THE ORGANIC COFFEE PRODUCTION AT PAKSONG BOLOVEN PLATEAU IN SOUTH OF LAO PDR

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หัวข้อวิทยานิพนธ์	การผลิตกาแฟอินทรีย์ที่ปากซองโบโลเวนปาโต ทางภาคใต้ของประเทศสาธารณรัฐประชาธิปไตยประชาชนลาว
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บทคัดย่อ

เชื้อสาเหตุโรคแอนแทรกโนส ที่แยกจากตัวอย่างโรคบนใบและผลกาแฟ นำมาจัดจำแนกโดยอาศัยลักษณะสัณฐานวิทยาและระดับโมเลกุล ยืนยันว่าเป็นเชื้อ *Colletotrichum gloeosporioides* ซึ่งนำมาทดสอบความสามารถในการเกิดโรคด้วย การนำสารออกฤทธิ์จากเชื้อรา *Chaetomium cupreum* CC3003 ในรูปของ crude hexane, ethyl acetate และ methanol พบว่ามีประสิทธิภาพดีในการยับยั้งเชื้อสาเหตุโรค *C.gloeosporioides* ซึ่งมีค่า ED_{50} เท่ากับ 13, 11 และ 28 ppm. ตามลำดับ การใช้ยาเชื้อชนิดผงที่ผลิตจาก *C. cupreum* มีประสิทธิภาพสูงสุดในการลดการเกิดโรคได้ 54.77% รองลงมาคือการใช้ nano-rotiorinal, nano-trichotoxin และการใช้สปอร์แขวนลอยของ *C. cupreum* ซึ่งลดการเกิดโรคได้ 46.23, 42.71 และ 18.9% ตามลำดับ เพื่อเปรียบเทียบกับไม่ใช้วิธีการใด ซึ่งเป็นรายงานครั้งแรกในการควบคุมโรคแอนแทรกโนสโดยชีววิธีในประเทศสาธารณรัฐประชาธิปไตยประชาชนลาวในการใช้ยาเชื้อที่ผลิตจากจุลินทรีย์และอนุภาคนาโนจากสารออกฤทธิ์จุลินทรีย์ *C. cupreum* ในการควบคุมเชื้อสาเหตุโรค *C. gloeosporioides*

*C. cupreum*CC3003 สามารถควบคุมเชื้อรา *C. gloeosporioides* สาเหตุโรคแอนแทรกโนสของกาแฟได้ ในการทดลองในอาหารเลี้ยงเชื้อร่วมกัน ซึ่งสามารถยับยั้งโคโลนีของเชื้อโรคได้ 29.89% และยับยั้งการสร้างสปอร์ได้ 38.61% หลังจากบ่มเชื้อไว้ 15 วัน

จากการทดสอบในต้นกาแฟอาราบีก้า อายุ 2 ปี ที่ปากช่อง แขวงจำปาศักด์ ประเทศลาว ซึ่งมีโรคแอนแทรกโนส จากการติดเชื้อธรรมชาติ พบว่าวิธีการปลูกกาแฟแบบอินทรีย์ มีผลผลิตคุณภาพดีกว่าวิธีการใช้สารเคมี เมื่อเปรียบเทียบกับไม่ใช้วิธีการใด วิธีการปลูกแบบอินทรีย์ได้เมล็ดกาแฟแห้งสูงกว่าวิธีการปลูกแบบเคมีและไม่ใช้วิธีการใด อย่างไรก็ตามวิธีการแบบอินทรีย์สามารถควบคุมและลดการเกิดโรคแอนแทรกโนสบนใบได้ 51.58 % ในขณะที่วิธีการทางเคมีลดการเกิดโรคได้ 33.35% และเมล็ดกาแฟที่ได้ตามวิธีการผลิตแบบอินทรีย์ โรคแอนแทรกโนส ลดลง 58.14% ในขณะที่วิธีการแบบเคมีโรคลดลง 45.18%

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ABSTRACT

Anthracnose pathogen was isolated from coffee leave and bean symptoms. Morphological and molecular phylogenetic data confirmed the species as *Colletotrichum gloeosporioides*. The pathogenicity of the isolate was also confirmed. Bioactive substances from *Chaetomium cupreum* CC3003 including crude hexane, ethyl acetate and methanol extracts showed good inhibition efficacy against *C. gloeosporioides*, with ED_{50} values of 13, 11 and 28 ppm, respectively. A powder bio-formulation of *C. cupreum* gave the highest disease reduction of 54.77 %, followed by nano-rotiorinol, nano-trichotoxin and a spore suspension of *C. cupreum* which produced disease reductions of 46.23, 42.71 and 18.59 %, respectively when compared with the inoculated control. This is the first report of bio-control of coffee anthracnose in the Lao PDR using bio-formulations and nano-materials of *C. cupreum* to control *C. gloeosporioides*.

Chaetomium cupreum CC 3003 was antagonized *Colletotrichum gloeosporiodes* causing anthracnose of coffee in bi-culture test which inhibited colony growth of 29.89 % and inhibited spore production of 38.61 % at 15 days incubation. It is clearly demonstrated that cultivation of coffee var Arabica aged 2 year trees in Paksong, Champasak Province, Lao PDR where seriously natural infested with anthracnose showing that organic method gave significantly better in cherry, wet parchment and dried beans than in chemical method as compared to the non-treated control in the field. Yield was gradually harvested for 6 times which resulted organic method was highest cherry and followed by chemical method when compared to the non-treated control. Organic method gave the highest in wet parchment and followed by chemical method when compared to the non-treated control. The dried coffee beans resulted significantly higher than chemical method

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and non-treated control. Moreover, organic method resulted a good disease control which reduced leave anthracnose in organic method of 51.58 % while it reduced disease in chemical method of 33.03 %.. Coffee bean anthracnose was reduced in organic method of 58.14 % while reduced in chemical method of 45.18 %.

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CHAPTER I

INTRODUCTION

Introduction.

The Lao People's Democratic Republic (Lao PDR) is a landlocked country, covering an area of 236,800 km^2 . The country is predominantly mountainous, with 80% of its land surface consisting of hills and mountains rising 100 to 3,000 meters above the plains of the Mekong River. These alluvial plains range in elevation up to about 200 meters above mean sea level. The remaining 20% of Lao PDR land area is comprised of the lowland plains. In the mountainous areas to the north and east, only the narrow river valleys and the plain of the jars are suitable for intensive agriculture. Lao PDR is located in Southeast Asia, bordered by Vietnam to the east, Cambodia to the south, Thailand to the west and south, and Myanmar and China to the north. Increasingly, it is being recognized that "landlocked" can be re-interpreted as "landlinked" changing the emphasis from " regional exclusion" to regional inclusion.

The country is divided administratively into 17 provinces. These are in turn divided in to 138 districts, 11,640 villages and 748,529 households (Ministry of Agriculture and Forestry, 1997). The country remains a predominantly rural economy, with about 83% of the population living in the rural areas and some 66% relying on subsistence agriculture.

Lao PDR is one country, which based on agriculture as the main factor of economic system. Agriculture is one of the most important sectors of the economy of the Lao PDR. It currently contributes 51% to gross domestic products (GDP) and accounts for 85.5% of the total force. Its contribution to national export earnings has been estimated at approximately 40%. Among the agricultural sub sectors, rice production is the single most important activity in the Lao PDR economy. Crops playing a role of more importance in Lao PDR are rice, coffee, maize, job'tear, cassava, mung-bean and soybean, etc.

The development history of the world coffee industry resulted from the horticultural skills of the Dutch, who moved the plants from Yemen to Batavia, and then to Amsterdam, where they were successful in producing an abundance of seeds to distribute to their colonial countries. The Catholic missions later played an important role in distributing the seeds and plants both to Africa and Latin America continents (Wrigley. 1982). Then commercial coffee production was distributed to many places around the world (Clifford and Willson, 1985). As a result, coffee is

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now one of the primary income sources and provides the most important single export product value for approximately 50 coffee producing countries in Latin America, Africa and Asia (Ridler, 1983).

Today several hundred million people in the world drink coffee, the Americans alone drinking around 430 million cups each day. The commercially important species of coffee, both of which originated in tropical Africa, are now grown in some 50 exporting countries, Judged by total value, coffee is one of the leading commodities in international trade, currently providing revenue of over $US 10 billion annually to the producing countries , and work for an estimated 20 million people who grow, process and distribute the crop throughout the world. For many South and Central American countries, including Colombia and EI Salvador, and many African nations, including Uganda, Burundi, Rwanda, and Ethiopia, coffee is their major source of foreign exchange (Wrigley, 1982).

1.1 Statement and Significance of the problem

Coffee production is the most important permanent crop, mainly grown on the Phouphieng Paksong Boloven Plateau. There are 24,000 ha of coffee grower, with 41,000 ha of coffee planted, especially in Saravan, Sekong, Champasak. There areas are suitable for growing the coffee. It is known that the region in a good coffee growing potential area. The government through MAF is trying to promote the planting of this crop as much as possible in order to support local and international needs (MAF, 1997).

The export price of Phouphiang Paksong Boloven Plateau coffee is 10% lower than the international market price due to poor quality. Law quality is mainly caused by poor husbandly, early harvesting and manual harvest processing from screening to drying . One of the important reasons for poor quality is that most of farmers and middlemen have limited concern regarding quality improvement since there is no price system based on quality. The price is fixed on the basis of weigh only. The coffee farms are very sensitive to market price fluctuations since coffee is cultivated in the farm of monoculture. Therefore, the recent low price on the international directly affects the coffee farm economy.

Coffee in Lao PDR. Coffee Arabica, comprising the varieties Typical and Bourbon was introduced into Lao PDR around 1920 and rapidly spread to the villages along side the colonial plantings in the uplands between 800 and 1,200 m above sea level. The coffee then spread to the plateau as a whole , down to a height of 600 m . Following leaf rust epidemics, the resistant coffea canephora , Coffea liberica and coffea excelsa species gradually replaced Arabica from

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1950 onwards. By the end of the 1960, the area was one third planted with Robusta coffee and two thirds Arabica, whereas it is now planted with over 95% Robusta with just a few patches of Arabica in the middle of Robusta , Liberica and Excelsa plantings. Production reaches 5,000 tons in colonial times, falling to under 1,500 tons in 1955 or there about. By 1970, it had climbed to 7,000 tons before another slump to around 3,000 tons as a result of the political situation. It was not until 1980 that the Laotian government realized the merits of coffee. In the 1999 to 2000 seasons Lao PDR produced almost 15,000 tons of coffee Robusta (96%) and Arabica (3%) are exported and Liberica (1%) is consumed locally. Coffee growing, which was once restricted to medium and high altitude areas, has now spread to zones between 300 and 400 m above mean sea level . There are many reasons for the increase in coffee out put. From 1980 on wards production was encouraged, but coffee was still marketed within an entirely state controlled commodity chain, at a price set by the authorities. In 1988 to 1989, the commodity chain was liberalized and exports intended to replay the country's debts were halted. Private operators set-up in the country and now work freely on the international market. The farm gate coffee price is now set by exporters rather than the state. The rise in the price paid to coffee grower has been accentuated by two factors;

1. The 1994 in Brazil, which led to a leap in prices that have not fallen since, due to a worldwide supply shortfall;
2. The drastic devaluation of the kip in relation to the dollar since the 1996 to 1997 season. In particular, farmers in upland areas now specialize in coffee and buy rice from the neighboring alluvial plains. State intervention and an initial evaluation of the Laotian coffee sector resulted in the introduction of a coffee rehabilitation and development programme. Mean yields increased from 250 to 300 kg / ha in 1983 to 400 to 450 kg/ha in 1990 to 1991. The project was launched once coffee growing was already expanding fast. Production leapt from 8,270 tons in 1996 to 1997 to 13,569 tons in 1997 to 1998, with an estimated mean yield of 550 to 600 kg/ Ha and a further 3,000 to 5,000 Ha planted each year.

The 15,000 tons of coffee currently produced in Lao PDR have little impact on a world scale, but they play a major role in the economy of the Phouphiang Paksong Boloven Plateau and the provinces of Champasak, Sekong and Saravan between which the plateau is split. The plateau is in the south of the country. Its agricultural activities are coffee, rice, cardamom, banana, others fruits, cabbages, potatoes and cattle raising and it supplies the Laotian and Thai markets. In value

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terms, coffee represents 63% of the 17,5 to 22,5 million dollars generated by agriculture each year. Since 1993, the coffee commodity chain has grown considerably, with 3,000 to 5,000 ha of new plantings each year and a resumption of upkeep in existing plantings, resulting in a marked shift towards monoculture and land saturation.

The Production systems and farmers strategies across the plateau reveal three main agricultural zones:

1. From 200 to 400 m above sea level, upland rice on cleared land is dominant (rice);
2. From 400 to 900 m above sea level, upland rice is gradually replaced by tree crops (coffee, fruit trees) as the altitude increases;.
3. Over 900 m above sea level, rice totally disappears, and is replaced by tree crops (coffee), vegetable (cabbages, potatoes) and coffee raising. This simplicity in fact masks a wide range of production systems.

The main differences between the cropping systems are the drops grown and the degree of land availability, largely due to the altitude sequence described the lowland area, between 200 and 400 m, is characterized by high land pressure ;and the upland area, above 900 m, by moderate to high land pressure due to the poor quality of some of the available land. Coffee is found in half the production systems described, and is grown between 400 and 1,200 m above sea level. New Robusta plantings are being set up with seedlings take from old planting, while seeds from the commercial plantings set up between 1993 and 1996 are being used for Catimor plantings. Improved planting materials requirements are very limited, which account for under 19% of the estimated 3,000 to 5,000 ha of spontaneous extension. Likewise, the owners of the large coffee estimates setup since 1996 choose to purchase their planting material from smallholders. There main types of systems have been identified according to the length of the fallow period, each of which can be split into sub-systems. However, coffee production systems in Champasak, Saravan and Sekong provinces are still of low productivity (JICA, 2001).

Coffee var. Arabica is distributed in Lao PDR to the growers in the uplands in 1920 which the sea level elevation of 800 and 1,200 m. (Clifford and Willson, 1985). Coffee becomes one of the primary sources of income and export product of 50 coffee producing countries in Latin America, Africa and Asia (Ridler, 1983). Coffee production is the most important perennial crop in the Lao PDR, mainly grown in the Phouphieng Paksong Boloven Plateau area. There are 41,000 ha of coffee plantations, especially in the Saravan, Sekong, Champasak provinces in the Lao PDR. The government has promoted the production of this crop to support local and

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international needs (MAF, 1997). However, the export price of coffee is 10% lower than the international market price due to its lower quality. The lower quality is caused by poor agronomic practices, early harvesting, and manual harvest processing from screening to drying, insects and disease problems. One of the important reasons for poor quality is that most of farmers have limited knowledge to improve quality, including control of insects and diseases especially anthracnose on coffee beans. The coffee growers apply many chemical fungicides for disease control but the anthracnose pathogen become resistant to chemical fungicides leading to an increase in anthracnose incidence on coffee beans (Soytong,2001; Vilavong and Soytong. 2013).

