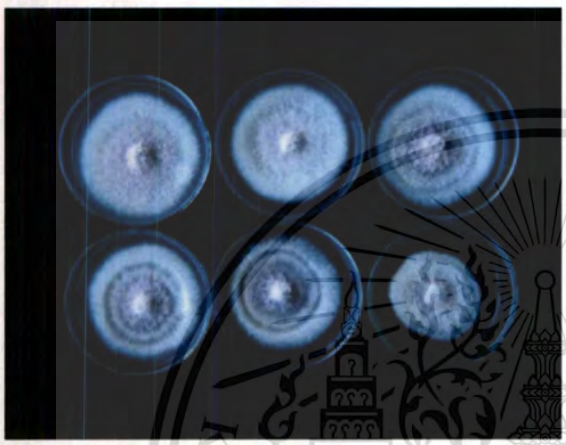


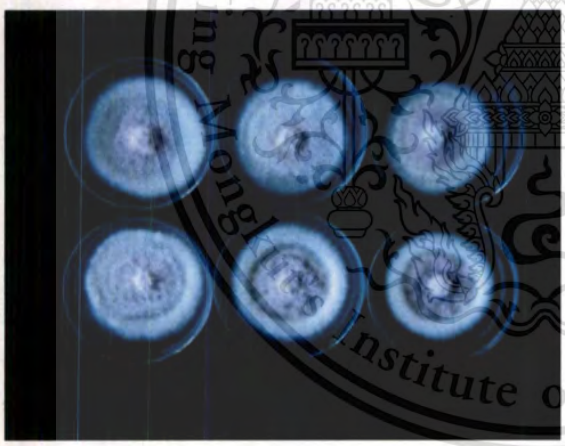
Figure 4.55. Five-day old colony of *F. oxysporum* f. sp. *lycopersici* NKSC02 from testing crude extracts of *E. nidulans* ; above row from left to right at 0, 10, and 50 µg/ml concentrations; below row from left to right at 100, 500, and 1000 µg/ml concentrations



Crude hexane



Crude ethyl acetate



Crude methanol

Figure 4.56 Five-day old colony of *F. oxysporum* f. sp. *lycopersici* NKSC02 from testing crude extracts of *E. rugulosa* ; above row from left to right at 0, 10, and 50 µg/ml concentrations; below row from left to right at 100, 500, and 1000 µg/ml concentrations

Table. 4.11. Assay of bioactive compounds against *Fusarium oxysporum* f. sp. *lycopersici*

Pure compounds	Inhibition of conidia production (%)[1]	ED_{50} µg/ml
Chaetoglobosin-C	89.00	5.94
Tajixanthone	68.37	167
CV (%)	9.50	

[1]Inhibition (%) = average number of conidia in control plate – average number of conidia in treated plate/ average number of conidia in control plate X 100.

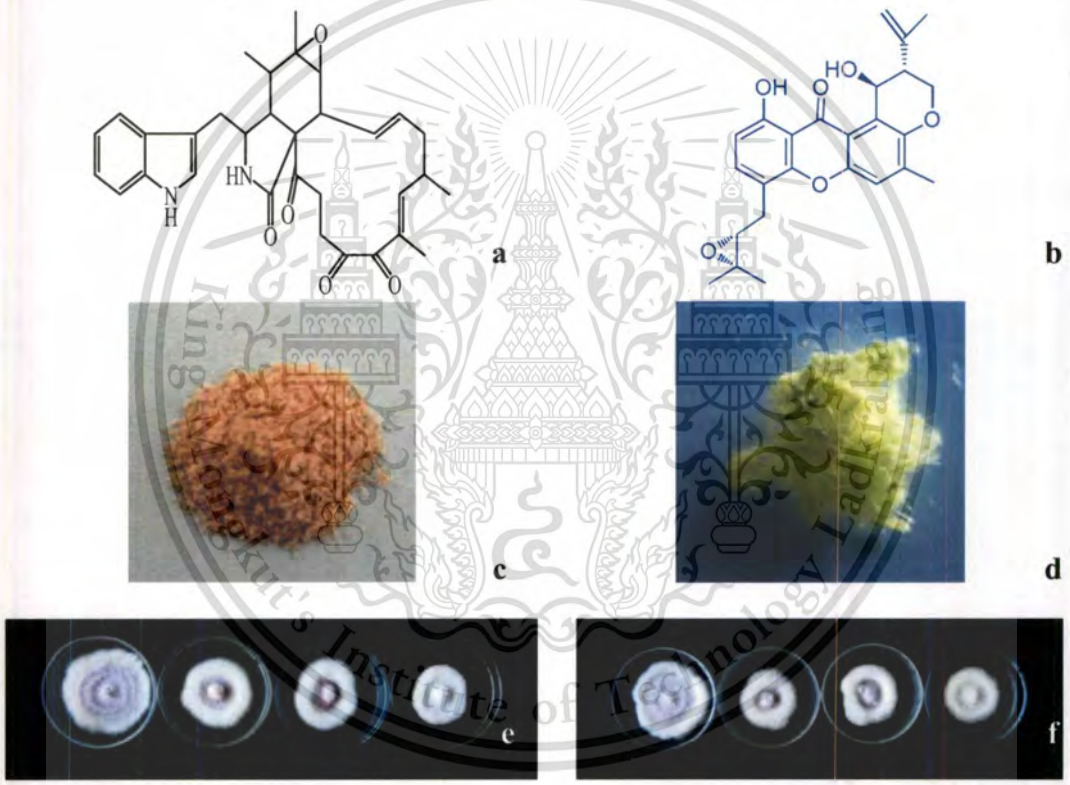


Figure 4.57 a. chemical structure of Chetoglobosin-C, b. chemical structure of tajixanthone, c. pure compound of Chetoglobosin-C, d. pure compound of tajixanthone, e. colony of *F.oxysproum* f. sp. *lycopersici* NKSC02 on mixed PDA with Chaetoglobosin-C (Source:Kanokmedhkul *et al.,* 2001), and f. colony of *F.oxysproum* f. sp. *lycopersici* NKSC02 on mixed PDA with tajixanthone. (Source: Moosophon *et al.,* 2007)

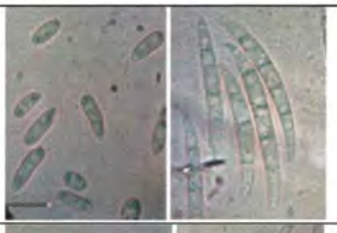
Pure compounds	Normal conidia	Abnormal conidia
Chaetoglobosin-C		
Tajixanthone		

Figure 4.58. Abnormal conidial lysis of *F. oxysporum* f. sp. *lycopersici* affecting to chaetoglobosin-C and tajixanthone.

Table 4.12. Effect of fungal metabolites to *Fusarium oxysporum* f. sp. *lycopersici* and its pathogenicity loss at 21 days after inoculation to tomato seedlings

Pure compounds	Concentrations µg ml^{-1}	DSI[1]
Chaetoglobosin-C	0	6.00a[2]
	10	1.00b
	50	1.00b
	100	1.00b
Tajixanthone	0	6.00a
	10	1.00b
	50	1.00b
	100	1.00b

[1]Disease Severity Index (DSI): 1 = no symptoms; 2 = plant showed 1–20% yellowing leaves and wilting, 3 = plant showed 21–40% yellowing leaves and wilting, 4 = plant showed 41–60% yellowing leaves and wilting, 5 = plant showed 61–80% yellowing leaves and wilting, and 6 = plant showed 81–100% yellowing leaves and wilting or death. [2]Average 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 4.13. Effect of crude EtOAc from *E. rugulosa* to induce disease immunity of wilt incidence in tomato var Sida

Treatments	Plant height(cm)	DSI[1]	Disease immunity[2] (%)
Fol[3]	20.7b[4]	5.25a	-
Fol+ crude EtoAC500µg/ml	40.75a	3.25b	30.09
Fol+ crude EtoAC 1000µg/ml	41.25a	1.00c	80.95
control	41.75a	1.00c	
CV (%)	9.42	20	

[1]Disease severity index (DSI) was scored at 21 days after inoculation. 1= no symptom; 2= plant showed yellowing leaves and wilting 1-20%, 3= plant showed yellowing leaves and wilting 21-40%, 4= plant showed yellowing leaves and wilting 41-60%, 5= plant showed yellowing leaves and wilting 61-80%, and 6= plant showed yellowing leaves and wilting or die 81-100%.[2]Disease immunity (%) = DSI in control – DSI in treatment/ DSI in control x 100. [3] Fol = *F. oxysporum* f. sp. *lycopersici*.
[4]Average of four replications, Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.01.

4.4 Evaluation of Bioformulations to control Fusarium wilt of tomato *in vivo*

4.4.1 Testing bioformulation of *Chaetomium elatum* ChE01to control Fusarium wilt of tomato

The disease severity index (DSI) of Fusarium wilt was lowest wilt incidence in application of oil and powder bioformulations (DSI 1.75 and 2.00) and followed by culture filtrate (DSI 2.5) which significantly differed from Prochoraz (DSI 3.25) and inoculated control (DSI 4.75). The non inoculated control was no wilt incidence. With this, application of oil bioformulation leaded to reduce wilt incidence and followed by application of powder bioformulation, culture filtrate and Prochoraz which reduced wilt incidence. Based on the result, oil bioformulation gave significantly highest in plant height (125cm) and followed by powder bioformulation, culture filtrate and Prochoraz which were 105.75, 100.50 and 87.50 cm, respectively when compared to the inoculated control (75.75 cm).

Plant weight showed the highest after apply oil bioformulation (184.25 g), and followed by powder formulation, culture filtrate and Prochoraz which were 169.25, 151.00 and 134.25 g, respectively when compared to the inoculated control (73.25 g).

With this regards, the root weights of oil and powder bioformulations gave significantly better than culture filtrate and Prochoraz treatments. Oil bioformulation gave significantly highest in fruit weight (327.5 g) and followed by powder bioformulation (279 g), culture filtrate (217.5 g) and Prochoraz(172 g) which significantly differed from the inoculated control (185 g). The number of fruits in oil bioformulation application was 21.5 fruits/plant which gave significantly higher than powder bioformulation (17.25 fruits/plant), culture filtrate (16 fruits/plant) and Prochoraz (11.75 fruits/plant) treatments which significantly differed from the inoculated control (11.75 fruits/plant) as shown in Table 4.14.

Table 4.14. Testing bioformulation of *Chaetomium elatum* to control Fusarium wilt of tomato var Sida in pot experiment.

Treatments	Plant height(cm)	Plant weight(g)	Root weight(g)	Fruit weight(g)	fruits/ plant	DSI
T1	100.50bc[1]	166.25b	10.25b	229.00c	15.00b	1.00d
T2	75.75d	73.25e	5.37c	185.00cd	11.75c	4.75a
T3	105.75b	169.25b	13.75b	279.00b	17.25b	2.25c
T4	125.00a	184.25a	24.25a	327.50a	21.50a	2.25c
T5	100.50bc	151.00c	10.75b	217.50cd	16.00b	3.25bc
T6	87.50cd	134.25d	10.25b	172.00d	11.75c	3.50b
CV (%)	7.72	1.99	15.37	9.64	8.21	17.44

T1=non-inoculated control, T2= inoculated control, T3=powder formulation, T4=oil formulation, T5=culture filtrate, and T6=chemical (Prochoraz). [1]Average of four replications. Means followed by the same letters were not significantly different by DMRT at P=0.01.

Table 4.15. Percent increasing in plant growth and disease reduction after application of bioformulations of *Ch. elatum*

Treatments	Plant height	Plant weight	Root weight	Fruit weight	Numbers of fruit/plant	DR[2]
Powder form	28.83[1]	56.72	60.94	33.69	31.88	46.31
Oil form	39.40	60.24	77.85	43.51	45.34	46.31
Filtrate culture	24.62	51.49	50.04	14.94	26.75	31.57
Prochoraz	13.42	45.43	47.60	-	-	26.31

[1]Incresing of plant growth parameters=each tested treatment-inoculated control/ tested treatment x 100.
[2]Disease reduction (DR) = disease severity index (DSI) of inoculated control – disease severity index (DSI) of treatment/disease severity index (DSI) of inoculated control x 100.

Figure 4.59 Testing bioformulation of *Chaetomium elatum* ChE01to control Fusarium wilt of tomato.

4.4.2 Testing bioformulation of *Emericella nidulans* EN01 to control Fusarium wilt of tomato

The disease severity index (DSI) of Fusarium wilt was lowest wilt incidence in oil and powder bioformulations (DSI 2.25) and followed by culture filtrate (DSI 2.75) which significantly differed from Prochoraz (DSI 3.5) and inoculated control (DSI 4.75). The non inoculated control was no wilt incidence. With this, application of oil bioformulation leaded to reduce wilt incidence and followed by application of powder bioformulation, culture filtrate and Prochoraz which also reduced wilt incidence respectively. Based on the result, oil bioformulation gave significantly highest in plant height (119.25 cm) and followed by powder bioformulation, culture filtrate and Prochoraz which were 109.75, 84.50 and 73.00 cm, respectively when compared to the inoculated control (62.75 cm).

Plant weight showed the highest after apply oil and powder bioformulations (98.5 and 91.75 g), and followed by culture filtrate and Prochoraz which were 68 and 67 g, respectively when compared to the inoculated control (64 g). With this, root weights of oil bioformulation (11.25 g) gave significantly better than powder bioformulation (10g), culture filtrate (6 g) and Prochoraz (7.75 g) treatments. Oil bioformulation gave significantly highest in fruit weight (218.5 g) and followed by powder bioformulation (179.5 g), culture filtrate (128 g) and Prochoraz (107 g) which significanyly differed from the inoculated control (83.75 g). The number of fruits in oil and power bioformulations application were 15 and 13.25 fruits/plant which gave significantly higher than Prochoraz (9.75 fruits/plant) and culture filtrate (8.5 fruits/plant) treatments which significantly differed from the inoculated control (8 fruits/plant) as shown in Table 4.16.

Table 4.16. Testing bioformulations of *Emericella nidulans* EN01 to control Fusarium wilt of tomato var Sida in pot experiment

Treatments	Plant height(cm)	Plant weight(g)	Root weight(g)	Fruit weight(g)	fruits/ plant	DSI
T1	101.75c[1]	78.00b	7.25abc	155.50c	13.50a	1.00e
T2	62.75f	64.00b	5.00c	83.75f	8.00b	4.75a
T3	109.75b	91.75a	10.00ab	197.50b	13.25a	2.00cd
T4	119.25a	98.50a	11.25a	218.50a	15.00a	1.75de
T5	84.50d	68.00b	6.0bc	128.00d	8.50b	2.75bc
T6	73.00e	67.00b	7.75abc	107.00e	9.75b	3.50b
CV (%)	3.21	8.06	22.58	9.16	11.80	16.43

T1=non-inoculated control, T2= inoculated control, T3=powder formulation, T4=oil formulation, T5=culture filtrate, and T6=chemical (Prochoraz).[1] Average of four replications. Means followed by the same letters were not significantly different by DMRT at P=0.01.

Table 4.17. Percent increaseing in plant growth and disease reduction after application of bioformulations of *Emericella nidulans* EN01

Treatments	Plant height	Plant weight	Root weight	Fruit weight	Numbers of fruit/plant	DR[2]
powder form	42.82[1]	30.24	50.00	57.59	39.62	57.89
oil form	47.37	35.02	55.55	61.67	46.66	63.15
filtrate	25.73	5.88	16.66	34.57	5.88	42.10
Prochoraz	14.04	4.47	35.48	21.72	17.94	34.72

[1]Incresing of plant growth parameters=each tested treatment-inoculated control/ tested treatment x 100.

[2]Disease reduction (DR) = disease severity index (DSI) of inoculated control – disease severity index (DSI) of treatment/disease severity index (DSI) of inoculated control x 100.

Figure 4.60 Testing bioformulation of *Emericella nidulans* EN01 to control Fusarium wilt of tomato

4.4.3 Testing bioformulation of *Emericella rugulosa* ER01 to control Fusarium wilt of tomato

The disease severity index (DSI) of Fusarium wilt was lowest wilt incidence in application of oil and powder bioformulations (DSI 2 and 1.75) and followed by culture filtrate (DSI 2.5) which significantly differed from Prochoraz (DSI 4.25) and inoculated control (DSI 5.00). The non inoculated control was no wilt incidence. With this, application of oil bioformulation leaded to reduce wilt incidence of 60 % and followed by application of powder bioformulation, culture filtrate and Prochoraz which reduced wilt incidence of 58, 50 and 15 %, respectively. Based on the result, oil bioformulation gave significantly highest in plant height (119.50 cm) and followed by powder bioformulation, culture filtrate and Prochoraz which were 105.25, 85.25 and 74.00 cm, respectively when compared to the inoculated control (65.75 cm). Plant weight showed the highest after apply oil bioformulation (182.35 g), and followed by powder formulation, culture filtrate and Prochoraz which were 168.50, 153.75 and 137 g, respectively when compared to the inoculated control (73.75 g). With this

regards, the root weights of oil and powder bioformulations gave significantly better than culture filtrate and Prochoraz treatments. Oil bioformulation gave significantly highest in fruit weight (584.25 g) and followed by powder bioformulation (540.75 g), culture filtrate (430 g) and Prochoraz (191.35g) which significanyly differed from the inoculated control (280 g). The number of fruits in oil bioformulation application gave significantly higher than powder bioformulation, culture filtrate and Prochoraz treatments which significantly differed from the inoculated control (Tables 4.18 and 4.19, Figures 4.61.)

Table 4.18. Testing bioformulations of *Emericella rugulosa* ER01 to control Fusarium wilt of tomato var Sida in pot experiment

Treatments	Plant height(cm)	Plant weight(g)	Root weight(g)	Fruit weight(g)	fruits/plant	DSI
T1	97.25c[1]	163.25b	6.62b	280.00d	19.50d	1.00c
T2	65.75f	73.75e	4.32c	141.25f	10.25f	5.00a
T3	105.25b	168.50b	12.25a	540.75b	29.25b	1.75bc
T4	119.50a[2]	182.25a	13.25a	584.25a	40.50a	2.00bc
T4	85.25d	153.75c	6.12bc	430.00c	24.50c	2.50b
T6	74.00e	137.00d	5.20bc	191.25e	15.00e	4.25a
CV (%)	3.1495	1.9912	15.1708	3.2257	8.7166	19.5449

T1=non-inoculated control, T2= inoculated control, T3=powder formulation, T4=oil formulation, T5=culture filtrate, and T6=chemical (Prochoraz). [1]Average of four replications. Means followed by the same letters were not significantly different by DMRT at P=0.01.

Table 4.19. Percent increasing in plant growth and disease reduction after application of bioformulations of *Emericella rugulosa* ER01

Treatments	Plant height	Plant weight	Root weight	Fruit weight	Numbers of fruit/plant	DR[2]
powder form	37.52[1]	56.23	64.73	73.87	64.95	65
oil form	44.97	59.53	67.39	75.82	74.69	60
filtrate	22.87	52.03	29.41	67.15	58.16	50
Prochoraz	11.14	46.16	16.92	26.14	31.66	15

[1]Incresing of plant growth parameters=each tested treatment-inoculated control/ tested treatment x 100.

[2]Disease reduction (DR) = disease severity index (DSI) of inoculated control – disease severity index (DSI) of treatment/disease severity index (DSI) of inoculated control x 100.

Figure 4.61. Testing bioformulation of *Emericella rugulosa* ER01 to control Fusarium wilt of tomato

CHAPTER V
DISCUSSION

The research findings indicated that tomato wilt collected from infested fields in Bangkok, Phetchabun, Tak, Nakhonratchasima, Burirum, Nongkhai, Sakonnakhon, and Khonkaen provinces yielded two isolates were isolated from Bangkok including BKRF01, BKRS01 isolates, 7 isolates PBRS101, PBRS102, PBRS103, PBRS104, PBRS201, PBRS202, PBRS203 were isolated from Phetchabun, 4 isolates MSRS01, MSRS02, TRS01, and TRS02 from Tak . Moreover, twelve isolates of *F. oxysporum*f. sp. *lycopersici*, which isolated from Burirum, Khonkaen, Nongkai, Nakhonratchasima and Sakonnakhon provinces in Thailand were obtained from Assist. Prof. Dr. Chamaiporn Charoenporn (Nakhonratchasima Rachabhat University, Nakhonratchasima, Thailand) as follows:- BRCO3, KK2, KSoC02, NKSC01, NKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03, and SSoC04 isolates. The morphological identification of the 12 isolates have been confirmed previously by Charoenporn *et al.* (2010) by sequencing the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA and a small portion of the 28S rDNA. Pure cultures of *F. oxysporum* f. sp. *lycopersici*were identified by morphological and molecular phylogeny as similar work of Charoenporn *et al.* (2010).

The pathogenicity tests performed on tomato seedlings in this study showed that the *F. oxysporum* f. sp. *lycopersici* NKRC02, NKRC04, SRC02 and NSC01 isolates were avirulent whereas Charoenporn *et al.* (2010) reported in a previous study as moderately virulent to the same variety of tomato. It revealed in this study that Isolate NKRC09, KSoC02, BRC03, SSoC03 and SSoC04 were also found to be avirulent, whereas a previous study showed low virulence (Charoenporn *et al.*, 2010). This suggested that repeatedly sub-cultured of *F. oxysporum* f. sp. *lycopersici* affected the stability of pathogenicity. *F. oxysporum* f. sp. *lycopersici* isolate NKSC02 was the most virulent isolate to cause wilt of tomato var. Sida and Cherry This observation is supported by *in vitro* studies of virulence by Soytong *et al.* (2001), Sibounnavong *et al.* (2009) and Charoenporn *et al.* (2010).

Total of 25 isolates of *F. oxysporum* f. sp. *lycopersici* were confirmed morphologically and based on molecular phylogeny. Results of the pathogenicity test

and AFLP analysis in this study revealed that 11 isolates were categorized as non-pathogenic or avirulent group and 14 isolates were categorized as pathogenic group which divided into 3 subgroups of low virulent (L), moderate virulent (M) and high virulent (H). As a result, the isolates of KSoC02, NKRC09, SSoC03 and SSoC04 were shown to be non-pathogenic isolates or avirulence but Charoenporn*et al* (2010) reported that these isolates were low virulent to cause wilt of tomato var. Sida. It can explain that the different varieties of tomatoes may affect to pathogenicity level of wilt disease infected by same isolate of *F. oxysporum* f. s p. *lycopersici* (Cai, 1999). Isolate KK2 with high virulent to tomato var. Sida as previous report (Charoenporn *et al*, 2010) become moderate virulent in tomato var Sida and Cherry in this study. Bunyatratchata *et al* (2005) reported that isolate KK2 isolated from Northeast part of Thailand had been tested its pathogenicity to cause wilt symptom on tomato varSida as the same disease level of Banny and UC82-L varieties which susceptible to standard tested isolate Fol 007 race 2 and concluded that KK2 was race 2. (Grttidge and O Brien, 1982 and Maiatt *et al,* 1996). Isolates NKRC02, NKRC04 and SCR02 with moderate virulent to tomato var. Sida as previous report (Charoenporn *et al,* 2010) but become non-pathogenic to tomato var. Cherry in present study. It was observed that those isolates were variable for pathogenicity to different varieties of tomatoes (Cherry and Sida varieties) from low and moderate virulent become non-pathogenic and from high virulent become moderate virulent. This phenomenon may explain that different varieties of tomatoes are affected with isolate of *F. oxysporum* f .sp. *lycopersici*, as also stated by Sibounnavong *et al* (2009) and continuing subculture of *F.oxysporum.* may lead to variable and lower degree of pathogenicity (Agrios,1997). It is interested that isolates NKSC01 and NKSC02 with high virulent to tomato var Sida as previous report (Charoenporn *et al.*, 2010), it was still expressed high virulent in tomato var. Cherry in this experiment. It can explain why the isolates were more stable than the other isolates or these isolates can infected both Cherry and Sida varieties (Sibounnavong *et al.*, 2010). However, Charoenporn *et al* (2010) reported previously that isolates KK2, KSoC02, NKSC01, NKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03 and SSoC04 were sequenced to confirm identification into species by using ITS sequences with the length of complete ITS1, 5.8S and ITS2 including small portions of 18S rDNA and 28 S rDNA. In this study, AFLP has been used as a powerful technique in molecular fingerprinting to study the relationship among fungal isolates and their pathogenicity as also showed by Brown (1996),

Janssen et al. (1996) and Majer *et al.* (1998). Regarding from result of the study, a total 81 polymorphic bands were amplified using three primers combination with EcoRI+G/MseI+ACG, EcoRI+G/MseI+CAC, EcoRI+ACG/MseI+Gat the 3' end of the primers on 25 isolates of *F. oxysporum* f. sp. *lycopersici.* The restriction enzymes, length and composition of selective nucleotides would help to determine complexity of the final AFLP fingerprint as reported by Janssen *et al.* (1996). Three nucleotides of primers combination for AFLP analysis can help to differentiate *Fusarium* spp causing root rot disease on wheat and gave good polymorphic bands (Mohammadi *et al.* 2009). The primer selectivity is related to genome size and good selectivity is found with primers of three selective nucleotides (El-Kazzaz, 2008). However, Gonnalez *et al.* (1998) stated that using two instead of three selective nucleotides in order to generate adequate number of fragments for AFLP analysis of *C. lindemuthianum* isolates. Primer selectivity is also good for primers with one or two selective nucleotides in simple genome such as bacteria, fungi and some plants, although selectivity is still acceptable with primers of three selective nucleotides.

Statistical analysis of AFLP data enabled the classification of *F. oxysproum* f .sp. *lycopersici* into two AFLP groups; non-pathogenic or avirulent and pathogenic or virulent groups. With this the pathogenic group was clearly divided into three subgroups which correlated with the result of pathogenicity.

In this study, 25 isolates of *F. oxysproum* f .sp. *lycopersici* were analyzed with primers to determine the distribution of genetic diversity among isolates which represents in different planting areas. Mohmed *et al* (2003) stated that the high-resolution genotyping method of AFLP analysis was suitable to study the genetic relationships within and between populations of *Fusarium* spp. In the present research however, it was not clearly relationship between provinces and distribution of pathogen. This result was similar to those of Charoenporn *et al,* (2010). In this study, it was showed that the phonetic dendrogram generated by UPGMA modified from neighbor procedure of PHYLIP version 3.5 based on genotypes in 8 populations as pop1:Khonkaen province, pop2:Bangkok province, pop3: Sakornnakon province, pop4:Burirum province, pop5: Nongkhai province, pop6: Nakhonratchasima, pop7: Tak province and pop8:Phetchabun province. A principal coordinate analysis (PCA) grouped all of the *Fusarium* spp. isolates into eight major clusters. No clear trend was detected between clustering in the AFLP dendrogram and geographic origin of the tested isolates as similar report of Mohmed *et al* (2003). However, it was observed

that pop 1: Khonkaen and pop 5: Nongkhai are located in the Northeast of Thailand where majority of planted areas of tomatoes which these geographical areas were found more moderate and high virulent isolates.

