UNIVERSITI PUTRA MALAYSIA

ISOLATION AND SCREENING OF POTENTIAL ANTAGONISTIC BACTERIA AGAINST Colletotrichum gloeosporioides, THE CAUSAL AGENT OF ANTHRACNOSE DISEASE IN PAPAYA

NUR AIMI RADI

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By

NUR AIMI BINTI RADI

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For my lovely parents,

Radi bin Yahaya and Ariyati bt Ahamad

For my wonderful siblings,

Mohammad Badri Bin Radi

Ahmad Fakhruddin Bin Radi

Nur Zawani Bte Radi

Nur Hanisah Bte Radi

Nur Afiqah Bte Radi

~ Thank you for your support and love~

ABSTRACT

The objective of this project is to screen the potential antagonistic bacteria to be used as a biological control for *Colletotrichum gloeosporioides,* the causal pathogen of anthracnose disease in papaya. Fifteen antagonistic bacteria were isolated from infected papaya fruit's surface and screened by dual and concomitant test. There is significant different of inhibitory effect at $p<0.05$ for dual culture assay test. Two isolates of bacteria (UPMKB4 and UPMKB6) had potential antagonist action against *C. gloeosporioides*. Mycelia growth test also showed a significant different for percentage of mycelia growth at $p<0.05$. Bacteria UPMKB4 and UPMKB6 inhibited the fungal growth by an average of 53.57 and 51.90%, respectively during *in-vitro* screening on PDA medium. Malformation of hyphae occurred in the presence of both bacteria. Hyphae were shrunk when treated with UPMKB6 and swelled when treated with UPMKB4. *C. gloeosporioides* treated with bacteria also showed the significant different at $p<0.05$ in reduction of the spore germination. Treatment with UPMKB3 showed the lowest percentage of germinated spore (45.43%). There is significant different at $p<0.05$ obtained in the fruits detached assay treatment. Antagonistic bacteria could reduce the growth and effect of *C. gloeosporioides*. UPMKB4 and UPMKB6 sprayed onto infected papaya showed the percentage of anthracnose disease severity of 53%, which categorized in the moderate condition, compared to 81% in control treatment. Therefore, *in-vitro* activities of UPMKB4 and UPMKB6 against *C. gloeosporioides* in this project suggested that these antagonistic bacteria can be used as an effective biological control agent.

ABSTRAK

Tujuan projek ini dijalankan adalah untuk menyaring bakteria yang berpotensi untuk digunakan sebagai kawalan biologi terhadap *Colletotrichum gloeosporioides*, kulat yang menyebabkan penyakit antraknos pada buah betik. Lima belas bakteria telah ditemui pada permukaan buah betik yang dijangkiti penyakit dan telah melalui ujian penyaringan. Terdapat perbezaan ketara pada $p<0.05$ terhadap kesan perencatan dalam ujian dwi kultur. Dua bakteria yang ditemui, (UPMKB4 dan UPMKB6) mempunyai tindakan antagonis yang berpotensi terhadap *C. gloeosporioide*s. Ujian pertumbuhan kulat juga menunjukkan perbezaan ketara pada $p<0.05$ terhadap peratusan pertumbuhan micelia. Bakteria UPMKB4 dan UPMKB6 merencat pertumbuhan kulat pada purata masing-masing 53.57 dan 51.90% semasa penyaringan *in-vitro* di atas media PDA. Perubahan bentuk pada hifa wujud dalam kehadiran kedua-dua bakteria. Hifa mengecut apabila dirawat dengan bakteria UPMKB6 dan mengembang apabila dirawat dengan bakteria UPMKB4. *C. gloeosporioides* yang dirawat dengan bakteria juga menunjukkan perbezaan ketara pada $p<0.05$ dalam pengurangan percambahan spora. Rawatan dengan UPMKB3 menunjukkan peratusan percambahan spora yang paling rendah (45.43%). Terdapat perbezaan ketara pada $p<0.05$ diperolehi dalam rawatan penyemburan pada buah. Bakteria antagonis dapat mengurangkan pertumbuhan dan kesan *C. gloeosporioides*. UPMKB4 dan UPMKB6 yang disembur pada buah betik yang dijangkiti penyakit menunjukkan tahap peratusan penyakit antraknos adalah 53%, dimana terkategori dalam keadaan sederhana, berbanding dengan 81% dalam rawatan kawalan. Oleh yang demikian, aktiviti *in-vitro* UPMKB4 dan UPMKB6 terhadap *C. gloeosporioides* dalam projek ini dapat dicadangkan bahawa bakteria antagonis boleh digunakan sebagai agen kawalan biologi yang efektif.

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APPROVAL

I certified that this research project report entitled "Isolation and Screening of Potential Antagonistic Bacteria against *Colletotrichum gloeosporioides*, the Causal Agent of Anthracnose Disease in Papaya" has been examined and approved as a partial fulfillment of the requirement for the degree of Bachelor of Science Bioindustry in the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Sarawak Campus.