Biological control of plant pathogens has interested for disease control in many crops. It is proved to be effective to reduce the pathogen inoculum and disease incidence in a number of economically plants. *Chaetomium* is a board spectrum biological fungicide used to control several plant pathogens, especially anthracnose caused by *Colletotrichum spp*. *Chaetomium* spp. has been reported to effectively control of chili anthracnose caused by *Colletotrichum capsici* (Ratanachredchai, et al.2001). The need to find alternative effective methods of disease control to safe human health and protect the environment is evident. Nanotechnology involves the building and re-structuring materials at the molecular level. Molecular nanotechnology refers to build organic materials into defined structures, atom by atom or molecule by molecule (Li and Wu, 2011). Agricultural applications of nanotechnology are recently explored and interested by growers (Soutter, 2012). Nanoparticles contain bioactive chemical scan transport through cuticles and tissues, and release the active substances into plant cells (Ditta, 2012). The popular shapes of nano-materials being used for biocide delivery are nanospheres, and nano capsules (Perlatti, et al.2013). This approach enables safe, economical, effective and rapid disease control in crop production (Soutter, 2012). Many research reports have indicated that fungal metabolites from *Chaetomium* spp. gave good control of plant pathogens (Soytong et al. 2001). Previous research stated that nanomaterial could be loaded with active compounds from *Chaetomium* spp.; methanol crude extract from *Chaetomium cupreum* was incorporated with polylactic acid and electropum at 25-30 kV and was pale orange in color. Scanning electron microscope images revealed that the nanomaterial from *C. cupreum* is 171 nanometers. Moreover, nanomaterial containing bioactive metabolites from *Trichoderma harzianum* PC01 at 5-10 ppm exhibited antifungal activity against *C. capsici* causing chili anthracnose (Dar and Soytong, 2014). Other research finding stated that nanomaterial containing bioactive metabolites from *Chaetomium* sp.

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at a concentration of 10 ppm reduced tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* race 2 after incubation for 7 days. The ED_{50} value of the nanomaterial was 0.0093 ppm (Dar and Soytong, 2013).

Coffee is one of the most economic plants in the world where peoples consume. The development history of the world coffee industry has resulted from the horticultural skills of the Dutch, who moved the plants from Yemen to Batavia, and Amsterdam, where are successful produced an abundance of coffee seeds to distribute to their colonial countries and later has played an important role in distributing the seeds and plants to Africa and Latin America continents (Willium et al. 2003). Thereafter the commercial coffee production was distributed to many countries around the world (Clifford and Willson, 1985). Coffee becomes one of the primary income sources and provides the most important single export product value for approximately 50 coffee producing countries in Latin America, Africa and Asia (Ridler, 1983). There are about hundred million people in the world drink coffee, the American alone has been drinking around 430 million cups each day. The commercially coffee is originated in tropical Africa, are now grown in some 50 exporting countries and leading commodities in international trade, currently providing revenue of over $US 10 billion annually to the producing countries and working for an estimated 20 million people who grow, process and distribute throughout the world eg. South and Central American countries, including Colombia and EI Salvador, and many African nations, including Uganda, Burundi, Rwanda, and Ethiopia (Willium et al., 2003). Coffee var. Arabica was introduced to Lao PDR in 1920 and rapidly spread to the villages along the side the colonial plantings in the uplands between 800 and 1,200 m above sea level. However, coffee production systems in Champasak, Saravan and Sekong provinces are still of low productivity (JICA,2001). Coffee production is the most important permanent crop, mainly grown in Paksong, Laos. There are 24,000 ha of coffee grower, with 41,000 ha of coffee planted, especially in Saravan, Sekong, Champasak provinces. The planting areas are suitable for growing the coffee which known as the region for a good coffee growing potential area. The government has been trying to promote the planting of this crop as much as possible in order to support local and international needs (MAF, 1997). The export price of coffee beans from Paksong coffee is 10% lower than the international market price due to poor quality. Low quality is mainly not only causing by poor management, but early harvesting, processing from screening to dry and diseases and insect pests as well. Soytong (2001) stated that application of chemical fungicides for disease control are recognized to cause environmental pollution and toxic chemical residues in

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agricultural products, and continuous apply chemical fungicides leads to pathogen resistant. *Chaetomium* spp as bio-control agent of plant disease has been successful controlled several plant diseases. and the research bio-products as agricultural in outs for organic crop production are successfully applied in several crops in Thailand eg. Organic biofertilizer, liquid biofertilizers, biological fungicides (Ketomium) for disease control and bioinsectice (Metarhizium and Beauveria).

1.2 Objectives of the study

1. To evaluate potential of *Chaetomium*-biofungicide to control anthracnose of coffee var. Arabica.
2. To comparison inputs used organic and chemical methods to cultivate coffee Arabica in the field.

1.3 Scope of the Study

Field experiment was set up at Paksong Boloven plateau, Champasak province, Lao PDR where seriously natural infested leaf anthracnose of coffee var Arabica caused by *Colletotrichum gloeosporiodes*. It was conducted by using Randomized Completely Block Design (RCBD) with 4 replications. Treatments were done as follows:-treatment 1 was non-treated control, treatment 2 was organic methods and treatment 3 was chemical method. With this, organic method were applied all organic certified agricultural inputs as organic fertilizer at the rate of 500 g per tree after harvesting and interval apply at every 2 month for 1 year, spraying liquid organic biofetilizer at 50 cc/ 20 L of water every 30 days until harvest, spraying *Chaetomium*-biofungicide (registered bioproduct at Department of Agriculture, Lao PDR) at the rate of 20-40 g/20 L of water and microbial extract to induce immunity at 50 cc/ 20 L of water every 30 days until harvest, spraying bio-insecticide (Metarhizium + Beauveria) at the rate of 50 cc/20 L of water and sulfur power at 10-20 g/20 L of water every 30 days until harvest. Chemical method was applied chemical fertilizers, chemical fungicide and insecticide as recommendation rate as well as chemical herbicide. Data were collected as cherry (g), wet parchment (g) and dried beans (g). Disease index of leaf anthracnose was rated as follows:- rate 1 = 1-25 % blighted symptom on leave , 2 = 26-50 % , 3 = 51-75% blighted symptom on leave, and 4 = 75-100 % blighted symptom on leave. Data were statistically computed analysis of variance (ANOV) in RCBD and treatment means were compared by using Duncan Multiple's Range Test (DMRT) at P=0.05 and 0.01.

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1.4 Time and place of the research work

The study was conducted at three location at:

1. Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang Bangkok 10520, Thailand.
2. Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok10502, Thailand.
3. Paksong Boloven Plateau district Champasak province LAO PDR research field.

Duration of thesis: start from June 11th 2012 until 2018

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CHAPTER II

LITERATURE REVIEW

2.1. Agricultural conditions in the southern region

2.1.1. Crop production

The study area is composed of some parts of the provinces of Champasak, Salavan and Sekong in the southern area of the country. The principal economic activity is farming in the provinces Champasak and Salavan which produce a surplus of rice for its consumption while Sekong province has a deficit in rice. The total share of the three provinces about 2,35% of rice production compared to the whole country on average in recent years. Among the crop production the provinces produce about 95% of the coffee and 90% of the cardamom production in the whole country, and about 50% of the tea, mostly from the Phouphiang Paksong Boloven Plateau. The total production of rice in the provinces amounted to 364,000 tons (paddy) in 1994. About 90% of the production is dependent on rain-fed lowland rice 9% is on slash-and-burn cultivation in the provinces in 1994 is rather low compared to the national total of about 33 percent (JICA,1995).

2.1.2. Champasak province

Most people in the province engage in lowland rice cultivation. They cultivate about 77,100 ha and produced 192,000 tons of rice on annual average for 1990 to 1994. There are also some 17,000 ha of coffee and other cash crops in the Phouphiang Paksong Boloven Plateau. There farmers in the Phouphiang Paksong Boloven Plateau do not grow rice to a significant scale, and buy almost all the rice they need from other parts of the province. The farmers who live a subsistence level depend on slash-and burn cultivation, mainly in the Phouphiang Paksong Boloven Plateau and hilly areas in the southeast of the province and west of the Mekong. The annual average area under slash-and-burn cultivation is estimated at about 4,300 ha (JICA, 1995).

2.1.3. Saravan province

Saravan province is characterized by the fertile foot plains of the Mekong and Xedone which produce a substantial surplus of rice. The average annual production of rice was about 103,000 tones for 1991 to 1994. Some two thirds of the people are rice farmers who live on the plains. In Laongam district there about 7,000 ha of coffee. Other field crops like cardamom, groundnut and cotton are grown in Salavan and Laongam district which occupy a part of the

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Phouphiang Pakxong Boloven Plateau. Recently banana cultivation has expanded in Laongam district (JICA,1995).

2.1.4. Sekong province

Sekong province was created in 1984 in order to improve the public services to population of the somewhat isolated and backward parts of Salavan province. Most of the people live in the eastern mountains and on the northeastern slopes of the Phouphiang Pakxong Boloven Plateau, near Thateng. The economy of the Sekong is entirely based on coffee, cardamom and subsistence farming. The people who live near Thateng grow coffee and cardamom, and generate the substantial part of the provincial income. The remainder of the production, except for a few rice farmers and the people who live in Sekong town, are slash-and burn cultivations who mostly live in the eastern mountains (JICA, 1995).

2.1.5. Coffee soil

Coffee is growing on various sites on soil varying from extremely acid (pH below 4.0) to slightly alkaline (pH up to 8.0).Neither of these extremes is suitable for economic high output production. A slightly acid soil is preferred; various writers suggest various ranges of pH. For example, Robinson (1964), pH 5,2 to 6,2 for Arabica, Acland (1971), pH 5,3 to 6,0 for Arabica, Forestier (1969), higher than pH4.5 .For Robusta. Robusta is more tolerant of neutral and slightly alkaline soils. Deuss (1969), reported that after clearance of forest and burning the soil pH rose to 8,0, compared to 5.7 without burning. Robustra coffee planted in the high pH soil gave a crop yield, averaged over the first five harvests, 47,2% greater than the yield from coffee planted in soil where the forest trash was not burnt.

Acidity within the correct range is not the only requirement. The necessary nutrient must be available at a reasonable level and in appropriate relative proportions. The more the nutrient levels depart from the optimum, the more costly will be fertilizer applications necessary to provide the correct nutrition for maximum yields.

2.1.6. Planting densities

Canephora (Robustra): from 1,000 to 2,000 trees/ha, depending on plant spacing as follows:

1) 4.0 x 2.5 m (1,100 trees/ha) allows for mechanization of the inter-row.
2) 3.0 x 2.5 m (1,330 trees/ha) previously a standard planting density.
3) 3.0 x 2.5 m (1,330 trees/ha) intermediate planting density for Robusta.
4) 3.0 x 2.0 m (1,660 trees/ha) intermediate planting density for Robusta.

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5) 3.0x1.7 m (1,960 trees/ha) high density, a new standard planting density for Robusta.

6) 2.5 x2.0 m (2,000 trees/ha) high density planting.

7) Arabica: from 1,000 to 2,500 trees /ha for typical, bourbon and undo novo varieties, depending on planting distances as follows;

8) 4.ox2.5m(1,000trees/ha)a former Brazilian standard with intercropping, Mechanization of the inter-row and perimeter of the crops is possible.

9) 3.0 x3.0 m approx. (1,100 trees/ha) a former East Africa standard of 10 feet by feet,

10) 2.74 x 2.74 m (1,329 trees/ha) a recently common East African standard.

11) 2.74 x 1.37 m (2,658 trees/ha) a recently common East African standard.

12) 2.0 x 2.0 m (2,500 trees/ha) (Cambrony, 1992).

2.1.7. Species

The species most used for commercial coffee production are coffee Arabica according for about 90% of world production, Coffae canephora at about 9%, and Coffea liberrica, at about 1% or less. Coffea canephora is increasingly used because it has been found to be valuable in the manufacture of soluble coffee. It is probable that the use of Coffea liberica will gradually disappear as it is distinctly large in size which makes it to difficult to handle in standard machinery , and it seems to have no outstanding advantages except that it is highly resistant to disease. The relative increasing sizes of the green coffee beans are coffee Arabica, coffea canephora, Coffea liberica (Cambrony, 1992).

2.1.8. Fertilization

The formula of complete chemical fertilizers, which are detailed on the bags, indicate the respective percentages of the useful elements which is equivalent to the number of fertilizing units per 100 kg of fertilizer contained in the bag. These units are given in order N,P,K and Mg, if present, followed by the trace elements contained in the mixture. The most commonly used fertilizer in coffee growing are 10-5-20, 10-10-20, 12-10-15, 12,8,8 and, most popular of all, 20-10-10. Their application is often accompanied by an additional dressing of a simple nitrogenous fertilizer (Cambrony, 1992) .

2.1.9. Location

Most of the world's coffee is grown within the torrid zone. Coffee cannot tolerate frost. In choosing a location, the chief consideration is altitude , other things being equal, one usually finds that the higher the altitude, the better the quality of the coffee. The linting altitude is the zone of frost danger (Cambrony, 1992).

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2.1.10. Cause of nutrient disorders and their cure

1) Nitrogen is the nutrient most commonly applied to coffee and the one to which there is the greatest response in most fertilizer trails.

   Criteria for the optimum range, for any nutrient, for the fourth pair of leaves taken bearing laterals

Element	Optimum range (%)
Nitrogen	2.50 – 3.00
Phosphorus	0.10 – 0.20
Potassium	1.50 – 2.50

Source: Clowes and Hill, (1982)

Provided that all the conditions are right for the coffee tree management, particularly soil moisture, and no elements being deficient, then the available nitrogen has the greatest effect on yield. Under most conditions where coffee trees are grown without shade and produce heavy crops, regular applications of nitrogen fertilizer are essential. Conversely, coffee growing under heavy shade responds to the application of nitrogen fertilizer.

The nitrogen deficiency symptoms result from a combination of low leaf nitrogen and light intensity and are most common on unshaded trees. The symptoms also appear after a heavy crop is taken from the trees.

A lack of nitrogen is first seen on the new growth, the young leaves appearing a pale green or yellow color all over all over. These chlorotic symptoms are more severe in high light intensities, i,e, in unshaded or lightly shaded coffee, and in the dry season when the nitrogen uptake by the surface roots is at a minimum. In severe cases not only is the number of levels reduced, but also they are often smaller and are carried on shorter internodes.

The translocation of nitrogen from the older leaves to the young growth and developing fruit is very efficient but irreversible, consequently, if this translocations are excessive the older leaves at the fruit-bearing nodes have been shed prematurely. This can be prevented by the application of a nitrogen fertilizer when a heavy crop is anticipated, supplemented by foliar

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sprays of nitrogen when a heavy crop is being carried in dry weather when the topsoil is dry and the feeder roots are not able to take up sufficient nitrogen. The leaves are then retained to provide photosynthates for the developing fruits.

Heavy mulching reduces this form of chlorosis, which occurs predominantly in the dry season, probably because the topsoil is kept moist and the roots are able to take up the nitrogen required by the tree, which they cannot do from hot, dry topsoil. Nitrogen fertilizer, sulphate of ammonia if the pH of the soil is 6.5 or more, area in the dry periods of the year or in overhead irrigation water, or calcium ammonium nitrate if the soil is tending to be too acid (i.e. below pH 5.2), will deal with the back of nitrogen.

The nitrogen should be just applied after the rains have started. At the early start of the rains, there is on upsurge in soil nitrogen (Hartly,1946), which will supply the plants' needs as growth restarts. It too much nitrogen fertilizer is applied at this time it can be leached out by the heavy rain. Ammoniums are held briefly in the topsoil until they are mineralized to nitrate. Because of this leaching in the heavy rains, some growers prefer to wait until the rain are ending before applying nitrogen fertilizer. The coffee tree needs a good supply of nitrogen as the fruit is developing but a very late application can encourage the production of side shoots, rather than flower buds the next season. In areas where heavy seasonal rainfall occurs, nitrogen application should be split, and where a heavy crop is set, part should be applied phosphorus in the last quarter of the wet season.

Many tropical soils immobilize phosphorus. In acid soil, iron and aluminum phosphates are formed, and in calcareous soils, calcium and magnesium phosphates. All these compounds are of low solubility. Coffee appears to have a remarkable ability to take up the amount of phosphorus it needs even when the level in the soil is low.