In conclusion, the genetic variation among isolates of *F. oxysproum* f .sp. *lycopersici* was clearly relationship between pathogenicity groups and AFLP groups. But it was not clearly correlated between AFLP and geographical areas. Moreover, this work provided new information on formae specialis of *F. oxysproum* f. sp *lycopersici* which could classify as race 2 that can cause wilt to different varieties of tomato e.g. Cheery and Sida varieties rather one variety. As Bunyatratchata *et al* (2006) reported that *F. oxysporum* f sp *lycopersici* race 2 can infected tomato var. Sida in Thailand as compared to standard race testing varieties of Bonny Best, UC82-L. There was a good correlation between AFLP groups and groups from result of pathogenicity test. Regarding from the result of the present study demonstrated clearly that the use of the AFLP is a powerful, simple and rapid technique to study the identification and genetic relationship between *F. oxysporum* and their pathogenicity. AFLP may therefore provide rich sources of molecular markers which are useful to study on the genetic variation for specific level.

Isolate NKSC02 is confirmed as a virulent to cause wilting of tomato var Sida which was previously reported by Sibounnavong *et al.* (2010) and this isolate was also confirmed by molecular phylogeny as *F. oxysporum* f. sp. *lycopersici* by Charoenporn *et al.* (2010) who sequenced the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA and a small portion of the 28S rDNA.

The antagonistic fungi namely: *Chaetomium brasilense* CB01, *Chaetomium cupreum* CC03, *Chaetomium elatum* ChE01, *Chaetomium lucknowense* CLT01, *Emericella nidulans* EN01 and *Emericella rugulosa* ER01 were proved to antagonize *F. oxysporum* f. sp. *lycopersici* NKSC02. The antagonism test demonstrated the antagonistic activity of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 to inhibit the conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 between 63 – 77 %. The result was in accordance with the study from Charoenpoen *et al.* (2010) who reported that *Ch. lucknowense* CLT significantly inhibited the mycelia growth and conidial production of *F. oxysporum* f. sp. *lycopersici* as 88.89 and 92.54 %, respectively. Furthermore, Sibounnavong *et al.* (2009) reported that *E. nidulans*

strongly inhibited colonial growth and sporulation of *F. oxysporum* f. sp. *lycopersici* in antagonism and crude extract tests. Tested antagonistic fungi of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 expressed were their abilities to inhibit the growth of *F. oxysporum* f.sp. *lycopersici* NKSC02. It is indicated that *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 significantly inhibited colony growth and inocula production of *F. oxysporum* f. sp. *lycopersici* NKSC02. Bioactivities tests of crude extracts and pure compounds from antagonistic fungi were also proved as a control mechanism.

To elucidate the control mechanism involved in the inhibition of *F. oxysporum* f. sp. *lycopersici*, crude extracts of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 were confirm for antifungal activity against of *F. oxysporum* f. sp. *lycopersici* NKSC02. The other control mechanism of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 involved in releasing antibiotic substances to inhibit *F. oxysporum* f. sp. *lycopersici*.

All tested crude extracts of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 were significantly inhibited conidia production of *F. oxysporum* f. sp. *lycopersici*. This result was similar to the report of Charoenpoen *et al.* (2010) who stated that crude hexane, crude ethyl acetate and crude methanol from *Ch. lucknowense* CLT inhibited *F. oxysporum* f. sp. *lycopersici* NKSC01 with the ED_{50} of 188, 209 and 212 µg/ml while in this study, those crude extracts inhibited the conidial production of different isolate of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED_{50} of 9.13, 18.10 and 1.63 µg/ml which were lower than those from previous report. Similar results were also reported by Srinon *et al.* (2006) and Sibounnavong *et al.* (2012), who stated that crude hexane, ethyl acetate and methanol extracts from *E. nidulans* inhibited the colony and sporulation of *F. oxysporum* f. sp. *lycopersici*.

Moreover, Soytong *et al.* (2005) reported that crude ethyl acetate extract of *Ch. globosum* CG at 1000 µg/ml inhibited conidia production of this pathogen. Crude ethyl acetate from *E. rugulosa* gave the highest of percent inhibition of conidial production of *F. oxysporum* f. sp. *lycopersici* in which ED_{50} value was 138 µg/ml while crude hexane and crude methanol presented their abilities to inhibit conidial

production at the ED_{50} values 313 and 1372 μg/ml, respectively. As a result, Sibounnavong *et al* (2009) reported that methanol crude extract from *E. nidulans* gave the highest inhibition of *F. oxysporum* f. sp. *lycopersici*. It is explained that ethyl acetate crude extract from *E. rugulosa* might have different antagonistic substances from methanol crude extract from *E. nidulans* as reported by Moosophon *et al.* (2007).

The crude extracts of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 were further isolated to pure compounds and the chemical structures were elucidated. It is clearly demonstrated that Chaetoglobosin C, a pure compound produced by *Ch. elatum* ChE01 and *Ch. lucknowense* CLT01, and tajixanthone, a pure compound produced by *E. rugulosa* ER01. Both pure compounds gave significantly of percent inhibited conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED_{50} of 5.94 and 167 μg/ml, respectively which were more effective than their crude extracts. It is suggested that Chaetoglobosin C and tajixanthone are expressed as a antibiotic substances to destroy the pathogen cells implies antibiosis. As previously reported by Soytong *et al.* (2001), Chaetoglobosin C from *Ch. globosum* inhibited several plant pathogens including *F. oxysporum* f. sp. *lycopersici*. Thohinung *et al.* (2010) also reported that *Ch. elatum* ChE01 produce Chaetoglobosin C that showed cytotoxicity against the human breast cancer and cholangiocarcinoma cell lines. In this study, we found that tajixanthone from *E. rugulosa* ER01 inhibited the tested plant pathogen. *E. rugulosa* ER01 was also reported by Moosophon *et al.* (2009) produced tajixanthone.

Inocula of *F. oxysporum* f. sp. *lycopersici* (1×10^7 spores/ml) were treated with pure compounds of Chaetoglobosin C and tajixanthone and inoculated to tomato seedlings caused no symptoms at day 21 while the treatment with pathogen alone showed significantly highest disease severity index . With this, no wilt incidences were appeared at all tested concentration of 10, 50 and 100 μg/ml of either Chaetoglobosin C or tajixanthone. It is stated that Chaetoglobosin C and tajixanthone affected directly to the pathogen inocula implies antibiosis which the occurrences of ruptured cells and abnormal conidia of pathogen. It is concluded that *Ch. elatum* ChE01, and *Ch. lucknowense* CLT01 are confirmed to produce Chaetoglobosin C and *E. rugulosa* ER01 produce tajixanthone. In this study, these two compounds exhibited antifungal activity against *F. oxysporum* f. sp. *lycopersici* NKSC02 at low concentration. In addition, Park *et al.* (2005) reported thatchaetoviridin-A purified

from *Ch. globosum* F0142 exhibited moderate control of tomato late blight at 125 μg/ml. Chaetoglobosin-C, which produced by *Ch. elatum* ChE01, was not only shown to exhibit cytotoxicity against the human pathogens (Thohinung *et al.,* 2010) but also inhibited the tomato wilt pathogen; *F. oxysporum* f. sp. *lycopersici* in this study. Moreover, this study demonstrated that either tajixanthone or Chaetoglobosin C mixed in a solution with pathogen cells of *F. oxysporum* f. sp. *lycopersici* caused cells ruptured and abnormal conidia. It is suggested that these pure compounds can lyse the cell wall of the pathogen and the protoplast becomes a plug inside the cells. These observations were similar to those reported by Sibounnavong *et al.* (2009) and Soytong (1992) who showed that the crude extracts of these antagonists ruptured the cells of the *F. oxysporum* f. sp. *lycopersici* inoculums. In this study, the abnormal conidia of pathogen cells affected by tajixanthone or Chaetoglobosin C leading to loss of its pathogenicity when inoculated to tomato seedlings var Sida and no symptoms were observed. Moreover, bioformulation of *E. nidulans* gave highly significant reduced *Fusarium* wilt of tomato var. Sida in a pot experiment (Sibounnavong *et al.,* 2010). It is questioned that these bioactive compounds should be further evaluated to determine their ability to control tomato wilt in pot and field trials. Thereafter, separation of crude extracts to get pure compound of tajixanthone, it is proved that tajixanthone can be actively express against *F. oxysporum* f. sp. *lycopersici* NKSC02 at lower concentration than crude extracts which the ED_{50} of 122 μg/ml to inhibit mycelia growth, 54 μg/ml to inhibit macrocondia and 42 μg/ml inhibit microconidia. Moosophon *et al* (2007) isolated pure compounds from *E. nidulans* as epishamixanthone, shamixanthone , emericellin, ergosta-6, 22-diene-3-ol-5, 8-epidioxy-(3β- 5α, 22*E*), sterigmatocystin and demethylsterigmatocystin which differed from isolation of pure compounds from *E. rugulosa* as found five new prenylxanthones, ruguloxanthones A-C, 14-methoxytajixanthone and tajixanthoneethanoate, one novel cyclooctadiene derivative, together with seven known, shamixanthone, tajixanthone, 14-methoxytajixanthone-25-acetate, tajixanthone hydrate, tajixanthonemethanoate, isoemericellin and ergosterol (Moosophon *et al.,* 2009). It is indicated that tajixanthone isolated from *E. rugulosa* firstly reported as a fungal metabolite to be actively against *F. oxysporum* f.sp. *lycopersici* implies antibiosis as a role of control mechanism.

The research findings indicated that treated tomato seedlings varSida with crude EtOAc of *E. rugulosa* at 1000 µg/ml gave significant lower DSI from treated with crude EtOAc of *E. rugulosa* at 500 µg/ml when compared to the inoculated with *F. oxysporum* f. sp. *lycopersici* NKSC02. Disease immunity to Fusarium wilt in Sida variety appeared the highest percent immunity of 80.95 % when treated with crude EtOAc at 1000 µg/ml and followed by treated with crude EtOAc at 500 µg/ml which showed immunity of 30.09 %. tajixanthone and Chaetoglobosin C may possible develop to be microbial elicitors to induce immunity in tomato plants against *F. oxysporum* f. sp. *lycopersici*. A part from the result, crude EtOAc of *E. rugulosa* with contains tajixanthone proved to be an microbial elicitor to induce immunity in tomato. As the inoculated tomato seedlings with *F. oxysporum* f. sp. *lycopersici* showed percent of disease immunity to Fusarium wilt in Sida varieiy of 80.95 %, where the wilt incidence was lower than the inoculated control. Hanh (1996) stated that elicitors are molecules that stimulate any of a number of defense responses in plants, such as synthesis of phytoalexins and pathogenesis-related proteins (PR-proteins). Such responses occurs after the binding of elicitor molecules to receptors normally located on the plant cell surface, promoting a signal transduction pathway that will lead to the activation of one or more defense mechanisms. The first characterized elicitors were oligosaccharide fragments from fungal cell walls, including oligochitin and oligochitosan.

The biological fungicides has been released and distributed to the growers over a decade. Kaewchai *et al.* (2009) stated that mycofungicides have been promoted for agricultural use because of their ability to control plant diseases and to increase crop production in an environmental friendly manner. The registered biological fungicide formulated from *Ch. cupreum* in Thailand could decrease disease incidence of tomato wilt and also increased in yield (Soytong, 1992). In this study, Fusarium wilt was lowest wilt incidence in oil and powder bioformulations from *Ch. elatum* ChE01, *E. nidulans* EN01 and *E. rugulosa* ER01 which significantly differed from Prochoraz and inoculated control. The application of oil bioformulation from *E. rugulosa* could reduce wilt incidence of 60 % and followed by application of powder bioformulation and Prochoraz which reduced wilt incidence of 58 and 15 %, respectively. As comparison to the work of Charoenporn *et al.* (2010) reported that oil bio-agent formulation from the other antagonistic fungi of *Ch. globosum* and *Ch. lucknowense* also showed their biological ability to control tomato wilt. The bio-agent

formulations namely N0802, CLT and PC01 gave significantly highest disease reduction of tomato wilt which were 44.68, 36.28 and 41.01%, respectively, followed by prochoraz (21.95%). Charoenporn *et al.* (2010) stated that all tested bio-agent formulations could significantly increase the yield of tomato when compared to prochoraz and inoculated control and concluded that *Ch. globosum*, *Ch. lucknowense* and *T. harzianum* developed as bio-agent formulations namely N0802, CLT and PC01 and showed their abilities to control tomato wilt. Based on the result, oil bioformulation from *E. rugulosa* gave significantly better for plant parameters in terms of plant height, plant weight, root weight, number of fruits and fruit weight than powder bioformulation and Prochoraz when compared to the inoculated control with *F. oxysporum* f. sp *lycopersici*. This result is similar to the report of Charoenporn *et al.* (2010) stated that all tested bio-agent formulations of antagonistic fungi; *Ch. globosum* and *Ch. lucknowense* could significantly reduce tomato wilt caused by *F. oxysporum* f. sp *lycopersici* and increase in yield of tomato when compared to prochoraz and inoculated control. However, bioformulations from *Ch. elatum* ChE01, *E. nidulans* EN01 and to *E. rugulosa* ER01 in these research finding revealed a good result to control wilt incidence of tomato caused by *F. oxysporum* f. sp *lycopersici.* Soytong *et al.* (2001) showed that the biological products consist of *Chaetomium* sp. (22 strains of *Ch. cupreum* and *Ch. globosum*) in biopellet and biopowder formulations which when applied to the soil could suppress the growth of *F. oxysporum* f. sp. *lycopersici* and reduce infection rate in tomato and those bioproducts has been released to the market. It is suggested that this new reports of bioformulations of *Ch. elatum* ChE01, *E. nidulans* EN01 and to *E. rugulosa* ER01 could be tested to control tomato wilt caused by *F. oxysporum* f.sp. *lycopersici* in the field trials.

CHAPTER VI
CONCLUSION

The research findings indicated that tomato wilt collected from infested fields in Bangkok, Phetchabun, Tak, Nakhonratchasima, Burirum, Nongkhai, SakonNakhon, and Khonkaen provinces are concluded as follows:- 2 isolates were isolated from Bangkok including BKRF01, BKRS01 isolates, 7 isolates PBRS101, PBRS102, PBRS103, PBRS104, PBRS201, PBRS202, PBRS203 were isolated from Phetchabun, 4 isolates MSRS01, MSRS02, TRS01, and TRS02 from Tak . Moreover, 12 isolates of *F. oxysporum* f. sp. *lycopersici*, which isolated from Burirum, Khonkaen, Nongkhai, Nakhonratchasima and Sakonnakhon provinces; BRCO3, KK2, KSoC02, NKSC01, NKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03, and SSoC04 isolates. *F. oxysporum* f. sp. *lycopersici* were confirmed identification by morphological and molecular phylogeny by sequencing the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA and a small portion of the 28S rDNA and AFLP.

The pathogenicity tests were performed on tomato seedlings in this study showed that the *F. oxysporum* f. sp. *lycopersici* NKRC02, NKRC04, SRC02, NSC01, NKRC09, KSoC02, BRC03, SSoC03 and SSoC04 isolates were avirulents. The pathogenicity test and AFLP analysis in this study revealed that 11 isolates were categorized as non-pathogenic or avirulent group and 14 isolates were categorized as pathogenic group which divided into 3 subgroups of low virulent (L), moderate virulent (M) and high virulent (H). As a result, the isolates of KSoC02, NKRC09, SSoC03 and SSoC04 were shown to be non-pathogenic isolates. Isolate KK2 isolated from Northeast part of Thailand was tested its pathogenicity to cause wilt symptom on tomato var Sida which susceptible to standard tested isolate Fol 007 race 2 and concluded that KK2 was race 2. In this study, AFLP has been used as a powerful technique in molecular fingerprinting to study the relationship among fungal isolates and their pathogenicity. It was not clearly relationship between provinces and distribution of pathogen. In this study, It was showed that the phonetic dendrogram generated by UPGMA modified from neighbor procedure of PHYLIP version 3.5 based on genotypes in 8 populations as pop1:Khonkaen province, pop2:Bangkok province, pop3: Sakornnakon province, pop4:Burirum province, pop5: Nongkai province, pop6: Nakhonratchasima, pop7:

Tak province and pop8:Phetchabun province. A principal coordinate analysis (PCA) grouped all of the *Fusarium* spp. isolates into eight major clusters. No clear trend was detected between clustering in the AFLP dendrogram and geographic origin of the tested isolates.

In conclusion, the genetic variation among isolates of *F. oxysproum* .f .sp. *lycopersici* was clearly relationship between pathogenicity groups and AFLP groups. But it was not clearly correlated between AFLP and geographical areas. Moreover, this work provided new information on formae specialis of *F. oxysproum* f. sp. *lycopersici* which could classify as race 2 that can cause wilt to different varieties of tomato e.g. Cheery and Sida varieties rather one variety. Regarding from the result of the present study demonstrated clearly that the use of the AFLP is a powerful, simple and rapid technique to study the identification and genetic relationship between *F. oxysporum* and their pathogenicity. AFLP may therefore provide rich sources of molecular markers which are useful to study on the genetic variation for specific level.

The antagonistic fungi namely: *Chaetomium brasilense* CB01, *Chaetomium cupreum* CC03, *Chaetomium elatum* ChE01, *Chaetomium lucknowense* CLT01, *Emericella nidulans* EN01 and *Emericella rugulosa* ER01 were proved to antagonize *F. oxysporum* f. sp. *lycopersici* NKSC02. The antagonism test demonstrated the antagonistic activity of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum*ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 to inhibit the conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 between 63 – 77 %.

Tested antagonistic fungi of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 expressed their abilities to inhibit the growth of *F. oxysporum* f. sp. *lycopersici* NKSC02. It is indicated that *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 gave significantly inhibited colony growth and inocula production of *F. oxysporum* f. sp. *lycopersici* NKSC02.

Bioactivities tests of crude extracts and pure compounds from antagonistic fungi were also proved as a control mechanism. To elucidate the control mechanism involved in the inhibition of *F. oxysporum* f. sp. *lycopersici*, crude extracts of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 were confirmed for antifungal activity

against of *F. oxysporum* f. sp. *lycopersici* NKSC02. The other control mechanism of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum*ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 involved in releasing antibiotic substances to inhibit *F. oxysporum* f. sp. *lycopersici*. All tested crude extracts of *Ch. brasilense* CB01, *Ch. cupreum* CC03, *Ch. elatum* ChE01, *Ch. lucknowense* CLT01, *E. nidulans* EN01 and *E. rugulosa* ER01 were significantly inhibited conidia production of *F. oxysporum* f. sp. *lycopersici*.

The crude extracts of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 were further isolated to pure compounds and the chemical structures were elucidated. It is clearly demonstrated that chaetoglobosin-C, a pure compound produced by *Ch. elatum* ChE01 and *Ch. lucknowense* CLT01, and tajixanthone, a pure compound produced by *E. rugulosa* ER01. Both pure compounds significantly inhibited conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02. It is suggested that chaetoglobosin-C and tajixanthone are expressed as a antibiotic substances to destroy the pathogen cells implies antibiosis.

Inocula of *F. oxysporum* f. sp. *lycopersici* (1×10^7 spores/ml) were treated with pure compounds of Chaetoglobosin C and tajixanthone and inoculated to tomato seedlings caused no symptoms at day 21 while the treatment with pathogen alone showed significantly highest disease severity index. With this, no wilt incidences were appeared at all tested concentration of 10, 50 and 100 µg/ml of either Chaetoglobosin C or tajixanthone. It is stated that Chaetoglobosin C and tajixanthone affected directly to the pathogen inocula implies antibiosis which the occurrences of ruptured cells and abnormal conidia of pathogen. It is concluded that *Ch. elatum* ChE01, and *Ch. lucknowense* CLT01 are confirmed to produce Chaetoglobosin C and *E. rugulosa* ER01 produce tajixanthone. In this study, these two compounds exhibited antifungal activity against *F. oxysporum* f. sp. *lycopersici* NKSC02 at low concentration. Moreover, this study demonstrated that either tajixanthone or Chaetoglobosin C mixed in a solution with pathogen cells of *F. oxysporum* f. sp. *lycopersici* caused cells ruptured and abnormal conidia. It is suggested that these pure compounds can lyse the cell wall of the pathogen and the protoplast becomes a plug inside the cells.

The research findings indicated that treated tomato seedlings varSida with crude EtOAc of *E. rugulosa* at 1000 µg/ml gave significant lower DSI from treated with crude EtOAc of *E. rugulosa* at 500 µg/ml when compared to the inoculated with

F. oxysporum f.sp. *lycopersici* NKSC02. Disease immunity to Fusarium wilt in Sida variety appeared the highest percent immunity when treated with crude EtOAc at 1000 µg/ml and followed by treated with crude EtOAc at 500 µg/ml which also expressed immunity. tajixanthone and Chaetoglobosin C may possible develop to be microbial elicitors to induce immunity in tomato plants against *F. oxysporum* f. sp. *lycopersici*. A part from the result, crude EtOAc of *E. rugulosa* with contains tajixanthone proved to be a microbial elicitor to induce immunity in tomato. As the inoculated tomato seedlings with *F. oxysporum* f. sp. *lycopersici* showed disease immunity to Fusarium wilt in Sida varieiy where the wilt incidence was much lower than the inoculated control.

Fusarium wilt was lowest wilt incidence in oil and powder bioformulations from *Ch. elatum* ChE01, *E. nidulans* EN01 and to *E. rugulosa* ER01 which gave significantly differed from Prochoraz and inoculated control. The application of oil bioformulation from *E. rugulosa* could reduce wilt incidence and followed by application of powder bioformulation, culture filtrate and Prochoraz which also reduced wilt incidence. Based on the result, oil bioformulation from *Ch. elatum* ChE01, *E. nidulans* EN01 and to *E. rugulosa* ER01 gave significantly better plant parameters in terms of plant height, plant weight, root weight, number of fruits and fruit weight than powder bioformulation and Prochoraz when compared to the inoculated control with *F. oxysporum* f. sp. *lycopersici*.

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Faculty of Agriculture National University of Laos (NUL), Nabong, Lao P.D.R. (5 years program of Bachelor degree)	B.Sc. (Agriculture)	3.07	2006
Department of Biology, Central Luzon State University , Philippines	M.Sc.(Biology)	1.5/3.5	2009
Biotechnology in Plant Pathology, International college, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.	Ph.D.	outstanding	2012

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Training Courses	**duration**	**Countries**
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Certificate of microbial biotechnology in agriculture	3/7/2006 to 3/5/2006	Thailand
Certificate of workshop on production of biological fungicide and a modern taxonomy of powdery mildew at Thai Mycological Association(TMA) , KMITL, Thailand	29 October 2006	Thailand
Certificate of Regional Training Program :Disease Management and Good Agricultural Practices in Vietnam (Supported by SIDA-SAREC, Sweden and MOSTE, Vietnam)	5-12 February 2007	Vietnam

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1. **Sibounnavong, P.** 2012. Screening of *Emericella nidulans* for biological control of tomato Fusarium wilt in Lao PDR. ***International Journal of Agricultural Technology 8(1):241-260. (TC index journal).***
2. **Sibounnavong, P.**, Sibounnavong, P.S., Kanokmedhakul S. and Soytong, K.(2012) Antifungal activities of *Chaetomium brasilense* CB01 and *Chaetomium cupreum* CC03 against *F. oxysporum* f. sp. *lycopersici* race 2. ***Journal of Agricultural Technology 8(3): 1029-1038. (TC index journal).***
3. **Sibounnavong, P.**, Kanokmedhakul, S. and Soytong, K. **2011**. Antifungal metabolites from antagonistic fungi used to control tomato wilt fungus *Fusarium oxysporum* f sp *lycopersici*. ***African Journal of Biotechnology 10(85):19714-19722. (ISI index journal).***
4. **Phoutthasone Sibounnavong**, Jintana Unartngam and **Kasem Soytong 2012**. Genetic variation of *Fusarium oxysporum* f. sp. *lycopersici* isolated From tomatoes in Thailandusing pathogenicity and AFLP markers. ***African Journal of Microbiology Research.*** **Accepted for publication** ***(ISI index journal).***
5. **Sibounnavong, P.**, Charoenporn, C., Kanokmedhakul, S., Soytong, K. 2012. A role of *Emericella rugulosa* as a biocontrol agent to control Tomato fusarium wilt. **African Journal of Agricultural Research. Accepted for publication** ***(ISI index journal)***.

APPENDIX

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Full Length Research Paper

Antifungal metabolites from antagonistic fungi used to control tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici*

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***Chaetomium elatum* strain ChE01, *Chaetomium lucknowense* strain CLT01 and *Emericella rugulosa* strain ER01, which were isolated from soil in Thailand, effectively controlled the most virulent isolate of *Fusarium oxysporum* f. sp. *lycopersici* NKSC02 causing wilt of tomato (*Lycopersicon esculentum* var Sida). Two antifungal substances were purified: Chaetoglobosin-C from *Ch. elatum* and *Ch. lucknowense* and tajixanthone from *E. rugulosa*. Chaetoglobosin-C showed greater antifungal activity against *F. oxysporum* f. sp. *lycopersici*, with an effective dose (ED_{50}) of 5.98 µg/ml, compared with tajixanthone (ED_{50} of 167 µg/ml). These results suggest that the disease control mechanism of these antagonistic fungi involves antibiosis. The inoculating tomato seedlings var. Sida with conidia of *F. oxysporum* f.sp. *lycopersici*, mixed with either a solution of chaetoglobosin-C or tajixanthone showed no wilt symptom. The conidia of *F. oxysporum* f. sp. *lycopersici* treated with these two compounds appeared abnormal and lost pathogenicity.**

Key words: Bioactivity test, *Chaetomium elatum*, *Chaetomium lucknowense*, *Emericella rugulosa*, *Fusarium oxysporum*.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely cultivated, popular and important vegetable crops in the world. *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen is one of the most common pathogen that causes wilt of tomato in areas of upland cultivation which can cause economic losses. *F. oxysporum* f. sp. *lycopersici* has become one of the most damaging and difficult to control wherever tomatoes are grown intensively, because it grows endophytically and persists in infested soils (Agrios, 1997). The disease control measures for this vascular wilt are either inefficient or difficult to apply the chemical fungicides. Over time tomatoes may develop resistance to some races of the pathogen; however, the pathogenic fungus may also develop resistance to chemical fungicides (Silva and Bettiol, 2005). *Chaetomium* spp. belong to the Ascomycota, and have been reported as antagonists against several plant pathogens (Soytong and Quimio, 1989; Soytong et al., 2001; Dhingra et al., 2003; Aggarwal et al., 2004; Park et al., 2005). Many species of *Chaetomium* with the potential to be biological control agents suppress the growth of bacteria and fungi through competition (for substrate and nutrients), mycoparasitism, antibiosis, or various combinations of these (Marwah et al., 2007; Zhang and Yang, 2007). *Chaetomium globosum* and *Ch. cupreum* in particular have been extensively studied and

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successfully used to control root rot disease of citrus, black pepper and strawberry, and have been shown to reduce damping off disease of sugar beet (Soytong et al., 2001; Tomilova and Shternshis, 2006). The plant disease control mechanism may involve in antibiosis, with the antagonistic fungus releasing antibiotic substances (Soytong et al., 2001; Kanokmedhakul et al., 2002, 2006; Park et al., 2005). Cullen and Andrews (1984) reported evidence of antibiosis by *Ch. globosum* against *Venturia inaequalis*, which causes apple scab.