Dr. Khairulmazmi bin Ahmad
Faculty of Agriculture and Food Sciences
Universiti Putra Malaysia Bintulu Sarawak Campus
(Supervisor)

Professor Dr. Japar Sidik bin Bujang
Dean
Faculty of Agriculture and Food Sciences
Universiti Putra Malaysia Bintulu Sarawak Campus

Date: 05 JUN 2009

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LIST OF ABBREVIATIONS

ANOVA	- Analysis of Variance
CRD	- Complete Randomized Design
FAMA	- Federal Agricultural Marketing Authority
HWT	- Hot Water Treatment
IPM	- Integrated pest Management
MARDI	- Malaysian Agricultural Research and Development Institute
MOA	- Ministry of Agriculture
NA	- Nutrient Agar
NB	- Nutrient Broth
PDA	- Potato Dextrose Agar
PIRG	- Percent Inhibition Radial Growth
USA	- United State of America
USD	- US Dollar

CHAPTER 1

INTRODUCTION

1.1 Background

Papaya (*Carica papaya* L.) is originated from Southern Mexico, Central America and Northern South America and now is cultivated in many countries with tropical climate such as Brazil, India, South Africa, Sri Lanka and Southeast Asia. In Malaysia, papaya is one of the most popular tropical fruits and has an excellent potential as an export crop. In 2002, Malaysia occupied the top position for export of papaya to Hong Kong and Singapore. Malaysia also ranked second in the world among the papaya exporter countries after Mexico. Malaysia's export of papaya was accounted for 28.5% of the total exports in 2002 (FAMA, 2006).

However, during storage and transportation, postharvest diseases may significantly lower the quality and value of this commodity (Rahman *et al.*, 2007). Anthracnose disease, which is caused by *Colletotrichum gloeosporioides* Penz. Sacc is a major postharvest disease in the tropical countries (Raable and Holtzman, 1964). Anthracnose in papaya can be controlled using postharvest treatment such as fungicides i.e. prochloraz and propiconazole, hot water treatment or HWT in combination with fungicides (Couey and Farias, 1979).

However, the use of fungicides for extended periods may cause emergence of strains of fungus to the types of fungicides used. In addition, the use of fungicide is becoming more restrictive due to health concerns (Wolfe *et al.*, 1940). Consumers are also demanding for a less chemical residue on products. Application of the heat

treatment will enhance the softening of papaya fruit. The response of papaya fruits to heat treatment varies with maturity and seasons. Papaya fruit being most susceptible to injury during cooler periods of HWT and may affect the ripening process in papaya (Rahman *et al.*, 2007). For these reasons, elucidating and searching for non-chemical control methods in reducing postharvest decay is becoming increasingly important. It is necessary to develop safer alternatives to synthetic fungicides that are effective and economically feasible as well as environmental friendly. Biological control seems to be an alternative method that shows a promising result in reducing the postharvest disease (Jubina and Girija, 1998; Janisiewiccz and Korsten, 2002).

Biological control is generally considered a more ecologically acceptable approach compared with chemical control and is more suited to modern concepts of integrated pest management (IPM) (Sigee, 1993). The use of antagonistic bacteria as a biological control agent has emerges as an important alternative in managing disease in recent years (Whipps, 1997; Boewn and Rovira, 1999). Many studies in the exploration of beneficial organisms have been carried out, such as *Pseudomonas fluorescens*, which is one of the examples used for the control of *Fusarium* wilt in tomato. Similarly, *P. fluorescens* were found to be an effective bio-control agent against *Phytophthora* disease in black pepper (Diby *et al.*, 2005).

1.2 Objectives

Hence, the objectives of this study were to:

1. Isolate and identify *C. gloeosporioides*, the causal agent of anthracnose disease in infected papaya fruit.
2. Isolate and screen the potential antagonistic bacteria *against C. gloeosporioides*.

CHAPTER 2

LITERATURE REVIEW

2.1 Papaya in Malaysia

Papaya is demanding for its sweet taste and high nutritional content. Malaysia has introduced papaya "*Eksotika*" variety which is a cross hybrid of Hawaii Sunrise Solo with local variety, Subang 6. It has gone through improvement process by MARDI, which has resulted in much firmer and sweeter fruit. Local breeder has developed another popular variety called "*Sekaki*". This variety has almost uniform size and the length is around one feet. In Malaysia, it is grown intensively in Johor, Perak and parts of Selangor and Pahang (MOA, 2009). Malaysia is now capable of producing up to 72,000 tonnes of papaya annually and this figure is expected to be surged to 300,000 tonnes in the year of 2010. Papaya was ranked first in terms of Malaysian export over the other tropical fruits in 2001. Malaysia has 22% of the world export product which has been growing in the international markets with some significant growth in Brunei (27%), Hong Kong (20%) and Singapore (14%) (MOA, 2009).