As seen from above the coffee tree requires comparatively little phosphorus, and few experiments have shown either response to phosphate application or phosphorus deficiency symptoms. Even though the amount may be small in comparisons with nitrogen and potassium, there is a relatively large demand for phosphorus by the developing flowers before and after an thesis, and available phosphorus is vital when the coffee tree is flowering and setting fruit (Mathew and Chokkana, 1961; Huxley, 1967; Cannell and Kimeu, 1977). Phosphorus is always associated with root growth and is therefore applied to newly planted coffee to encourage good root development, and the level is maintained during the productive life of the trees. Applications are made early in the first wet season following the harvest period after harvest is complete.

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Serious actual deficiencies to occur in Papua New Guinea and the old Kiva province of the Belgian Congo (Zaire).Seasonal or physiological deficiencies occur when the demand is high but soils are either too dry or too wet for adequate uptake. When deficiency symptoms do occur, the oldest leaves become mottled, and irregular lemon-yellow patches with a reddish or purplish tinge development (autumn tints). The whole leaf may take up this high color. The younger leaves are darker than normal, blue-green in color, and hang downwards and backwards from the primary, This condition appears to occur when a heavy crop is being matured under drought conditions, in the absence of irrigation or mulch, when the topsoil is dry . In a severe case, there can be a serious loss of leaves from the fruit-bearing branches. Phosphorus deficiency has also been recorded on the Kilimanjaro red clay loan during prolonged periods of heavy rainfall when the trees because temporarily waterlogged. The condition was not changed by heavy application of double superphosphate but cleared up when normal soil moisture condition returned (Robinson, 1964). Low levels of phosphorus are associated with low levels of sulphur and a dressing of single superphosphate assists in dealing with both. The soil organic matter is an important reservoir of phosphorus, nitrogen and sulphur, and it is probably better, where practical, to apply and maintain the soil phosphorus level in the organic from by the use of cattle manure and mulch, Superphosphate will correct the deficiency on most soils, but where the soil is below pH 5,6, either triple superphosphate or another phosphate fertilizer with a low sulphur content should be used. Diammonium phosphate is another useful fertilizer for mildly acid soils, in that it supplies both nitrogen and phosphorus. Foliar application of soluble diammonium phosphate has not proved of great benefit, in terms of improved phosphorus nutrition, as the amount absorbed by the leaves is insufficient but the nitrogen constituent is of value (Robinsion, 1964), Phosphoric acid applied in applied in this way is liable to scorch the leaves.

2.1.11. Potassium.

Like many tropical fruit crops, coffee has a high level of potassium uptake. Many of the virgin soils on which coffee was originally planted were adequately supplied with potassium but this needs replenishment over the years. Where mulching is practiced or cattle manure used, soil levels of potassium are maintained and even enhanced.

The sequence of leaf symptoms which develop when potassium is deficient starts with chlorotic spots, principally at the distal ends of the older leaves. These spots either coalesce along the leaf margin as they because necrotic or become joined by marginal faint yellow coloration or are joined by chlorotic margins. Continuous marginal chlorosis develops at the distal and of the

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leaf, which is more advanced in the areas of the chlorotic spots. Before the leaves become completely necrotic they are shed, usually along the whole length of the lateral. Salt damage produces very similar symptoms, distinguishable by foliar analysis.

Potassium usually becomes inadequate when the trees are bearing a heavy crop. There is a striking response of such coffee trees to a heavy application of potassium fertilizer, supplied either as marinate (potassium chloride with 50-60% K_2O) or sulphate (40-52% K_2O and about 18% sulphur).

2.1.12. Altitude

Altitude relates to temperature. Therefore in equatorial areas Arabica is a highland crop growing at 1,000 meters to 2,000 meters, with Robusta growing from sea level to 700 meters. As the distance from the equator increases, so Robusta will become uneconomic, whereas Arabica will continue to be a valuable crop at decreasing altitudes until restricted by frequent or lengthy frosts.

2.1.13. Site aspect and topography

These factors must be considered together in some degree. Steep slopes raise problems of erosion control and access. In the sub-tropics north or south-facing slopes, depending on the hemisphere, will receive more sun, which may be important where conditions are marginal. In area with a risk of frost, valley bottoms should be avoided, whilst planting on slopes will assist the movement of cold air way from the coffee. Wind is an important factor, exposed sites will need windbreaks. Site close to the sea may suffer from the effects of salt spray. Areas with a fair risk of flooding at periods of heavy rainfall should be avoided.

2.1.14. Climate and soil

2.1.15. Rainfall

All coffee species are evergreen so transpiration is continuous. However the natural of coffee is the understory of rainforest and therefore there has not been an incentive to develop a mechanism to reduce water in times of stress. Accordingly the plants will lose water continuously and the rate of loss will be dependent entirely on the meteorological conditions.

The rate of loss of water by evaporation from an open water surface is dependent on air temperature, relative humidity, wind speed and the amount of radiant solar energy arriving at the evaporating surface. Penman (1948) derived an equation by which evaporation from an open water surface (open-pan evaporation) can be calculated from solar radiation, mean air temperature, mean temperature of dew point and mean run of wind. If the instrumentation

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necessary to measure solar radiation directly is not available, hours of bright sunshine can be used in its place. Ripley (personal communication, quoted by McCulloch, 1965), published a series of tables from which evaporation can be calculated. These tables are intended for tropical conditions and include the adjustment for altitude.

The rate loss of water from plants (transpiration) is always less than evaporation. The actual evaporation for any crop can be determined by measurement of the water-balance using a lysimeter (Pereira and McCulloch, 1062). The results are normally expressed as the annual evapotranspiration per evaporation ratio. McCulloch (1956) quotes some results. Evergreen montane rain forest has evapotranspiration per evaporation ratio equal to 0.9 mature pine 0.8, pasture 0.8 and mature bamboo forest 0.7. The ratio is usually between 0.5 and 0.9. For a complete vegetative cover. For a full discussion of this subject see Pereira (1973).

The water requirement of Arabica coffee was studied in Kenya by Wallis (1963) and Blore (1966). The ratio evapotranspiration per evaporation ratio varied from 0.5 in dry months to 0.8 in wet months. The lower values in dry months arose because coffee was planted at the then standard spacing which left gaps between the tree canopies. The inter-rows would be either bare ground or mulched, these surfaces lose little moisture when dried out in the dry season. The average annual requirement of water over twelve years was found to be 951 mm. The monthly evapotranspiration varied between 60 and 115 mm. Achtnich (1958) calculated the water the water requirement for coffee in relation to its latitude. Where the dry season (or season) is not too long and the soil has a high water retention capacity, Arabica coffee could, theoretically, be grown satisfactorily without irrigation where the average total rainfall is about 1,100 mm. In practice, imperfect rainfall distribution and adverse soil and weather condition create a considerable risk of failure where average annual rainfall is less than about 1,300 mm. As conditions become more adverse a higher rainfall will be necessary but soil water capacity will become limiting when the dry season is prolonged. The length of the dry season may be critical; for example Robinsion (1964) recommended "Not more than a maximum of four months dry weather should occur at any one stretch, unless a fair production of this period is cloudy and per or dull and cool." Cultural methods, e.g. mulching to minimize water loss from the soil, become more important as conditions become more adverse. Similar considerations apply to Robusta coffee, but as it more suited to lower altitudes where temperatures are higher, the total water requirement will be higher. Forestier (1969) quotes a minimum annual rainfall of 1,250 mm and states that rainfall within the range 1,550 mm to 2,000 mm is preferable.

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The relationship between coffee crop and rainfall has been studied in several countries. Sylvain (1959) reviewed the relationship between coffee and water. Physiological effects are discussed. A relationship between transpiration and light intensity for Brazil reported by Franco (1947) is quoted, also a comparison of monthly on such very shallow soils, excessive rainfall can have an adverse effect. The author found, in the western Highlands of Papua New Guinea, that there was a reduction of yield when rainfall from November to July exceeded 1,500 mm. The greater the amount by which rainfall exceeded 1,500 mm, the greater was the reduction in yield. This effect is presumably a consequence of water logging which will reduce root efficiency, although low light levels may some effect by reducing photosynthesis, and heavy rainfall at the commencement of the wet season may reduce pollination, Kumar (1982) reported that wet conditions affected hormone production; flowering abnormalities ensured which reduced crop yield.

2.1.16. Temperature

The optimum temperature ranges for the various species will be similar to those of their natural habitat. For Arabica the range is 24 ^{o}C to above 25°C. The photosynthetic rate is reduced and leaves are damaged by continuous exposure to high temperatures (over 30°C). Leave exposed continually to high irradiance develop chlorotic symptoms (Huxley,1967). Low temperatures produce a white or yellow discoloration of the leaves. The discolored areas are not uniform, although the initial effect is often limited to the leaf margins. Severely affected leaves are reduced in size, often distorted and mottled and may eventually scorch and fall. The symptoms are more severe when a high temperature, as in bright sunlight, is followed by a low temperature. Franco (1947) induced these symptoms by cooling seedling to 3°C in a refrigerator. In severe cases excessive branching to secondary and tertiary stems occur and shoot tips blacken, distort and shrived. Because of the effect of wide diurnal temperature variations the condition is known as "hot-and-cold disease". Frost destroys Arabica leaves and fruit.

For Robusta coffee the temperatures are higher, the optimum range is 24 ^{o}C to 30 ^{o}C. The tree will withstand and occasional temperature as low as 7°C but long periods of 15°C are harmful. Fruit and leaves are destroyed at 5 ^{o}C.

2.1.17. Humidity and cloud cover

Atmospheric humidity is an important factor in determining the loss of moisture by evapotranspiration. High humidity will reduce water loss, whilst low humidity will increase it.

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The humidity level during the dry season is therefore important; high humidity reduces the stress on the plant and extends rainless period through the plants will survive without damage.

Cloud cover commonly results in increased humidity in addition to lowering temperature and can therefore be advantageous during a long dry season. The low assimilation rate of coffee. In extreme cases of severe reduction of light cloud will not have a significant effect. In extreme cases of severe reduction in light intensity lower leaves will not receive sufficient light to achieve their normal assimilation rate, this may be partly compensated by the upper leaves which will not reach temperatures at which their activity is reduced.

Cloud at ground level, i.e. mist, often adds to the water in the soil from condensation on the trees. In some marginal areas this water can be of critical importance. Pasons (1960) recorded about 250 mm of drip water from a pine tree near San Francisco.

2.1.18. Wind

Excessively strong winds can cause physical damage to the trees. At wind speeds below those necessary to break stems, wind increases water loss by evapotranspiration and therefore moisture stress in the trees. If the wind is cold the effects of low temperature are accentuated so that 'hot-and-cold disease' is more severe. If the wind is hot, exposed leaves may wilt and even die. In either case vegetative growth and crop yield are reduced. Good windbreaks are essential in exposed situations.

2.1.19. Sun drying

Sun drying is the traditional method used for over three centuries in Ethiopia and the Yemen to remove the unwanted mucilage, pulp, skin and parchment and so obtain the dry coffee beans, In Ethiopia and the Yemen, the crop ripened in the dry season an sun drying is no problem. La Roque (1751) in this description of the ancient methods of coffee culture and preparation in the Yemen, said that the berries were left to dry on the trees, and when the outer covering began to shrivel, the trees were shake, causing the fully matures fruits to drop upon cloths spread out to receive them. They were next exposed to the sun on drying mats, and then husked by 'heavy stones and spars of wood they afterwards roll over them'. The beans were given a further drying in the sun and were the submitted to a winnowing process, for which large fans were used, the flat roofs of the houses are still for drying coffee.

Most of the Arabica coffee grown in Brazil and part of the crops of many other countries are sun dried, including a third of Bugishu Arabica crop in Uganda. Coffee is sun dried in Brazil because the very large crop is picked in a very short period from May to June onwards, varying

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with the regions, and there is insufficient water, time, equipment or labor to pulp the cherry, which has been stripped from the trees at a single pass irrespective of its stage of ripeness. This is said to give the Brazilian coffee its characteristic flavor. Sun dried and hulled Arabica is known in the trade as 'unwashed Arabica'. With the increase of population, the shortage of water, and the problem of river pollution dealt with later, other areas may have to sun dry more coffee even at the expense of quality.

Robusta coffee is largely sun dried, as quality has never been a consideration, although Robusta was commonly pulped on the plantations in Zaire. Similarly, the estates in Indonesia mainly pulped, whereas the smallholders sun dried. About 90% of the crop is now sun dried (Jobin, 1983). In Uganda wet processing of Robusta was done on a small scale, about 4,000 tones year in 1960 (Rowe, 1063), but very little, if any, washed Robusta is currently (1985) produced as there is no longer sufficient premium over sun dried Robusta. Some washed Robusta is produced in Papua New Guinea.

Sun drying is very difficult in tropical zones where there are two long rainy seasons and more cloud cover throughout the year than at the tropics. Smallholders spreads their harvested cherries in the sun, usually on have swept ground, where they become contaminated with dirt or stones. After heavy rain the bare ground cannot be used until it has completely dried out; also this may give the coffee an undesirable smell or flavor. Grass mats or hessian are often placed under the coffee which stops soil contamination and makes it easier to carry the drying coffee away when the dew comes down in the evening. Better still are the concrete barbecues built with a very smooth surface, if possible colored black to take up the beat. The surface should have a slight slope to a drainage hole in one corner so that rainwater does not accumulate in the wet season and the edge of the floor raised slightly to prevent the drying coffee flowing over the edge.

Coffee which is being dried close to the ground is a haven for chickens,, goats, even pigs, which can be seen picking over the coffee. In Uganda, raised coffee drying tables made from elephant grass reeds were encouraged. These allowed air to flow through the coffee which was kept high above domestic animals. The more prosperous growers replaced the reeds with fine wire-meshing.

Drying cherries should be spread in as thin a layer as possible, ideally not deeper than 2 or 3 cm and turned over regularly with wooden rakes or shovels. At the height of the picking season the coffee can be twice as deep, provided that it is turned regularly, otherwise it becomes moldy or rots. In a thin layer it is hardly likely to not even if not moved about.

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A square meter of barbecue will accommodate 40 kg of cherries at a depth of 4 to 6 cm. For the area in square meters of drying floor required. Coste (1968), give a rough figure, as used in Brazil, of 1 per 20th the number of trees. A hectare of coffee planted 2.5 x 2.5 m, i.e. 1,600 trees/ha, on this basis requites 80 m^2, and a coffee plantation of 200,000 trees (125ha) will require a drying area the size of a football pitch. The area required will depend upon the amount of rainfall and cloud expected at harvest time. In fine weather cherries will dry in under 10 days, but near the equator it often takes three weeks or longer before the cherries are completely dried pot. In Spanish Guines (Equatorial Guinia sinze 1968) Nosti Nava (1953) recommended not more than 25kg/ m^2 for Liberica and this was usually dry in 12 days.

In the first day or so the cherry ferments a little but fermentation stops as the moisture content of the cherry is reduced. Fermentation, which reduces the quality of the finished product, can easily occur if the cherry dries only slowly. During drying the red color of the pericarp turns blacks and the beans and husk shrink and harden. Some of the mesocarp or mucilage may penetrate the silverskin, which turns a darker brown than when the wet method is used. The silverskin also adheres closer to the bean. The beans prepared by the dry method have a yellow-brown color instead of the blue-green color of beans prepared correctly by the wet method.

By moving the cherry around the heat can get into the berries much better. As the pulp is hygroscopic the coffee should be pushed together and kept under cover at to retain the heat. Similar action is necessary when it rains. Plastic sheets are cheap covering material but do not take up moisture (Wrigley,1982).