Di Pietro et al. (1992) reported that *Ch globosum* can produce chetomin, which effectively inhibited *Pythium ultimum*, which caused damping-off of sugar beet. *Ch. globosum* strain KMITL 0802 has been shown to produce chaetoglobosin – C, which inhibits some pathogens (Kanokmedhakul et al., 2002). Park et al. (2005) also reported that *Ch. globosum* F0142 can produce chaetoviridin A to control rice blast, wheat leaf rust and tomato late blight. Soytong (1992) and Soytong et al. (2001) showed that a specific isolate of *Chaetomium cupreum* produced secondary metabolites that significantly suppressed tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* in the tomato fields in Thailand, and later found that this isolate of *Ch. cupreum* produced rotiorinols A to C and rotiorin, which exhibited antifungal activity against *Candida albicans* (Kanokmedhakul et al., 2006). *Chaetomium cochlioides*- strains VTh01 and CTh05 have been shown to exhibit antimicrobial activity against a *Phytophothora* sp. that causes root rot, the anthracnose fungus *Colletotrichum gloeosporioides*, and *F. oxysporum* f. sp. *lycopersici*.

Among the compounds isolated from *Ch. cochlioides* strains VTh01 and CTh05, Phonkerd et al. (2008) identified four new dimeric spiro-azaphilones; (cochliodones A to D), two new azaphilones; (chaetoviridines E and F), and a new *epi*-chaetoviridin A. The isolate of the *Chaetomium elatum* strain ChE01 used in this study has been reported to produce a chaetoglobosin V, prochaetoglobosin III and prochaetoglobosin III_{ed}, chaetoglobosins B to D, F and G, and isochaetoglobosin D, which have been shown to exhibit cytotoxicity against a human breast cancer cell line. It should be noted that *Ch. elatum* ChE01 can also produce chaetoglobosin-C as a major compound, comprising up to 2% of the dried mycelial mat when grown in liquid culture (Thohinung et al., 2010). Moreover, the isolate of Ascomycete *Emericella rugulosa* used in this study has been shown to produce five prenylxanthones, rugulox-anthones A to C, 14-methoxytajixanthone,-tajixanthone ethanoate, a bicycle(3.3.1)-nona-2, 6-diene derivative named rugulosone, shamixanthone, tajixanthone, 14-methoxytajixanthone-25-acetate, tajixanthone hydrate, tajixanthone methanoate, isoemericellin and ergosterol. Among these, the bicycle (3.3.1)–nona-2,6-diene derive-ative has been shown to exhibit antimalarial and antimycobacterial activity and cytotoxicity against three cancer cell lines (Moosophon et al., 2009). Another species of *Emericella* (*Emericella nidulans*) has also been reported to antagonize *F. oxysporum* f. sp. *lycopersici* (Sibounnavong et al., 2009). The antimicrobial activity of chaetoglobosin-C, which is produced by *Ch. elatum* ChE01, and *Ch. lucknowense* CLT01, and of tajixanthone, which is produced by *E. rugulosa* ER01, could be involved in the disease control mechanism of these antagonistic fungi against the tomato wilt fungus *F. oxysporum* f. sp. *lycopersici*. The objectives of research findings were to isolate *F. oxysporum* f. sp. *lycopersici* causing tomato wilt and test for it pathogenicity and to investigate antagonistic fungi namely Ch. elatum strain ChE01, Ch. lucknowense strain CLT01 and *E. rugulosa* strain ER01 for antagonism of *F. oxysporum* f. sp. *lycopersici*. Crude extracts and pure compounds, chaetoglobosin-C from Ch. elatum and Ch. lucknowense and tajixanthone from *E. rugulosa* were tested for their antibiosis. The effects of chaetoglobosin-C and tajixanthone for pathogenicity loss of *F. oxysporum* f. sp. *lycopersici* were also investigated.

MATERIALS AND METHODS

Isolation and pathogenicity test

Disease samples were collected from infested soil from tomato fields in Bangkok, Pechaboon, Tak, and Chaingmai provinces in Thailand. The pathogen was isolated by transferring surface-sterilized plant tissue to a potato dextrose agar (PDA) medium and using soil plate techniques following the methods used by Agrios (1997). Pure cultures of *F. oxysporum* f. sp. *lycopersici* were identified by morphological characteristics under a binocular compound microscope, maintained on PDA slants and deposited at the Biocontrol Research Unit and Mycology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Eleven isolates of *F. oxysporum* f. sp. *lycopersici*, which were isolated from Burirum, Khonkaen, Nongkhai, Nakhonratchasima and Sakon-nakhon provinces in Thailand were obtained from Asst. Prof. Dr. Chamaiporn Charoenporn (Nakhonratchasima Rachabhat University, Nakhonrat-chasima, Thailand) as follows: isolates BRCO3, KK2, KSoC02, NKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03, and SSoC04. The morphological identification of the 11 isolates has been confirmed previously by Charoenporn et al. (2010) by sequencing the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA as well as a small portion of the 28S rDNA.

All *F. oxysporum* f. sp. *lycopersici* isolates were tested for pathogenicity to tomato seedlings using Koch's postulates. Briefly, all isolates were sub-cultured and multiplied on PDA and incubated for 7 to 10 days at room temperature approximately (30 to 32°C). The inoculum of pathogen was adjusted to 1×10^7 spores/ml before inoculating to 20–day–old tomato seedlings var. Sida. The roots of tomato seedlings were washed under running sterilized water and cut at five points on the root tips before dipping the roots into a 20 ml spore suspension for 15 min. A control was performed by dipping seedling roots into sterile distilled water. The seedlings were then potted in sterilized soil. After 10 days, symptoms of

isease were recorded using the Disease Severity Index (DSI) and ated according to Sibounnavong et al. (2009, 2010) as follows: 1 = o symptoms, 2 = 1 to 20% of leaves yellow and wilted, 3 = 21 to 0% of leaves yellow and wilted, 4 = 41 to 60% of leaves yellow nd wilted, 5 = 61 to 80% of leaves yellow and wilted, and 6 = 81 to 00 % of leaves yellow and wilted. The experiment was conducted sing a completely randomized design (CRD) with six replications f each treatment. The experiment was repeated twice. Virulence as categorized according to the DSI, following the method used y Charoenporn et al. (2010) as follows: non-pathogenic (DSI =1), w virulence (DSI ≤ 3.50), moderate virulence (DSI > 3.50 to 4.50), nd highly virulence (DSI > 4.50). The most virulent isolate was elected for further experiments.

ntagonism test

ntagonistic fungi were isolated from soil in Thailand, namely *Ch. latum* strain ChE01, *Ch. lucknowense* strain CLT01 and *E. ugulosa* strain ER01. These isolates were tested to determine their bility to antagonize the *F. oxysporum* f. sp. *lycopersici* isolate dentified as the most virulent in the pathogenicity test. The test was onducted using the methods of Soytong (1992), Sibounnavong et l. (2009) and Charoenporn et al. (2010). The antagonistic fungi nd pathogen were separately cultured on PDA at room emperature (30 to 32°C) for seven days. A 0.5 cm diameter terilized cork borer was used to remove agar plugs from the ctively growing edge of cultures of the pathogenic fungus and of he antagonistic fungi and used to inoculate 9 cm diameter PDA plates: an agar plug of the pathogen was placed on one side of the plate opposite an agar plug of an antagonistic fungus. Plates inoculated with a single plug of an antagonistic fungus or of the pathogen acted as the controls. The plates were incubated at room temperature (30 to 32°C) for 30 days. The experiment was performed using a completely randomized design (CRD) with four replications. Data were collected regarding colony diameter (cm) and the number of conidia produced by the pathogen. A haemcytometer was used to count the number of conidia. Percentage inhibition of pathogen colony growth and of conidia production was calculated using the following formula:

$$\% \text{ inhibition} = \frac{A - B}{A} \times 100$$

Where, A is the colony diameter or number of conidia produced by the pathogen on the control plate and B is the colony diameter or number of conidia produced by the pathogen when inoculated opposite an antagonistic fungus. Analysis of variance was statistically analyzed and treatment means were compared using Duncan's Multiple Range Test (DMRT) at p = 0.05 and 0.01. The experiment was repeated twice.

Crude extracts and pure compounds from antagonistic fungi

Crude extracts from each antagonistic fungus were obtained from the method used by Kanokmedhakul et al. (2006), Moosophon et al. (2009) and Thohinung et al. (2010). The fungi were cultivated in potato dextrose broth at room temperature (30 to 32°C) for 30 days. The dried fungal biomass of each antagonistic fungus was ground and sequentially extracted with hexane, ethyl acetate, and methanol. The solvents were then evaporated *in vacuo* to yield crude hexane, crude ethyl acetate (EtOAc), and crude methanol (MeOH) extracts, respectively. The extracts were separated and purified using chromatographic methods to obtain the compounds. The structures of these compounds were identified by spectroscopic methods, IR, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR (COSY, HMQC, HMBC, and NOESY).

Crude extract bioassay

The crude extracts were assayed for inhibition of the most virulent isolate of *F. oxysporum* f. sp. *lycopersici*. The experiment was conducted by using a factorial experiment in CRD with four replications. Factor A represented the different solvents: A1 = crude hexane, A2 = crude ethyl acetate and A3 = crude methanol. Factor B represented the different concentrations: B1 = 0 µg/ml (control), B2 = 50 µg/ml, B3 = 100 µg/ml, B4 = 500 µg/ml and B5 = 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 30 min. To perform the assay, a sterilized 3 mm diameter cork borer was used to remove agar plugs from the actively growing edge of the pathogen culture. An agar plug was transferred to the center of 5 cm diameter Petri dishes of PDA containing crude extract at each concentration and incubated at room temperature (30 to 32°C) until the pathogen on the control plates had grown over the plate. Data were collected regarding the number of conidia produced by the pathogen and used to calculate the percentage of conidia inhibition. The effective dose (ED_{50}) was calculated using Probit analysis. The experiment was repeated twice.

Bioactive compound assay

Pure compounds of chaetoglobosin-C from *Ch. elatum* and *Ch. lucknowense* and tajixanthone from *E. rugulosa* (Figure 1) were separately tested for their antifungal activities against *F. oxysporum* f. sp. *lycopersici*. To perform the assay, a sterilized 3 mm diameter cork borer was used to remove agar plugs from the actively growing edge of the pathogen culture. An agar plug was transferred to the center of 5 cm diameter Petri dishes of PDA containing either pure compounds of Chaetoglobosin–C or tajixanthone at each concentration and incubated at room temperature (30 to 32°C) until the pathogen on the control plates grows over the plate. The experiment was performed using a CRD with four replications. Treatments comprised four different concentrations: 0, 10, 50 and 100 µg/ml. The experiment was repeated twice. Data were collected regarding the number of conidia produced by the pathogen and calculated for percentage conidial inhibition. The ED_{50} was calculated using Probit analysis

Effect of fungal metabolites on disease incidence

The roots of 20– day– old tomato seedlings var. Sida were washed under running sterilized water and cut at five points on the root tips before dipping the roots into each treatment a 20 ml spore suspension of 1×10^{7} spores/ml mixed with different concentration of pure compounds for 15 min. The experiment was conducted by using a factorial experiment in CRD with four replications. Factor A represented the pure compounds: A1 = tajixanthone, and A2 = chaetoglobosin –C. Factor B represented the different concentrations: B1 = 0 (control), B2 = 10, B3 = 50, and B4 = 100 µg/ml. A control was performed by dipping seedling roots into sterile distilled water. The seedlings were then planted in pots which contained sterilized soil. The experiment was repeated twice. Disease incidence was recorded using the Disease Severity Index used previously in the pathogenicity test.

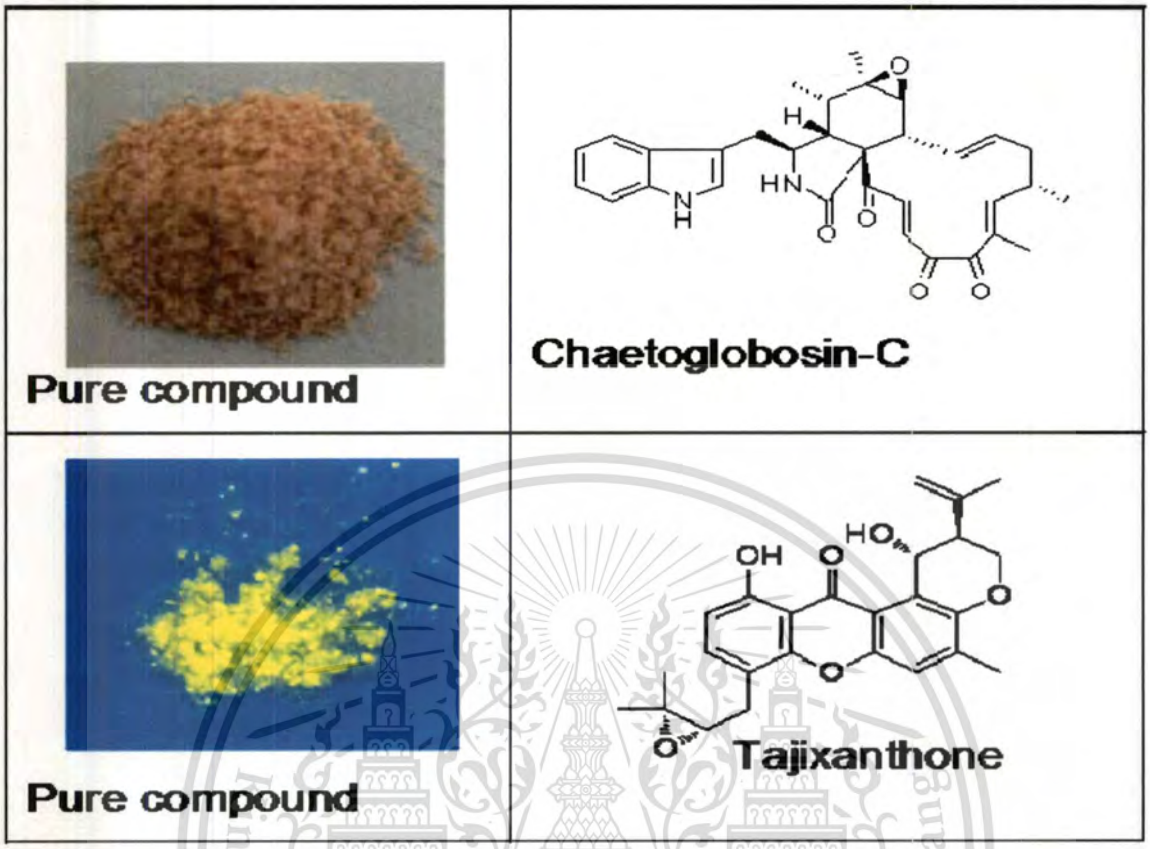

Figure 1. The pure compounds chaetoglobosin-C and tajixanthone: powder (left) and chemical structures (right).

Table 1. Isolates of *Fusarium* spp. and their pathogenicity group.

Provinces	Isolates	DSI[1]	Pathogenicity group[3]
Bangkok	BKRS01	1.87^{cde2}	L
	BKKRS02	1.25^{cde}	L
Pechaboon	PBRS01	1.62^{cde}	L
	PBRS02	1.50^{cde}	L
	PBRS03	1.50^{cde}	L
	PBRS04	1.62^{cde}	L
	PBrS01	2.12^{cd}	L
	PBrS02	1.50^{cde}	L
	PBSS01	1.87^{cde}	L
Tak	MSRS01	1.50^{cde}	L
	MSrS01	1.62^{cde}	L
	TrS01	2.50^{c}	L
	TRS01	2.63^{c}	L
	TSS01	2.37^{c}	L
Chaingmai	CMRS01	1.50^{cde}	L
	CMRS02	1.62^{cde}	L

Table 1. Continue

Burirum	BRC03	1.00[e]	A
	KK2	4.25[b]	M
KhonKaen	KSoC02	1.00[de]	A
Nongkhai	NKSC02	5.25[a2]	H
	NKRC02	1.00[e]	A
	NKRC04	1.00[e]	A
	NKRC09	1.00[e]	A
Nakhonratchasima	NSC01	1.25[d]	L
Sakon-nakhon	SRC02	1.00[e]	A
	SSoC03	1.50[cde]	L
	SSoC04	1.75[cde]	L
	control	1.00[e]	A

[1]Tomato plants were assessed for disease symptoms 21 days after inoculation using the Disease Severity Index (DSI): 1 = No symptoms; 2 = Plant showed 1 to 20% yellowing leaves and wilting, 3 = Plant showed 21 to 40% yellowing leaves and wilting, 4 = Plant showed 41 to 60% yellowing leaves and wilting, 5 = Plant showed 61 to 80% yellowing leaves and wilting, and 6 = Plant showed 81 to 100% yellowing leaves and wilting or death. [2]Average 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. [3]The pathogenicity group of the isolates was determined according to the DSI: A = Avirulent (DSI = 1), L = Low virulence (DSI ≤ 3.50), M = Moderate virulence (DSI ≥ 3.50 to 4.50), H = High virulence (DSI > 4.50).

RESULTS

Isolation and pathogenicity test

Sixteen isolates of *Fusarium* spp. (BKRS01, BKKRS02, PBRS01, PBRS02, PBRS03, PBRS04, PBrS01, PBrS02, PBSS01, MSRS01, MSrS01, TrS01, TRS01, TSS01, CMRS01 and CMRS02) were obtained from isolation work, and 11 isolates of *F. oxysporum* f. sp *lycopersici* (BRCO3, KK2, KSoC02, NKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03, and SSoC04) were grouped for pathogenicity according to disease severity index as shown in Table 1. The results showed that six of the isolates were avirulent (NKSC02, NKRC04, NKRC09and SRC02), 19 isolates showed a low level of pathogenicity (BKRS01, BKKRS02, PBRS01, PBRS02, PBRS03, PBRS04, PBrS01, PBrS02, PBSS01, MSRS01, MSrS01, TrS01, TRS01, TSS01, CMRS01 and CMRS02, NSC01, SSoC03 and SSoC04), one isolate was moderately virulent (KK2). Only isolate NKSC02 was highly virulent (Table 1 and Figure 2). Therefore, the highly virulent isolate NKSC02 was chosen for further studies.

Antagonism test

The result shows that *Ch. elatum* ChE01 significantly inhibited mycelial growth and the production of conidia by *F. oxysporum* f. sp. *lycopersici* NKSC02 of 74.95 and 77.12% inhibition, respectively followed by *Ch. Lucknowense* CLT01 and *E. rugulosa* ER01, which inhibited mycelial growth by 48.64 and 40.20%, respectively and inhibited conidial production by 58.41 and 63.28%, respectively (Table 2).

Crude extract bioassay

All tested crude extracts of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 significantly inhibited the conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 as shown in Table 3. Among the three types of crude extracts, methanol extract from all antagonistic fungi showed the highest inhibitory activity with ED50 ranged from 1.50 to 7.66 µg/ml. With this, the crude hexane, crude ethyl acetate (EtOAc) and crude methanol (MeOH) extracted from *Ch. elatum* ChE01 inhibited the conidial production of isolate NKSC02 with the ED_{50} of 9.10, 8.90 and 1.50 µg/ml respectively. The crude hexane, crude EtOAc and crude MeOH extracted from *Ch. lucknowense* CLT01 inhibited the conidial production of isolate NKSC02 with the ED_{50} of 9.1, 18.0and 1.63 µg/ml, respectively. Moreover, the crude hexane, crude EtOAc and crude MeOH extracted from *E. rugulosa* ER01 inhibited the conidial production of isolate NKSC02 with the ED_{50} of 20.8, 9.8 and 7.6 µg/ml,

Figure 2. Pathogenicity test. (a) Uninoculated tomato seedlings (control), (b). tomato seedlings inoculated with *Fusarium oxysporum* f sp. *lycopersici* isolate NKSC02.

Table 2. Mycelial and conidia inhibition of *Fusarium oxysporum* f. sp. *lycopersici* by antagonistic fungi at 30 days.

Antgonistic fungi	Mycelial inhibition (%)[1]	Conidia inhibition (%)[1]
Ch. elatum ChE01	74.95[a]	77.12[a]
Ch. lucknowense CLT01	48.64[b]	58.41[c]
E. rugulosa ER01	40.20[b]	63.28[b]

[1]Average 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 = 0.05.

Table 3. Bioassay of crude extracts against *Fusarium oxysporum* f. sp. *lycopersici*.

Antagonistic fungi	Crude extracts	Conidial inhibition (%)	ED_{50} µg/ml
	Hexane	92.00[ab1]	9.10
Ch. elatum ChE01	Ethyl acetate	94.00[a]	8.90
	Methanol	97.00[a]	1.50
	Hexane	90.29[b]	9.13
Ch. lucknowense CLT01	Ethyl acetate	90.00[b]	18.10
	Methanol	95.00[a]	1.63
	Hexane	79.00[c]	20.83
E. rugulosa ER01	Ethyl acetate	92.00[ab]	9.86
	Methanol	91.00[ab]	7.66

[1]Average 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.

respectively (Table 3). The blue highlight should be deleted.

Bioactive compound assay

Chaetoglobosin-C was isolated from *Ch. elatum* ChE01 and *Ch. luckowense* CLT01 and tajixanthone isolated from *E. rugulosa* ER01 which elucidated using chromatographic methods to obtain these compounds. The structures were identified by spectroscopic methods, IR, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR (COSY, HMQC, HMBC, and NOESY) as shown in Figure 1. Chaetoglobosin-C, a pure compound produced by *Ch. elatum* ChE01 and *Ch.*

Table 4. Assay of bioactive compounds against *Fusarium oxysporum* f. sp. *lycopersici.*

Pure compound	Conidial inhibition (%)	ED_{50} µg/ml
Chaetoglobosin-C	89.00	5.94
Tajixanthone	68.37	167

Table 5. Effect of fungal metabolites on disease incidence of tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici*

Pure compound	Concentrations µg mL^{-1}	DSI[1]
Chaetoglobosin-C	0	6^{a2}
	10	1^{b}
	50	1^{b}
	100	1^{b}
Tajixanthone	0	6^{a}
	10	1^{b}
	50	1^{b}
	100	1^{b}

[1]Tomato plants were assessed for disease symptoms 21 days after inoculation using the Disease Severity Index (DSI): 1 = No symptoms; 2 = Plant showed 1 to 20% yellowing leaves and wilting, 3 = Plant showed 21 to 40% yellowing leaves and wilting, 4 = Plant showed 41 to 60% yellowing leaves and wilting, 5 = Plant showed 61 to 80% yellowing leaves and wilting, and 6 = Plant showed 81 to 100% yellowing leaves and wilting or death. [2]Average 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.

luckowense CLT01, significantly inhibited conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED50 value of 5.94 µg/ml. Moreover, tajixanthone, a pure compound produced by *E. rugulosa* ER01, significantly inhibited conidia production of *F. oxysporum f. sp. lycopersici* NKSC02 with an ED50 value of 167 µg/ml (Table 4).

Effect of fungal metabolites on disease incidence

Inoculum of *F. oxysporum* f. sp. *lycopersici* (1×10^7 spores/ml) treated with pure compounds of chaetoglobosin-C and tajixanthone before inoculating to tomato seedlings caused no symptoms at day 21 while the treatment with pathogen alone showed significantly high disease severity index as shown in Table 5. No disease incidences were observed at all tested concentration of 10, 50 and 100 µg/ml of either chaetoglobosin-C or tajixanthone which significantly differed from the control. It revealed that the antibiotic substances of chaetoglobosin-C and tajixanthon affected directly to the pathogen conidial inoculum which implies antibiosis mechanism of control. Moreover, the occurrences of ruptured cells and abnormal conidia thereafter mixing with each pure compound of chaetoglobosin-C and tajixanthon were observed under the microscope (Figure 3).

DISCUSSION

The pathogenicity tests performed on tomato seedlings in this study showed that the *F. oxysporum* f. sp. *lycopersici* NKRC02, NKRC04, SRC02 and NSC01 isolates were avirulent whereas Charoenporn et al. (2010) reported in a previous study as moderately virulent to the same variety of tomato. It revealed in this study that isolate NKRC09, KSoC02, BRC03, SSoC03 and SSoC04 were also found to be avirulent, whereas a previous study showed low virulence (Charoenporn et al., 2010). This suggested that repeatedly sub-cultured of *F. oxysporum* f. sp. *lycopersici* affected the stability of pathogenicity. *F. oxysporum* f. sp. *lycopersici* isolate NKSC02 was the most virulent isolate to cause wilt of tomato var. Sida. This observation is supported by *in vitro* studies of virulence by Soytong et al. (2001), Sibounnavong et al. (2009) and Charoenporn et al. (2010).

The antagonism test demonstrated the antagonistic activity of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 to inhibit the conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 between 63 to 77%. The result was in accordance with the study from Charoenpoen et al. (2010) who reported that *Ch lucknowense* CLT significantly inhibited the mycelial growth and conidial production of *F. oxysporum* f. sp. *lycopersici* as 88.89 and 92.54%, respectively. Further-

Pure compounds	Normal conidia	Abnormal conidia
Chaetoglobosin-C		
Tajixanthone		

Figure 3. Abnormal conidial lysis of *F.oxysporum* f. sp. *lycopersici* owing to chaetoglobosin-C and tajixanthone

more, Sibounnavong et al. (2009) reported that *E. nidulans* strongly inhibited colonial growth and sporulation of *F. oxysporum* f. sp. *lycopersici* in antagonism and crude extract tests.