2.2 Anthracnose Disease in Papaya

Anthracnose is considered as one of the principal postharvest diseases in papaya, occurring in all production regions in the world. The fungus infects various parts of the plant but its greatest importance occurs in fruits, which is becoming unfit for the commercialization. The latent of infection is not detected at harvest, but more prominent in the market (Wolfe and Lynch, 1940).

2.2.1 Symptoms of Anthracnose Infection

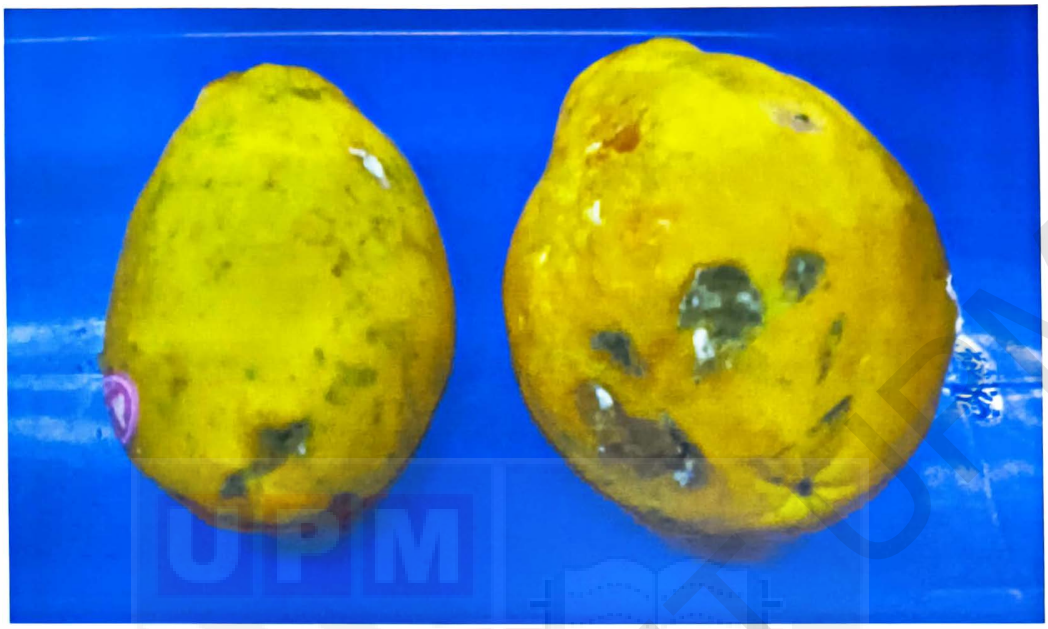

Figure 2.1: Symptoms of anthracnose disease in papaya fruit

The symptoms of anthracnose disease are usually appearing in small dark round on the ripening portion of the fruits. As the fruits ripen, these spots enlarge rapidly, forming circular and slightly sunken lesions. The lesions enlarge as the fruits mature and may reach a size of as much as five cm in diameter (Figure 2.1). Frequently, a large number of lesions may be found on a fruit and as the lesions enlarge, they may cover major portion of the fruit. The margins also will appear dark in colour while the centre portion of the lesion turns into brown or black. As the disease develops, it will frequently produce large mass of spores in the centre portions of the lesion, causing them to turn into light orange or pink. The spores sometimes are produced in concentric rings, giving the lesion an appearance of a bull's eye. In addition, the fungus is also advance into fruits, producing a rot of the affected tissues, causing the tissues to become firm with a white-greyish discoloration that turns into brown and

darker in colour (Wolfe and Lynch, 1940). All of these symptoms of infection will prevent the marketing of the infected fruits.

Although this disease usually appears on the ripening portions of the fruits, occasionally, the green portions of the fruit may become infected (Dickman and Alvarez, 1983). The disease appears first as small lesions, but soon due to the penetration by the fungus, the latex from the fruit oozes out in a stick mounds or horns. The lesions enlarge very slowly and rarely become larger than one cm in diameter as long as the fruit remain green.

2.2.2 Epidemiology of *C. gloeosporioides*

C. gloeosporioides also attacks the petioles of the lower leaves as they begin to die and are shed from the plant. Infection of these petioles is important as it may act as a source of potential inoculums for the infection of the fruits (Plortz *et al.*, 1994). In the field, the production of conidia which is occurs in large principally in the petioles of senescent leaves and in mature fruits being considered as a major source of inoculums.

When the conidia germinate, the perfect stage of the anthracnose fungus produces ascospores, which become airborne and lodge on the fruit surface. They later penetrate the cuticle by enzymatic action and the hyphae remaining sub-cuticles in a latent state until the beginning of maturation of the fruits. At this stage, the symptoms of the disease will appear. Stanghellini and Aragaki (1966) observed that spores applied on unwounded mature fruit resulted in disease only if the fruits were detached. The disease is mostly favoured by wet weather conditions.