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CHAPTER III

RESEARCH METHODOLOGY

3.1 Isolation of coffee anthracnose pathogen

The anthracnose pathogen was isolated by the tissue transplanting technique from symptomatic leaves and beans of coffee var. Arabica. The diseased plant parts were cut at the advanced margin of lesions into small pieces (5mm×5mm) and surface disinfected with 10% sodium hypochlorite for 1 min, followed by rinsing with sterile distilled water two times, and transferred to water agar (WA). The mycelia growing out of the plant tissue were sub-cultured to potato dextrose agar (PDA), and incubated at room temperature (approximately 28-30 ^{o}C) for 7-10 days. Single spore isolation was done to obtain a pure culture. The isolate was morphologically identified to species under a compound microscope.

3.2 Pathogenicity test

Pathogenicity was done by the plug inoculation method using a modified protocol from Rattanacherdchai (12). Coffee leaves were surface disinfected with 10 % sodium hypochlorite and left to air-dry in the laboratory. Leaves were wounded by gently pricking with a sterilized needle; two 0.5 cm-diameter wounds were made per leaf, 0.5 cm diameter. Inoculums was prepared by culture on potato dextrose agar (PDA) at room temperature (approximately 28-30 ^{o}C) for 15 days. The agar plugs of the pathogen (0.3cm-diameter) were cut from actively growing areas of the colony by sterilized cork borer, and transferred to the wound sites on the leaves. The control treatment was wounded and inoculated with a PDA plug without the pathogen. The inoculated leaves were maintained in a moist chamber at room temperature. The experiment was done by Completely Randomized Design (CRD) with four replications. Lesion diameter (mm) was recorded and analysis of variance (ANOVA) was computed. Treatment means were statistically compared by Duncan's Multiple Range Test (DMRT) at P =0.05 and 0.01.

3.3 Evaluation of bioactive substances of *Chaetomium* sp. against anthracnose pathogen of coffee var. Arabica

The crude extraction from antagonistic fungi was performed using the method of Kanokmedhakul (6). *Chaetomium cupreum* CC3003 was cultured in PDB at room temperature for 30 days. Fungal biomass was moved from PDB, filtered through cheesecloth and air-dried overnight. Fresh and dried fungal biomass were recorded. Dried fungal biomass was ground with

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an electrical blender, extracted with 200 ml hexane, and shaken for 24 hour at room temperature. The ground fungal biomass was separated by filtration through Whatman No. 4 filter paper. The marc was extracted again with hexane using the method described above. The filtrates were evaporated *in vacuo* to yield crude extracts. The marc was further extracted with ethyl acetate and methanol using the same procedure as hexane. Each crude extract was weighed, and then kept at 5 ^{o}C until use. The crude extracts of *C. cupreum* CC3003 were tested for inhibition of the anthracnose pathogen. The experiment was done by using a 3x6 factorial Completely Randomized Design (CRD) with four replications. Factor A represented crude extracts which consisted of crude hexane, ethyl acetate and methanol extracts and factor B represented extract concentrations of 0, 10, 50, 100, 500, and 1,000 ppm. Each crude extract was dissolved in 2% dimethyl sulfoxide, and mixed into PDA before autoclaving at 121 ^{o}C, 15 lbs/in.^{2} for 30 min. The tested pathogen was cultured on PDA and incubated at room temperature for 5 days and 3 mm-diameter agar plugs were removed from the colony margin using a sterilized cork borer. The agar plug of pathogen was transferred to the middle of a 5.0 cm-diameter PDA plate containing each extract concentration and incubated at room temperature (28-30 ^{o}C) for 4 days. Colony diameter and number of conidia were recorded. Means were computed by ANOVA and compared by DMRT at P□ 0.05 and P□0.01. The effective dose (ED_{50}) was computed by using probity analysis. The morphology of normal and abnormal conidia were observed and recorded under a compound microscope.

3.4 Antagonism of *Chaetomium* sp to control coffee anthracnose *in vitro*

Bi-culture antagonistic test was performed by following the method of Soytong (1992). The experiment was designed in Completely Randomized Design (CRD),with 4 replications. The active peripheral colony of *Chaetomium cupreum* and *Colletotrichum* sp were cut with 3 mm diameter with sterilized cork borer. The agar plug of each fungus was moved to PDA plate at one side (2.0 cm from center) and other into opposite site. For control plates, either agar plug of pathogen or antagonist was placed on PDA plate at center of the medium. The tested plates were incubated at room temperature (28-30 ^{o}C) for 5-7 days or more. Data were collected as colony diameter and spore number of pathogen. Percentage of growth and spore inhibition of pathogen was computed as the following formula: Inhibition (%) = A – B/A X 100, where A = colony diameter or conidial number of pathogen in control and B = colony diameter or conidial number

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of pathogen in dual culture plate. All data were statistically computed for analysis of variance (ANOVA). Treatment means were compared by with Duncan's New Multiple Range Test.

3.5 Testing bio-formulations to control coffee anthracnose in a pot experiment

One-year-old coffee var. Arabica plants were inoculated with a 1×10^{6} spores/ml suspension of the anthracnose pathogen. Ten wounds on leaves/seedling with the fifth leaf from the top; a wound was puncture with sterilized needles 10 times. There after, the following treatments were applied at 15-days intervals:- T1 was inoculation with anthracnose pathogen, T2 was a spore suspension of *C. cupreum* CC3003 at a concentration of 1×10^{6} spore/ml, T3 was a bio-formulation in powder of *C. cupreum* CC3003 at a concentration of 10 g/20 L of water, T4 was nano-trichotoxin-A50 and T5 was Nano-rotiorinol. Nano-trichotoxin-A50 and nano-rotiorinol were produced by Dr. Kasem Soytong and Joselito Dar at the Biocontrol Research Laboratory, Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand, which were designed from bioactive substances of *C. cupreum* CC3003 producing rotiorinol (Kanokmedhakul, *et al.*, 2006), and *Trichoderma harzianum* PC01 producing trichotoxin A50 (Suwan, *et al.*,2000) as previous works.

Data were collected using the following disease severity index: level 1 was no symptoms, level 21-25 % symptoms, level 3 was 26-50%, level 4 was 51-75% and level 5 was over 75%. Disease incidence was assessed at 90 days after treatment based on a disease incidence scale (23) as follows:- 1=no infected leaves, 2=1–25% infected leave per plant, 3=26–50%, 4=51–75% and 5=76–100%. Therefore, disease reduction (%) = disease rating in inoculated control – disease rating in treatment/ disease rating in inoculated control × 100. The experiment was arranged in Randomized Complete Block Design (RCBD) with four replications and five treatments. Means were computed using ANOVA and treatment means were compared using DMRT at P□0.05 and P□0.01.

3.6 Comparison of organic and chemical methods to cultivate coffee Arabica in the field

Field experiment was set up at Paksong, Champasak Province, Lao PDR where seriously natural infested leave anthracnose of coffee var Arabica caused by *Colletotrichum gloeosporiodes*. It was conducted by using Randomized Completely Block Design (RCBD) with 4 replications. Treatments were done as follows:-treatment 1 was non-treated control, treatment 2 was organic methods and treatment 3 was chemical method. With this, organic method were

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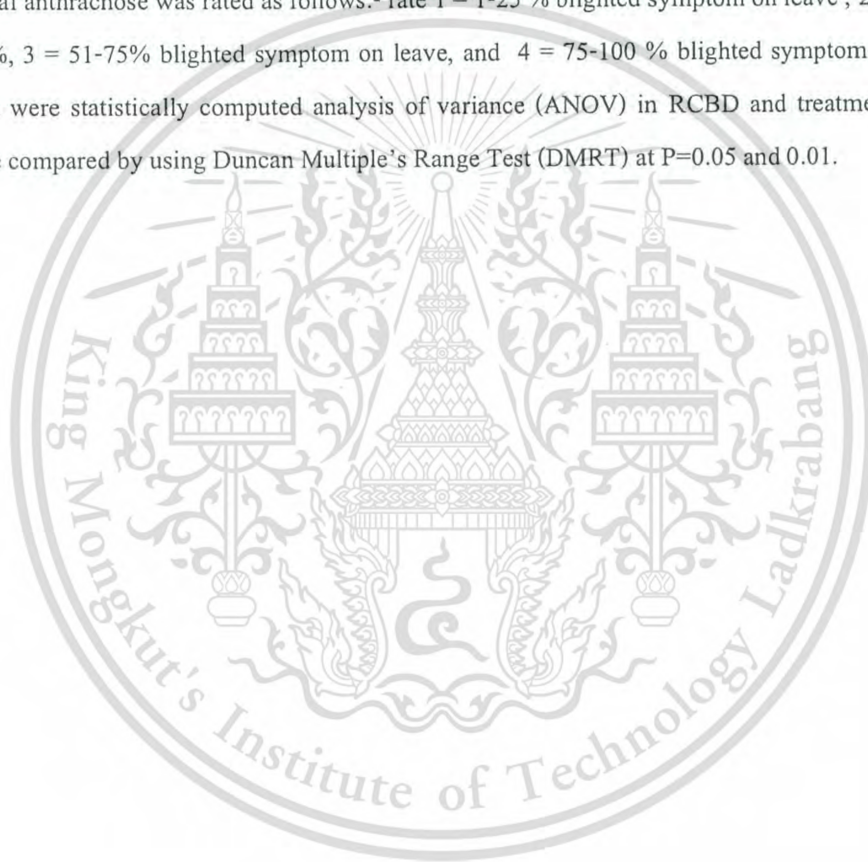
applied all organic certified agricultural inputs as organic fertilizer at the rate of 500 g/tree after harvesting and interval apply at every 2 month for 1 years, spraying liquid organic biofetilizer at 50 cc/ 20 L of water every 30 days until harvest, spraying *Chaetomium*-biofungicide (registered bioproduct at department of Agriculture, Lao PDR) at the rate of 20-40 g/20 L of water and microbial extract to induce immunity at 50 cc/ 20 L of water every 30 days until harvest, spraying bio-insecticide (Metarhizium + Beauveria) at the rate of 50 cc/20 L of water and sulfur power at 10-20 g/20 L of water every 30 days until harvest. Chemical method was applied chemical fertilizers, chemical fungicide and insecticide as recommendation rate as well as chemical herbicide. Data were collected as cherry (g), wet parchment (g) and dried beans (g). Disease index of leaf anthracnose was rated as follows:- rate 1 = 1-25 % blighted symptom on leave , 2 = 26-50 %, 3 = 51-75% blighted symptom on leave, and 4 = 75-100 % blighted symptom on leave. Data were statistically computed analysis of variance (ANOV) in RCBD and treatment means were compared by using Duncan Multiple's Range Test (DMRT) at P=0.05 and 0.01.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CHAPTER IV

RESULTS

4.1 Isolation of coffee anthracnose pathogen

Colletotrichum gloeosporioides was isolated from anthracnose symptom on leaf and coffee beans var. Arabica. Pure culture was morphologically identified (Fig 4.1) in the basis of Sutton (1980) and Bailey and Jeger (1992).

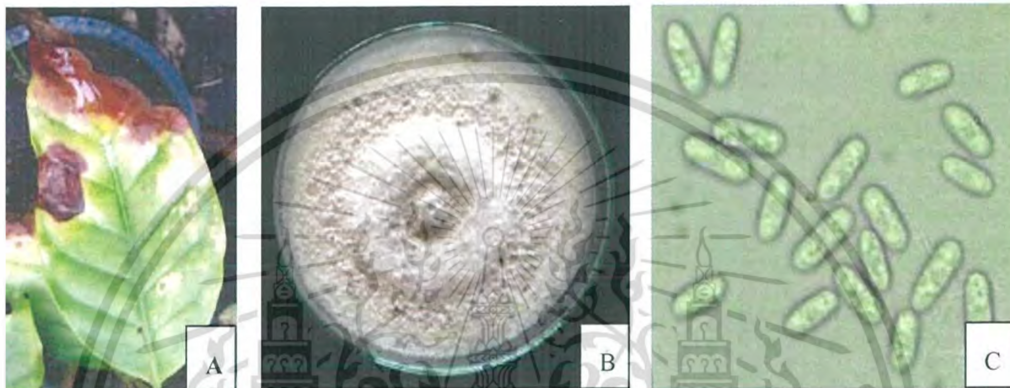

Figure 4.1. Leaf anthracnose of coffee var. Arabica caused by *Colletotrichum gloeosporioides* (A) ; pure culture on PDA at 20 days (B) and conidia, 400 X (C).

4.2 Pathogenicity test

Pathogenicity was proved the isolate was virulent to cause disease on coffee leave. Thenon-inoculated control was no infection and inoculated leaves *Colletotrichum gloeosporioides* was significantly clearly invaded lesion on leaves after 20 days inoculation as seen in (Fig 4.2). It is firstly reported by Vilavong and Soytong (2013) that coffee anthracnose var. Arabica in Lao PDR caused by C. *gloeosporioides*.

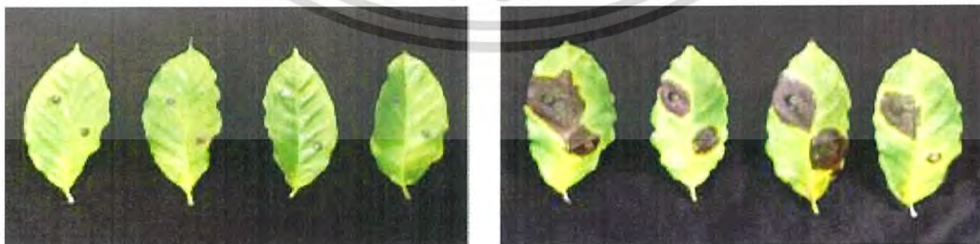

Figure 4.2. Pathogenicity test of *Colletotrichum gloeosporioides* on Arabica variety coffee. The inoculated control (left) and inoculated leaves (right) after 20 days.

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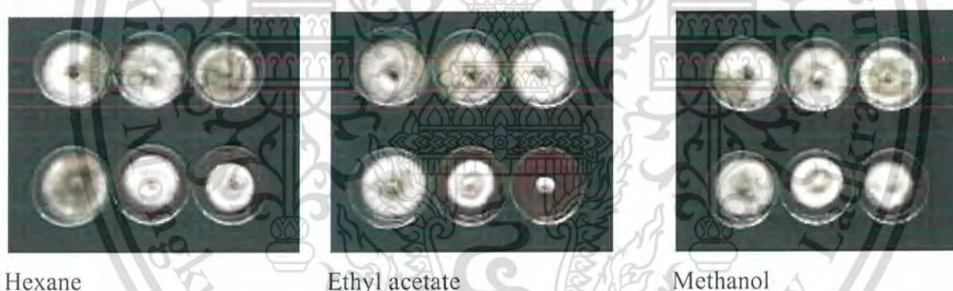
4.3 Evaluation of bioactive substances of *Chaetomium* sp. against anthracnose pathogen of coffee var. Arabica

The results showed that the crude ethyl acetate extract of *C. cupreum* CC3003 showed the highest inhibition of *C. gloeosporioides* with an ED_{50} of 11.03 ppm, followed by the crude hexane and methanol extracts which exhibited ED_{50} of 23.42 and 28.26 ppm, respectively. Colony growth and conidial production were inversely related to bioactive substance concentration. At 1,000 ppm, the crude ethyl acetate extract showed the significantly highest inhibition of spore production of *C. gloeosporioides* (96%), followed by the crude methanol (94 %) hexane extracts (89%) (Table 4.1). The crude ethyl acetate extract significantly inhibited colony growth by 61 %, while the crude hexane and methanol extracts produced a 21 % reduction (Figure 4.3). The bioactive substances of *C. cupreum* CC3003 extracted by hexane, ethyl acetate and methanol clearly showed that antifungal control mechanism was lysis and antibiosis as can be seen in the abnormal appearance of spores under the compound microscope. All concentrations of bioactive substances of *C. cupreum* CC3003 caused abnormal spore morphologies which were apparently related to loss of pathogenicity (Figure 4.4).