To elucidate the control mechanism involved in the inhibition of *F. oxysporum* f. sp. *lycopersici*, crude extracts of these antagonistic fungi were tested for antifungal activity against *F. oxysporum* f. sp. *lycopersici* NKSC02. The control mechanism of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 revealed releasing antibiotic substances to inhibit *F. oxysporum* f. sp. *Lycopersici.*

All tested crude extracts of *Ch. elatum* ChE01, *Ch. lucknowense* CLT01 and *E. rugulosa* ER01 significantly inhibited conidial production of *F. oxysporum* f. sp. *lycopersici.* This result is similar to the report of Charoenpoen et al. (2010) who stated that crude hexane, crude ethyl acetate and crude methanol from *Ch. lucknowense* CLT inhibited *F. oxysporum* f. sp. *lycopersici* NKSC01 with the ED_{50} of 188, 209 and 212 µg/ml, while in this study, those crude extracts inhibited the conidial production of different isolate of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED_{50} of 9.13, 18.10 and 1.63 µg/ml which were lower than those from previous report. Similar results were also reported by Srinon et al. (2006) and Sibounnavong et al. (2009), who stated that crude hexane, ethyl acetate and methanol extracts from *E. nidulans* inhibited the colony and sporulation of *F. oxysporum* f. sp. *lycopersici.* Moreover, Soytong et al. (2005) reported that crude ethyl acetate extract of *Ch. globosum* CG at 1000 µg/ml inhibited conidial production of this pathogen.

The crude extracts were further isolated to pure compounds and the chemical structures were elucidated. It is clearly demonstrated that chaetoglobosin-C, a pure compound produced by *Ch. elatum* ChE01 and *Ch. lucknowense* CLT01, and tajixanthone, a pure compound produced by *E. rugulosa* ER01. Both pure compounds significantly inhibited conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 with the ED_{50} of 5.94 and 167 µg/ml, respectively which were more effective than their crude extracts. It is suggested that chaetoglobosin-C and tajixanthone are expressed as an antibiotic substances to destroy the pathogen cells implies antibiosis. As previously reported by Soytong et al. (2001), chaetoglobosin-C from *Ch. globosum* inhibited several plant pathogens including *F. oxysporum* f. sp. *lycopersici.* Thohinung et al. (2010) also reported that *Ch. elatum* ChE01 produce chaetoglobosin-C that showed cytoxicity against the human breast cancer and cholangiocarcinoma cell lines. In this study, we found that tajixanthone from *E. rugulosa* ER01 inhibited the tested plant pathogen. *E. rugulosa* ER01 was also reported by Moosophon et al. (2009) to produce tajixanthone. It is concluded that *Ch. elatum* ChE01 and *Ch. lucknowense* CLT01 are confirmed to produce chaetoglobosin-C and *E. rugulosa* ER01 produce tajixanthone. In this study, these two compounds exhibited antifungal activity against *F. oxysporum* f. sp. *lycopersici* NKSC02 at low concentration. In addition, Park et al. (2005) reported that chaetoviridin-A purified from *Ch. globosum* F0142 exhibited moderate control of tomato late blight at 125 µg/ml. In addition, Chaetoglobosin-C produced by *Ch. elatum* ChE01, was not only shown to exhibit cytotoxicity against the human pathogens (Thohinung et al., 2010) but also inhibited the tomato wilt pathogen; *F. oxysporum* f. sp. *lycopersici* in this study.

However, this study demonstrated that either tajixanthone or chaetoglobosin-C mixed in a solution with pathogen cells of *F. oxysporum* f. sp. *lycopersici* caused cells rupture and abnormal conidia. It is suggested that these pure compounds can lyse the cell wall of the pathogen and the protoplast becomes a plug inside the cells. These observations were similar to those reported by Sibounnavong et al. (2009) and Soytong (1992) who showed that the crude extracts of these antagonists

uptured the cells of the *F. oxysporum* f. sp. *lycopersici* noculums. In this study, the abnormal conidia of patho- ɟen cells affected by tajixanthone or chaetoglobosin-C eading to loss of its pathogenicity when inoculated to omato seedlings var Sida and no symptoms were ɔbserved. Moreover, formulated *E. nidulans* as a ɔiological fungicide significantly reduced *Fusarium* wilt of omato var. Sida in a pot experiment (Sibounnavong et ɑl., 2010). It is questioned that these bioactive com- ɔounds should be further evaluated to determine their ɑbility to control tomato wilt in pot and field trials. Tajixanthone and chaetoglobosin-C may possible develop o be microbial elicitors to induce immunity in tomato ɔlants against *F. oxysporum* f. sp. *lycopersici*.

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Genetic variation of *Fusarium oxysporum* f. sp. *lycopersici* isolated from tomatoes in Thailand using pathogenicity and AFLP markers

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Abstract

Genetic variation among 25 isolates of *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt was determined using pathogenicity test and AFLP markers. The isolates were collected from 8 provinces in Thailand. Based on the pathogenecity result, all isolates divided into two groups depended on non-pathogenic and pathogenic isolates. Cluster analysis based on AFLP also grouped the pathogenic isolates into 3 subgroups as low, moderate and high virulence. A dendrogram resulting from a cluster analysis showed two main distinct groups, designated as group 1, non-pathogenic isolates and group 2, pathogenic isolates rooting from outgroup.Eighty one polymorphic bands were analyzed using the computer software. The results showed that the genetic differentiation occurred among populations (*Gst* = 0.5898). However, the populations in

the same geographical area, Khonkaen and Nong khai, Tak and Phechaboon were more closely related genetically than another populations based on Nei's genetic distance, indicating the movement of the fungal conidia between these areas. This work provided new information on formae specialis of *F. oxysproum* f sp *lycopersici* NKSC01 and NKSC02 were high virulent which could classify as race 2 causing wilt of tomato var. Cheery.

Key words: AFLP marker, tomato, Pathogenecity, *Fusarium oxysporum* f. sp. *lycopersici*

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely cultivated, popular and important vegetable crops in the world. It is usually infected by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen causing wilt in lowland cultivation that can cause economic losses. The disease management is very difficult due to their endophytic growth and persistence in soil (Agrios, 1997). It has become one of the most damaging disease wherever tomatoes are grown intensively due to the pathogen persists in the infested soils (Silva and Bettiol, 2005). Most of *Fusarium* spp is known as plant pathogenic strain, that cause many diseases such as wilt, root rot and crown rot diseases on a various variety of crops (Nelson et al., 1981). Many researches on *Fusarium* spp. have been focused on studying for plant pathogenic isolates (Mohammadi et al., 2004; Pasquali et al., 2004). However, the nonpathogenic groups represent a significant proportionality of the isolates found and keep most genetic

diversity within this species complex (Bao et al., 2002). There is a large deal of genetic relationships between pathogenic and non-pathogenic *F. oxysporum* isolates (Baayen et al., 2000). Skovgaard et al. (2002) suggested that particular pathogenic isolates might germinate from non pathogenic strains by mutations affecting a few loci. Some nonpathogenic isolates have been studied to change from pathogenic isolates through loss of virulence (Skovgaard et al., 2002). James et al. (2000) reported that some isolates of *Fusarium oxysproum* were highly virulent, whereas others were nonpathogenic fungi. Moreover, both highly virulent isolates and nonpathogenic isolates are not different based on morphological study. Therefore, methods are needed and importance to identify and quantify population of highly virulence of *F. oxysporum* Baayen et al. (2000) and Mayek et al. (2001) stated that molecular markers have been used to study genetic relationships for pathogenicity in many group of fungi. Using the histone-H3 encoding gene and amplified fragment length polymorphisms (AFLPs) could be used for studying genetic differences between highly virulence, low virulence and nonpathogenic isolates of *F. oxysporum*. These previous results suggested that molecular marker can be used to separate these two phenotypes and compare the phylogenetic relationships of highly virulent *Fusarium* spp. (Donaldson et al., 1995). Amplified fragment length polymorphisms (AFLP) is a powerful technique in molecular marker for studying relationships among isolates of fungi between population and species levels (Cunningham, 1997; Kauserud and Schumacher, 2003; Nelson et al., 1983; Skovgaard et al., 2003). Moreover, AFLP analysis has been used to investigated genetic variation within and between among different *Fusarium* spp. (Adb-Elsalam et al., 2002; Kiprop et al., 2002; Sivaramakrishnan et al., 2002).

The objectives in this study were to determine the genetic variation and differentiation of *F. oxysporum* f.sp. *lycopersici* populations isolated from tomato wilt and to find out the correlation among pathogenic isolates (low, moderate, high virulence) and non-pathogenic isolates of *Fusarium* spp. using AFLP and pathogenicity markers. Moreover, genetic differentiation among population of geographical areas was analyzed.

Materials and methods

Isolation and pathogenicity test

Pure cultures of *Fusarium oxysproum* were isolated from the root samples of tomato wilt disease by tissue transplanting technique_from Bangkok, Phechaboon, Tak, Burirum, Khonkaen, Nongkhai, Nakhonratchasima and Sakon Nakhon provinces in Thailand. The diseased root samples were cut into 1 cm long, then surface disinfected by 10 % sodium hypochlorite for 3 minutes and washed through sterilized distilled water before moving to water agar (WA). The hyphal tip was sub-cultured onto potato dextrose agar (PDA) to get pure culture. Pure culture in each isolate was done single spore isolation and maintained on PDA slants and deposited at the Biocontrol Research Unit and Mycology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

All isolates were tested on tomato seedlings of Cherry variety for pathogenicity using Koch's postulates: Tomato seedlings at 20–day–old were washed under running sterilized water and cut at five points on the root tips before dipping the roots into a 20 ml spore suspension (1×10^7 spores/ml) for 15 min. A control was performed by dipping seedling roots into sterile distilled water. The seedlings were then potted in sterilized soil. After 15 days, symptoms of disease were recorded using the Disease Severity Index

(DSI) and rated according to Sibounnavong et al. (2009, 2010) as follows: 1 = no symptoms, 2 = 1–20% of leaves yellow and wilted, 3 = 21–40% of leaves yellow and wilted, 4 = 41–60 % leaves yellow and wilted, 5 = 61–80% of leaves yellow and wilted, and 6 = 81–100 % of leaves yellow and wilted. The experiment was conducted using a completely randomized design (CRD) with six replications in each treatment. The experiment was repeated twice. Pathogenic isolates or non-pathogenic isolates was recorded. The non-pathogenic isolates were categorized as avirulence (A) and pathogenic isolates were categorized as degree of virulence according to the DSI, modified the method of Charoenporn et al. (2010): avirulence (DSI =1), low virulence (DSI ≤ 3.50), moderate virulence (DSI > 3.50 – 4.50), and high virulence (DSI > 4.50).

DNA extraction

50 mg of grounded fungal biomass was used for genomic DNA extraction with 0.5 ml of extraction buffer (50mM Tris-HCl, 850mM NaCl, 100 mM EDTA, and 1% SDS) and incubated at 65°C for 30 min then added with Phenol ($^1/_2$ vol) and Chloroform:IAA (24:1) ($^1/_2$ vol). After centrifugation at 13000 rmp for 10_min, the upper aqueous phase was deproteinized by additional 1vol of Chloroform:IAA (24:1). After centrifugation at 13000 rmp for 10 min, the DNA molecules were added with 2 vol of absolute ethanol and incubated at -20°Cfor 1 hour. After centrifugation at 13000 rmp) for 10 min, the DNA molecules were washed by 70% ethanol and centrifugation at 13000 rmp for 10 min twice. The end product of DNA molecules were dissolved in 100 µl of TE (10mM Tris HCl 8.0, 1mM EDTA). The DNA concentration was measured using on 1% agarose gel electrophoresis.

Fingerprinting analysis using AFLP marker

The AFLP reactions were performed as described by Vos et al. (1995) with the following modifications: Genomic DNA (500 ng.) was digested with a combination of restriction enzymes *Eco* RI (50 Units) and *Tru* 9I (*Mse* I) (10 uints) in a mix of 10x ligase buffer, 0.5 M Nacl and BSA. The digested DNA fragments were ligated to their respective adapter pair of both enzymes in a reaction of T4 DNA ligase (1u) and T4 DNA ligase buffer (1x) and incubated at 37° C for 3 hours.

After the restriction-ligation products were diluted 10 fold with TE buffer (10 mM tris, 0.1 mM EDTA,pH 8.0). The first amplifications were carried out with 1 selective nucleotide at 3' end of each primer in volume of 25 µl of PCR buffer containing PCR buffer (1x), dNTP (0.2mM), each primer (E+A/M+G, E+G/M+A, E+C/M+G and E+G/M+C) 5pmole, $MgCl_2$ (2.5mM), Taq polymerase (0.5u). This preamplification was carried out in a thermal cycler programmed for 20 cycles of 30 sec at 94°C, 60 sec at 56 °C, 60 sec at 72 °C and hold 16 °C for 15 min. The selective amplifications were performed using selected combinations of primers with two or three selective nucleotides (Table 1). All seventeen combination primers were screened to investigate the most suitable primers. They were carried out in volumes of 20 µl of PCR buffer containing 5 µl diluted preamplified DNA, PCR buffer (1x), dNTP (0.2mM), each primer 5 pmole , MgCl2 (2.5mM) and Taq polymerase (1u). The PCR amplifications were performed with an initial denaturation at 94°C for 30 sec followed by 12 cycles of 94°C for 30sec, annealing at 65 °C each cycle was reduced by 1°C for 30 sec and extension step at 72 °C for 60sec. in each of the following 10 cycles, the annealing temperature

was reduced by 1 ºC. The next 30 PCR cycles continued of 94 ºC for 30 sec, 56 ºC for 30 sec and 72 ºC for 60 sec.

For gel analysis, the amplification reaction products were mixed with 10 µl of formamide dye (98% formamide, 10 mM EDTA pH 8.0, 0.3% bromo phenol blue and 0.3% xylene cyanol) and heat at 95 ºC for 3 min and quickly cooled on ice. Each sample (2 µl) was examined on a 5% polyacrylamide gel plus 7M urea on a Model S2 sequencing gel electrophoresis apparatus. Electrophoresis was performed at constant power 50 W for 3 hr. After electrophoresis, the gel plate was removed, fixed in 10% acetic acid for 30 min, and washed in distilled water 3 times for 2 min. The gels plate were stained in silver solution (1 g of silver nitrate and 1.5 ml of 37% formaldehyde per liter) for 30 min and rinsed with distilled water. After staining, the gels were developed in a cool developer solution (30 g of sodium carbonate, 1.5 ml of 37% formaldehyde and 0.01 g of sodium thiosulfate) until the bands appeared. The staining was stopped by adding 10% acetic acid (fixed solution) for 1-2 min, rinsed with distilled water for 2 min and dried under fume hood overnight.

Data analysis

The fingerprint patterns were scored for both monomorphic and polymorphic bands as binary data by 1 (present) or 0 (absent). The binary data was analyzed with the computer program NTSYS pc version 2.02 (Rohlf, 1993). An unweighted pair group arithmetic mean method (UPGMA) cluster analysis was performed using the DICE's similarity coefficient. Dendrogram was generated with the tree option (TREE) and a cophenetic value distance matrix was derived from dendrogram with a COPH program in NTSYSpc. The cophenetic value distance matrix was compared for level of

correlation with the original matrix with the MXCOMP NTSYS program. Bootstrap values were calculated with 1000 replications by Winboot program (Yap and Nelson, 1996).

All the polymorphic bands were recorded in the GenAlex6 format. A principle coordinate plot based on genetic distances between all pairs of AFLP genotypes was generated in GenAlex6 and was used to generate a two-dimensional principal coordinate analysis was based on the population of AFLP genotypes in the PCA plot. Neighbor joining tree based Nei's (1978) genetic distance was generated using UPGMA modified from neighbor procedure of PHYLIP version 3.5.

Table 1. Primers combination were used for screening

EcoRI primer + selective base		MseI primer + selective base	
+A	+AC	+GTA	+GT
+G	+ACG	+ACG	+G
+G	+ACT	+AAC	+G
+G	+AGC	+AGC	+G
+C	+GCG	+GTA	+C
+G	+GTC	+CTA	+C
+G	+CGC	+CGC	+G
+G	+CTG	+CAC	+G
+AG		+GT	

Results

Isolation and pathogenicity test

25 isolates of *F. oxysporum* were obtained from Bangkok (BKRS01 and BKRF01), Phetchaboon (PBRS101, PBRS102, PBRS103, PBRS104, PBRS201, PBRS202 and PBRS203), Tak (MSRS01, MSRS01, TRS01 and TRS02), Burirum (BRC03), Khonkaen (KK2 and KSoC02),

Nongkai (NKSC01, NKSC02, NKRC02, NKRC04 and NKRC09), Nakhonratchasima (NSC01) and Sakonnakorn (SRC02, SSoC03 and SSoC04). These isolates were pathogenically reconfirmed by Kock's postulate method to 20 days old tomato seedling var. Sida. Result showed that 11 isolates, BKRS01, BKRF01, BRC03, KSoC02, NKRC02, NKRC04, NKRC09, NSC01,SRC02, SSoC03 and SSoC04 were non-pathogenic or avirulent group (DSI = 1). The pathogenic isolates showed 11 isolates were low virulent (L), one isolate was moderate virulent (M) and two isolates were high virulent (H) as shown in Table 2.

Table 2. Isolates of *Fusarium* spp. and their pathogenicity group in tomato var. Cherry

Provinces	Isolates	DSI[1]	Pathogenic or non-pathogenic isolates
Bangkok	BKRS01	1.00 e[2]	Non-pathogenic
	BKRF01	1.00 e	Non-pathogenic
Phechaboon	PBRS101	2.00 d	Low virulence
	PBRS102	2.00 d	Low virulence
	PBRS103	2.00 d	Low virulence
	PBRS104	2.00 d	Low virulence
	PBRS201	2.00 d	Low virulence
	PBRS202	2.00 d	Low virulence
	PBRS203	2.00 d	Low virulence
Tak	MSRS01	2.00 d	Low virulence
	MSRS02	2.00 d	Low virulence
	TRS01	2.00 d	Low virulence
	TRS02	2.00 d	Low virulence
Burirum	BRC03	1.00 e	Non-pathogenic
Khonkaen	KK2	4.25 c	Moderate virulence
	KSoC02	1.00 e	Non-pathogenic
Nongkhai	**NKSC01**	4.75 b	High virulence
	NKSC02	5.50 a	High virulence
	NKRC02	1.00 e	Non-pathogenic
	NKRC04	1.00 e	Non-pathogenic
	NKRC09	1.00 e	Non-pathogenic
Nakonratchasima	NSC01	1.00 e	Non-pathogenic
Sakon nakon	SRC02	1.00 e	Non-pathogenic
	SSoC03	1.00 e	Non-pathogenic
	SSoC04	1.00 e	Non-pathogenic

[1]DSI = Disease severity index:- avirulence (DSI =1), low virulence (DSI ≤ 3.50), moderate virulence (DSI > 3.50 – 4.50), and high virulence (DSI > 4.50).

2 Average of two repeated experiments from eight replications. Means followed by a common letter were significantly different by DMRT at P=0.01.

DNA fingerprint analysis using AFLP marker

Seventeen combination primers were screened on five isolates for investigating suitable primers combination use for study. Result showed that there were only three primers combination including EcoRI+G/MseI+ACG, EcoRI+G/MseI+CAC, EcoRI+ACG/MseI+G gave highly number of polymorphic bands when compared with others primer combination which resolved 22, 22.4 and 20.5 polymorphic bands, respectively as shown in Figs 1 and Table 3. The three primers were chosen for further screening on 25 isolates of *F. oxysporum* f. sp. *lycopersici*. Then, a total 81 polymorphic bands were amplified using primers combination with EcoRI (E)+3 and MseI (M+1) and EcoRI (E)+1 and MseI (M+3) at the 3' end of the primers on 25 isolates of *F. oxysporum* f sp *lycopersici*. The polymorphic bands were analyzed using NTSYS program. Cluster analysis divided all the isolates into two major groups at 30% Dice' coefficient similarity. Group 1 described as non-pathogenic isolate group (avirulence) which consisted of KS0C02, BKRF01, SSoC04, SRC02, BKRS01, BRC03, SSoC03, NKRC09, NKRC02, NKRC04 and NSC01. Group 2 described as pathogenic isolate group which divided into 3 subgroups as follows:- subgroup 1 was low virulent isolates of MSRS01, MSRS02, PBRS102, PBRS203, TRS01, PBRS201, PBRS103, PBRS101, PBRS 104, TRS02 and PBRS202; subgroup 2 was moderate virulent isolates of KK2, and subgroup 3 was high virulent isolates of NKSC02 and NKSC01. A UPGMA tree was resulting from AFLP cluster analysis showed 85.4% bootstrap value of Isolates NKSC01 and NKSC02 which high virulent isolates were causing wilt disease of tomato var. Cherry. Among the pathogenic isolates that grouped into low virulence (L), AFLP cluster analysis showed over 60% of bootstrap. Moreover, 99.5% of bootstrap value for non-pathogenic or avirulent group (Fig 2). Thus, there was very clearly demonstrated the relationship between degree of degree of virulence and their genetic relationship. Moreover, It is also clearly shown that the phenetic dendrogram generated by UPGMA on genotypes in 8 populations as pop1:Khonkaen province, pop2:Bangkok province, pop3: Sakorn nakorn province, pop4:Bururum province, pop5: Nongkai province, pop7: Tak province and pop8:Pechaboon province. With this, a principal coordinate analysis (PCA) grouped all of the *Fusarium* spp. isolates into eight major clusters. It observed that pop 1: Khonkaen and pop 5: Nongkai is located in the Northeast of Thailand where majority of planted areas of tomatoes in which these geographical areas were found more moderate and high virulent isolates.

Discussion

Total of 25 isolates of *F. oxysporum* f. sp. *lycopersici* were confirmed morphologically and based on molecular phylogeny. Results of the pathogenicity test and AFLP analysis in this study revealed that 11 isolates were categorized as non-pathogenic or avirulent group and 14 isolates were categorized as pathogenic group which dividied into 3 subgroups of low virulent (L), moderate virulent (M) and high virulent (H). As a result, the isolates of

KSoC02, NKRC09, SSoC03 and SSoC04 were shown to be non-pathogenic isolates or avirulence but Charoenporn et al (2010) reported that these isolates were low virulent to cause wilt of tomato var. Sida. It can explain that the different varieties of tomatoes may affect to pathogenicity level of wilt disease infected by same isolate of *F. oxysporum* f. sp. *lycopersici* (Cai 2003). Isolate KK2 with high virulent to tomato var. Sida as previous report (Charoenporn et al ,2010) become moderate virulent in tomato var Sida in this study. Bunyatratchata et al (2005) reported that isolate KK2 isolated from Northeast part of Thailand had been tested its pathogenicity to cause wilt symptom on tomato var Sida as the same disease level of Banny and UC82-L varieties which susceptible to standard tested isolate Fol 007 race 2 and concluded that KK2 was race 2. (Grttidge,1982 and Maiatt et al, 1996). Isolates NKRC02, NKRC04 and SCR02 with moderate virulent to tomato var. Sida as previous report (Charoenporn et al ,2010) but become non-pathogenic to tomato var. Cherry in present study. It was observed that those isolates were variable for pathogenicity to different varieties of tomatoes (Cherry and Sida varieties) from low and moderate virulent become non-pathogenic and from high virulent become moderate virulent. This phenomenon may explain that different varieties of tomatoes are affected with isolate of *F. oxysporum f sp lycopersici*, as also stated by Sibounnavong et al (2009) and continuing subculture of Fusarium may lead to variable and lower degree of pathogenicity (Agrios,1997). It is interested that isolates NKSC01 and NKSC02 with high virulent to tomato var Sida as previous report (Charoenporn et al ,2010), it was still expressed high virulent in tomato var. Cherry in this experiment. It can explain why the isolates were more stable than the other isolates or these isolates can infected both Cherry and Sida varieties (Sibounnavong et al, 2010). However, Charoenporn et al (2010) reported previously that isolates KK2,

KSoC02, NKSC01, KKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03 and SSoC04 were sequenced to confirm identification into species by using ITS sequences with the length of complete ITS1, 5.8S and ITS2 including small portions of 18S rDNA and 28 S rDNA.

In this study, AFLP has been used as a powerful technique in molecular fingerprinting to study the relationship among fungal isolates and their pathogenicity as also showed by Brown (1996), Janssen et al. (1996) and Majer et al. (1998).

Regarding from result of the study, a total 81 polymorphic bands were amplified using three primers combination with EcoRI+G/MseI+ACG, EcoRI+G/MseI+CAC, EcoRI+ACG/MseI+G at the 3' end of the primers on 25 isolates of *F. oxysporum* f sp *lycopersici*. The restriction enzymes, length and composition of selective nucleotides would help to determine complexity of the final AFLP fingerprint as reported by Janssen et al. (1996). Three nucleotides of primers combination for AFLP analysis can help to differentiate *Fusarium* spp causing root rot disease on wheat and gave good polymorphic bands (Mohammad et al. 2009). The primer selectivity is related to genome size and good selectivity is found with primers of three selective nucleotides (El-Kazzaz, 2008). However, Gonnalez et al. (1998) stated that using two instead of three selective nucleotides in order to generate adequate number of fragments for AFLP analysis of *C. lindemuthianum* isolates. Primer selectivity is also good for primers with one or two selective nucleotides in simple genome such as bacteria, fungi and some plants, although selectivity is still acceptable with primers of three selective nucleotides.

Statistical analysis of AFLP data enabled the classification of *F. oxysproum* f sp *lycopersici* into two AFLP groups; non-pathogenic or avirulent and pathogenic or virulent

groups. With this the pathogenic group was clearly divided into three subgroups which correlated with the result of pathogenicity.

In this study, 25 isolates of *F. oxysproum* f sp *lycopersici* were analyzed with primers to determine the distribution of genetic diversity among isolates which represents in different planting areas. Mohmed et al (2003) stated that the high-resolution genotyping method of AFLP analysis was suitable to study the genetic relationships within and between populations of *Fusarium* spp. In the present research however, It was not clearly relationship between provinces and distribution of pathogen. This result was similar to those of Charoenporn et al, (2010). In this study, It was showed that the phenetic dendrogram generated by UPGMA modified from neighbor procedure of PHYLIP version 3.5 based on genotypes in 8 populations as pop1:Khonkaen province, pop2:Bangkok province, pop3: Sakorn nakon province, pop4:Burirum province, pop5: Nongkai province, pop7: Tak province and pop8:Pechaboon province. A principal coordinate analysis (PCA) grouped all of the *Fusarium* spp. isolates into eight major clusters. No clear trend was detected between clustering in the AFLP dendrogram and geographic origin of the tested isolates as similar report of Mohmed et al (2003). But it observed that pop 1: Khonkhaen and pop 5: Nomhkai is located in the Northeast of Thailand where majority of planted areas of tomatoes which these geographical areas were found more moderate and high virulent isolates.