2.3 Management of Anthracnose Disease

Anthracnose disease can be controlled through a continuous spraying programme in the field. Removal of infected leaves is essential for sanitation purposes. Spraying of maneb (0.2%) has been recommended by Raable and Holtzman (1964) and Harkness (1967). In addition, field spraying of bavistin (0.1%) and daconil (0.2%) at 15 days of interval has been found to be superior over maneb sprays (Rawal *et al.*, 1983).

The postharvest phase of anthracnose can be controlled by hydrothermal treatment. This treatment process involves the submerging of the fruits into hot water with the temperature of 43 to 49^{0}C for 20 minutes shortly after harvest (Couey *et al.*, 1984). However, this treatment leads to the unevenly de-greening of the fruits (Patel *et al.*, 1973). For exportation purposes, a treatment with fungicide is recommended to be applied after hydrothermal treatment to increase the control effectiveness of the fungal diseases.

2.4 Biology of *C. gloeosporioides*

Kingdom	:	Fungi
Phylum	:	Ascomycota
Subphylum	:	Deuteromycotina
Class	:	Coelomycetes
Order	:	Melanconiales
Family	:	Melanconiaceae
Genus	:	*Colletotrichum*
Species	:	*C. gloeosporioides*

C. gloeosporiodes which is responsible for many diseases, also referred to as anthracnose on many tropical fruits including banana, avocado, papaya, coffee, passion fruit, and others. *C. gloeosporioides* colonies on potato dextrose agar medium, PDA were grey to salmon patches, powdery to velutine, profusely sporulated and with abundant production of conidiomata. Mycelium is grey-white in colour with concentric rings (Svidzinski *et al.,* 1998).

Patel *et al.*, (1973) reported the occurrence of the perfect stage (*Glomerella cingulata*), with perithecia on various parts of the host, solitary or aggregated, globule to obpyriform, dark brown to black, clavate to cylindrical, with ascospores narrowly oval to cylindrical to fusifirm.

The pathogens are favouring with specific temperature with 28^0C is being optimal, together with high humidity. Spores must have free water to germinate as germination is negligible below 97% relative humidity. Spores are only released from acervuli when there is an abundance of moisture. Splashing from rain is a common means of spread. Severity of disease is related to weather and the fungus is relatively inactive in dry weather. Sunlight, low humidity and extremes temperature (below 18 or greater than 25^0C) rapidly inactivate the spores.

2.5 Antagonistic Bacteria as a Biological Control

Biological control involves the control of one organism by another antagonistic organism, defined as a biological agent with the potential to interfere in the life processes of plant pathogens (Fravel, 1998). Biological control of plant disease normally involves the external application of specific microorganisms (antagonists)

to the plant or its environment to limit the initiation and progression of the disease. The antagonists can act at various points in the disease cycle, including the survival of the pathogen in the external environment, build-up of pathogen on the host surface, entry of pathogen into the host and transmission of the pathogen between hosts (Sigee, 1993).

Biological agents are able to limit the growth and activity of plant pathogens in many ways. The ability to inhibit the growth of fungi has been described for bacteria (Sigee, 1993). The inhibitory effects rely on different principles. These may include the competition for nutrients and space (Droby *et al.*, 1998), or the production of anti-microbial compound, such as antibiotic, bacteriocins and siderophores (El-Tarabily *et al.*, 1994). Antimicrobial compounds are indicated by the clear inhibition zone around the centre of antagonist colony. Natural compounds produced by antagonistic bacteria are generally regarded as the source of bio-pesticide and special conditions are required for extraction and optimization of antibiotic metabolite substances from antagonistic bacteria (Dikin *et al.*, 2005). Optimization of antibiotic production by bio-control agents may enhance their capacity to control diseases. Cultural conditions such as nutritional factors, temperature and pH of the culture media can affect the growth of bio-control agents and accumulation of antibiotics (Roitman *et al.*, 1990; Slininger and Jackson, 1992).

Effects of pathogen inhibition exhibited by a particular metabolite in the laboratory cannot always be translated directly into biological control in the field, as the conditions experienced in the field are much more complex. However, there are a number of studies demonstrating a direct effect of particular antimicrobial

metabolites on pathogen numbers in soil. One such example is the suppression of root pathogens by plant-growth-promoting rhizobacteria. In another case, the population size of antibiotic produced by *Pseudomonas fluorescens* was shown to correlate positively with a reduction in the number of lesions on wheat roots caused by *Gaeumannomyces graminis var. tritici.*

For *Pseudomonas* spp., it was shown that small lipopeptides, the pseudomucins with antibiotic activity against fungi, were produced (Diby *et al.*, 2005). These peptides contain unusual amino acids such as hydroxyaspartic acid and diaminobutyric acid. Other antagonistic bacteria such as *Enterobacter agglomerans* produce chitinolytic enzymes with the ability to degrade fungal cell walls. *Bacillus subtilis* is able to produce antifungal peptides, the iturins, which are under discussion for the biological control against the peach brown rot caused by *Monilinia fructicola* (Sigee, 1993).