Hexane Ethyl acetate Methanol

Figure 4.3. Effects of crude hexane, ethyl acetate and methanol extracts from *Chaetomium cupreum* on the colony growth of *Colletotrichum gloeosporioides*

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Table 4.1. The effect of crude extracts of *Chaetomium cupreum* CC3003 against *Colletotrichum gloeosporioides* causing coffee anthracnose at 7 days after inoculation

Crude extracts	concentration (ppm)	Colony diameter (cm.)	Spore number	% colony inhibition	% spore inhibition	ED_{50} (ppm)
Hexane	0	5.0a[1]	24.3a	0.0i	0.0i	
	10	4.8ab	17.6c	3.7ghi	27.7h	
	50	4.7ab	8.1ef	4.2gh	66.6ef	23.42
	100	4.7bc	3.8gh	5.2fg	84.1bcd	
	500	4.5de	3.0hi	9.8de	87.7abc	
	1,000	3.9g	2.6hi	21.4b	89.2abc	
Ethyl acetate	0	5.0a	21.5b	0.0i	0.0i	
	10	4.8ab	11.1d	3.1ghi	47.6g	
	50	4.5cde	6.5fg	9.0def	69.8e	11.03
	100	4.4e	4.2gh	10.6d	80.4cd	
	500	4.2f	2.8hi	15.5c	86.2abc	
	1,000	1.9h	0.8i	61.5a	96.0a	
Methanol	0	5.0a	24.2a	0.0i	0.0i	28.26
	10	4.9a	16.5c	0.5hi	31.7h	
	50	4.6bcd	10.0de	6.3efg	58.8f	
	100	4.5cde	6.3fg	9.1def	73.8de	
	500	4.1f	2.0hi	17.5c	91.7ab	
	1,000	3.9g	1.3hi	21.9b	94.3ab	
	C.V. (%)	2.27%	14.93%	18.1%	8.85%	

1/ Means of four replication, means in each column followed by a common letter are not significantly different by one way ANOVA and (DMRT) at P□0.01.

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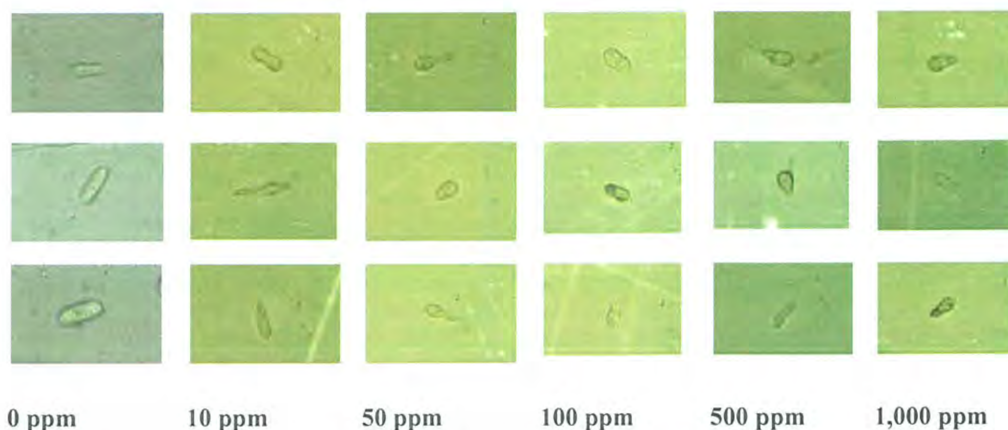

Figure 4.4. Abnormal spore morphologies of *Colletotrichum gloeosporioides* caused by different concentrations of hexane (top), ethyl acetate (middle) and methanol (bottom) crude extracts of *Chaetomium cupreum*.

4.4 Antagonism of *Chaetomium* sp to control coffee anthracnose *in vitro*

A dual culture test was done between effective antagonistic fungus, *C. cupreum* CC3003 (Figure 4.5) and pathogen isolate, *C. gloeosporiodes* showed that in the dual culture plates the colony diameter of pathogen was 9.00 cm in average, while in dual culture plate it was 6.28 cm in average and has a 30% of colony inhibition. (Figure 4.6) It was interesting that in the dual culture plate the average number of pathogen spores was 15.13X10^6 and in the control plate the average was 26.25 X10^6 spores, and the inhibition percentage was 42.60% after incubation for 30 days. (Table2)

Table 4.2. *Chaetomium cupreum* against *Colletotrichum gloeosporiodes* causing anthracnose of coffee in Bi-culture test.

	Control	Bi-culture	GI[2]	C.V. (%)
Colony diameter(cm)	9.00a[1]	6.28b	30.00	3.56%
Spore number (10^6 cfu/ml)	26.25a	15.13b	42.60	15.78

[1/] Means of four replication. Means followed by a common letter are not significantly different by DMRT at P □ = 0.01, [2/] Growth inhibition (GI) = (R1-R2) / R1 × 100 ; R1 = colony diameter of *C. gloeosporiodes* in Control, R2 = colony diameter of *C. gloeosporiodes* in Bi-culture plate.

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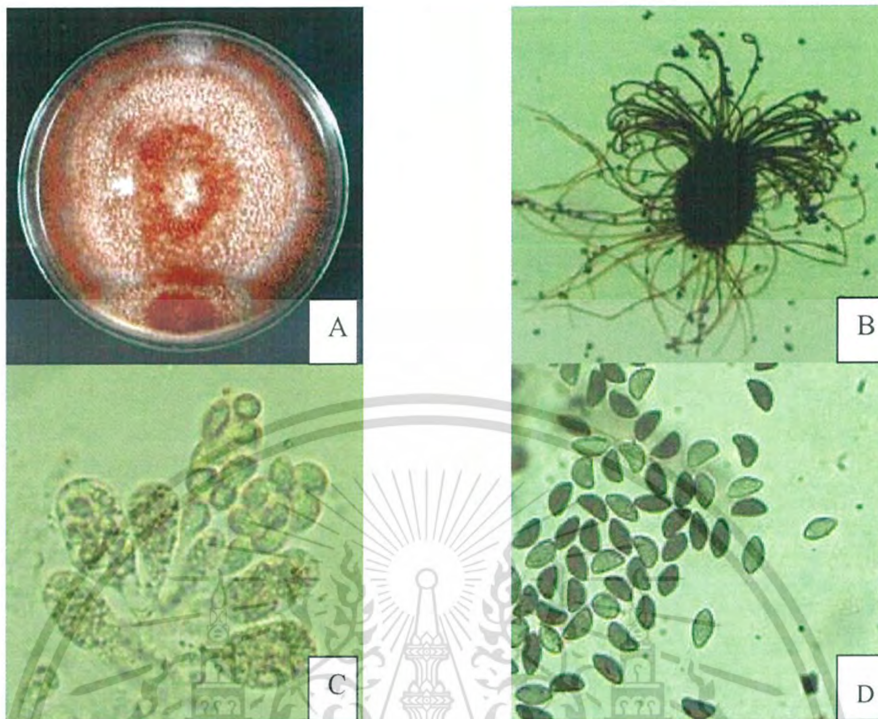

Figure 4.5. *Chaetomium cupreum*, A = Pure culture of *Chaetomium cupreum* on PDA at 30 days , B = Perithecium and Terminal hair (100X),C = Asci (400) and D = Ascospores (400 X)

Figure 4.6. Dual culture test.

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4.5 Testing bio-formulations to control coffee anthracnose in a pot experiment

The results showed that the powder bio-formulation of *C.cupreum* CC3003 at a concentration of 10 g/20 L of water gave the significantly highest control of coffee anthracnose *gloeosporioides* with a disease index of 0.90, followed by nano-rotiorinol, nano-trichotoxin and spore suspension of *C. cupreum* with disease indices of 1.07, 1.14 and 1.62, respectively when compared with the inoculated control (1.99). that the powder bio-formulation of *C. cupreum* CC3003 produced the highest disease reduction of 54.77 %, followed by nano-rotiorinol, nano-trichotoxin and spore suspension of *C. cupreum* CC3003 with disease reductions of 46.23, 42.71 and 18.59 %, respectively when compared with the inoculated control as seen in (Figure 4.7) and (Table 4.3).

Figure 4.7. The effect of bio-formulation of *Chaetomium cupreum* CC3003 coffee anthracnose caused by *Colletotrichum gloeosporioides* at 60 days after inoculation, where T1 was inoculated with *C. gloeosporioides,* T2 was a spore suspension of *C. cupreum* CC3003 at a concentration of $1x10^6$ spore/ml, T3 was a powder bio-formulation of *C. cupreum* CC3003 at a concentration of 10 g/20 L of water, T4 was nano-trichotoxin-A50 and T5 = Nano-rotiotinol

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Table 4.3. Effect of bio-formulation of *Chaetomium cupreum* CC3003 on control of leaf anthracnose caused by *Colletotrichum gloeosporioides* in coffee var. Arabica at 30 days after inoculation

Treatments	Disease index	Disease reduction (%)
Inoculated control	1.99a	0.00
Spore suspension of *C. cupreum*	1.62ab	18.59
Bioformulation of *C. cupreum*	0.90b	54.77
Nano-trichotoxin	1.14b	42.71
Nano-rotiorinol	1.07b	46.23
C.V. (%)	33.39%	-

[1]Means of four replications, means in column followed by a some common letter are not significantly different one way ANOVA by (DMRT) at P□0.01.[2] Disease reduction (%) = disease rating in inoculated control – disease rating in treatment/ disease rating in inoculated control × 100

4.6 Comparison of organic and chemical methods to cultivate coffee Arabica in the field

It was clearly demonstrated that cultivation of coffee var Arabica aged 2 years trees in Paksong, Champasak Province, Lao PDR where seriously natural infested with anthracnose caused by *C. gloeosporiodes* showing that organic method gave significantly better in cherry, wet parchment and dried beans than in chemical method as compared to the non-treated control in the field. Yield was gradually harvested for 6 times, as result the organic method gave highest cherry (2,421 g/tree) and followed by chemical method (1,340 g/tree) when compared to the non-treated control (1,072 g/tree) as seen in (Table 4.4). Organic method resulted to get the highest in wet parchment (1,081 g/tree), and followed by chemical method (490 g/tree) when compared to the non-treated control (436 g/tree), (Table 4.5). It is interested that dried coffee beans (507.25 g/tree) revealed significantly higher than chemical method (213.50 g/tree) and non-treated control (211.50 g/tree) (Table 4.6).The natural infestation of anthracnose disease caused by *C. gloeosporiodes* that infected to leaves, twigs and coffee beans during the experiment were evaluated. Result showed that organic method gave a good disease control which reduced leave anthracnose in organic method of 51.58 % while it reduced disease in chemical method of 33.03 %. as seen in (Table 4.7)

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Table 4.4. Cherry yield (g/tree) in comparison of organic and chemical methods to cultivate coffee Arabica in the field.

Treatments	Harvest 1	Harvest 2	Harvest 3	Harvest 4	Harvest 5	Harvest 6	Total
Non-treated	95b	55b	200a	370a	177a	175b	1,072
Organic	475a	252a	425a	662a	262a	345a	2,421
Chemical	190ab	62 b	275a	650a	82a	90b	1,340
CV(%)	22.41	23.28	29.05	23.17	25.15	25.57	-

1/ Means of four replication, means followed by a common letter are not significantly different by DMRT at P = 0.01.

Table 4.5. Wet parchment yield (g/tree) in comparison of organic and chemical methods to cultivate coffee Arabica in the field

Treatments	Harvest 1	Harvest 2	Harvest 3	Harvest 4	Harvest 5	Harvest 6	Total
Non-treated	44b	24 b	95 a	130 a	68 ab	75 a	436
Organic	197a	106a	198 a	265a	140 a	175 a	1,081
Chemical	38 b	27 b	107 a	180a	37b	101 a	490
CV(%)	20.47	26.78	21.06	23.34	21.61	22.62	-

1/ Means of four replication, means followed by a common letter are not significantly different by DMRT at P = 0.01

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Table 4.6. Dried bean yield(g/tree) in comparison of organic and chemical methods to cultivate coffee Arabica in the field.

Treatments	**Harvest 1**	**Harvest 2**	**Harvest 3**	**Harvest 4**	**Harvest 5**	**Harvest 6**	**Total**
Non-treated	25.25b	12.50b	45.25a	60.00a	31.25ab	37.50ab	211.50
Organic	98.75a	46.25a	98.75a	124.75a	68.75a	70.00a	507.25
Chemical	42.50ab	12.25b	53.00a	80.75a	18.25b	6.25b	213.00
CV(%)	27.05	26.28	24.36	27.09	29.81	26.65	-

1/ Means of four replication, means followed by a common letter were not significantly different by DMRT at P = 0.01.

Table 4.7. Anthracnose disease of coffee Arabica leaves

Treatments	before	After	DR (%)[2]	Bean	DR(%)
Non-treated	1.80 a[1]	2.21 a	---	2.70 a	---
Organic	1.83 a	1.07 c	51.58	1.13 b	58.14
Chemicals	1.71 a	1.48 b	33.03	1.48 b	45.18
CV(%)	8.05	14.36	---	8.59	---

1/ Means of four replication, means followed by a common letter were not significantly different by DMRT at P = 0.01 and Disease reduction (%) = disease rating in non-treated control – disease rating in treatment/ disease rating in non-treated control X 100.

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CHAPTER V

DISCUSSION

Colletotrichum gloeosporioides was isolated from coffee var Arabica leaves and beans symptomatic of coffee anthracnose, identified morphologically and by molecular phylogeny, its pathogenicity was confirmed on that host. We previously reported and confirmed through molecular and morphological tests that anthracnose on coffee var. Arabica in Lao PDR is caused by *C. gloeosporioides* (Bailey and Jeger, 1992; Sutton, 1980, Vilavong and Soytong. 2013). Previous research indicated that crude extracts of *C. cupreum* CC3003 significantly inhibited *C. gloeosporioides* causing anthracnose of mango (Noiaium and Soytong, 1999) chili (Soytong, *et al*. 2001), grape (Soytong *et al*. 2005) and *Fusarium* sp (Soytong, *et al*.2013). *C. cupreum* is reported to produce antagonistic substances that break down the pathogen cells resulting in loss of viability and pathogenicity (Soytong,1992b). Moreover, The mechanism of plant disease control by *C. cupreum* CC3003 involves the production of antibiotics including rotiorinol A, and C, rotiorinol and epi-isochromophilone II that exhibited antifungal activity against *Candida albicans* with IC_{50} values of 10.5, 16.7, 24.3 and 0.6 ppm, respectively .Moreover, *C. cupreum* CC3003 could inhibit several plant pathogens, eg. *Pyricularia oryzae* (Soytong, K. 1992a), *Colletotrichum* spp. (Soytong *et al*. 2005 and Noiaium, and Soytong, 19990, *Fusarium oxysporum*, *Phytophthora palmivora,* (Pechprome and Soytong, 1997). In the current research bio-formulations *C. cupreum* CC3003 showed effective reduction of coffee anthracnose. In addition a microbial elicitor from *Chaetomium* was shown to induce immunity against anthracnose caused by *Colletotrichum capsici* in chili by production of the phytoalexin capsidiol. It is interesting that new roles for nano-rotiorinol and nano-trichotoxin-A50 in plant disease control were also identified. But our research found that nano-rotiorinol and nano-trichotoxin were less effective in disease suppression than the powder bio-formulation *C. cupreum* CC3003. This may due to the concentration used and/or physiological conditions. Further study should examine possible phytoalexin production in coffee by bio-formulations of *C. cupreum* CC3003 in coffee and concentration optimizattion.