In conclusion, the genetic variation among isolates of *F. oxysproum* f sp *lycopersici* was clearly relationship between pathogenicity groups and AFLP groups. But it was not clearly correlated between AFLP and geographical areas. Moreover, this work provided new information on formae specialis of *F. oxysproum* f. sp *lycopersici* which could

classify as race 2 that can cause wilt to different varieties of tomato e.g. Cheery and Sida varieties rather one variety. As Bunyatratchata et al (2006) reported that *F. oxysporum* f sp *lycopersici* race 2 can infected tomato var. Sida in Thailand as compared to standard race testing varieties of Bonny Best, UC82-L. There was a good correlation between AFLP groups and groups from result of pathogenicity test. Regarding from the result of the present study demonstrated clearly that the use of the AFLP is a powerful, simple and rapid technique to study the identification and genetic relationship between *F. oxysporum* and their pathogenicity. AFLP may therefore provide a rich source of molecular markers which are useful to study on the genetic variation for specific level.

Table 3. Total number of polymorphic bands of screening primer pairs

EcoRI primer	MseI primer	No. of bands
A	GTA	17
G	ACG	22
G	AAC	12.6
G	AGC	18.8
C	GTA	17
G	CTA	11.2
G	CGC	12.2
G	CAC	22.4
AG	GT	8.2
AC	GT	11.8
ACG	G	20.5
ACT	G	15.8
AGC	G	17
GCG	C	15.2
GTC	C	16
CGC	G	8
CTG	G	11.8

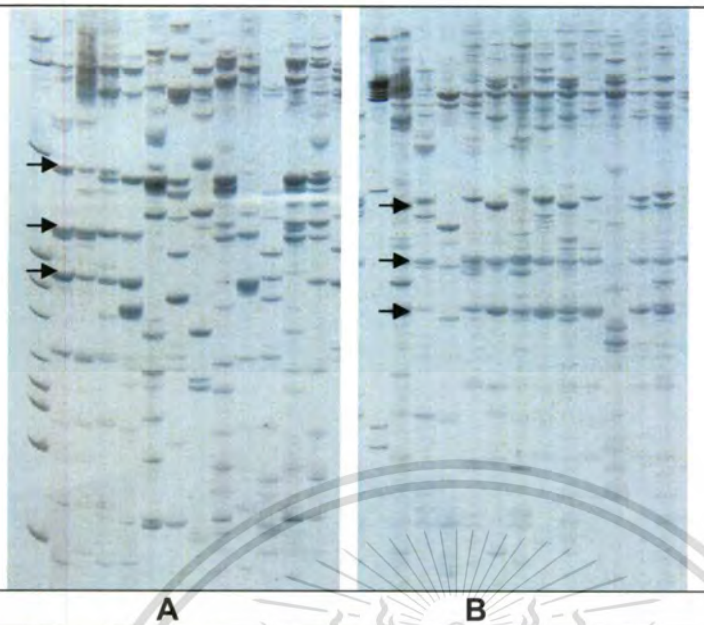

Figure 1. DNA fingerprint of *Fusarium oxysporum* f. sp. *lycopersici* by AFLP markers using E+ACG/M+G (A) and E+G/M+CAC (B) primers. The polymorphic bands shown by arrows and lane 1 is 100 bp plus DNA Ladder (Fermentas).

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This project is a part of a Ph.D. thesis (Biotechnology in Plant Pathology). I would like to express my sincere thank to the Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA), Philippines for funding a Ph.D. research fellow. I also thank the Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen campus for supporting the facilities of this research work.

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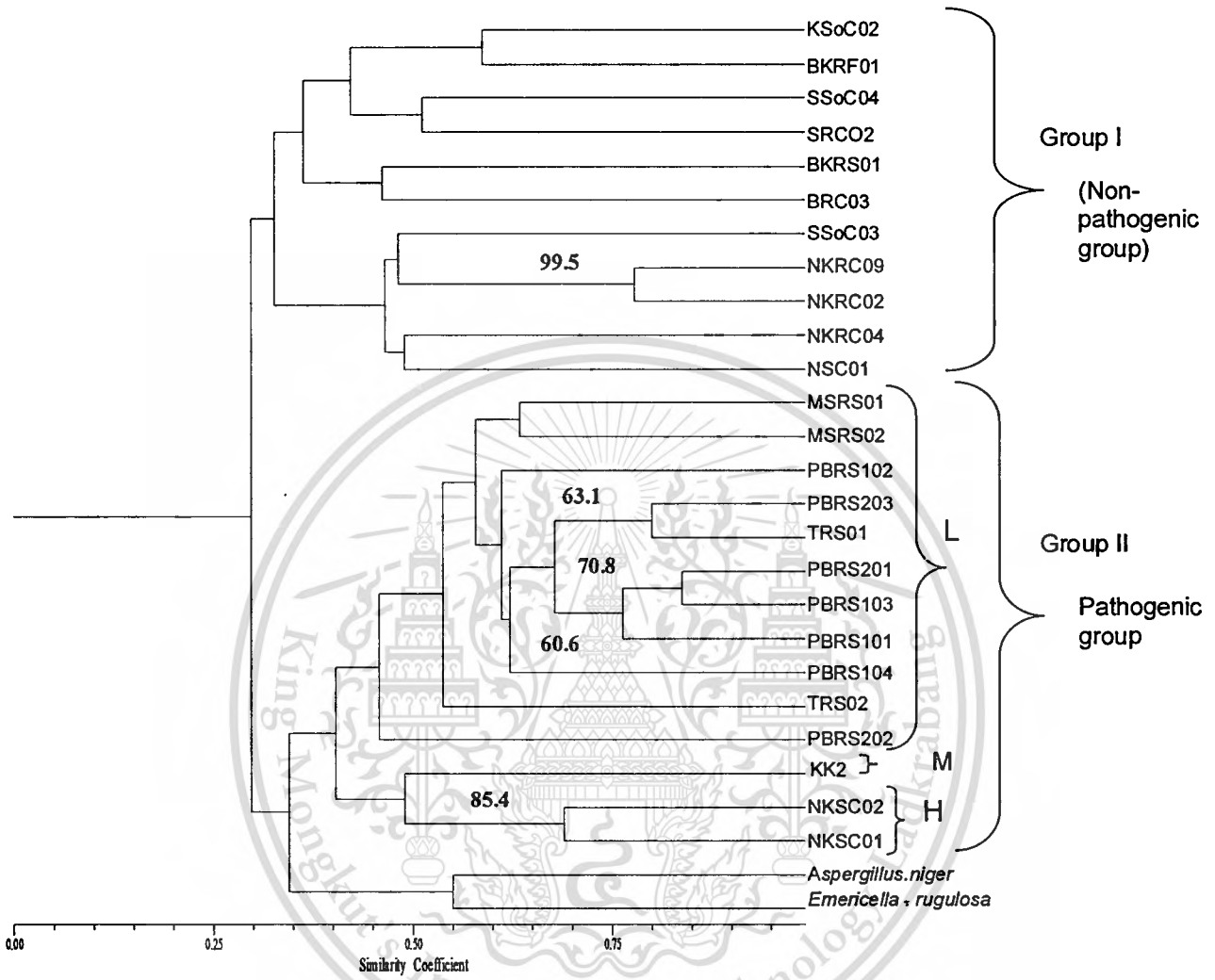
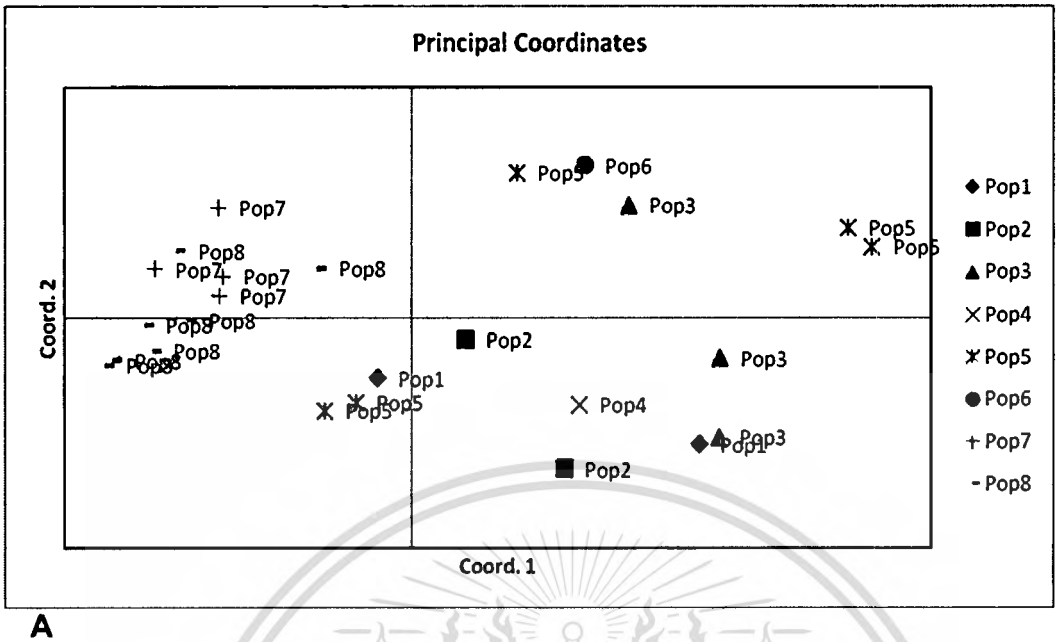
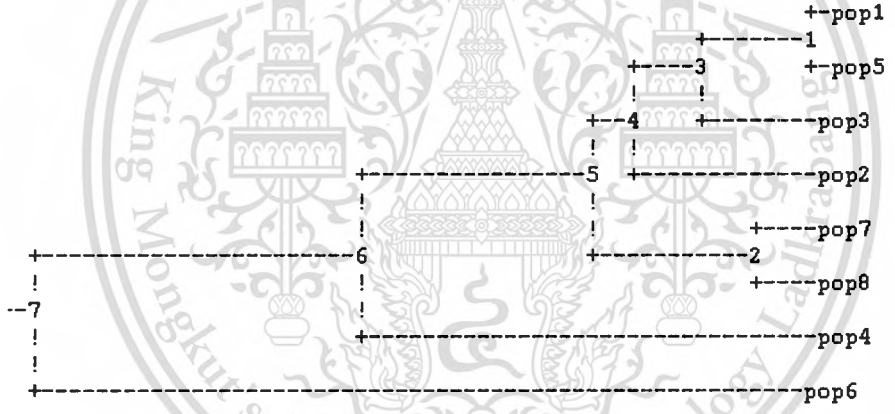


Fig 2. Phenetic dendrogram of *Fusarium oxysporum* f. sp. *lycopersici* isolates based on the binary matrix of polymorphic bands, using the UPGMA algorithm and Dice's similarity coefficient (NTSYS program). Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branch.



A



B

Fig.3. A, Principle coordinate analysis plot of AFLP genotypes of 25 isolates of *Fusarium oxysporum* f. sp. *lycopersici* from Thailand based on genetic Nei's (1978) genetic distance between genotypes. **B**, Dendrogram based Nei's genetic distance using UPGMA modified from neighbor procedure of PHYLIP version 3.5 based on AFLP genotypes in 8 populations, pop1: Khonkaen; pop2: Bangkok; pop 3: Sakon Nakhon; pop4: Burirum; pop5: Nongkhai; pop6 Nakhon Ratchasima; pop7: Tak and pop8: Phetchaboon.

pop ID	1	2	3	4	5	6	7	8
1	****	0.9272	0.9292	0.7791	0.9882	0.6440	0.8757	0.9169
2	0.0756	****	0.8846	0.8511	0.8885	0.6205	0.8618	0.8848
3	0.0734	0.1226	****	0.7726	0.9347	0.6458	0.8526	0.8510
4	0.2496	0.1612	0.2580	****	0.7989	0.5679	0.7151	0.7538
5	0.0119	0.1183	0.0675	0.2245	****	0.7292	0.8978	0.9214
6	0.4401	0.4772	0.4373	0.5658	0.3159	****	0.6721	0.6841
7	0.1327	0.1487	0.1594	0.3353	0.1078	0.3973	****	0.9630
8	0.0868	0.1224	0.1613	0.2826	0.0819	0.3796	0.0377	****

Table 4 Nei's unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal) of AFLP genotypes of *Fusarium oxysporum* f. sp. *lycopersici* populations in Thailand

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Cover Letter

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Dear Editor,

I am very much appreciated to re-submit the revised manuscript AJAR 12-076 entitled '**A role of *Emericella rugulosa* as a biocontrol agent to control tomato fusarium wilt**' for publication in African Journal of Agricultural Research as the reviewers suggested. However, I would like again to confirm that the present manuscript has not been published elsewhere or under editorial review for publication elsewhere. I am also change authors and addresses of author to be corrected.

Thank you very much for your kind consideration.

I am looking forward to hear from you.

Yours Sincerely,

Kasem Soytong

Corresponding author: Kasem Soytong

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A role of *Emericella rugulosa* as a biocontrol agent to control tomato fusarium wilt

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Key words: *Emericella rugulosa, Fusarium oxysporum* f. sp. *lycopersici*, crude extracts, Tajixanthone, bioformulation

Abstract

Fusarium oxysporum f.sp. *lycopersici* isolate NKSC02 showed the highest virulent for wilt incidence of tomato var Sida. Culture of the antagonistic fungus *Emericella rugulosa* on PDB+CWDB at pH5-8 gave significantly highest fresh weight of fungal biomass served to be appropriate medium to increase the number of spores to produce bioformulation. The ED_{50} of crude ethyl acetate from *E. rugulosa* against *F. oxysporum* f.sp. *lycopersici* isolate NKSC02 was 138 µg/ml while crude hexane and crude methanol were 313 and 1372 µg/ml, respectively. Tajixanthone, a pure compound of *E. rugulosa* expressed antifungal activity against mycelial growth, macrocondia and microconidia of *F. oxysporum* f.sp. *lycopersici* NKSC02 which the ED_{50} of 122, 54 and 42 µg/ml., respectively. It implies a role of antibiosis. Disease immunity to Fusarium wilt in Sida variety showed the highest immunity of 80.95 % when treated with crude EtOAc at 1000 µg/ml and followed by crude EtOAc at 500 µg/ml that showed immunity of 30.09 %. Bio-formulations produced from *E. rugulosa* in powder and oil bases gave highly significant different in DSI when compared to the non-treated control. Oil based bioformulation gave significantly higher in growth parameters and yield than powder based formulation, culture filtrate of *E. rugulosa* and chemical fungicide treatment and lower wilt incidence of tomato than the others.

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely cultivated, popular and important vegetable crops in the world. *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen is one of the most common pathogen that causes wilt of tomato in areas of upland cultivation which can cause economic losses. *F. oxysporum* f. sp. *lycopersici* has become one of the most damaging and difficult to control wherever tomatoes are grown intensively because it grows endophytically and persists in infested soils (Agrios, 1997). The disease control measures for this vascular wilt are either inefficient or it is difficult to apply the chemical fungicides. Over time tomatoes may develop resistance to some races of the pathogen; however, the pathogenic fungus may also develop resistance to chemical fungicides (Silva and

Bettiol, 2005). *Emericella* spp. belong to the Ascomycota, and have been reported as antagonists against plant pathogen (Sibounnavong et al. 2010). *Emericella rugulosa* used in this study has been shown to produce five prenylxanthones, ruguloxanthones A-C, 14-methoxytajixanthone,-tajixanthone ethanoate, a bicyclo[3.3.1]-nona-2,6-diene derivative named rugulosone, shamixanthone, tajixanthone, 14-methoxytajixanthone-25-acetate, tajixanthone hydrate, tajixanthone methanoate, isoemericellin and ergosterol. Among these, the bicyclo[3.3.1]–nona-2,6-diene derivative has been shown to exhibit antimalarial and antimycobacterial activity and cytotoxicity against three cancer cell lines (Moosophon et al. 2009). Another species of *Emericella* (*Emericella nidulans*), has also been reported to antagonize *F. oxysporum* f. sp. *lycopersici* (Sibounnavong et al. 2009a; 2010). The antimicrobial activity tajixanthone, which is produced by *E. rugulosa* ER01, could be involved in the disease control mechanism of these antagonistic fungi against the tomato wilt fungus *F. oxysporum* f. sp. *lycopersici*. The objectives of this study were to evaluate the antagonistic fungus, *Emericella rugulosa* as a new antagonist against *F. oxysporum* f. sp. *lycopersici* causing tomato wilt and to elucidate its control mechanism as antibiosis for a role of biocontrol of wilt disease.

MATERIALS AND METHODS

Pathogen and Pathogenicity test

Pure cultures of *F. oxysporum* f. sp. *lycopersici* were isolated from Burirum, Khonkaen, Nongkhai, Nakhonratchasima and Sakon-nakhon provinces in Thailand and culture on potato dextrose agar (PDA) as follows:- isolates BRCO3, KK2, KSoC02, NKSC01, NKSC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03, and SSoC04. These isolates were previously confirmed by sequencing the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA and a small portion of the 28S rDNA (Charoenporn et al. 2010). Pathogenicity was reconfirmed by inoculating the pathogen to 15 day old tomato seedling var. Sida using root-dipped method (Marlatt et al., 1996) with conidial suspension of pathogen $1x10^7$ conidia/ml. Disease severity index (DSI) was scored at 21 days after inoculation based on the modified disease severity scale as follows:- 1= no symptom; 2= plant showed

yellowing leaves and wilting 1-20%, 3= plant showed yellowing leaves and wilting 21-40%, 4= plant showed yellowing leaves and wilting 41-60%, 5= plant showed yellowing leaves and wilting 61-80%, and 6= plant showed yellowing leaves and wilting 81-100%or die. Pathogenicity test was conducted twice for each isolate. All tested isolates were recorded for non-pathogenic and pathogenic isolates. Pathogenicity group was categorized according to DSI as non-pathogenic (DSI =1), low (DSI ≤ 3.50), moderate (DSI > 3.50 - 4.50), and high (DSI > 4.50). The most aggressive isolate was selected for further experiment.

Growth of *Emericella rugulosa* ER01 culture in liquid media and pH levels

Emericella rugulosa ER01 isolated from cultivated soil was used as antagonistic fungus. The fungus was sub-cultured on PDA for examination the morphological characteristics for using in further study. Three media namely Potato dextrose broth (PDB), coconut water dextrose broth (CWDB) and mixed between PDB and CWDB (1:1) were prepared and used in this experiment. Potato dextrose broth (PDB) was prepared by boiling 200g of potato in 1000 ml of water and mixed with 20g of dextrose, for CWDB was prepared by boiling 1000 ml of coconut water and mixed with 20g of dextrose. The media were separated in 20 ml of medium in each flask and adjusted the pH levels by adding either HCl or NaOH to get the required pH levels after that, the media were sterilized by autoclaving at 121° C, 15 lbs/inch^2 for 20 min. Then an agar plug (0.3 cm diameter) of *E. rugulosa* was transferred into each Petri dish and incubated for 14 days at room temperature (approximately, 30-32 °C). After 14 days, the culture on each Petri dish was separately filtered using Whatman filter paper No 4 to get the fresh fungal biomass. The fungal biomass was air dried at room temperature for 48 hours. Fresh fungal biomass was weighted (g) using electrical balance. The experiment was set up using two factorial experiment in Completely Randomized Design (CRD). The two factors were as follows: - three kinds of media and the four pH levels of the media. Each treatment was consisted with four replications. The following are the treatments of the study:Factor A - kinds of media a1 = PDB (Potato Dextrose Broth); a2 = CWDB (Coconut Water Dextrose Broth); a3 = mixed between PDB and CWDB, Factor B – pH levels b1 = 5, b2 = 6, b3 = 7 and b4 = 8. The most suitable medium and pH level for the

fungus growth was used as medium to culture the antagonist for preparing the fungal biomass to formula the biofungicides to control Fusarium wilt of tomato in the pot experiment.

Crude extract bioassay against *F. oxysporum* f. sp. *lycopersici*

Crude extraction from *Emericella rugulosa* was done by following the method of Kanokmedhakul et al. (2006), Moosophon et al. (2009), and Thohinung et al. (2010). *E. rugulosa* was cultivated in potato dextrose broth (PDB) at room temperature for 30 days to yield fresh fungal biomass and dried overnight. The dried fungal biomass was ground and sequentially extracted with hexane, ethyl acetate, and methanol. The crude filtrate was evaporated *in vacuo* to separate solvent and then yielded crude hexane, crude ethyl acetate (EtOAc), and crude methanol (MeOH) extracts, respectively.

The crude extracts were assayed for inhibition of the most virulent isolate of *F. oxysporum* f. sp. *lycopersici*. The experiment was conducted by using a factorial experiment in CRD with four replications. Factor A represented crude extracts, a1 = crude hexane, a2 = crude ethyl acetate and a3 = crude methanol. Factor B represented the different concentrations: b1 = 0 µg/ml (control), b2 = 50 µg/ml, b3 = 100 µg/ml, b4 = 500 µg/ml and b5 = 1,000 µg/ml. Each crude extract was mixed with PDA before autoclaving at 121°C (15 psi) for 30 min. A sterilized cork borer was used to remove agar plugs from the actively growing edge of the pathogen culture at 3 mm diameter. An agar plug was transferred to the center of 5 cm diameter of each Petri dish on PDA containing crude extract at each concentration and incubated at room temperature until the pathogen on the control plates had grown over the plate. Data were collected regarding the number of conidia produced by the pathogen and used to calculate the percentage of conidia inhibition. The effective dose (ED_{50}) was calculated using Probit analysis. The experiment was repeated twice. The most effective crude extract was used for study on effect of fungal metabolites on disease incidence.

Pure compound bioassay against *F. oxysporum* f. sp. *lycopersici*

Tajixanthone, pure compound from chromatographic separation of the crude hexane extract from *E. rugulosa*. Its structure was identified by spectroscopic method.

Dried mycelium mat of *E. rugulosa* was ground into powder and then extracted with hexane for 3 times. The solvent extract was evaporated *in vacuo* to get crude hexane, extract. The crude hexane extract was separated by chromatographic methods. The structures of the isolated compound were elucidated base on IR, ^{1}H NMR, ^{13}C NMR and 2D NMR. The experiment was conducted using CRD with four replications. Treatments were the concentrations of 0 µg/ml (control), 50 µg/ml, 100 µg/ml and = 500 µg/ml. The method was done as same as crude extract bioassay.

Effect of crude extracts for disease immunity of wilt incidence in tomato var Sida

The experiment was conducted by using a CRD with four replications. Treatments were conducted as follows: T1= control; non-inoculated with conidia of pathogen; T2= control; inoculated with conidia of the pathogen; T3= inoculated with pathogen mixed with 500 µg/ml of the most effective crude extract and T4= inoculated with pathogen mixed with 1000 µg/ml of the most effective crude extract. The roots of 20– day– old tomato seedlings var. Sida were washed under running sterilized water and cut at five points on the root tips before dipping the roots into each treatment. A 20 ml spore suspension of 1×10^{7} spores/ml mixed with different concentrations of crude extract for 15 min. The seedlings were then planted in pots which contained sterilized soil. The experiment was repeated twice. DSI was scored as previous experiment and disease immunity (%) was computed as follows:- DSI in control – DSI in treatment/ DSI in control X 100.

Testing bioformulation of *E. rugulosa* to control Fusarium wilt of tomato

Bioformulations were separately formulated as powder, oil based formulation according to the method of Soytong (2001) by using fungal biomass of *E. rugulosa*. The antagonistic fungus was cultured in the PDB mixed CWDB at pH 5-6 and incubated at room temperature for 30 days. The number of antagonistic spores in bioormulation was adjusted to 2×10^{7} spores/ml before added either in sterilized palm's oil for oil based bioformulation or sterilized talcum for powder based bioformulation.

Bioformulations of *E. rugulosa* were tested for their abilities to control tomato wilt caused by *F. oxysporum* f. sp *lycopersici in vivo*. Tomato seedlings var. Sida at 30 days

old were inoculated with conidial suspension of *F. oxysporum* f. sp. *lycopersici* at concentration of $1x10^7$conidia/ml by dipping root for 15 min and transplanted into plastic pot contained with sterilized mix soil (soil:sand:compost, 4:1:1). Sterilized mix soil was sterilized at 121°C, 15 lbs/inch^2 for 1 h in two consecutive days. Randomized Completely Block Design (RCBD) was performed with four replications. Treatments were designed as follows:-, non-inoculated control,(T_1), inoculated with pathogen and non-treated bio-agent formulation (T_2), culture filtrate from antagonist (T_3), powder bioformulation (T4), oil liquid bioformulation (T_5) and Chemical fungicide (prochoraz 50% WP) (T_6). Each treatment were separately applied at the rate of 20 ml/ 20 L of water while powder bioformulation and prochoraz 50% WP chemical fungicide was applied at the rate of 20 g/ 20 l of water at every 2 weeks by spraying around rhizosphere soil and above plants. Data were collected as disease severity index (DSI), plant height (cm), plant fresh (g), fruit weight (g), fruit per plant and root weight (g). Disease severity index (DSI) was scaled as previous experiment. Percentage of disease reduction was analyzed using formula: % disease reduction = (Disease severity index of control – Disease severity index of treatment)/ Disease severity index of control x 100.. Percent increased in yield was analyzed using formula: (yield per plant in treatment – yield per plant in control)/ yield per plant in treatment x 100. All data were subjected to analysis of variance (ANOVA). Treatment means were statistical compared with Duncan's New Multiple Range Test (DMRT) at $P \leq 0.05$ to separate means. The experiment was repeated two times.

RESULTS

Pathogen and pathogenicity test

It was shown that the isolate NKSC02 gave significantly highest disease index of 5.50 level which resulted to high virulent to cause wilting of tomato var Sida and followed by isolate NKSC01 and KK2 which DSI were 4.75 and 4.25 respectively (Table1). The isolates BRC03, KSoC02, NKRC02, NKRC04, NKRC09, NSC01, SRC02, SSoC03 and SSoC04 were non-pathogenic isolates. Isolate NKSC02 was then selected for further experiment.

Growth of *Emericella rugulosa* ER01 culture in liquid media and pH levels

Results showed that *E. rugulosa* could grown very well in mixed media of PDB and CWDB in the range of pH levels ; 5, 6, 7 8 which gave significantly produced fresh weight of fungal biomass at 1.70, 1.57, 1.60 and 1.70g / Preti dish when compared with other treatments respectively as seen in Table 2. This result suggested that mixed media between potato dextrose broth (PDB) and coconut water dextrose broth (CWDB) at pH levels range from 5 - 8 are more suitable medium and pH levels for mycelial production of *E. rugulosa* than other treatments. It is concluded that PDB+CWDB, pH5-8 gave significantly highest weight of fresh fungal biomass, followed by PDB; pH7-8, CWDB; pH6, respectively.