Biological control using antagonistic bacteria has been successful in controlling many postharvest pathogens as well as they are environmental friendly. *Burkholderia cepacia* and other *Pseudomonas* species are some of the most promising bio-control microorganisms (Cartwright and Benson, 1995).

B. cepacia has shown antagonistic action against a broad range of plant pathogens, including the postharvest disease in papaya (Rahman *et al.*, 2007). The bacteria is known to produce a wide range of secondary metabolites such as pyrrolnitrin, phenazine, cepabactin and other unidentified volatile or non-volatile compounds (Roitman *et al.*, 1990; Cartwright and Benson, 1995). Several studies suggested that pyrrolnitrin production by *B. cepacia* as well as other *Pseudomonas* spp. is closely

associated with bio-control of plant diseases (Burkhead *et al.*, 1994; Cartwright and Benson, 1995; Hwang *et al.*, 2002).

Several microbial agents have been used for biological control of postharvest diseases, even on fresh fruits (Janisewiz and Korsten, 2002). For example, *P. fluorescens* isolate A506 is a commercially available microbial pesticide registered on pome fruit for the control of fire blight caused by *Erwinia amylovora* (Stockwell *et al.*, 1997). The other commercial bio-control products were registered in US and are sold as Aspire with a yeast active ingredient for biological control of postharvest disease in apple (Droby *et al.*, 1998). Some strains of *Streptomyces* spp. are also commercially available as microbial pesticides registered on some crops for biological control of some diseases (Jones and Samac, 1996).

CHAPTER 3

MATERIALS AND METHODS

3.1 Isolation and Identification of *C. gloeosporioides*

Pure culture of *C. gloeosporioides* was previously isolated from infected papaya fruits. Surface tissues with the disease symptoms were cut into one cm^2 pieces, placed into 10% of sodium hypochlorite solution for three minutes and rinsed with sterile distilled water. The infected tissues were then blotted dry and placed on PDA containing streptomycin sulfate (50 mg/L). The petri dishes were then incubated at 25°C for five days for assessment period. Isolated fungus was sub-cultured to obtain pure culture of the isolates. Pure cultures were maintained in refrigerator at 4°C for further study. The fungus was confirmed based on their morphological characteristics (Montesino and Bonaterra, 1996).

3.2 Sample and Collection

Infected papaya fruits which bought from Bintulu markets were used in obtaining the fungus and antagonistic bacteria.

3.3 Isolation of Antagonistic Bacteria

Pieces of fruit's surface tissue with $1cm^2$ were cut from the healthy and infected lesion areas. The pieces were then disinfected with 1% of sodium hypochlorite and rinsed with distilled water. Fifteen g of sliced tissues were then soaked in 100 mL of sterilized water and rotary shaking at 200 rpm for 24 hours at room temperature (28 ± 2^0C). Serial dilutions of suspension (10^{-1} up to 10^{-5}) were made. After that, 0.5 mL of 10^{-5} diluted suspension was streaked on nutrient agar medium (NA) plates.

Plates were then incubated at 31±2°C for 48 hours. Bacterial colonies were then further isolated by sub-culturing single bacteria colony on NA medium plates. Pure cultures of bacteria were kept in refrigerator at 4°C for further study. Pure cultures of bacteria were screened for antagonistic bacteria based on dual culture assay (Montesino and Bonaterra, 1996).

3.4 Bacterial Gram Staining

One droplet of distilled water was dropped onto a slide and smeared with bacteria. The bacterial smear was air dried and heated. The slide was then stained with crystal violet for one to two minutes. The slide was flooded with iodine for one to two minutes after stained with distilled water. The iodine was immediately decolourize by washing the slide with acetone alcohol. The slide was then washed thoroughly with distilled water to remove the acetone alcohol. Safranin was flooded onto the slide for two minutes and followed by distilled water to remove safranin colour. The excess water was blotted and dried in hand over Bunsen flame. The slide was then examined under compound microscope to identify the cell morphology and group of the bacteria.