Coffee anthracnose was identified and done pathogenicity test in Arabica variety which caused by *Colletotrichum gloeosporioides*. As previous report, the anthracnose pathogen was isolated from coffee leaf and bean symptoms. Morphological and molecular phylogenetic data confirmed the species as *Colletotrichum gloeosporioides* .Bi-culture antagonistic test is clearly

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shown that *Chaetomium cupreum* CC3003 inhibit the colony and spore production of *Colletotrichum gloeosporiodes* causing coffee anthracnose. Similar works had been reported that *Chaetomium* species can be antagonistic against various plant pathogens eg. *Fusarium* and *Helminthosporium* (Tveit and Moore, 1954). *Chaetomium* spp reported to control other anthracnose pathogen like in grape (Soytong et al (2005), mango (Noiaium *et al.* 1999), chill pepper (Ratancherdchai *et al.* 2010). It is possible that *Ch. cupreum* CC3003 expressed control mechanism as antibiosis to inhibit coffee anthracnose in this study. Isolate of *Ch. cupreum* CC3003 used in this study was reported by Kanokmedhakul et al. (2006) who studied that *C. cupreum* CC3003 produced the new azaphilones namely rotiorinols A-C, two new stereoisomers, (-)-rotiorin and a known compound, rubrorotiorin. These compounds exhibited antimicrobial activity against *Candida albicans* with IC_{50} values of 10.5,16.7,24.3, and 0.6 ug/mL, respectively.

It was clearly demonstrated that cultivation of coffee var Arabica trees aged 2 years in Paksong Bolloven Plateau, Champasak Province, Lao PDR where seriously natural infested with anthracnose caused by *Colletotrichum gloeosporiodes* showing that organic method gave significantly better in cherry, wet parchment and dried beans than in chemical method as compared to the non-treated control in the field. As result, organic method were applied powder organic fertilizer, liquid organic biofetilizer, *Chaetomium*-biofungicide, microbial extract to induce immunity, bio-insecticide (Metarhizium + Beauveria) and sulfur power every 30 days until harvest.

In this research findings, coffee var Arabica is successfully cultivated by using organic method when applied those agricultural inputs as mentioned above. It is demonstrated that organic method get the highest in wet parchment and followed by chemical method when compared to the non-treated control and dried coffee beans significantly higher than chemical method and non-treated control. The organic method showed a good disease control which significantly better reduced leave anthracnose in organic method than the chemical method, that the powder bio-formulation of *C. cupreum* significantly resulted to reduce anthracnose disease of 54.77 %. The application of nano-rotiorinol, nano-trichotoxin and a spore suspension of *C. cupreum* reduced anthracnose incidence of 46.23, 42.71 and 18.59 %, respectively ,while the inoculated control had high anthracnose disease. The application of bioformulation of *C. cupreum* in powder form, nanorotiorinol, and nano-trichotoxin to reduce coffee anthracnose.

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CHAPTER VI

CONCLUSIONS

Coffee anthracnose pathogen was isolated from leaves and beans of coffee and identified *as Colletotrichum gloeosporioides*, then proved for pathogenicity. Bioactive substances as crude hexane, crude ethyl acetate and crude methanol from *Chaetomium cupreum* CC3003 showed good efficacy to inhibited *C. gloeosporioides.* which he ED_{50} values were 13, 11 and 28 ppm. Bio-fomulation in powder of *Ch. Cupreum* gave the highest disease reduction of 54.77 %, and followed by nano- *Chaetomium,* nano- trichotoxin and spore suspension of *Ch. cupreum* which disease reduction were 46.23,42.71 and 18.59 %, respectively when compared with the inoculated control. It is the first report on bio-control of coffee anthracnose in Lao PDR using bio-formulation and nano-materials of *Ch. Cupreum* to control *C. gloeosporioides* causing coffee anthracnose in Arabica variety.

Colletotrichum gloeosporioides is proved to be pathogenic isolate causing anthracnose disease of coffee var Arabica in Lao PDR. *Chaetomium cupreum* CC3003 inhibited sporulation of *C. gloeosporiodes* by 42.60 % in 30 days. The tested nanoCCH, nanoCCE and nanoCCM derived from *C. cupreum* CC3003 significantly inhibited C. *gloeosporioides* causing coffee anthracnose at low concentrations if 3-15 ppm. The tested nano-particles applied to inoculated coffee seedlings significantly reduced coffee anthracnose. Research and development on nanoparticles constructed from fungi are needed to search new strategy for plant disease control. *Chaetomium cupreum* CC3003 was antagonized *Colletotrichum gloeosporioides* causing anthracnose of coffee in bi-culture test which inhibited colony growth of 29.89 % and inhibited spore production of 38.61 % at 15 days incubation. It is clearly demonstrated that cultivation of coffee var Arabica aged 2 years trees in Paksong Boloven Plateau, Champasak Province, Lao PDR where seriously natural infested with anthracnose showing that organic method gave significantly better in cherry, wet parchment and dried beans than in chemical method as compared to the non-treated control in the field. Yield was gradually harvested for 6 times which resulted organic method was highest cherry and followed by chemical method when compared to the non-treated control. Organic method gave the highest in wet parchment and followed by chemical method when compared to the non-treated control. The dried coffee beans resulted significantly higher than chemical method and non-treated control. Moreover, organic method resulted a good disease control which reduced leaf anthracnose in organic method of 51.58 % while it reduced disease in chemical method of

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33.03 %.Coffee bean anthracnose was reduced in organic method of 58.14 % while reduced in chemical method of 45.18 % .

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4.1.1 Somlit, V and K. Soytong. 2014. Application of *Chaetomium cupreum* for biocontrol of *Colletotrichum coffeanum* causing coffee anthracnose in Arabica verities in Laos. The 3th International Conference on Integration of Science and Technology for Sustainable Development (ICIST 2014). November 28-29, 2014, Pakse district Champasak province LAO PDR.

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4.2.Reseach publication

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APPENDIX

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Application of A New Bio-Formulation of *Chaetomium cupreum* For Biocontrol of *Colletotrichum gloeosporioides* Causing Coffee Anthracnose on Arabica Variety in Laos

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ABSTRACT

The anthracnose pathogen was isolated from coffee leave and bean symptoms. Morphological and molecular phylogenetic data confirmed the species as *Colletotrichum gloeosporioides*. The pathogenicity of the isolate was also confirmed by detached leaf method which inoculated the virulent isolate into coffee leaves. The crude extracts with hexane, ethyl acetate and methanol solvents from *Chaetomium cupreum* CC3003 resulted significantly inhibited *C. gloeosporioides* that the ED_{50} values of 13, 11 and 28 ppm, respectively. The bioactive substances of *C. cupreum* CC3003 expressed antifungal activity against *C. gloeosporioides* as can be seen in the abnormal appearance of spores. A powder bio-formulation of *C. cupreum* significantly resulted to reduce anthracnose disease of 54.77 %. The application of nano-rotiorinol, nano-trichotoxin and a spore suspension of *C. cupreum* reduced anthracnose incidence of 46.23, 42.71 and 18.59 %, respectively while the inoculated control had high anthracnose disease. The application of bio-formulation of *C. cupreum* in powder form, nano-rotiorinol, and nano-trichotoxin to reduce coffee anthracnose was reported for the first time in Lao PDR.

Keywords: anthracnose; coffee; nano-elicitors; plant immunity

INTRODUCTION

Coffee var. Arabica is distributed in Laos to the growers in the uplands in 1920 which the sea level elevation of 800 and 1,200 m (Clifford & Helm, 1985). Coffee becomes one of the primary sources of income and export product of 50 coffee producing countries in Latin America, Africa and Asia (Ridler, 1983). Coffee production is the most important perennial crop in the Lao People's Democratic Republic (PDR), mainly grown in the Phouphieng Paksong area. There are 41,000 ha of coffee plantations, especially in the Saravan, Sekong, Champasak provinces in the Lao PDR. The government has promoted the production of this crop to support local and international needs (Ministry of Agriculture and Forestry, 1997). However, the export price of coffee is 10 % lower than the international market price due to its lower quality, poor agronomic practices and early and manual harvest processing for drying. The important reason for poor quality is limited knowledge by coffee growers to improve quality, control of insects and diseases on coffee beans. The coffee growers have been repeatedly applied chemical fungicides to eliminate anthracnose disease and the pathogen become resistant to chemical fungicides (Soytong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001).

Application of biocontrol agents for disease control has increasingly extended to plant pathologists. The effective biocontrol agents can reduce the pathogen inoculum and disease incidence. *Chaetomium* was reported as biological fungicide to control several plant pathogens. The need to find alternative effective methods of disease control to safe human health and protect the environment is evident. Nanotechnology involves the building and re-structuring materials at the molecular level. Molecular nanotechnology refers to build up the organic or inorganic particles into defined nanometers by molecule (Li, Huang, & Wu, 2011). Agricultural applications of nanotechnology are recently explored and interested by growers (Soutter, 2012). Plant cells are easily and vastly absorbed the nanoparticles through cuticles and tissues (Ditta, 2012). The nanospheres, and nanocapsules are the popular shapes of nano-particles to be used for biocide (Perlatti, de Souza Bergo, Fernandes da Silva, Fernandes, & Forim, 2013).

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This approach enables safe, economical, effective and rapid disease control in crop production (Soutter, 2012).The metabolites from *Chaetomium* spp. were published to control the plant pathogens (Soytong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001). Previous research stated that nano-particles loaded with active metabolites of *C. cupreum* with polylactic acid and electropun at 25-30 kV showed pale orange color. The nano-particle size from *C. cupreum* is averaged as 171 nanometers after loaded in scanning electron microscope. Moreover, nanomaterial containing bioactive metabolites from *Trichoderma harzianum* PC01 at 5-10 ppm exhibited antifungal activity against *C. capsici* causing chili anthracnose (Dar & Soytong, 2014). The current research aimed to isolate the causal agent of coffee anthracnose and evaluate bio-formulations to control anthracnose of coffee var. Arabica.

MATERIALS AND METHODS

Coffee Anthracnose Pathogen and Pathogenicity Test

The causal agent isolated from anthracnose symptoms on leaf and bean of coffee var. Arabica by the tissue transplanting method. The advanced margin of symptom between disease and healthy parts were cut to small pieces (5 × 5 mm), and surface disinfected with 10 % clorox for 1 minute, washed in sterilized water for two times, and moved onto water agar (WA). The hyphal tips growing from infected tissue were moved by sterilized needle and placed onto potato dextrose agar and incubated at 28-30 °C for 7 days. Pure culture derived from single spore isolation techniques. The isolate was morphologically and molecular phylogenic identified into species level. The molecular phylogenic identification was conducted by DNA extraction using polymerase chain reaction (PCR). The extraction of genomic DNA from culture growing in potato dextrose broth (PDB) was done with DNA easy Plant Mini kit (Qiagen, Hilden, Germany). PCR amplification of the internal transcribed spacer, ITS1 5.8S and ITS2 regions was used the primer pairs PN3 and PN16 (modified from Neuvéglise, Brygoo, Vercambre, & Riba, 1994). The 25 µl volumes contained 1 µl genomic DNA, 0.5 µl dNTPs, 1 µl of each primer and 0.2 µl *Taq* DNA polymerase in 2.5 µl buffer were reacted. The initial denatured step of 95 °C for 5 minutes, 35 cycles of 94 °C for 1 minutes, 60 °C for 2 minutes, then 72 °C for 3 minutes and a final extension of 72 °C for 5 minutes were amplified. The separation of PCR products were shown in agarose gel to yield the purified for DNA for sequencing by the U-gene gel Extraction Kit II (U-gene Biotechnology Co., P.R. China). The related species based on the ITS including 5.8S gene of DNA sequencing database was retrieved from GenBank.

Pathogenicity was done by the plug inoculation method. The healthy coffee leaves were sterilized by 10 % sodium hypochlorite and air-dried. The surface sterilized leaves were made wounds by a sterilized needle, 0.5 cm-diameter wound was done per leaf. PDA medium was used to culture the pathogen and incubated at room temperature for 15 days. The plugs of the pathogen (0.3 cm-diameter) were cut from peripheral colony by sterilized cork borer, and placed to the wounds on the leaves. The control was wounded and transferred a PDA plug without the pathogen. The leaves were inoculated that kept in moist box at room temperature. The experiment was done using Completely Randomized Design (CRD) and repeated 4 times. Data was recorded as lesion diameter (mm). The statistical analysis was computed analysis of variance. Duncan's Multiple Range Test (DMRT) at P =0.05 and 0.01 was used to compare the different.

Evaluation Bioactivity from *Chaetomium* sp. Against Coffee Anthracnose Pathogen

The crude extracts of *Chaetomium cupreum* was prepared as the method of Kanokmedhakul et al. (2006). *Chaetomium* sp. was cultured in PDB at 28-30 °C for 30 days and filtered through cheese cloth to get the specimen and dried overnight. Then, specimen was put into an electrical blender to get ground specimen. It was processed to extract in a solvent of 200 ml hexane, shaken for 24 hours, then filtrated with filter paper to get filtrate. The extracted marc with hexane was serially also done with the solvents of hexane and methanol to get crude extracts. Data were collected as fresh and dry weight (g), and kept in refrigerator at 5 °C for experiment. All crude extracts were evaluated to inhibit the tested pathogen. The experiment was designed as 3 x 6 factor factorial CRD, and repeated four times. Factor A was hexane, ethyl acetate and methanol crude extracts of *C. cupreum*. Factor B was 0, 10, 50, 100, 500 and 1,000 ppm. The 2 % dimethyl sulfoxide used to dissolve each crude extract in PDA, then sterilized in autoclave for 30 minutes. The colony margin of pathogen culture at 5

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days was cut by a sterilized borer (3 mm-diameter) to get agar plugs. The pathogen's agar plug was moved to the center of a 5.0 cm-diameter PDA plate, and maintained at 28-30 °C room for 4 days. Data collection was done as diameter of colony and count the conidia using haemacytometer. Probit analysis was computed the effective dose (ED_{50}). Thenormal and abnormal conidia were recorded under a binocular compound microscope.

Testing Bio-formulations to Control Coffee Anthracnose in Pot Experiment

One-year-old coffee var. Arabica plants were inoculated with a 1×10^{6} spore ml^{-1} suspension of the anthracnose pathogen. Ten wounds on leaves/seedling with the fifth leaf from the top, a wound was puncture with sterilized needles 10 times. The experiment was designed as Randomized Complete Block Design (RCBD) and repeated four times. The following treatments were applied at 15-day intervals. T1 was inoculation with anthracnose pathogen. T2 was a spore suspension of *C. cupreum* CC3003 at a concentration of 1×10^{6} spore ml^{-1}. T3 was a bio-formulation in powder of *C. cupreum* CC3003 at a concentration of 10 g 20 L^{-1} of water. T4 was nano-trichotoxin-A50 and T5 wasnano-rotiorinol. Nano-trichotoxin-A50 andnano-rotiorinol were produced by Dr. Kasem Soytong and Joselito Dar at the Biocontrol Research Laboratory, KMITL, Bangkok, Thailand, which designed from metabolites of *C. cupreum* CC3003 producing rotiorinol (Kanokmedhakul et al., 2006) and *Trichoderma harzianum* PC01 producing trichotoxin A50 (Suwan et al., 2000) as previous works. The data were statistical analyzed and compared treatment difference by DMRT.

Disease index (DI) scored as level 1 was no symptoms, level 2 was 21-25 % blight, level 3 was 26-50 % blight, level 4 was 51-75 % blight and level 5 was over 75 % blight.

Reduction of disease (%) was calculated as

$$\frac{\text{DI in inoculated control} - \text{DI in treatment}}{\text{DI in inoculated control}} \times 100$$

RESULTS AND DISCUSSION

Coffee Anthracnose Pathogen and Pathogenicity Test

Colletotrichum sp. designated as "CC" was isolated from coffee leaves and beans of var. Arabica exhibiting anthracnose symptoms (Fig. 1). The pathogenicity of the isolate was confirmed, the isolate produced typical anthracnose symptoms on coffee leaves 20 days after inoculation (Fig. 2).