Crude extract bioassay against *F. oxysporum* f. sp. *lycopersici*

Crude extracts of the antagonist; *E. rugulosa* could inhibit conidial production (macroconidia and microconidia) of the pathogen at the concentrations ranged from 10 to 1,000µg/ml as shown in Table 3. Crude extracts of tested antagonists showed the highest properties to inhibit conidial production of *F. oxysporum* f.sp. *lycopersici* NKSC02 at 1,000µg/ml. Crude ethyl acetate of *E. rugulosa* was the most effective crude extract gave highly significant different of colony diameter and number of conidia production by the pathogen at the lowest when compared to other treatments which were 3.67 cm and $2.15x10^7$ spore/ml while control plate that did mixed with the crude extract produce conidia by the pathogen at $17.6x10^7$ spore/ml followed by crude hexane that gave colony diameter at 4.29 cm and number of conidia at $4.22x10^7$ spore/ml. While crude methanol gave 4.62 cm of colony diameter and $7.54x10^7$ spore/ml of conidia. The highest conidial inhibition was presented by crude ethyl acetate at the concentration of 1000µg/ml followed by crude hexane and crude methanol which were at 87.55, 76, and 55.41%, respectively. Crude ethyl acetate showed the highest inhibition of conidial production of the pathogen in which ED_{50} value was 138 µg/ml while crude

hexane and crude methanol presented their abilities to inhibit conidial production at the ED_{50} values 313 and 1372 µg/ml, respectively.

Pure compound bioassay against *F. oxysporum* f. sp. *lycopersici*

A pure compound of Tajixanthone from *E. rugulosa* is elucidated the structures base on IR, ^{1}H NMR, ^{13}C NMR and 2D NMR. Result confirmed that tajixanthone expressed antifungal activity against mycelial growth, macrocondia and microconidia of *F. oxysporum* f.sp. *lycopersici* NKSC02 which the ED_{50} of 122, 54 and 42 µg/ml., respectively (Table 4).

Effect of crude extracts for disease immunity of wilt incidence in tomato var Sida

Result found that treated tomato seedlings var Sida with crude EtOAc of *E. rugulosa* at 1000 µg/ml gave significant lower DSI from treated with crude EtOAc of *E. rugulosa* at 500 µg/ml when compared to the inoculated with *F. oxysporum* f.sp. *lycopersici* NKSC02 as shown in Table 5. Disease immunity to Fusarium wilt in Sida varieiy showed the highest immunity of 80.95 % when treated with crude EtOAc at 1000 µg/ml and followed by treated with crude EtOAc at 500 µg/ml which showed immunity of 30.09 %.

Testing bioformulation of *E. rugulosa* to control Fusarium wilt of tomato

The disease severity index (DSI) of Fusarium wilt was lowest wilt incidence in oil and powder bioformulations (DSI 2 and 1.75) and followed by culture filtrate (DSI 2.5) which significantly differed from Prochoraz (DSI 4.25) and inoculated control(DSI 5.00). The non inoculated control was no wilt incidence. With this, application of oil bioformulation leaded to reduce wilt incidence of 60 % and followed by application of powder bioformulation, culture filtrate and Prochoraz which reduced wilt incidence of 58, 50 and 15 %, respectively. Based on the result, oil bioformulation gave significantly highest in plant height (119.50 cm) and followed by powder bioformulation, culture filtrate and Prochoraz which were 105.25, 85.25 and 74.00 cm, respectively when compared to the inoculated control (65.75 cm). Plant weight showed the highest after

apply oil bioformulation (182.35 g), and followed by powder formulation, culture filtrate and Prochoraz which were 168.50,153.75 and 137.00 g, respectively when compared to the inoculated control (73.75 g). With this regards, the root weights of oil and powder bioformulations gave significantly better than culture filtrate and Prochoraz treatments. Oil bioformulation gave significantly highest in fruit weight (584.25 g) and followed by powder bioformulation (540.75 g), culture filtrate (430 g) and Prochoraz(191.35 g) which significanyly differed from the inoculated control (280 g). The number of fruits in oil bioformulation application gave significantly higher than powder bioformulation, culture filtrate and Prochoraz treatments which significantly differed from the inoculated control (Tables 6 and 7).

DISCUSSION

Isolate NKSC02 is confirmed as a virulent to cause wilting of tomato var Sida which was previously reported by Sibounnavong et al. (2010) and this isolate was also confirmed by molecular phylogeny as *F. oxysporum* f. sp. *lycopersici* by Charoenporn et al. (2010) who sequenced the internal transcribed spacer (ITS) region ITS1, 5.8S and ITS2 and a small portion of 18S rDNA and a small portion of the 28S rDNA. *E. rugulosa* could grown very well in mixed media of PDB and CWDB in the range of pH levels from 5- 8 which optimum condition for the growth of *E. rugulosa.* The result was similar to the report of Sibounnavong et al. (2009b) who stated that mixed medium between PDB and CWBD at pH levels 5-8 was the optimum condition for the growth of *E. nidulans*.

Crude ethyl acetate from *E. rugulosa* gave the highest inhibition of conidial production of *F. oxysporum* f. sp. *lycopersici* in which ED_{50} value was 138 µg/ml while crude hexane and crude methanol presented their abilities to inhibit conidial production at the ED_{50} values 313 and 1372 µg/ml, respectively. As a result, Sibounnavong et al (2009a) reported that methanol crude extract from *E. nidulans* gave the highest inhibition of *F. oxysporum* f. sp. *lycopersici*. It is explained that ethyl acetate crude extract from *E. rugulosa* might have different antagonistic substances from methanol crude extract from *E. nidulans* as reported by Moosophon et al (2006).

Thereafter, separation of crude extracts to get pure compound of tajixanthone, it is proved that tajixanthone can actively express against *F. oxysporum* f.sp. *lycopersici*

NKSC02 at lower concentration than crude extracts which the ED_{50} of 122 µg/ml to inhibit mycelia growth, 54 µg/ml to inhibit macrocondia and 42 µg/ml., to inhibit microconidia. Moosophon et al (2006) isolated pure compounds from *E. nidulans* as epishamixanthone, shamixanthone , emericellin, ergosta-6, 22-diene-3-ol-5, 8-epidioxy-(3β- 5α, 22*E*), sterigmatocystin and demethylsterigmatocystin which differed from isolation of pure compounds from *E. rugulosa* as found five new prenylxanthones, ruguloxanthones A-C, 14-methoxytajixanthone and tajixanthone ethanoate, one novel cyclooctadiene derivative, together with seven known, shamixanthone, tajixanthone, 14-methoxytajixanthone-25-acetate, tajixanthone hydrate, tajixanthone methanoate, isoemericellin and ergosterol (Moosophon et al, 2009). It is indicated that tajixanthone isolated from *E. rugulosa* firstly reported as a fungal metabolite to be actively against *F. oxysporum* f.sp. *lycopersici* implies antibiosis as a role of control mechanism.

A part from the result, crude EtOAc of *E. rugulosa* with contains tajixanthone proved to be an microbial elicitor to induce immunity in tomato. As the inoculated tomato seedlings with *F. oxysporum* f.sp. *lycopersici* showed disease immunity to Fusarium wilt in Sida varieiy of 80.95 % where the wilt incidence was much lower than the inoculated control. Hanh (1996) stated that elicitors are molecules that stimulate any of a number of defense responses in plants, such as synthesis of phytoalexins and pathogenesis-related proteins (PR-proteins). Such responses occurs after the binding of elicitor molecules to receptors normally located on the plant cell surface, promoting a signal transduction pathway that will lead to the activation of one or more defense mechanisms. The first characterized elicitors were oligosaccharide fragments from fungal cell walls, including oligochitin and oligochitosan (Hahn, 1996).

The biological fungicides has been released and distributed to the growers over a decade. Kaewchai et al (2009) stated that mycofungicides have been promoted for agricultural use because of their ability to control plant diseases and to increase crop production in an environmental friendly manner. The registered biological fungicide formulated from *C. cupreum* in Thailand could decrease disease incidence of tomato wilt and also increased in yield (Soytong, 1992).

In this study, Fusarium wilt was lowest wilt incidence in oil and powder bioformulations from *E. rugulosa* which significantly differed from Prochoraz and

inoculated control. The application of oil bioformulation from *E. rugulosa* could reduce wilt incidence of 60 % and followed by application of powder bioformulation and Prochoraz which reduced wilt incidence of 58 and 15 %, respectively.

As comparison to the work of Charoenporn et al. (2010) reported that oil bio-agent formulation from the other antagonistic fungi of *Chaetomium globosum* and *Ch. lucknowense* also showed their biological ability to control tomato wilt. The bio-agent formulations namely N0802, CLT and PC01 gave significantly highest disease reduction of tomato wilt which were 44.68, 36.28 and 41.01%, respectively, followed by prochoraz (21.95%). Charoenporn et al. (2010) stated that all tested bio-agent formulations could significantly increase the yield of tomato when compared to prochoraz and inoculated control. It is concluded that *C. globosum*, *C. lucknowense* and *T. harzianum* developed as bio-agent formulations namely N0802, CLT and PC01 and showed their abilities to control tomato wilt. Based on the result, oil bioformulation from *E. rugulosa* gave significantly better plant parameters in terms of plant height, plant weight, root weight, number of fruits and fruit weight than powder bioformulation and Prochoraz when compared to the inoculated control with *F. oxysporum* f. sp *lycopersici*.

This result is similar to the report of Charoenporn et al. (2010) stated that all tested bio-agent formulations of antagonistic fungi; *Chaetomium globosum* and *Ch. lucknowense* could significantly reduce tomato wilt caused by *F. oxysporum* f. sp *lycopersici* and increase in yield of tomato when compared to prochoraz and inoculated control. However, bioformulation from *E. rugulosa* in this research finding revealed a good result to control wilt incidence of tomato caused by *F. oxysporum* f. sp *lycopersici* Soytong et al. (2001) showed that the biological products consist of *Chaetomium* sp. (22 strains of *C. cupreum* and *C. globosum*) in biopellet and biopowder formulations which when applied to the soil could suppress the growth of *F. oxysporum* f.sp. *lycopersici* and reduce infection rate in tomato and those bioproducts has been released to the market. It is suggested that this new bioformulation of *E. rugulosa* could be used for further biofungicide to control tomato wilt caused by *F. oxysporum* f.sp. *lycopersici*.

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Table 1. Isolates of *Fusarium* spp. and their pathogenicity groups in Sida variety

Sources	Isolates	DSI[1]	Pathogenicity groups
Burirum	BRC03	1.00d[2]	Non-pathogenic
KhonKaen	KK2	4.25c	Moderate virulent
	KSoC02	1.00d	Non-pathogenic
Nongkhai	NKSC01	4.75b	High virulent
	NKSC02	5.50a	High virulent
	NKRC02	1.00d	Non-pathogenic
	NKRC04	1.00d	Non-pathogenic
	NKRC09	1.00d	Non-pathogenic
Nakhonratchasima	NSC01	1.00d	Non-pathogenic
Sakon Nakhon	SRC02	1.00d	Non-pathogenic
	SSoC03	1.00d	Non-pathogenic
	SSoC04	1.00d	Non-pathogenic

[1]Disease severity index (DSI) was scored at 21 days after inoculation. 1= no symptom; 2= plant showed yellowing leaves and wilting 1-20%, 3= plant showed yellowing leaves and wilting 21-40%, 4= plant showed yellowing leaves and wilting 41-60%, 5= plant showed yellowing leaves and wilting 61-80%, and 6= plant showed yellowing leaves and wilting or die 81-100%.

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

[3] Pathogenicity group was categorized according to DSI as non-pathogenic (DSI =1), low (DSI ≤ 3.50), moderate (DSI > 3.50 - 4.50), and high (DSI > 4.50).

Table 2. Fresh weight of fungal biomass of *E. rugulosa* in different liquid media and pH levels

Media	pH	Fresh weight (g)
PDB	5	1.30cd
	6	1.19d
	7	1.54ab
	8	1.29cd
CWDB	5	1.14d
	6	1.39bc
	7	1.15d
	8	1.13d
PDB:CWDB	5	1.70a[1]
	6	1.57a
	7	1.60a
	8	1.70a
CV(%)		6.57

[1]Average of four replications . Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.01.

Table 3. ED_{50} of crude extracts from *Emericella rugulosa* to inhibit *Fusarium oxysporum* f.sp. *lycopersici* isolate NKSC02 at 7 days

Crude extracts	Concentrations (µg/ml)	Number of conidia x10^7	Conidial inhibition (%)	ED_{50} (µg/ml)
Crude hexane	0	17.60a	-	
	10	16.67abc	5.12h	
	50	15.45def	12.18efg	
	100	12.33g	29.86d	313
	500	7.92h	55.06c	
	1000	4.22i	76.00b	
Crude EtOAc	0	17.50ab	-	
	10	15.65cde	9.43fgh	
	50	14.47ed	16.03ef	138
	100	8.66h	49.75c	
	500	4.50i	73.87b	
	1000	2.15j	87.55a	
Crude MeOH	0	17.00ab	-	
	10	16.37bcd	6.43gh	1372
	50	15.44def	11.6efgh	
	100	14.33f	17.89e	
	500	15.05g	25.26d	
	1000	7.54h	55.41c	
CV (%)		4.77	-----	

[1]Average of four replications . Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.01.

Table 4. ED_{50} of tajixanthone, a pure compound of *Emericella rugulosa* to inhibit *Fusarium oxysporum* f.sp. *lycopersici* isolate NKSC02 at 7 days

Inhibition	ED_{50} (µg/ml)
Colony	122
Macroconidia	54
Microconidia	42

Table 5. Effect of crude extracts to induce disease immunity of wilt incidence in tomato var Sida

Treatments	Plant height (cm)	DSI[1]	Disease immunity[3] (%)
T1 inoculated with pathogen	20.7b[2]	5.25a	-
T2 treated with crude EtOAc of ER at 500 µg/ml	40.75a	3.25b	30.09
T3 treated with crude EtOAc of ER at 1000 µg/ml	41.25a	1.00c	80.95
T4 non inoculated control	41.75a	1.00c	-
CV(%)	9.42	20	

[1]Disease severity index (DSI) was scored at 21 days after inoculation. 1= no symptom; 2= plant showed yellowing leaves and wilting 1-20%, 3= plant showed yellowing leaves and wilting 21-40%, 4= plant showed yellowing leaves and wilting 41-60%, 5= plant showed yellowing leaves and wilting 61-80%, and 6= plant showed yellowing leaves and wilting or die 81-100%.
[2]Average of four replications. Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.01.
Disease immunity (%) = DSI in control – DSI in treatment/ DSI in control X 100.

Table 6. Testing bioformulations to control Fusarium wilt of tomato *in vivo*

Treatments	Plant height(cm)	Plant weight(g)	Root weight(g)	Fruit weight(g)	fruits/plant	DSI[1]
non inoculated control	97.25c	163.25b	6.62b	280.00d	19.50d	1.00c
inoculated with pathogen	65.75f	73.75e	4.32c	141.25f	10.25f	5.00a
powder bioformulation	105.25b	168.50b	12.25a	540.75b	29.25b	1.75bc
Oil bioformulation	119.50a[2]	182.25a	13.25a	584.25a	40.50a	2.00bc
culture filtrate	85.25d	153.75c	6.12bc	430.00c	24.50c	2.50b
Prochoraz	74.00e	137.00d	5.20bc	191.25e	15.00e	4.25a
CV(%)	3.14	1.19	15	3.22	8.71	19.54

[1]Disease severity index (DSI) was scored at 21 days after inoculation. 1= no symptom; 2= plant showed yellowing leaves and wilting 1-20%, 3= plant showed yellowing leaves and wilting 21-40%, 4= plant showed yellowing leaves and wilting 41-60%, 5= plant showed yellowing leaves and wilting 61-80%, and 6= plant showed yellowing leaves and wilting or die 81-100%.
[2]Average of four replications. Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.01.

Table 7 Percent increased in plant growth and disease reduction after apply bioformulations

Treatments	Plant height	Plant weight	Root weight	Fruit weight	Numbers of fruit/plant	DR[2]
powder bioformulation	37.25[1]	56.23	64.73	73.87	64.95	58
Oil bioformulation	44.56	61.17	67.39	75.82	74.69	60
culture filtrate	19.35	52.03	29.41	67.15	58.16	50
Prochoraz	11.48	46.16	16.92	26.14	31.66	15

[1]Increased in plant growth parameters = treatment – inoculated control / treatment X 100.
[2]Disease reduction (DR) = disease index of inoculated control - disease index of treatment/disease index of inoculated control X 100.

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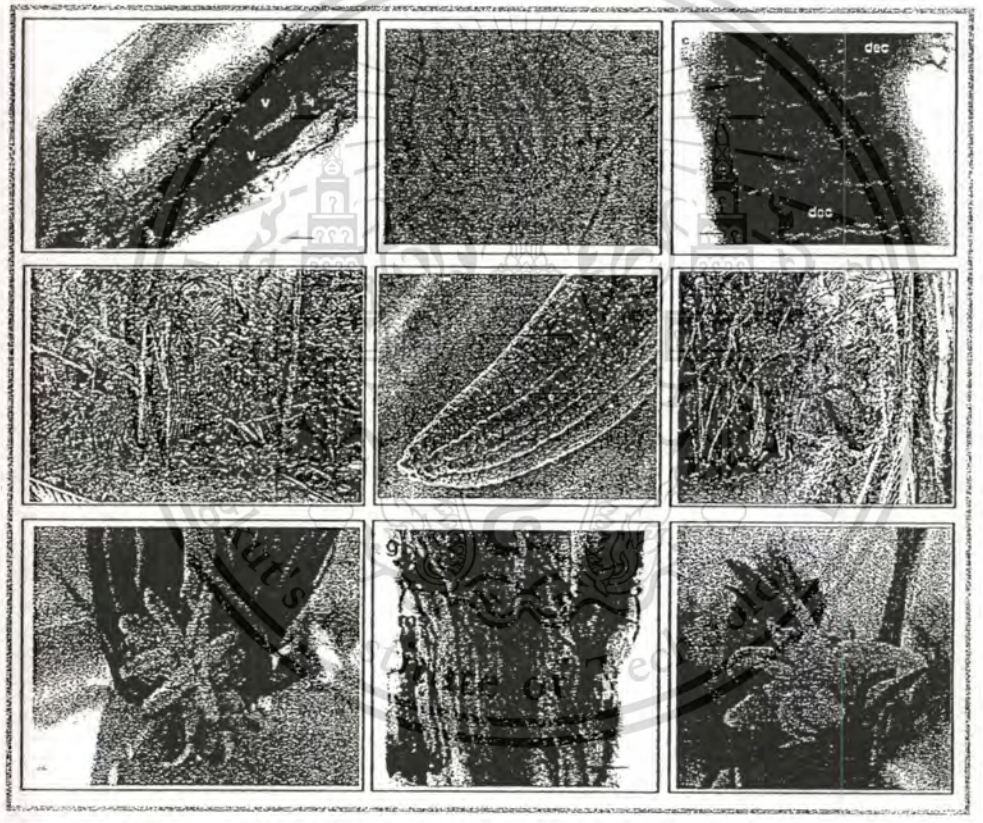

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Screening of *Emericella nidulans* for biological control of tomato *Fusarium* wilt in Lao PDR

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The isolate VTS16 was significantly highest disease index of tomato wilt caused by *F. oxysporum f sp lycopersici* var Sida which categorized as high virulent. *E. nidulans* isolate L01 is screened to be the most potential antagonistic fungus against *F. oxysporum* f sp *lycopersici* which inhibited spore production of 82.05 %. Crude methanol of *E. nidulans* isolate L01 expressed antifungal activity against *F. oxysporum* f sp *lycopersici* at the ED_{50} of 112 µg/ml, and follwed by crude ethyl acetate and crude hexane which were 379 and 915 µg/ml, respectively. Thereafter, *E. nidulans* L01 cultured on PDB at pH 8 and mixed PDB and CWDB at pH6 produced the highest fungal biomass and suitable to propagate for spore production. Disease index in oil based formulation produced from *E. nidulans* isolate L01 gave the lowest wilt incidence of tomato var Sida (DSI 1.75) and followed by powder based formulation (DSI 2.00), culture filtrate (DSI 2.75) and prochoraz (DSI 3.50) when compared to inoculated control (DSI 4.75). It is shown that oil based formulation showed significantly better plant height (119.25 cm) than powder based formulation which plant height was 109.75 cm and followed by culture filtrate and prochoraz which plant height were 84.50 and 73.00 cm, respectively when compared to the inoculated control (62.75 cm). Powder and oil based formulations gave the plant weight of 91.75 and 98.50 g/plant which better in plant weight than culture filtrate and prochoraz (68.00 and 67.00 g/plant) which non-significantly differed when compared the inoculated control (64.00 g/plant). Results in root weight and fruit number/plant were similar to those in plant weight. Tomato treated with oil and powder based formulation of *E. nidulans* isolate L01 gave the highest yields (fruit weight) of 218.50 and 197.50 g/plant, respectively and followed by treated with culture filtrate and prochoraz which yielded 128.00 and 107.00 g/plant, respectively when compared to the inoculated control (83.75 g/plant). As a result, it is indicated that power and oil based formulations increased in plant growth parameters 30-60 % when compared to inoculated control. Oil and powder based formulations reduced the wilt incidence of 63.15 and 57.89 % and followed by culture filtrate and prochoraz which reduced wilt incidence of 42.10 and 36.31 %, respectively.

Key words: *Emericella nidulans, Fusarium oxysporum* f. sp. *lycopersici*, crude extracts, bio-agent formulations

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Introduction

Tomato (*Lycopersicon esculentum* Mill.) is widely cultivated as vegetable crops in the world. *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen causes wilt of tomato mostly in upland areas which cause economic losses. *F. oxysporum* f. sp. *lycopersici* causing tomato wilt has become difficult to control wherever tomatoes are intensively grown and persisted in the infested soils (Agrios, 1997). The disease controls are managed either inefficient or difficult by using the chemical fungicides. Over time tomatoes have been reported to develop resistant to some races of *F. oxysporum* f. sp. *lycopersici* or the pathogen may also develop resistant to chemical fungicides (Silva and Bettiol, 2005). The excessive and misuse of a wide range of fungicides has led to harmful to environment and increase resistant pathogen populations (Soytong *et al.*, 2001).

The available control methods for Fusarium wilt of tomato are either inefficient or difficult to apply (Alabouvette *et al.*, 1998). Biological control of plant disease using of beneficial microorganisms would help to reduce the chemical fungicide application and decrease cost of production. It has been proved the effective biological control agents through antibiosis, competition, suppression, direct parasitism and induced resistance. (Haggag and Mohamed, 2007 and Larkin and Fravel, 1998). Reports on biological control of Fusarium wilt have been increased using *Trichoderma harzianum*, *Pythium oligandrum*, *Achromobacter xylosoxydans*, *Penicillium oxalicum*, non-pathogenic *Fusarium oxysporum*, *Chaetomium globosum* and *Chaetomium cupreum* (Floch *et al.*, 2003; De Cal *et al.*, 2000; Moretti *et al.*, 2008; Silva and Bettiol, 2005; Soytong *et al.*, 2001). Moreover, Larkin and Fravel (1998) reported that non-pathogenic *Fusarium* spp. could reduce the incidence of tomato wilt between 50–100% while *Trichoderma* spp and *Gliocladium* spp. reduced wilt incidence between 37–75% and *Pseudomonas* spp. reduced wilt incidence of 30–63% in field trial. Suwan *et al.* (2000) mentioned that mycelial extracts of *Trichoderma harzianum* PC01 which produce Trichotoxin acted as a potent biological control agent. The extract could inhibit mycelial growth and sporangial production of *Phytophthora palmivora* with ED_{50} values of 2.2 and 0.45 mg/ml. The mechanism of antibiosis plays the important role of production of secondary metabolites against plant pathogens. Some specific isolates of *Trichoderma* spp have reported to produce volatile and non-volatile antifungal substances, such as 6-n-pentyl-6H-pyran-2-one (6PP), gliotoxin, viridin, harzianopyridone, harziandione and peptaibols. *Trichoderma* spp. was also reported to be enhanced plant defense responses to pathogen attack (Vinale *et al.*, 2008). *C. globosum* produces cell wall hydrolases (such as chitinase and

glucanase) and antibiotics (such as chaetoglobosin and chaetomanone), which inhibit fungal plant pathogens (Kanokmedhakul *et al.*, 2002; Park *et al.*, 2005). These are involoved in biocontrol mechanism. Soytong *et al.* (2001) stated that Chaetoglobosin C, the bioactive compound extracted from *C. globosum*, act as alien substance which induced a localized and sub-systemic oxidative burst in tomato, tobacco, potato, and carrot. This possibility acts as an induction of plant immunity for disease resistance. Moreover, crude extract of *C. cupreum* inhibited spore production of *F. oxysporum* f. sp. *lycopersici* causing tomato wilt var Sida at 85.14% (Soytong, 1992).

Emericella spp. belongs to the Ascomycota, and have been reported as antagonistic fungi against *F. oxysporum* f. sp. *lycopersici* (Sibounnavong *et al.*, 2010). *Emericella nidulans* and *Emericella rugulosa* has been reported to produce antibiotic substances, five prenylxanthones, ruguloxanthones A-C, 14-methoxytajixanthone,-tajixanthone ethanoate, a bicycle[3.3.1] -nona-2,6-diene derivative named rugulosone, shamixanthone, tajixanthone, 14-methoxytajixanthone-25-acetate, tajixanthone hydrate, tajixanthone methanoate, isoemericellin and ergosterol. Among these, the bicyclo[3.3.1]–nona-2,6-diene derivative exhibited not only antimalarial, antimycobacterial activity and cytotoxicity against three cancer cell lines (Moosophon *et al.*, 2009) but also antifungal against Fusarium wilt pathogen (Sibounnavong *et al.*, 2009). *E. nidulans* has been firsltly reported as a new biological control agent to antagonize *F. oxysporum* f. sp. *lycopersici* causing tomato wilt (Sibounnavong *et al.*, 2010).

The objectives of research project were to isolate *F. oxysporum* f. sp. *lycopersici* causing tomato wilt and pathogenicity proved to screen the virulent isolate for pathogenicity. Isolation and screening of *Emericella* spp. as antagonistic fungi against *F. oxysporum* f. sp. *lycopersici* were done. Bi-culture antagonistic and crude extract tests against *F. oxysporum* f. sp. *lycopersici* were proved it control mechanism. The research finding for optimum growth of effective isolate *Emericella* spp. in different media and pH levels were investigated for mass production. Bio-agent formulations of effective isolate *Emericella* spp were performed and evaluated their efficacy. Testing bio-agent formulations of the effective *Emericella* spp. to control Fusarium wilt of tomato var. Sida in pot experiment was also evaluated.