3.5 Screening of Antagonistic Bacteria

Three different screening techniques were used to screen potential antagonistic bacteria against *C. gloeosporioides* in this study. The techniques used were as follows:

3.5.1 Dual Culture Assay Test

A mycelia plug was taken from the edge of six day old culture of *C. gloeosporioides* and placed at the centre of a PDA plate. Bacterial isolate was streaked on PDA medium with a distance of 2.5 cm from *C. gloeosporioides* agar plug. All the test antagonistic pairings were incubated for seven days at 28±2°C. The PDA plates were then observed for the percent inhibition radial growth (PIRG) between fungus and bacterial isolates .The PIRG was calculated based on the bottom formula. Each treatment was replicate thrice.

$$\text{PIRG (\%)} = \left[\frac{A-B}{A}\right] \times 100$$

Where,

A = diameter of control hyphal growth,

B = diameter of treated hyphal growth. (Tops and Wain, 1957)

3.5.2 Mycelia Growth Test

A mycelia plug was taken from the edge of six day old culture of *C. gloeosporioides* and dipped into selected bacterial suspension of 10^{-9} cfu/mL for 30 minutes and air dried in the lamina airflow chamber. Treated mycelia plugs were placed on petri dishes containing PDA medium. Fungal plugs dipped in sterile distilled water served as a control. The PDA plates were then incubated at 28±2°C for seven days. Each treatment was replicate thrice. The diameter of mycelia growth was measured after six days of incubation (Rahman *et al.*, 2007). Hyphal strands at the end of the fungal colony were removed and examined under a compound microscope for abnormalities.

3.5.3 Spore Germination Test

One hundred µl of 10^{-5} cfu/mL spore suspension of *C. gloeosporioides* was spread over the PDA plate using a sterile bent glass rod. Two sterilized filter paper discs with 1 cm diameter were placed at 3 cm apart on the agar. After that, 50 µL of 10^{-9} cfu/ml bacterial suspension was pipette onto each of the paper disc. Discs received 50 µL of sterilized nutrient broth (NB) served as a control. Spore occurring around each disc were examined after 24 hours of incubation at 28±2°C (Rahman *et al.*, 2007).

3.6 Fruit Detached Assay

Fifteen healthy papaya fruits were tested in this study. Five hundred ml of spore suspension of *C. gloeosporioides* was prepared. Based on the statistical analysis, two antagonistic bacteria isolates i.e. UPMKB4 and UPMKB6 were tested. Bacterial suspension of UPMKB4 and UPMKB6 were prepared. Three treatments were carried out in this test; T1 (spore suspension + distilled water), T2 (spore suspension + UPMKB4) and T3 (spore suspension + UPMKB4 + UPMKB6). Fiftenn healthy papaya fruits were grouped into three. Each treatment consists of five papaya fruits served as replication. Each papaya fruit in the first group was sprayed with spore suspension followed by distilled water using hand sprayer. The same spraying technique and concentration was applied to treatment 2 (T2) and treatment 3 (T3). The fruits were then air dried and sealed using transparent plastic bags and incubated at room temperate. The observation of percentage of disease severity was carried after five days of incubation. Rating process was carried out based on the following disease rating scale index:

Table 3.1: Disease rating scale index developed based on the degree of infection appeared on the fruit surface

Disease severity (%)	Severity	Rating scale
80 – 100	Very severe	4
60 – 79	Severe	3
40 – 59	Moderate	2
1 - 39	Mild	1

(Bowen, 2004)

The percentage of disease severity for each treatment was calculated using the following formula:

$$\%DS = \frac{X_1 + X_2 + ... + X_n}{Y \times \text{maximum rating scale}} \times 100$$

Where,

X_n = source of disease severity of each fruit,

Y = total number of fruits in the same treatment.

3.7 Preparation of 10^{-9}cfu/mL of Bacterial Suspension

Twenty five ml of NB was prepared for each 150 mL conical flask. NB in the conical flasks was then autoclaved and cooled. One loop of each bacteria screened from the dual culture assay test was transferred into each conical flask and shake for 24 hours at 160 rpm. Serial dilutions of 10^{-1} up to 10^{-7} were made. After that, 0.1 mL of suspension from 10^{-5}, 10^{-6}, and 10^{-7} were streaked onto PDA plates. Colony count plate was carried out after 24 hours of incubation at 31 ± 2^{0}C, by using the following formula:

$$\text{cfu/mL} = \frac{number\ of\ colony\ after\ 24hrs}{X\ ml} \times DF$$

Where,

DF = dilution fraction,

X = mL used to be pour onto each PDA.

cfu/mL of 10^{-9} obtained from the calculation for each bacterium was used in mycelia growth and spore germination test.

3.8 Preparation of 10^{-5} cfu/mL of Spore Suspension

Five hundred ml of distilled water was added in a 1 L beaker. Then, 10 mL of distilled water was poured on a fungal plate and the spore was scraped using spatula. Five plates with fully grown of *C. gloeoprorioides* were prepared to collect the spores. Spore suspension was then transferred into prepared beaker and mixed using magnetic stir plate for 10 minutes to homogenize the mixture. Serial dilutions of 10^{-1} up to 10^{-5} were made. After that, 0.1 mL of spore suspension from 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were streaked onto PDA plates. Colony count plate was carried out after three days of incubation at 28±2^{0}C by using the following formula:

$$\text{cfu/mL} = \frac{number\ of\ colony\ after\ 24hrs}{X\ ml} \times DF,$$

Where,

DF = dilution fraction,

X = mL used to be pour onto each PDA.