The non-inoculated leaves remained anthracnose-free. The molecular phylogeny of the coffee anthracnose pathogen was determined the species of *Colletotrichum* isolate CC as *Colletotrichum gloeosporioides*. *Chaetomium globosum* was compared as the outgroup. Bio Edit, version 7.0.2 was used for sequencing assembly, aligned with Clustal X, version 1.83. The phylogenetic tree was performed a heuristic search by using neighbour joining. The search was done by using PAUP* 4.0b8 (Fig. 3). The previously reports were confirmed through molecular and morphological characters that anthracnose on coffee var. Arabica in Laos was caused by *C. gloeosporioides* (Bailey & Jeger, 1992; Sutton, 1980).

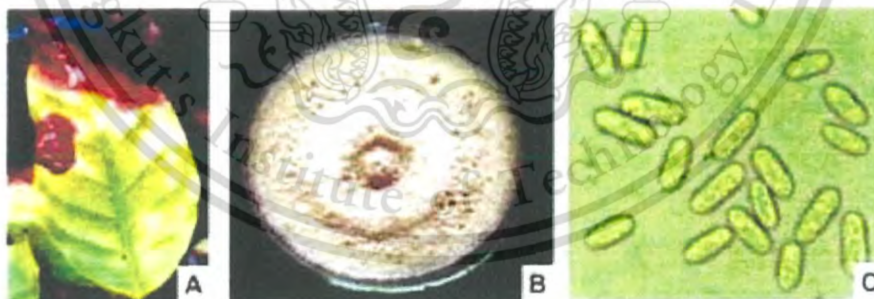

Fig. 1 Leaf anthracnose of coffee var. Arabica caused by *Colletotrichum gloeosporioides* (A), pure culture on PDA at 20 days (B), and conidia, 400 X (C)

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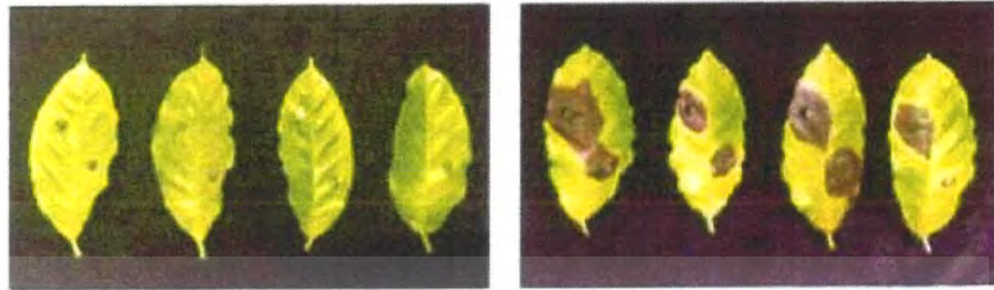

Fig. 2. Pathogenicity test of *Colletotrichum gloeosporoides* on Arabica variety coffee. The inoculated control (left) and inoculated leaves (right) after 20 days

Fig. 3. Phylogenetic tree of *Colletotrichum gloeosporoides* isolate CC from coffee anthracnose compared to GenBank data. Distance-based analysis construction of the ITS1 and 5.8S regions of rDNA. Numbers at the branches indicated the percentage of bootstrap values after 1000 replications. *Chaetomium globosum* was out group.

Evaluation Bioactivity from *Chaetomium* sp. against Coffee Anthracnose Pathogen

The results showed that the crude ethyl acetate extract of *C. cupreum* CC3003 showed the highest inhibition of *C. gloeosporoides* with an ED_{50} of 11.03 ppm, followed by the crude hexane and methanol extracts which exhibited ED_{50} of 23.42 and 28.26 ppm, respectively. Colony growth and conidial production were inversely related to bioactive substance concentration. Crude ethyl acetate extract at 1,000 ppm showed significantly highest inhibited sporulation of *C. gloeosporoides* (96 %), followed by the crude methanol (94 %) hexane extracts (89 %) (Table 1). The crude ethyl acetate extract significantly inhibited colony growth by 61 %, while the crude hexane and methanol extracts produced a 21 % reduction (Fig. 4).

Previous research indicated that crude extracts of *C. cupreum* CC3003 significantly inhibited *C. gloeosporoides* causing anthracnose of chili (Soytong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001). The bioactive substances of *C. cupreum* CC3003 extracted by hexane, ethyl acetate and methanol clearly showed that antifungal control mechanism was lysis and antibiosis as can be seen in the abnormal appearance of spores under the compound microscope. All concentrations of bioactive substances of *C. cupreum* CC3003 caused abnormal spore morphologies which were apparently related to loss of pathogenicity (Fig. 5). *C. cupreum* was reported to produce antagonistic substances that break down the pathogen cells resulting in loss of viability and pathogenicity (Heye & Andrews, 1983). Moreover, the mechanism of plant disease control by *C. cupreum* CC3003 involves the production of antibiotics including rotiorinol A, and C, rotiorinol and epi-isochromophilone II that expressed to inhibit the growth of *Candida albicans* which IC_{50} were 10.5, 16.7, 24.3 and 0.6 ppm, respectively (Kanokmedhakul, et al., 2006; Soytong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001). Moreover, *C. cupreum* CC3003 is reported to inhibit *C. capsici* causing chilli anthracnose (Ratanacherdchai, Wang, Lin, & Soytong, 2010) and other antagonistic *Chaetomium* sp reported to inhibit *Botrytis cinerea* (Kohl, Molhoek, van der Plas, & Fokkema, 1995) and *Helminthosporum victoriae* (Tveit & Moore, 1954).

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Table 1. The effect of crude extracts of *Chaetomium cupreum* CC3003 against *Colletotrichum gloeosporioides* causing coffee anthracnose at 7 days after inoculation

Crude extracts	Concentration (ppm)	Colony diameter (cm)	Spore number	% colony inhibition	% spore inhibition	ED_{50} (ppm)
Hexane	0	5.0a	24.3a	0.0i	0.0i	
	10	4.8ab	17.6c	3.7ghi	27.7h	
	50	4.7ab	8.1ef	4.2gh	66.6ef	23.42
	100	4.7bc	3.8gh	5.2fg	84.1bcd	
	500	4.5de	3.0hi	9.8de	87.7abc	
	1000	3.9g	2.6hi	21.4b	89.2abc	
Ethyl acetate	0	5.0a	21.5b	0.0i	0.0i	
	10	4.8ab	11.1d	3.1ghi	47.6g	
	50	4.5cde	6.5fg	9.0def	69.8e	11.03
	100	4.4e	4.2gh	10.6d	80.4cd	
	500	4.2f	2.8hi	15.5c	86.2abc	
	1000	1.9h	0.8i	61.5a	96.0a	
Methanol	0	5.0a	24.2a	0.0i	0.0i	
	10	4.9a	16.5c	0.5hi	31.7h	
	50	4.6bcd	10.0de	6.3efg	58.8f	28.26
	100	4.5cde	6.3fg	9.1def	73.8de	
	500	4.1f	2.0hi	17.5c	91.7ab	
	1000	3.9g	1.3hi	21.9b	94.3ab	
	C.V. (%)	2.27%	14.93%	18.1%	8.85%	

Remarks: Statistical analysis and treatment means of four replications in each column are shown significantly different with common letters by DMRT at P = 0.01

Fig. 4. Effects of metabolites of *Chaetomium cupreum* on the colony growth of *Colletotrichum gloeosporioides*

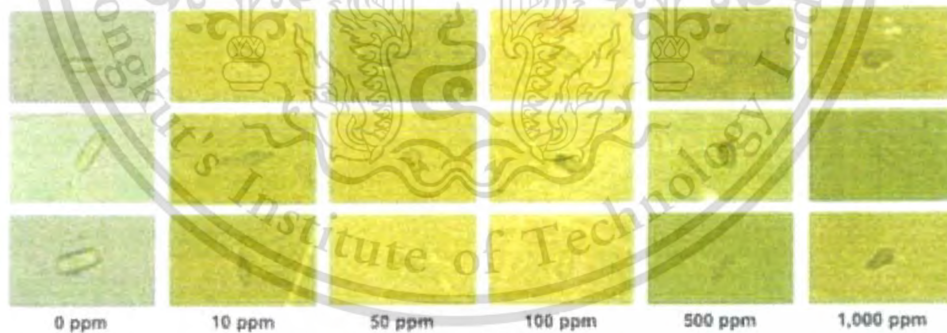

Fig. 5. Abnormal spore morphologies of *Colletotrichum gloeosporioides*. Crude hexane (top), crude ethyl acetate (middle) and crude methanol (bottom) of *Chaetomium cupreum*

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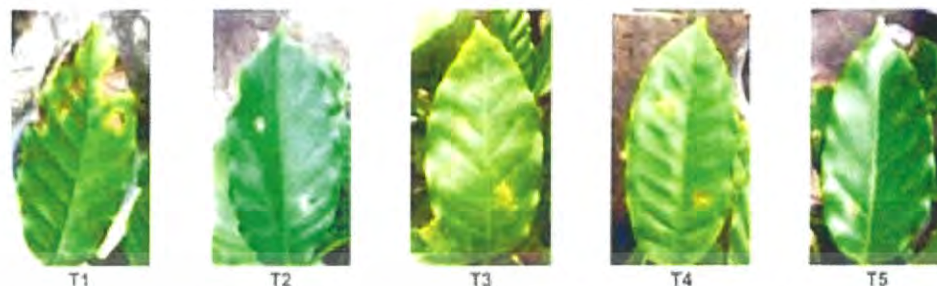

Fig. 6. The effect of bio-formulation of *Chaetomium cupreum* to control coffee anthracnose caused by *Colletotrichum gloeosporioides* at 60 days after inoculation, where T1 was inoculated with *C. gloeosporioides*, T2 was a spore suspension of *C. cupreum* CC3003 (1 x 10^6 spore ml^{-1}), T3 was a powder bio-formulation of *C. cupreum* (10 g 20 L^{-1} of water), T4 was nano-trichotoxin-A50 and T5 = Nano-rotiotinol

Table 2. Effect of bio-formulation of *Chaetomium cupreum* CC3003 on control of leaf anthracnose caused by *Colletotrichum gloeosporoides* in coffee var. Arabica at 30 days after inoculation

Treatments	Disease index	Disease reduction (%)[2]
Control	1.99a[1]	0.00
Spores(*C. cupreum*)	1.62ab	18.59
Bioformulation (*C. cupreum*)	0.90b	54.77
Nano-trichotoxin	1.14b	42.71
Nano-rotiorinol	1.07b	46.23
C.V. (%)	33.39%	-

Remarks: Statistical analysis and treatment means of four replications in column was shown significantly different with common letters by DMRT at P = 0.01. Disease reduction (%) = DI in inoculated control – DI in treatment/ DI in inoculated control × 100

Testing Bio-formulations to Control Coffee Anthracnose in Pot Experiment

The powder bio-formulation of *C. cupreum* CC3003 at a concentration of 10 g 20 L^{-1} of water gave the significantly highest to control coffee anthracnose caused by *C. gloeosporioides* with a disease index (DI) of 0.90.Thenano-rotiorinol, nano-trichotoxin and spore suspension of *C. cupreum* showed DI of 1.07, 1.14 and 1.62, respectively when compared to the control (1.99). With this, the powder bio-formulation of *C. cupreum* CC3003 revealed the highest disease reduction of 54.77 %. The application of nano-rotiorinol reduced anthracnose of 46.23 %. Thenano-trichotoxin and spore suspension of *C. cupreum* reduced the disease of 42.71 and 18.59 % when compared to the control (Fig. 6 and Table 2). In the current research bio-formulations *C. cupreum* CC3003 showed effective reduction of coffee anthracnose. In addition, a microbial elicitor from *Chaetomium* was shown to induce immunity against anthracnose caused by *Colletotrichum capsici* in chili by production of the phytoalexin (Soytong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001). It is interesting that new roles for nano-rotiorinol and nano-trichotoxin-A50 in plant disease control were also identified. But our research found that nano-rotiorinol and nano-trichotoxin were less effective in disease suppression than the powder bio-formulation *C. cupreum* CC3003. This may due to the concentration used and/or physiological conditions. Further study should examine possible phytoalexin production in coffee by bio-formulations of *C. cupreum* CC3003 in coffee and concentration optimization.

CONCLUSION

The molecular phylogeny of the coffee anthracnose pathogen was determined the species of *Colletotrichum* isolate CC to be *C. gloeosporioides*. The pathogenicity of the isolate was confirmed, the isolate produced typical anthracnose symptoms on coffee leaves for 20

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days after inoculation. Crude ethyl acetate extract at 1,000 ppm resulted the highest spore inhibition of *C. gloeosporioides* (96 %), followed by the crude methanol (94 %) crude hexane extracts (89 %). The bioactive substances of *C. cupreum* showed antifungal control mechanism as can be seen in the abnormal appearance of spores. In pot experiment, the powder bio-formulation of *C. cupreum* showed the highest disease reduction (54.77 %). The testednano-rotiorinol, nano-tricholoxin and spore of *C. cupreum* reduced anthracnose 46.23, 42.71 and 18.59 %, respectively, when compared to the control.

ACKNOWLEDGEMENT

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Potenza, 13 July 2018

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Date: 13 July 2018
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We are pleased to inform you that your paper entitled **"Nano-particle derived from chaetomium cuprem cc3003 against Anthracnose of Coffee var.Arabica"** has been accepted for publication in our journal and will be included in its forthcoming issue (Volume 9, Issue 1, 2018) with its assigned doi: 10.4081/pb.2018.7736).

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Nano-particle derived from *chaetomium cuprem* cc3003 against Anthracnose of Coffee var.Arabica

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Keywords: coffee anthracnose, nano-particles, *Chaetomium*

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Contribution :Mr Somlit Vilavong performed most of experimental work, data collecting and analyzing, manuscript writing, supervised experimental work in laboratory, helped correct the paper, performed the English language of the paper.

Disclosure statement : No potential conflict of interest was reported by the authors

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Abstract

Colletotrichum gloeosporioides is proved to be a pathogenic isolate causing anthracnose disease on coffee var.Arabica in Lao PDR. *Chaetomium cupreum* CC3003 inhibits sporulation of *C. gloeosporiodes* by 42.60 % in 30 days. The tested nano CCH, nano CCE and nanoCCM derived from *C. cupreum* CC3003 significantly inhibits *C. Gloeosporioides* that cause coffee anthracnose at low concentrations of about 3-15 ppm. The tested nano-particles applied to inoculated coffee seedlings significantly reduce coffee anthracnose. Research and development on nano-particles extracted from fungi are necessary to discover new strategies to control plant disease.

I. Introduction

The Lao People's Democratic Republic is located in Southeast Asia. The country predominantly depends on rural economy, with about 83% of the population living in the rural areas and some 66% relying on subsistence agricultureMAF.[1]Economically, coffee is one of the most important crops that resulted from the horticultural skills of the Dutch.[2] Coffee is distributed to many places around the world.[3] As a result, coffee is now one of the most important exported commodities from approximately 50 countries in Latin America, Africa, Asia and Lao PDR.[4,5] Coffee anthracnose caused by *Colletotrichum gleosprorioides* was confirmed by molecular phylogeny that invaded coffee beans and leading to economic damage of coffee plantations in Lao PDR .Crude extracts from *Chaetomium cupreum* CC3003 inhibited *C. gloeosporioides*. The bio-formulation of *C. cupreum* decreased the incidence ofanthracnose.[6]In recent years, nanotechnology has started to investigate plant disease control measures for food safety concerns in agriculture.[7] Within creasing nanotechnology for plant disease protections that have been developed by some researchers, nano-particles from natural products may become the new approach for plant disease control. Nanotechnology in crop production is to build up the materials at the molecular level into nano particles.[8] Some scientists have been investigating organic nano materials and different kinds of nano-particles for biological properties.[9,10]Nano formulations have been started to produce from natural products to control insects.[4] Bioactive compounds from natural products derived from *C. cupreum* CC3003 were investigated to be active against several plant pathogens.[11] The objective of the research was to study the nano-particles from *C. cupreum* CC3003 to control anthracnose of coffee var. Arabica.