Materials and methods

Pathogen and pathogenicity test

Fusarium oxysporum f. sp. *lycopersici* was isolated from tomato cultivation in Laos PDR by using tissue transplanting and soil plate method according to the work of Soytong (1992). All isolates were tested for pathogenicity using tomato seedling var. Sida by inoculating root-dipped with conidial suspension of pathogen $1x10^7$ conidia/ml. Disease severity index (DSI) was scored at 21 days after inoculation based on the modified disease severity of Sibounnavong *et al* (2010) as follows:- 1= no symptom; 2= plant showed yellowing leaves and wilting 1-20%, 3= plant showed yellowing leaves and wilting 21-40%, 4= plant showed yellowing leaves and wilting 41-60%, 5= plant showed yellowing leaves and wilting 61-80%, and 6= plant showed yellowing leaves and wilting 81-100% or die. The most virulent isolate was selected for further experiment. The test was done using Completely Randomized Design (CRD) with four replications and repeated twice. Data collection was computed analysis of variance and means were compared using Duncan's Multiple Range Test (DMRT) at P = 0.05 and 0.01.

Emericella nidulans as antagonistic fungus

Emericella spp. were isolated from forest soil in Lao PDR using soil plate technique on glucose asparagines nitrate agar. The isolates were cultured on poato dextrose agar and tested to screen its biological control potential to inhibit the most virulence isolate of *F. oxysporum* f. sp. *lycopersici*. The most effective antagonistic fungus of *E. nidulans* was sub-cultured on potato dextrose agar (PDA) for further study.

Bi-culture antagonistic test

The test was conducted by using the method of Soytong (1992) and Sibounnavong *et al*. (2008 and 2009a). The most promising antagonistic fungus *E. nidulans* and the virulent isolate of *F. oxysporum* f. sp. *lycopersici* were made bi-culture on PDA for 7 days, incubated at the room temperature (28-30°C). The edge of active colony growth of pathogenic fungus and promising antagonistic fungus was taken with 0.5 mm diameter by the sterilized cork borer and one agar plug of each fungus was transferred to the opposite sides on the PDA plates of 9 cm diameter and separately culture of antagonistic fungi and pathogen served as a controls, incubated at the room temperature (28-30°C) for three to four weeks. Data were collected as colony diameter (cm) and spore

production counted on Haemacytometer under binocular compound microscope. The experiment was done using CRD with four replications. Data collection were recorded as colony diameter (cm), number spore production of tested pathogen, and computed the analysis of variance (ANOV), then compared treatment means using Duncan's Multiple Range Test (DMRT) at P = 0.05 and 0.01. The experiment was repeated in two times.

Growth of Emericella nidulans culture in different media and pH levels

The most effective of *E. nidulans* was further studied on the optimum growth for bio-agent formulation. The experiment was conducted using two factor factorial experiment in Completely Randomized Design (CRD). Factor A - kinds of media, A1 = PDB (Potato Dextrose Broth); A2 = CWDB (Coconut Water Dextrose Broth); A3 = mixed between PDB and CWDB, Factor B – pH levels, B1 = 5, B2 = 6, B3 = 7 and B4 = 8. PDB was prepared by boiling 200g of potato in 1000 ml of water and mixed with 20g of dextrose. CWDB was prepared by boiling 1000 ml of coconut water and mixed with 20g of dextrose. The media were separated in 20 ml of medium in each flask and adjusted the pH levels by adding either HCl or NaOH to get the required pH levels, then sterilized by autoclaving at 121° C, 15 lbs/inch^2 for 20 min. An agar plug of *E. rugulosa* was transferred into the middle of medium in each Petri dish and incubated for 7 days at room temperature (approximately, 28-30 °C). After 7 days, the culture on each treatment was separately filtered using Whatman filter paper No 4 to get fresh fungal biomass. The fungal biomass was air dried at room temperature for 48 hours. Fresh and dried fungal biomass were weighted (mg) using electrical balance. The most suitable medium and pH level for the fungus growth was used as based medium to culture *E. nidulans* for preparing the fungal biomass to formula the biofungicide to control Fusarium wilt of tomato in the pot experiment.

Crude extract test against pathogen

It was also tested the mechanism of control in term of antibiosis of the most effective *E. nidulans.* The crude extracts from *E. nidulans* was performed followed the method of Kanokmedhakul *et al.* (2006), Moosophon *et al.* (2009), and Thohinung *et al.* (2010). *E. nidulans* was cultured in potato dextrose broth (PDB) at room temperature (30–32°C) for 30 days. The dried fungal biomass was ground and sequentially extracted with hexane, ethyl acetate, and methanol. The solvents were then evaporated *in vacuo* to yield crude hexane, crude ethyl acetate (EtOAc), and crude methanol (MeOH)

extracts, respectively. The crude extracts were assayed for inhibition of the most virulent isolate of *F. oxysporum* f. sp. *lycopersici*. The experiment was conducted by using a factorial experiment in CRD with four replications. Factor A represented the different solvents: A1 = crude hexane, A2 = crude ethyl acetate and A3 = crude methanol. Factor B represented the different concentrations: B1 = 0 µg/ml (control), B2 = 50 µg/ml, B3 = 100 µg/ml, B4 = 500 µg/ml and B5 = 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 30 minutes. To perform the assay, a sterilized 3-mm diameter cork borer was used to remove agar plugs from the actively growing edge of the pathogen culture. An agar plug was transferred to the center of 5 cm diameter Petri dishes of PDA containing crude extract at each concentration and incubated at room temperature (30–32°C) until the pathogen on the control plates had grown over the plate. Data were collected regarding the number of conidia produced by the pathogen and used to calculate the percentage of conidia inhibition. The effective dose (ED_{50}) was calculated using Probit analysis. The experiment was repeated twice. The most effective crude extract was used for study on effect of fungal metabolites on disease incidence.

Testing bio-agent formulations to control Fusarium wilt of tomato

Bio-agent formulations were separately formulated as powder and oil based formulation according to the method of Soytong (2001) by using fungal biomass of *E. nidulans* which culture on the optimum pH and medium test from previously experiment. The culture of *E. nidulans* was incubated at room temperature (28-32°C) for 30 days. The ascospores of *E. nidulans* was collected and adjusted to 2×10^7 spores/ml, then added into sterilized palm oil as to formulate the based oil bio-agent form or added to sterilized talcum as based powder bio-form. Bio-agent formulations of *E. nidulans* were evaluated for their abilities to control tomato wilt by inoculated *F. oxysporum* f. sp *lycopersici* to tomato seedlings var. Sida. Tomato seedlings at 30 days old were inoculated with conidial suspension of *F. oxysporum* f. sp. *lycopersici* at concentration of 1×10^7conidia/ml by dipping root for 15 min before transplanting into plastic pot contained with sterilized mix soil (soil:sand:compost, 4:1:1). Soil mixture was sterilized at 121 C, 15 lbs/inch^2 for 1 h in two consecutive days.

The experiment was conducted using Randomized Completely Block Design (RCBD) with four replications. Treatments were designed as follows:- non-inoculated control (T_1), inoculated with pathogen and non-treated bio-agent formulation (T_2), culture filtrate of *E. nidulans* (T_3), powder bio-agent

formulation (T4), oil bio-agent formulation (T_5) and chemical fungicide, prochoraz 50% WP (T_6). Each treatment was separately applied at the rate of 20 ml/ 20 L of water, while powder bio-agent formulation and prochoraz 50% WP were applied at the rate of 20 g/ 20 l of water at every 2 weeks by spraying around rhizosphere soil and above plants. Data were collected as disease severity index (DSI), plant fresh weight (g), and fruit weight or yield (g). Disease severity index (DSI) was scaled as previous experiment. Percentage of disease reduction was analyzed using formula: % disease reduction = (Disease severity index of control – Disease severity index of treatment)/ Disease severity index of control x 100. Plant fresh and dry weights (g) and fruit weight (g) were recorded at harvested day. Percent increased in yield was analyzed using formula: (yield per plant in treatment – yield per plant in control)/ yield per plant in treatment x 100. All data were subjected to analysis of variance (ANOVA). Treatment means were statistical compared with Duncan's New Multiple Range Test (DMRT) at $P \leq 0.05$ to separate means. The experiment was repeated two times.

Results

Pathogen and pathogenicity test

Twenty isolates of *F. oxysporum* was encountered and cultured on PDA for morphologically study under binocular compound microscope. In general, culture on PDA appears as fast-growing colony, reaching 9 cm diameter in 7-15 days at room temperature. Aerial mycelium sparse to abundant and becoming felted, whitish purple or vaiable in color in some isolates. Philophores are short and formed singly and branched. Macroconidia shape is fusiform, slightly curved, 3-5 septate, 13.0-34.0 x 2.5-3.5 µm. Microconidia abundant, 0-1 septate, elliptical or cylindrical, straight, 4.0 x 1.5 µm and chamydospores are in chain with either terminal and intercalary (Figure 1) as similar to Domsch and Gams (1993).

Pathogenicity showed that isolate VTS16 was significantly highest disease index of tomato wilt caused by *F. oxysporum f sp lycopersici* var Sida which categorized as high virulent and followed by isolate VTS17 which showed medium virulent. The majority of isolates showed low virulent group as follows:- VTS 01, 02, 03,04,05,06,07,08,09,10,11,12,13,14,15,18 and 19. Only isolate of VTS 20 was avirulence as shown in Table 1.

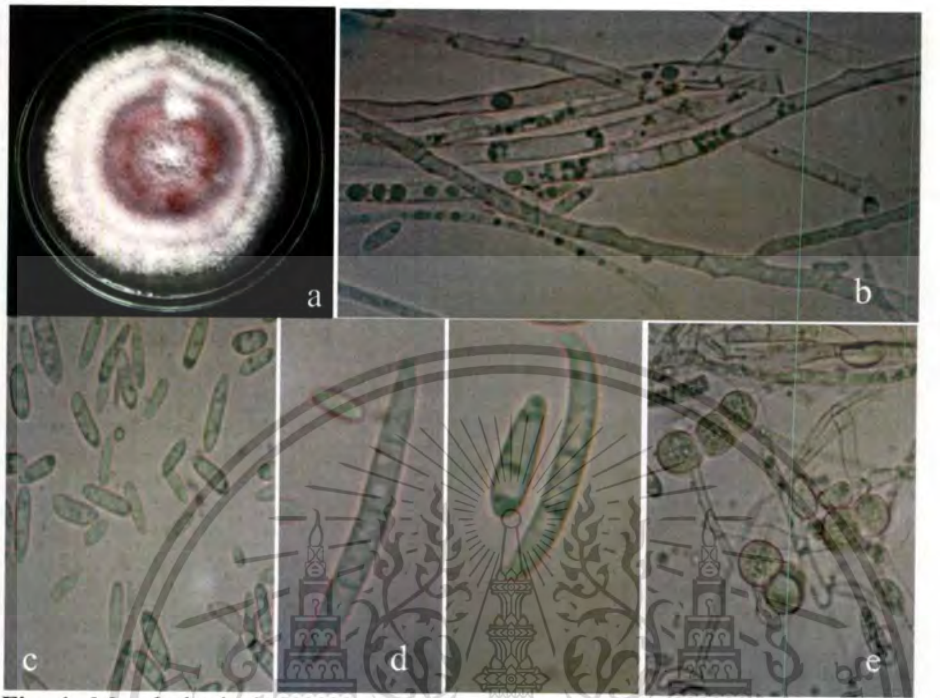


Fig. 1. Morphological character of *Fusarium oxysporum* f.sp. *lycopersici* at 15 days.
a. Colony on PDA, b. mycelium, c. microconidia, d. macroconidia, e. chlamydospores.

Emericella nidulans as antagonistic fungus

All isolates of *Emericella nidulans* were tested to screen it biiocontrol potential against *F. oxysporum* f sp *lycopersici*. Each isolate was cultured on PDA for identification work. It is observed that all encountered isolates appear imperfect or anamorphic stage namely *Aspergillus nidulans* (Eidam) Winter. Colony diameter on PDA averaged 5-6 cm in two weeks at room temperature (28-30°C), green from conidia becoming brownish when mature to produce abundant Cleistothecia, dull yellow, globoseor oval sharp 122 x 345.0 µm in diameter, surrounded by hülle cells. Hülle cells ellipsoidal to globose, 9.0-20.0 µm in diameter. Asci are globose to subglobose, 8-spored. Ascospores are red to purple, smooth, 2.5-4.0 × 3.4-5.5 µm, with two narrow longitudinal furrows as similar described by Domsch and Gams (1993) as shown in Figure 2.

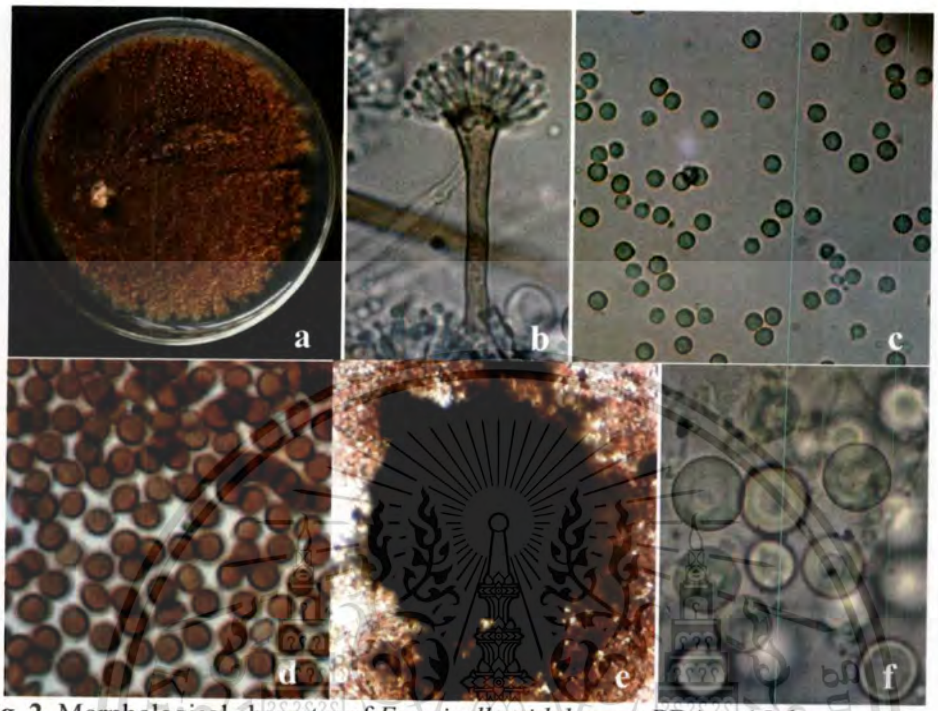


Fig. 2. Morphological character of *Emericella nidulans* on PDA at 15 days
a. Emericella nidulans on PDA, b. Thallus in imperfect stage, c. conidia, d. ascospores in perfect stage, e. Cleistothecium or ascomatum , f. hülle cells

Bi-culture antagonistic test

The colony diameter of *F. oxysporum f sp lycopersici* isolate VTS16 in bi-culture plate averaged 5.83 cm while in control plate was 9.00 cm. The conidial number of *F. oxysporum* f sp *lycopersici* VTS16 in bi-culture plate averaged 0.69 x 10^7 conidia/ml while in control plate was 2.70x10^7 conidia/ml. It showed that *E. nidulans* isolate L01 significantly inhibited colony growth of 35.25 % and inhibited spore production of 82.05 % as shown in Figure 3. The other isolates of *E. nidulans* that no potent to inhibit the conidial production were screened out.

Growth of E. nidulans culture in different media and pH levels

Results showed that *E. nidulans* isolate L01 which cultured in PDB at pH 8 and mixed media of PDB and CWDB at pH6 produced more fungal biomass which gave highly significant in fresh weight of fungal biomass than other treatments. However, CWDB medium at pH 5, 6, 7, and 8 was not suitable for

growth of *E. nidulans* which gave lowest of fungal biomass in the medium at pH levels of 5, 6, 7, and 8 respectively (Table 2). This result suggested that the *E. nidulans* isolate L01 required specific media and pH levels to prduce spore as required for developing the bio-agent formulation.

Crude extract test against pathogen

It revealed that crude methanol of *E. nidulans* isolate L01 at 1000 µg/ml was significantly inhibited *F. oxysporum* f sp *lycopersici* 84.40 %, and followed by crude ethyl acetate and crude hexane which were 64.40 and 60.28 %, respectively. Crude methanol of *E. nidulans* isolate L01 expressed antifungal activity against *F. oxysporum* f sp *lycopersici* VTS16 at the ED_{50} of 112 µg/ml, and follwed by crude ethyl acetate and crude hexane which were 379 and 915 µg/ml, respectively (Table 3).

Testing bio-agent formulations to control Fusarium wilt of tomato

Disease index in oil based formulation produced from *E. nidulans* isolate L01 gave the lowest wilt incidence (DSI 1.75) of tomato caused by *F. oxysporum* f sp *lycopersici* VTS16 and followed by powder based formulation (DSI 2.00), culture filtrate (DSI 2.75) and prochoraz (DSI 3.50) when compared to inoculated control (DSI 4.75). It is shown that oil based formulation showed significantly better plant height (119.25 cm) than powder based formulation which plant height was 109.75 cm and followed by culture filtrate and prochoraz which plant height were 84.50 and 73.00 cm, respectively when compared to the inoculated control (62.75 cm). Powder and oil based formulations gave the plant weight of 91.75 and 98.50 g/plant which better in plant weight than culture filtrate and prochoraz (68.00 and 67.00 g/plant) which non-significantly differed when compared the inoculated control (64.00 g/plant). Results in root weight and fruit number/plant were similar to those in plant weight. Tomato var Sida treated with oil and powder based formulation *E. nidulans* isolate L01 gave the highest yields (fruit weight) of 218.50 and 197.50 g/plant, respectively and followed by treated with culture filtrate and prochoraz which yielded 128.00 and 107.00 g/plant, respectively when compared to the inoculated control (83.75 g/plant) as shown in Table 4. As a result, it is indicated that power and oil based formulations increased in plant growth parameters 30-60 % when compared to inoculated control. Oil and powder based formulations reduced the wilt incidence of 63.15 and 57.89 % and followed by culture filtrate and prochoraz which reduced wilt incidence of 42.10 and 36.31 %, respectively as shown in Table 5.

Discussion

F. oxysporum f. sp. *lycopersici* VTS16 was isolated from infested tomato fields in Lao PDR and proved to be the most virulent isolate causing wilt of tomato var. Sida as confirmed by Sibounnavong *et al.* (2010). It is shown that *E. nidulans* isolate L01 which cultured in PDB at pH 8 and mixed media of PDB and CWDB at pH6 produced more fungal biomass which gave highly significant in fresh weight of fungal biomass than other treatments. This was similar to the report of Sibounnavong *et al.* (2009b) who reported that the mixed medium between PDB and CWBD at pH levels 5-8 are suitable for culture of *E. nidulans*. This result suggested that mixed media between PDB and CWDB at pH levels range from 5 - 8 are most optimum medium and pH levels for the growth of *Emericella* spp. than other treatments.

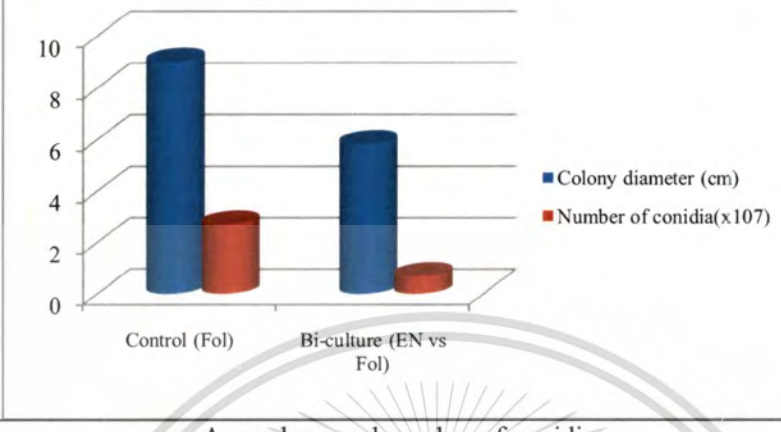
In this research finding, crude methanol of *E. nidulans* isolate L01 at 1000 µg/ml significantly inhibited *F. oxysporum* f sp *lycopersici* 84.40 %, and followed by crude ethyl acetate and crude hexane which were 64.40 and 60.28 %, respectively. These results are reportedby Soytong *et al.* (2005) who stated that the potentail antagonistc fungi would produce some antagonistic substances to express antifungal activity against plant pathogens, and reported that antagonistic substances from *C. globosum* CG extracted with ethyl acetate and *T. harzianum* PC01 extracted with ethyl acetate at 500 µg/ml could inhibit conidia production of *Colletotrichum gloeosporioides* WMF01 causing grape anthracnose at ED_{50} of 2 and 7 µg/ml, respectively. The result of this study showed that crude methanol of *E. nidulans* isolate L01 expressed antifungal activity against *F. oxysporum* f sp *lycopersici* at the ED_{50} of 112 µg/ml, and follwed by crude ethyl acetate and crude hexane which were 379 and 915 µg/ml, respectively. The tested antagonistic fungus showed antibiotic mechanism to inhibit growth of Fusarium wilt pathogen which was supported by Soytong (1992) who stated that antagonistic substance from *C. cupreum*, *C. globosum* and *T. harzianum* could inhibit the growth and break the cells of *F. oxysporum* f.sp. *lycopersici*. Moreover, Park *et al.* (2005) stated that liquid culture of *C. globosum* F0142 could suppress the development of disease more than 80% and can exhibit antifungal activity against *Phytophthora infestans* in tomato at moderate level *in vivo*. However, the related species of *Emericella rugulosa* reported to produce Prenylxanthones and a bicycle(3.3.1) nona-2,6-diene that expressed against human pathogens (Moosophon, *et al.*, 2009). However, Moosophone *et al.* (2006) reported that the separation of crude hexane and ethyl acetate extracts from *Emericella nidulans* isolate EN01 yielded six compounds, epishamixanthone (1), shamixanthone (2), emericellin (3), ergosta-6, 22-diene-3-ol-5, 8-epidioxy-(3β- 5α, 22*E*) (4), sterigmatocystin

(5) and demethylsterigmatocystin (6). With this, some of these pure compounds expressed antifungal activity against plant pathogens.

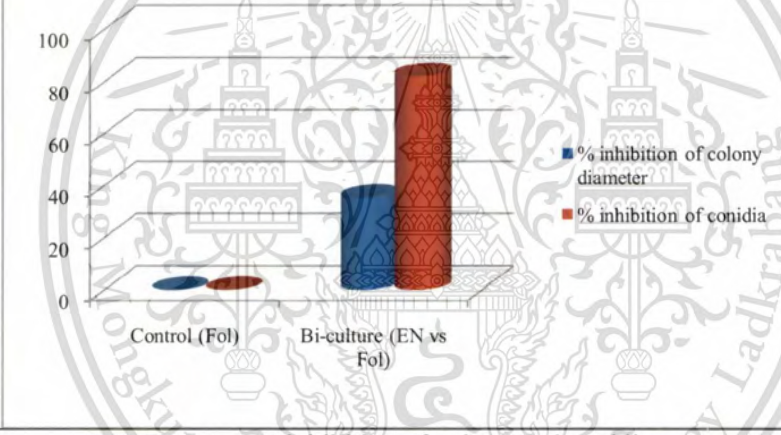
As a result, it is indicated that power and oil based formulations of *E. nidulans* L01 increased in plant growth parameters 30-60 % when compared to inoculated control. The oil and powder based formulations reduced the wilt incidence of 63.15 and 57.89 % and followed by culture filtrate and prochoraz which reduced wilt incidence of 42.10 and 36.31 %, respectively.

The bio-agent formulations of powder and oil forms produced *E. nidulans* are clearly demonstrated effective control of Fusarium wilt of tomato caused by *F. oxysporum* f. sp *lycopersici*. Kaewchai *et al.* (2009) stated that mycofungicide produced from potential effective antagonists gave a good control of plant disease as the same alevel as chemical fungicides. However, Charoenporn *et al.* (2010) reported that oil bio-agent formulation *Chaetomium globosum* and *Ch. lucknowense* showed their biological ability to control tomato wilt cause by *F. oxysporum* f. sp *lycopersici*. The result of bio-agent formulation from *E. nidulans* L01 is similarly reported by Soytong (1992) as stated that registered bio-fungicide formulated from *C. cupreum* could decrease disease incidence of tomato wilt caused by *F. oxysporum* f. sp *lycopersici* and also increased in yield. Soytong et al. (2001) who showed that the biological products consist of *Chaetomium* sp. (22 strains of *C. cupreum* and *C. globosum*) in biopellet and biopowder formulations which when applied to the soil could suppress the growth of *F. oxysporum* f.sp. *lycopersici* and reduced infection rate in tomato. The effective of powder based bio-agent formulations also supported the previous work of Soytong *et al.* (2005) who stated that *Chaetomium* bio-products formulated from *C. globosum* and *C. cupreum* as powder formulation gave a good control Bottle palm rot caused by *Thielaviopsis paradoxa* in the field and reduced disease incidence of 75%.

It is concluded that this reaserch finding of *E. nidulans* isolate L01 developed as bio-agent formulation would be feasible to extend this biological fungicide to control tomato wilt in different tomato varieties where susceptible wilt incidence, especially in the field.



A = colony and number of conidia



B= per cent inhibition of colony and conidia

Fig. 3. Bi-culture antagonistic test between *Emericella nidulans* L01 and *Fusarium oxysporum* f.sp. *lycopersici* VTS16

Table 1. Isolates of *Fusarium oxysporum* f. sp. *lycopersici* and their pathogenicity group

Isolates	Samples	DSI[1]	Pathogenicity group
VTS16	Rhizosphere soil	5.25 a[2]	High
VTS17	Rhizosphere soil	4.25 b	Medium
VTS01	Root	2.37 c	Low
VTS02	Root	2.37 c	Low
VTS03	Root	2.37 c	Low
VTS04	Root	2.12 cd	Low
VTS05	Root	1.87 cde	Low
VTS06	Root	1.87 cde	Low
VTS07	Root	1.75 cde	Low
VTS08	Root	1.62 cde	Low
VTS09	Root	1.62 cde	Low
VTS10	Root	1.62 cde	Low
VTS11	Root	1.50 cde	Low
VTS12	Rhizosphere soil	1.50 cde	Low
VTS13	Rhizosphere soil	1.50 cde	Low
VTS14	Rhizosphere soil	1.50 cde	Low
VTS15	Rhizosphere soil	1.50 cde	Low
VTS18	Rhizosphere soil	1.50 cde	Low
VTS19	Rhizosphere soil	1.25 de	Low
VTS20	Rhizosphere soil	1.00 e	Availurent
CV(%)	-	24.00	-

[1]Disease severity index (DSI) was scored at 21 days after inoculation. 1= no symptom; 2= plant showed yellowing leaves and wilting 1-20%, 3= plant showed yellowing leaves and wilting 21-40%, 4= plant showed yellowing leaves and wilting 41-60%, 5= plant showed yellowing leaves and wilting 61-80%, and 6= plant showed yellowing leaves and wilting or die 81-100%.