Spore concentration at 10^{-5} cfu/mL obtained from the calculation was used in the spore germination test.

3.9 Statistical Analysis

The experiments in dual culture assay test, mycelia growth test, spore germination test and fruit detached assay were conducted in Complete Randomized design (CRD). The significant data was determined using Tukey ($p<0.05$). The percentage data were transformed into Arcsine transformation before subjected to ANOVA.

CHAPTER 4

RESULTS

4.1 Isolation and Identification of *C. gloeosporioides*

C. gloeosporioides was successfully isolated from infected papaya fruit and sub-cultured onto PDA medium for further study. The morphology of *C. gloeoporioides* was observed under compound microscope at 100 magnifications. A lot of oblong and slightly curved conidia were observed (Figure 4.1). The hyphae were straight with no shrink end or vacuolar in it.

Figure 4.1: Conidia of *C. gloeosporioides* after stained with Lacto phenol Blue dye at 100 magnifications by compound microscope

4.2 Isolation of Antagonistic Bacteria

Fifteen antagonistic bacteria were successfully isolated from the surface of infected papaya. The separation of bacterial isolates was carried out on the basis of morphological characteristics such as colony colour, elevation, the margin of colony, and colony surface. The results of observation under compound microscope were presented in Table 4.1. The pure culture of each isolate was maintained for screening test against *C. gloeosporioides*. From a statistical analysis, UPMKB4 and UPMKB6 have the highest inhibitory action against *C. gloeosporioides*. Therefore, bacteria of UPMKB4 and UPMKB6 were selected to be observed. Gram staining was carried out to determine the morphology of UPMKB4, UPMKB6 and UPMKB9 (Figure 4.2). Both selected antagonistic bacteria were gram negative with rod-shaped.

Table 4.1: The graming and shape of isolated bacteria from infected papaya fruit

Isolated Bacteria	Gram	Shape
UPMKB1	Negative	Rod
UPMKB2	Negative	Rod, coccus
UPMKB3	Negative	Rod
UPMKB4	Negative	Rod
UPMKB5	Negative	Rod
UPMKB6	Negative	Rod
UPMKB7	Negative	Rod
UPMKB8	Negative	Rod
UPMKB9	Positive	Rod
UPMKB10	Negative	Rod
UPMKB11	Negative	Rod
UPMKB12	Negative	Rod
UPMKB13	Negative	Rod
UPMKB14	Negative	Rod
UPMKB15	Negative	Rod