II. Materials and methods

2.1. Culture of pathogen and *Chaetomium cupreum* CC3003

Infected Coffee fruits by anthracnose of the Arabica variety was collected from Paksong Highland, Champasak Province, Lao PDR. Pure

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cultures of *Colletotrichum gloeosporioides* and *Chaetomium cupreum* CC3003 were isolated from both leaves and fruits and results were confirmed using molecular phylogenic.[6] They were separately transferred onto potato dextrose agar (PDA), incubated at temperature between 27-35 °C for 15 days. Morphological developments were recorded and photographed.

2.2. Pathogenicity tests

Pathogenicity tests were done by detached leaf method. The agar plug of pathogen was inoculated into wounded leaf and kept in a moist chamber. An agar plug of PDA alone onto wounded leaf was served as a control. Lesion size (mm) was recorded. The experiment was repeated four times.

2.3. Dualculture test

Chaetomium cupreum CC3003 produce antibiotic substances used in this study as reported by Kanoknedhakul *et al.*[12] An agar plug of 0.5 cm with mycelia of *C. cupreum* CC3003 was moved to one side of PDA plate and the other agar plug of pathogen was placed in the opposite side at an equal distance from each other in the tested plates. Control plates were cultivated either with pathogen or with *C. cupreum* CC3003 alone. The colony diameter (cm) and number of pathogen spores in dual culture plate and control plate were counted. The experiment was performed using CRD (Completely Randomized Design), and compared treatment means using DMRT(Duncan's Multiple Range Test). It was repeated four times.

2.4. Effect of nano-particles derived from *Chaetomium cupreum* CC3003 against *Colletotrichum gloeosporiodes* in 7 days

The nano-particles used in this research finding derived from *C. cupreum* CC3003 were 171 nano meters in average.[13] The experiment was performed using a Completely Randomized Design (CRD) with 2 factors. Different kinds of nano-particles, nano-CCH, nano-CCe and nano-CCM were investigated with different concentrations of 0, 3, 5, 10 and 15 ppm. The experiment was repeated four times. Colony diameter and number of spores were collected and a computer analysis of variance was performed, then compared with Duncan Multiple Range Test (DMRT). The effective dose at 50% was calculated using probity analysis program. The normal and abnormal spores from each treatment were also recorded.

2.5. Evaluation of bio formulation produced from spores of *Chaeomium cupreum* CC3003 and nano-particles to control anthracnose of coffee var Arabica in pot experiment

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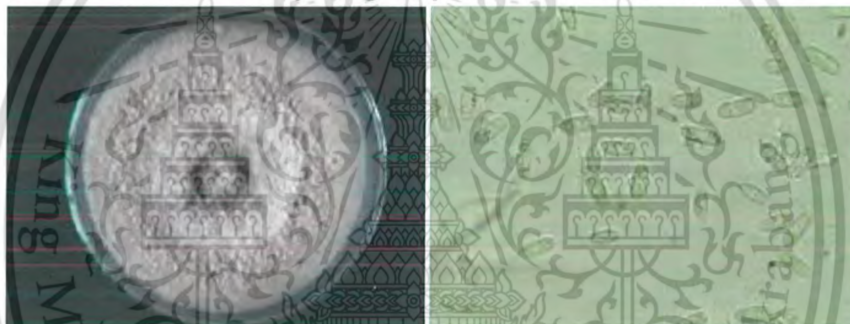
The six months seedlings of coffee va Aribaca was used to inoculate *C. gloeosporioides* on wounded leaves at a concentration of 1x106 spores/ml, each wound inoculated with1 ml. *Chaetomium*-bio formulation was prepared according to the method of Soytong *et al.*[11] and applied at the rates of 10 g and 20 g/20 water and nano-particles 15 ppm., then the wounded lesions were sprayed after inoculation for 24 hours. Lesion size was recorded and calculated using ANOVA and DMRT comparison tests. The experiment was tested using RCBD and repeated four times.

III. Results

3.1. Culture of pathogen

C. gloeosporioides causing anthracnose of coffee was cultured on PDA for 15 days and the observed colony had a greyish white colour , and a single cell of conidia (Figure 1).

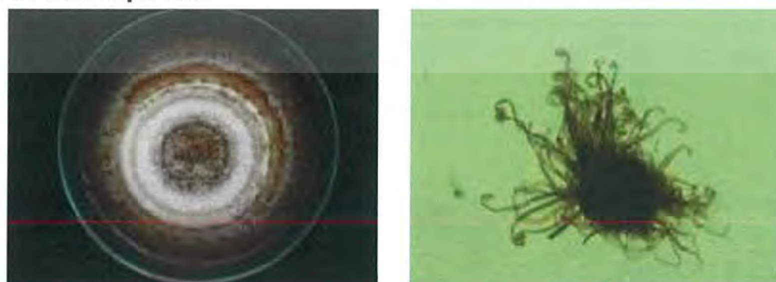
Figure 1. Culture of *Colletotrichum gloeosporioides* causing anthracnose of coffee on PDA for 15 days, right = pure culture and left = conidia



3.1.1. *ChaetomiumcupreumCC3003*

The isolate was cultured on PDA for 3 weeks to produce perithecia, as and ascospores (Figure 2).

Figure 2. Culture of *Chaetomium cupreum* CC3003 on PDA for 3 weeks, upper right = pure culture, upper left = perithecium, lower right = as and lower left = ascospores



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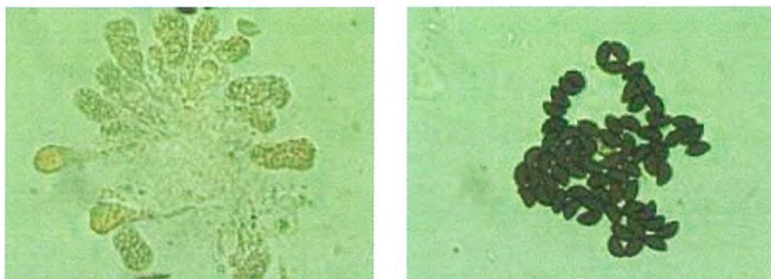

A pathogenicity test was completed to reconfirm the pathogenic isolate of Arabica coffee, which was observed in 15 days after inoculation. The inoculated lesions showed the pathogen infected lesions with an average of 2.17 cm when compared to the control that shows no symptoms as you can observe in the Figure 3.

3.2. Pathogenicity test

Figure 3. Pathogenicity test of *C. gloeosporioides* causing anthracnose of coffee, upper = non-inoculated control and lower = inoculated with pathogenic isolate

3.3. Dual Culture Test

A dual culture test was done between effective antagonistic fungus, *C. cupreum* CC3003 and pathogenic isolate, *C. gloeosporiodes* showed that in

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the dual culture plates the colony diameter of pathogen was 9.00 cm in average, while in dual culture plate it was 6.28 cm in average and has a 30% of colony inhibition. It was interesting that in the dual culture plate the average number of pathogen spores was 15.13X106 and in the control plate the average was 26.25 X106 spores, and the inhibition percentage was 42.60% after incubation for 30 days (Figure 4).

Figure 4. Dual culture test between *C. cupreum* CC3003 and *C. gloeosporiodes*, right = *C. cupreum* CC3003, middle = dual cultures and left = *C. gloeosporiodes*

3.4.* Effect of nano-particles derived from *Chaetomium cupreum* CC3003 against *Colletotrichum gloeosporioides

Results showed that at the concentration of 15 ppm., nano CCM showed a significant lower colony growth (3.32 cm.) than nano CCE and nano CCH. The colony diameters were respectively 3.79 and 3.83 cm when compared to the non-treated control (5 cm.). Moreover, at the concentration of 15 ppm., nano CCE showed a significant lower spore number (0.88 x 106) than nano CCH and nano CM, which were respectively2.13 x 106 and 2.5 x 106 spores (Table 1). Nano CCM showed the highest colony inhibition of 33.75%, and followed by nano CCE and nano CCH which were respectively24.09% and 23.34%. However, nano CCE showed a significantly high inhibition of sporulation of 93.57%, and followed by nano CCM, which were 83.59%and nano CCH which were 82.24 %, respectively. The results concluded that nano CCE expressed the highest antifungal activity against *Colletotrichum gloeosporioides* at the ED_{50} of 10.09 ppm., and followed by nan oCCE and nano CCH which the ED_{50}were 37.29 and 40.42 ppm, respectively. All tested nano-particles derived from *Chaetomium cupreum* CC3003, nano CCH, nano CCE and nano CCM at concentrations of 3, 5, 10, and 15 ppm, actively expressed the control mechanism of antibiosis that the pathogen cells were abnormal and showed apossible loss of pathogenicity (Figure 5).

Table 1.Nano-particles derived from *Chaetomium cupreum* CC3003 against *Colletotrichum gloeosporioides* in 7 days

Nanoparticles	Conc.	Colony diameter	Number of	Colony[2] inhibition	spore[2] inhibition	ED_{50} (ppm.)

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		(cm)[2]	spores (X10^6)			
Nano CCH	0	5.00a[1]	12.23bc	0.00f	0.00f	40.42
	3	4.85ab	8.63de	3.10ef	28.24e	
	5	4.76bc	6.63ef	4.85de	52.00cd	
	10	4.49d	2.13gh	10.25c	82.27ab	
	15	3.83e	2.13gh	23.34b	82.42ab	
Nano CCE	0	5.00a	13.88ab	0.00f	0.00f	37.29
	3	4.93ab	9.88cd	1.45ef	28.45e	
	5	4.80bc	6.63ef	4.00de	52.00cd	
	10	4.64c	4.25fg	7.10d	69.35bc	
	15	3.79e	0.88h	24.09b	93.57a	
Nano CCM	0	5.00a	15.88a	0.00f	0.00f	10.90
	3	4.92ab	7.75de	1.50ef	49.66d	
	5	4.80bc	6.38ef	3.95de	58.77cd	
	10	4.35d	3.25gh	13.00c	79.92ab	
	15	3.32f	2.50gh	33.75a	83.59ab	
C.V. (%)		1.79%	18.93%	18.75%	18.47%	

[1/] Means of four replication which followed by a common letters are not significantly different by DMRT at P = 0.05.

[2/] Inhibition (%) = (R1-R2)/R1 × 100 where R1 = colony or spores of pathogen in control, R2 = colony or spores in treatment

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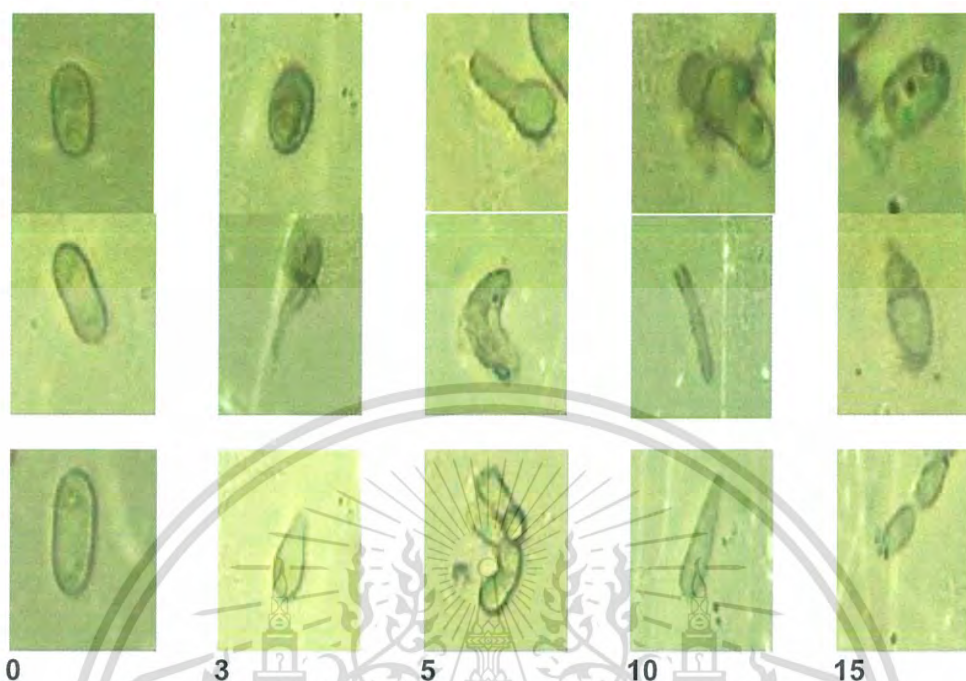
Figure 5. Abnormal conidia after treated with Nano CCH, nano CCE and nano CCM



3.5. Evaluation of bio-formulation produced from spores of *Chaeomium cupreum* CC3003 and nano-particles to control anthracnose of coffee var. Arabica in pot experiment

Results showed that coffee seedlings inoculated with *C. gloeosporioides* treated with nano-particles for six months showed a decreaseof35.40%in terms of disease incidence. While the one treated with *Chaetomium*-bioformulation 20 g/20 L of water and *Chaetomium*-biofor mulation 10 g/20 L of water showed a disease reduction of 0.83% and 1.13%, respectively, after treatment for 30 days in a pot experiment.

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Table 2. Symptoms and disease reduction after treated with bio formulation and nano-particles derived from *Chaetomium cupreum* CC3003 in 30 days

Treatments	Lesions (mm.)	Disease reduction[2]
Inoculated	1.45a[1]	----
Chaetomium-bioformulation 10 g/20 L of water	1.13b	28.31
Chaetomium-bioformulation 20 g/20 L of water	0.83ab	26.55
Nano-particles15 ppm	0.73a	35.40
C.V. (%)	78.62%	----

[1/] Means of four replications which followed by a common letters are not significantly different by DMRT at P =0.05
[2/] Disease reduction (%) = Lesion size in inoculated control – Lesion size in treatment/ Lesion size in inoculated control × 100

IV. Discussion and conclusion

Anthracnose of coffee var. Arabica proved to be caused by *C. gloeosporioides* as previously reported by Vilavong *et al.*[6]and the pathogenic isolate was confirmed by molecular phylogeny. The isolate used of *C. cupreum* CC3003 was effective as Kanoknedhakul *et al.*[12]reported that it released(+) Rotiorin the active compound against Candidaalbican at IC50 of 0.6 ppm. It is possible that *C. cupreum* CC3003 inhibits sporulation of *C. gloeosporiodes* by 42.60% within 30days in dual culture test. The research finding was similar to the results of Vilavong *et al.*[6]

As a result, the nano-particles of *Chaetomium cupreum* CC3003, nano CCH, nano CCE and nano CCM inhibits *C. gloeosporioides* causing coffee anthracnose at concentration of 3-15 ppm. at concentrations of 3, 5, 10, and 15 ppm, similar results found from Tann*et al.*[14] who used these nano-particles as a treatment to control *Curvularialunata* that cause leaf blight of rice in Cambodia.

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The research findings of using nano-particles to treat coffee seedlings inoculated with *C. gloeosporioides* reduced anthracnose disease of coffee. *Tannet al.*[14] reported that bio-formulation of *C. cupreum* and nano product from *C. Cupreum* showed a good control of blight disease in the rice pot experiment. Bio-formulation of *C. cupreum*, and nano products from *C. cupreum* reduced the incidence of disease by 58.33%. The use of bioactive compounds from *Chaetomium* species were proved to be an effective anti fungal against plant pathogens.[11] Research on nano-particles loaded with bioactive substances from effective fungi will need more investigation and development to ensure safe crop production.

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