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

[3] Pathogenicity group of tested isolates was categorized as Avirulent (A), Low (L), medium (M) and high virulent.

Table 2. Fresh weight of fungal biomass of *E. nidulans* in different liquid media and pH levels

Media	pH	Fresh weight (g)
PDB	5	1.22c
	6	1.26 bc
	7	1.14cd
	8	1.57 a
CWDB	5	0.49f
	6	0.58f
	7	0.54f
	8	0.64f
PDB:CWDB	5	0.87e
	6	1.42 ab
	7	1.29 bc
	8	0.99de
CV(%)		9.1

[1]Average of four replications . Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.01.

Table 3. Bioassay of crude extracts from *E. nidulans* at different concentrations to inhibit *Fusarium oxysporum* f.sp. *lycopersici* isolate VTS16 at 7 days.

Crude extracts	Concentrations (µg/ml)	Number of conidia ($x10^7$)	Conidial inhibition (%)	ED_{50} (µg/ml)
Crude hexane	0	14.6ab	-	
	10	14.07b	4.03i	
	50	12.78c	12.88h	
	100	11.91d	18.8g	915
	500	10.11f	31.1ef	
	1000	5.82h	60.28c	
Crude EtoAC	0	15a	-	
	10	12.31cd	17.9gh	
	50	11.11e	25.88f	
	100	10.11f	32.55e	379
	500	7.13g	52.40d	
	1000	5.33h	64.40c	
Crude MeoH	0	14.10b	-	
	10	11.81d	16.16gh	
	50	10.08f	28.39ef	
	100	5.70h	59.46c	112
	500	3.77i	73.17b	
	1000	2.61j	84.44a	
CV (%)		3.59	7.07	

[1]Average of four replications. Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.01.

Table 4. Testing bio-agent formulations to control Fusarium wilt of tomato *in vivo*

Treatments	Plant height (cm)	Plant weight (g)	Roots weight (g)	Numbers of fruit/plant	Yield	DSI[1]
non inoculated control	101.75c	78.00b	7.25abc	13.50a	155.50b	1.00e
inoculated control	62.75f	64.00b	5.00c	8.00b	83.75d	4.75a
powder formulation	109.75b	91.75a	11.25a	13.25a	197.50a	2.00 cd
Oil formulation	119.25a[2]	98.50a	10.00ab	15.00a	218.50a	1.75de
culture filtrate	84.50d	68.00b	6.00 bc	8.50b	128.00bc	2.75bc
prochoraz	73.00e	67.00b	7.75 abc	9.75b	107.00cd	3.50b
C.V.(%)	3.21	8.03	22.58	11.80	9.16	16.34

[1]Disease severity index (DSI) was scored at 21 days after inoculation. 1= no symptom; 2= plant showed yellowing leaves and wilting 1-20%, 3= plant showed yellowing leaves and wilting 21-40%, 4= plant showed yellowing leaves and wilting 41-60%, 5= plant showed yellowing leaves and wilting 61-80%, and 6= plant showed yellowing leaves and wilting or die 81-100%.
[2]Average of four replications. Means with the same common letters in each column are not significantly different according to Duncan's multiple range test at P = 0.01.

Table 5. Increased in percentage of growth parameters from testing bio-agent formulations to control Fusarium wilt of tomato *in vivo*

Treatments	Plant height[1]	Plant weigh	Roots weight	Numbers of fruit/plant	Yield	DR[2]
Powder formulation	42.82	30.24	55.55	39.62	57.59	57.89
Oil formulation	47.37	35.02	50.00	46.66	61.67	63.15
culture filtrate	40.37	5.88	16.66	5.88	34.60	42.10
prochoraz	14.04	4.47	35.48	17.94	21.72	26.31

[1]Increased in plant growth parameters = inoculated control – treatment / inoculated control X 100.

[2]Disease reduction (DR) = disease index of inoculated control - disease index of treatment/disease index of inoculated control X 100.

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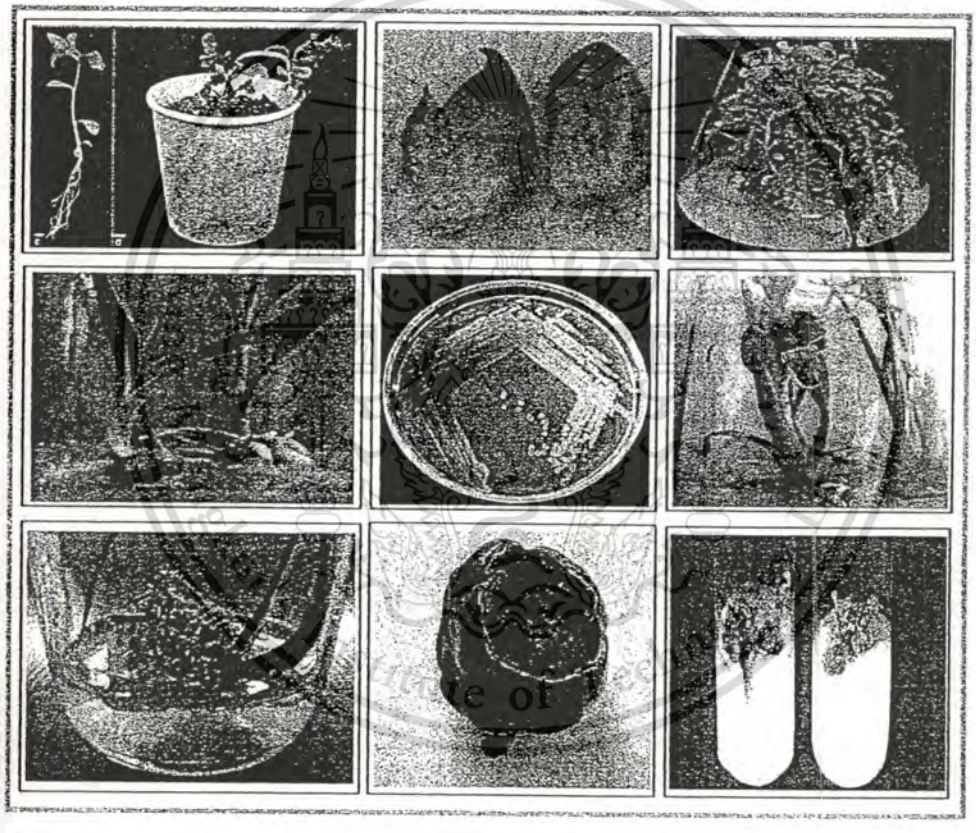

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Antifungal activities of *Chaetomium brasilense* CB01 and *Chaetomium cupreum* CC03 against *Fusarium oxysporum* f.sp. *lycopersici* race 2

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Sibounnavong, P., Sibounnavong, P.S., Kanokmedhakul S. and Soytong, K.(2012) Antifungal activities of *Chaetomium brasilense* CB01 and *Chaetomium cupreum* CC03 against *F. oxysporum* f. sp. *lycopersici* race 2. Journal of Agricultural Technology 8(3): 1029-1038.

The antagonistic fungi of *Chaetomium brasilense* CB01 and *Chaetomium cupreum* CC03 were proved to antagonize *F. oxysporum* f.sp. *lycopersici* NKSC02 race 2 caused tomato wilt of sida abd cherry varieties. The bioactivities test demonstrated the antagonistic activity of *Ch. brasilense* CB01 and *Ch. cupreum* CC03 to inhibit the conidial production of *F. oxysporum* f. sp. *lycopersici* race 2. To elucidate the control mechanism involved in the inhibition of *F. oxysporum* f. sp. *lycopersici*, crude extracts of *Ch. brasilense* CB01 and *Ch. cupreum* CC03, were confirmed for antifungal activity against of *F. oxysporum* f. sp. *lycopersici* race 2. The other control mechanism involved in releasing antibiotic substances to inhibit *F. oxysporum* f. sp. *lycopersici* race 2. All tested crude extracts of *Ch. brasilense* CB01 and *Ch. cupreum* CC03, were significantly inhibited conidia production of *F. oxysporum* f. sp. *lycopersici* race 2. It is indicated that crude extracts from hexane, EtOAc and MeOH from *Ch. brasilense* CB01 inhibited *F. oxysporum* f.sp. *lycopersici* race 2 at the ED_{50} of 29.87, 38.99 and 2.99 µg/ml, respectively. Crude extracts from hexane, EtOAc and MeOH from *Ch. cupreum* CC03 inhibited *F. oxysporum* f.sp. *lycopersici* race 2 at the ED_{50} of 2.33, 2.38 and 2.65 µg/ml, respectively.

Key words: *Chaetomium brasilense*, *Chaetomium cupreum*, *F. oxysporum* f.sp. *lycopersici* race 2 , fungal metabolites

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Introduction

Researches on natural products for antimicrobial against plant pathogens have been reported from several recent works. There are many new species of promising antagonists that can be used to control Fusarium wilt of tomatoes. The biocontrol agents and their bioactive compounds extracted from different species of antagonistic fungi were reported to inhibit the growth of many plant pathogenic fungi, including Fusarium wilt of tomato (Kanokmedhakul *et al.*, 2006 and 2003; Thongsri and Soytong, 2004; Srinon *et al.* 2004, Suwannapong and Soytong, 2002 and Sibounnavong *et al.* 2009ab). The bioactive compounds, Trichotoxin A50 extracted from *Trichoderma harzianum* PC01; and Chaetoglobosin C extracted from *Chaetomium globosum*. These compounds have also been reported to elicit resistance or immunity in plants by inducing oxidative burst in plant cells (Nuchdonrong *et al.* 2004; Soytong *et al.* 2001). The metabolites from fungi become one of potent antifungal against several plant pathogens. Crude extracts of *Trichoderma hamatum* WS01 and *Penicillium* sp.WS01 were reported to inhibit *Fusarium oxysporum* f.sp. *cucumerinum* and *F. oxysporum* f.sp. *lycopersici* isolated from wilt of cucumber and tomato (Srinon *et al.* 2006). Crude extracts from P. chrysogenum could protect cotton plants against wilt disease (*F. oxysporum* f. sp. *vasinfetum* and *Verticillium dahlidae*) and increases yield under field condition. (Dong *et al.* 2005, 2003; Dong and Cohen, 2001; Saidkarimov and Cohen, 2003) and *Colletotrichum gloeosporioides* (Soytong *et al.* 2005), *Phytophthora parasitica* (Meepeung and Soytong, 2004) and De Cal (2004) studied *P. oxalicum* to inhibit *F. oxysporum* f.sp. *lycopersici* and *Botrytis cinerea*. In addition, *Gliocladium virens* produced gliotoxin (Lumsden *et al.* 1992) and its properties against wood attacking fungi; *Postia placenta* and *Neolentinus lepideus* and *Trametes versicolor* and *Phlebia brevisspora* (Terry *et al.*,1996). Chulalak and Soytong (2006) reported that the bioactive compound extracted from *Chaetomium cochliodes* and *Ch. cupreum* inhibited plant pathogenic fungi, *Phytophthora palmivora* (root rot of pomelo) and *Fusarium oxysporum* f. sp. *lycopersici* (tomato wilt). Soytong *et al.* (2001) reported that the bioactive compound from *Ch. cupreum* inhibited the spore production of *F. oxysporum* which the ED_{50} was 113.43 µg/ml and inhibited the spore production of *P. palmivora* which the ED_{50} was 53.46 µg/ml. Moreover, the bioactive compounds revealed that *Ch. cupreum* could reduce the sporulation of *P. palmivora* which the ED_{50} was 279.67 µg/ml. With this, the ED_{50} of crude extracts from *Ch. cochliodes* was 323.01 µg/ml to inhibited *F. oxysporum* and the ED_{50} of crude henae and ethyl acetate from *Ch. cochliodes* inhibited *F. oxysporum* were 203.64 and 416.41 µg/ml, respectively. A mechanism of antibiosis can occur during interactions involving low-molecular-weight

diffusible compounds or production of antibiotics by biological control agents (Benítez *et al.* 2004). With this, the effective biological control agents produce several types of antibiotics to play important role in disease control (Lewis *et al.* 1989; Handelsman and Stabb, 1996). Specific species of fungi can produce specific metabolite that either impede spore germination as fungistasis, or kill the cells as antibiosis (Benítez *et al.* 2004). *T. harzianum* PC01 reported to produce trichotoxin A50 that it would induce resistant to mamy crops like tomato and potato etc. (Suwan *et al.* 2000). *Ch. globosum* can produce Chaetoglobusin C (Soytong *et al.* 2001) and *Ch. cupream* can produce rotiorinol (Kanokmedhakul *et al.* 2006). Antibiotics chaetoglobosin C and rotiorinol were reported to inhibit several plant pathogen e.g. *F. oxysporum* f. sp. *lycopersici*, *C. gloeosporides* and *Phytopthora* spp. (Soytong *et al.* 2001). The objective was to evaluate the biological activities of antagonists against *F. oxysporum* f. sp. *lycopesici* race 2 caused tomato wilt of sida abd cherry varieties.

Materials and methods

Pathogen to be tested:- *Fusarium oxysporum* f.sp. *lycopersici* NKSC02 race 2 which pathogenic causing wilt to tomato var. Sida and Cherry from previous reports were used.

Effective antagonists to be tested:- *Chaetomium brasilense* CB01 and *Chaetomium cupreum* CC03 offered from Assoc. Prof. Dr. Kasem Soytong, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Ladkrabang, Bangkok, Thailand were used.

Crude extraction:- Crude extracts from each antagonistic fungus were done follwed the method of Kanokmedhakul *et al.* (2006) and Moosophon *et al.* (2009). The fungi were cultured in potato dextrose broth (PDB) at room temperature for 30 days. The dried fungal biomass of each antagonistic fungus was ground and sequentially extracted with hexane, ethyl acetate and methanol. The solvents were then evaporated in vacuo to yield crude hexane, crude ethyl acetate (EtOAc), and crude methanol (MeOH) extracts, respectively.

Bioassays:- Crude extracts were assayed for inhibition of the most virulent isolate of *F. oxysporum* f. sp. *lycopersici* NKSC02 race 2. The experiment was conducted by using a factorial experiment in Completely Randomized Design (CRD) with four replications. Factor A represented the different solvents: A1 = crude hexane, A2 = crude ethyl acetate and A3 = crude methanol. Factor B represented the different concentrations: B1 = 0 µg/ml (control), B2 = 50 µg/ml, B3 = 100 µg/ml, B4 = 500 µg/ml and B5 = 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 30 minutes. To perform the assay,

a sterilized 3-mm diameter cork borer was used to remove agar plugs from the actively growing edge of the pathogen culture. An agar plug was transferred to the center of 5 cm diameter Petri dishes of PDA containing crude extract at each concentration and incubated at room temperature until the pathogen on the control plates had grown over the plate. Data were collected regarding the number of conidia produced by the pathogen and used to calculate the percentage of conidia inhibition. The effective dose (ED_{50}) was calculated using Probit analysis. The experiment was repeated twice.

Results

Chaetomium brasilense CB01 and *Chaetomium cupreum* CC03 at different concentrations of 0, 10, 50, 100, 500, and 1,000 g/ml were tested for inhibition of *F. oxysporum* f. sp. *lycopersici* NKSC02 which obtained from previous experiment. Hexane crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 3.67, 3.19, 2.67, 2.37 and 1.94 cm, respectively when compared to the control (0 µg/ml) of 5 cm. EtOAc crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 3.05, 2.92, 2.64, 2.27 and 2.22 cm, respectively when compared to the control (0 µg/ml) of 5 cm. MeOH crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 3.67, 3.50, 2.97, 2.77 and 2.22 cm, respectively when compared to the control.

Hexane crude extract from *Ch.cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 5.00, 4.87, 4.47, 4.45 and 4.12 cm, respectively when compared to the control. EtOAc crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02 which were 5.00, 3.92, 3.67, 3.54 and 3.40 cm, respectively when compared to the control. MeOH crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in colony diameter of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 4.47, 4.12, 3.74, 3.54 and 3.25 cm, respectively when compared to the control (Table 1 and 2).

Table 1. Effect of crude extracts from antagonistic fungi on mycelia growth of *Fusarium oxysporum* f.sp. *lycopersici* NKSC02

Crude extracts	Colony diameter (cm) of *Fusarium oxysporum* f.sp. *lycopersici* at each concentration (µg/ml))					
	0	10	50	100	500	1000
C. brasilense						
Hexane	5a[1]	3.67b	3.19c	2.67f	2.37g	1.94h
EtOAc	5a	3.05cd	2.92de	2.64f	2.27g	2.22g
MeOH	5a	3.67b	3.50b	2.97de	2.77ef	2.22g
C. cupreum						
Hexane	5a	5.00a	4.87a	4.47b	4.45b	4.12b
EtOAc	5a	5.00a	3.92d	3.67ef	3.54f	3.40g
MeOH	5a	4.47b	4.12c	3.74e	3.54f	3.25 h

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

Table 2. Effect of crude extracts from antagonistic fungi for percentage of colony inhibition growth of *Fusarium oxysporum* f.sp. *lycopersici* NKSC02

Crude extracts of	Colony inhibition of *Fusarium oxysporum* f.sp. *lycopersici* (%)				
	10	50	100	500	1000
C. brasilense					
Hexane	26.5g[1]	36.0f	46.5c	52.5b	61.0a
EtOAc	39.0ef	46.0cd	47.0c	54.5b	55.5b
MeOH	26.5 g	30.0 g	40.5 def	44.5 cde	55.5b
C. cupreum					
Hexane	0.0h	2.5h	10.5g	11.0g	17.5f
EtOAc	0.0h	21.5 e	26.5 cd	29.0 bc	32.0 ab
MeOH	10.5 g	17.5 f	25.0 d	31.0 b	35.0 a

[1]Average 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 from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in spore production of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 21.5 $x10^7$, 15.93 $x10^7$, 14.0 $x10^7$ and 2.16 x 10^7 spore/ml, respectively.when compared to the control (0 µg/ml) of 35.78 $x10^7$ spore/ml. EtOAc crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in spore production of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 26.71 $x10^7$, 19.61 $x10^7$, 11.48 $x10^7$, 5.35 $x10^7$ and 4.40 $x10^7$ spore/ml, respectively when compared to the control (0 µg/ml) of 36.24 $x10^7$ cm. MeOH crude extract from *Ch. brasilense* CB01 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in spore production of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 11.83 $x10^7$,

9.84 x10^7, 8.52 x10^7, 4.28 x10^7 and 1.07 x10^7 spore/ml, respectively when compared to the control (0 µg/ml) of 35.72 x10^7 spore/ml. Hexane crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in spore production of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 15.93 x10^7, 8.64 x10^7, 6.82 x10^7, 5.94 x10^7 and 3.18 x10^7 spore/ml, respectively when compared to the control (0 µg/ml) of 39.50 x10^7 spore/ml. EtOAc crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in spore production of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 14.43 x10^7, 8.87 x10^7, 7.68 x10^7, 4.48 x$10^7$and 2.40 x10^7 spore/ml, respectively when compared to the control. MeOH crude extract from *Ch. cupreum* CC03 at the concentrations of 10, 50, 100, 500 and 1000 µg/ml gave significantly different in spore production of *F. oxysporum* f. sp. *lycopersici* NKSC02which were 13.93 x10^7, 8.43 x10^7, 6.16 x10^7, 2.86 x$10^7$and 1.07 x10^7 spore/ml, respectively when compared to the control (Table 3).

Table 3. Effect of crude extracts from antagonistic fungi against conidia production of *Fusarium oxysporum* f.sp. *lycopersici* NKSC02

Crude extracts of	Number of conidia(x10^7) of *Fusarium oxysporum* f.sp. *lycopersici* at each concentration (µg/ml)					
	0	10	50	100	500	1000
C. brasilense						
Hexane	35.78a[1]	21.51c	15.93d	14.50d	4.10gh	2.16hi
EtOAc	36.24a	26.71b	19.61c	11.48e	5.35g	4.40gh
MeOH	35.72a	11.83e	9.84ef	8.52f	4.28gh	1.07i
C. cupreum						
Hexane	39.50b	15.93d	8.64fg	6.82hi	5.94i	3.18k
EtOAc	38.47c	14.43e	8.87f	7.68gh	4.48j	2.40k
MeOH	41.00a	13.93e	8.43fg	6.16i	2.86k	1.07 l

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

It revealed that crude extract at 1000 µg/ml from MeOH of *Ch. brasilense* CB01 gave significantly better inhibited spore production of *F. oxysporum* f.sp. *lycopersici* as 96.98 % better than crude extracts from EtOAc and MeOH which were 55.50 %. Crude extract at 1000 µg/ml from MeOH of *Ch. cupreum* CC03gave significantly better inhibited spore production of *F. oxysporum* f.sp. *lycopersici* as 97.37 % better than crude extracts from hexane and EtOAc which were 93.75 and 91.92 %, respectively. It is indicated that crude extracts from hexane, EtOAc and MeOH from *Ch. brasilense* CB01 inhibited *Fusarium oxysporum* f.sp. *lycopersici* race 2 at the ED_{50} of 29.87, 38.99 and 2.99 µg/ml, respectively (Table 4). Crude extracts from hexane, EtOAc and MeOH from

Ch. cupreum CC03 inhibited *Fusarium oxysporum* f.sp. *lycopersici* race 2 at the ED_{50} of 2.33, 2.38 and 2.65 µg/ml, respectively (Table 4).

Table 4. Effect of crude extracts from antagonistic fungi for percentage of conidia inhibition of *Fusarium oxysporum* f.sp. *lycopersici* NKSC02

Crude extracts	Conidia inhibition of *Fusarium oxysporum* f.sp. *lycopersici* (%)					
	10	50	100	500	1000	ED_{50} µg/ml
C. brasilense						
Hexane	39.83k[1]	55.44i	59.47h	88.53c	93.95b	29.87
EtOAc	33.17l	45.88j	68.29g	85.22d	87.85c	38.99
MeOH	66.87g	72.38f	76.09e	87.98c	96.98a	2.99
C. cupreum						
Hexane	59.64j	78.23g	82.71de	84.38de	91.92b	2.33
EtOAc	62.46i	76.92g	82.17ef	88.34c	93.75b	2.38
MeOH	65.99h	79.41fg	85.49d	93.13b	97.37a	2.65

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

Discussion

The antagonistic fungi *Ch. brasilense* CB01 and *Ch. cupreum* CC03, were proved to antagonize *F. oxysporum* f.sp. *lycopersici* NKSC02 race 2 causing wilt to tomato var. Sida and Cherry. The antagonism test demonstrated the antagonistic activity of *Ch. brasilense* CB01 and *Ch. cupreum* CC03, to inhibit the conidial production of *F. oxysporum* f. sp. *lycopersici* NKSC02 between 63 – 77 %. Similar result was in accordance with the study from Charoenpoen *et al.* (2010) reported that *Chaetomium lucknowense* CLT significantly inhibited the mycelia growth and conidial production of *F. oxysporum* f. sp. *lycopersici* as 88.89 and 92.54 %, respectively. Furthermore, Sibounnavong *et al.* (2009) reported crude extacrts of *Emericella nidulans* strongly inhibited colonial growth and sporulation of *F. oxysporum* f. sp. *lycopersici*. Crude extracts of *Ch. Brasilense* CB01 and *Ch. cupreum* CC03 were confirmed for antifungal activity against of *F. oxysporum* f. sp. *lycopersici* NKSC02 race 2. The other control mechanism of *Ch. brasilense* CB01 and *Ch. cupreum* CC03 involved in releasing antibiotic substances to inhibit *F. oxysporum* f. sp. *lycopersici*. All tested crude extracts of *Ch. brasilense* CB01 and *Ch. cupreum* CC03 were significantly inhibited conidia production of *F. oxysporum* f. sp. *lycopersici*. This result was similar to the report of Charoenpoen *et al.* (2010) who stated that crude hexane, crude ethyl acetate and crude methanol from *Ch. lucknowense* CLT inhibited *F. oxysporum* f. sp. *lycopersici* NKSC01 with the ED_{50} of 188, 209 and 212 µg/ml while in this study, crude extracts from methanol, ethyl acetate and hexane *Ch. brasilense*

CB01 inhibited the conidial production of different isolate of *F. oxysporum* f. sp. *lycopersici* NKSC02 race 2 with the ED_{50} of 29.87, 38.99 and 2.99 µg/ml, respectively and crude extracts from methanol, ethyl acetate and hexane *Ch. cupreum* CC03 inhibited the conidial production of different isolate of *F. oxysporum* f. sp. *lycopersici* NKSC02 race2 with the ED_{50} of 2.33, 2.38 and 2.65 µg/ml. Similar results were also reported by Srinon *et al.* (2006) and Sibounnavong *et al.* (2009) who stated that crude hexane, ethyl acetate and methanol extracts from *E. nidulans* inhibited the colony and sporulation of *F. oxysporum* f. sp. *lycopersici*. Moreover, Soytong *et al.* (2005) reported that crude ethyl acetate extract of *Ch. globosum* CG at 1000 µg/ml inhibited conidia production of this pathogen. As a result, Sibounnavong *et al.* (2009) reported that methanol crude extract from E. nidulans gave the highest inhibition of *F. oxysporum* f. sp. *lycopersici*. It is explained that ethyl acetate crude extract from *E. rugulosa* might have different fungal mrtabolites from methanol crude extract of *E. nidulans* as reported by Moosophon *et al.* (2006).

It concluded that *Ch. cupreum* CC03 can be produced some metabolites to inhibit *F. oxysporum* f. sp. *lycopersici* race 2 which Kanokmedhakul *et al.* (2006) found antifungal azaphilones from *Ch. cupreum* CC3003 effectively inhibited some human pathogens. Moreover, in this study *Ch. brasilense* CB01 proved to produce antifungal metabolites against *F. oxysporum* f. sp. *lycopersici* race 2 cause tomato wilt which it is the same isolate of reported *Ch. brasilense* CB01 by Khumkomkhet *et al.* (2009) found four new depsidones, mollicellins K-N which exhibited antimalarial activity against Plasmodium falcipurum and mollicellin K exhibited antmycobacterial activity against *Mycobacterium tuberculosis* and antifungal activity against *Candida albicans* and some cancer cell lines. The result of research finding would extend for testing to cotrol tomato wilt in the fields and further study would convey to apply these bioactive compounds as microbial elicitors to induce plant immunity.

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