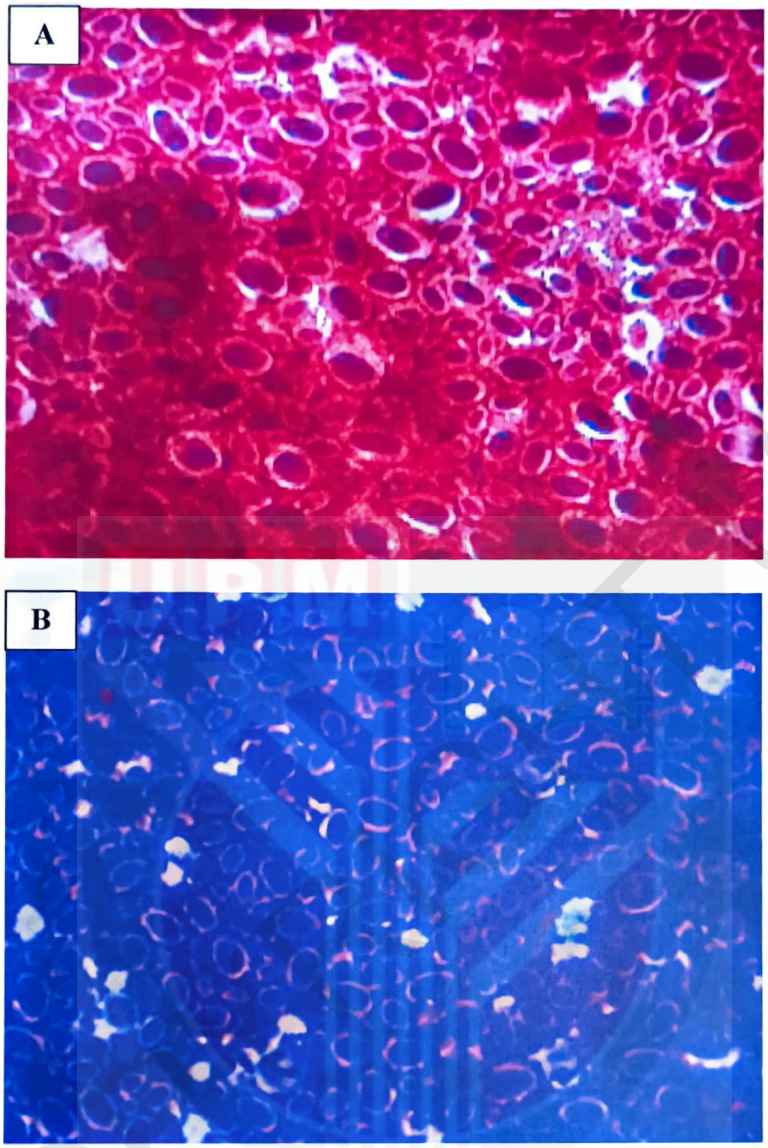

Figure 4.2: Gram staining of UPMKB4 (A) and UPMKB9 (B). Observed with 100 magnifications by compound microscope

4.3 Dual Culture Assay Test

Isolated bacteria with inhibitory characteristics were selected and screened by means of dual culture assay test. Out of 15 bacteria isolated, eight of them were showed inhibitory effect by showing clearing zone towards *C. gloeosporioides* on PDA media (Figure 4.3). The eight potential antagonistic bacteria were UPMKB1, UMPKB2, UMPKB3, UPMKB4, UPMKB6, UPMKB7, UPMKB9 and UPMKB10. On the other hand the other five bacteria isolate UPMKB5, UPMKB8, UPMKB11, UPMKB12, UPMKB13 and UPMKB15 did not show any inhibitory effect (Figure 4.4). Interestingly, five isolates namely UPMKB1, UPMKB2, UPMKB3, UPMKB4 and UPMKB10 had produced a significantly ($p<0.05$) higher inhibitory effect than the other isolates as presented in Table 4.2. The PIRG of UPMKB1, UPMKB2, UPMKB3, UPMKB4 and UPMKB10 were 38.53, 30.30, 35.06, 29.87 and 42.86%, respectively, with respect to the control after seven days of incubation.

Table 4.2: Effect of antagonistic bacteria against *C. gloeosporioides* based on PIRG value

Isolate number	Inhibitory effect (PIRG)*
UPMKB1	38.53[a]
UPMKB2	30.30[a]
UPMKB3	35.06[a]
UPMKB4	29.87[a]
UPMKB6	16.88[ab]
UPMKB7	27.62[ab]
UPMKB9	18.62[ab]
UPMKB10	42.86[a]

Means in a column with the same letter(s) do not show significantly different at $p<0.05$ according to Tukey. *Percentage inhibition zone of radial growth of *C. gloeosporioides* after seven days of incubation

Figure 4.3: Clear inhibition zone of *C. gloeosporioides* as treated with UPMKB1, UPMKB2, UPMKB3, UPMKB4, UPMKB6, UPMKB7, UPMKB9 and UPMKB10 after seven days of incubation

Figure 4.4: No clear inhibition zone of *C. gloeosporioides* as treated with UPMKB5, UPMKB8, UPMKB11, UPMKB12, UPMKB13, UPMKB14 and UPMKB15 after even days of incubation

4.4 Mycelia Growth Test

Results of this study showed that all isolates of antagonistic bacteria of UPMKB1, UPMKB2, UPMKB3, UPMKB7, UPMKB9 and UPMKB10 were not significantly different ($p<0.05$) as compared to the control treatment. However, two antagonistic bacteria isolates i.e. UPMKB4 and UPMKB6 were significantly ($p<0.05$) suppressed the diameter of fungal growth on PDA medium after seven days of incubation with respect to the control (Table 4.3 and Figure 4.5). The lowest suppression means of mycelia diameter was 3.25 cm and the highest value was 4.05 cm. The percentage of inhibited mycelia treated with UPMKB4 and UPMKB6 were 53.57 and 51.90% respectively.

Table 4.3: Effect of the antagonistic bacteria on the diameter of *C. gloeosporioides*

Antagonistic bacteria	Mean of mycelia diameter of *C. gloeosporioides* (cm)	Mycelia inhibition (%)
Control	4.250^{a}	39.29^{b}
UPMKB1	3.950^{ab}	43.57^{ab}
UPMKB2	3.783^{ab}	45.95^{ab}
UPMKB3	3.833^{ab}	45.24^{ab}
UPMKB4	3.250^{b}	53.57^{a}
UPMKB6	3.367^{b}	51.90^{a}
UPMKB7	3.633^{ab}	48.10^{ab}
UPMKB9	4.050^{ab}	42.14^{ab}
UPMKB10	3.967^{ab}	43.34^{ab}

Means in a column with the same letter(s) do not significantly different at $p<0.05$ according to Tukey

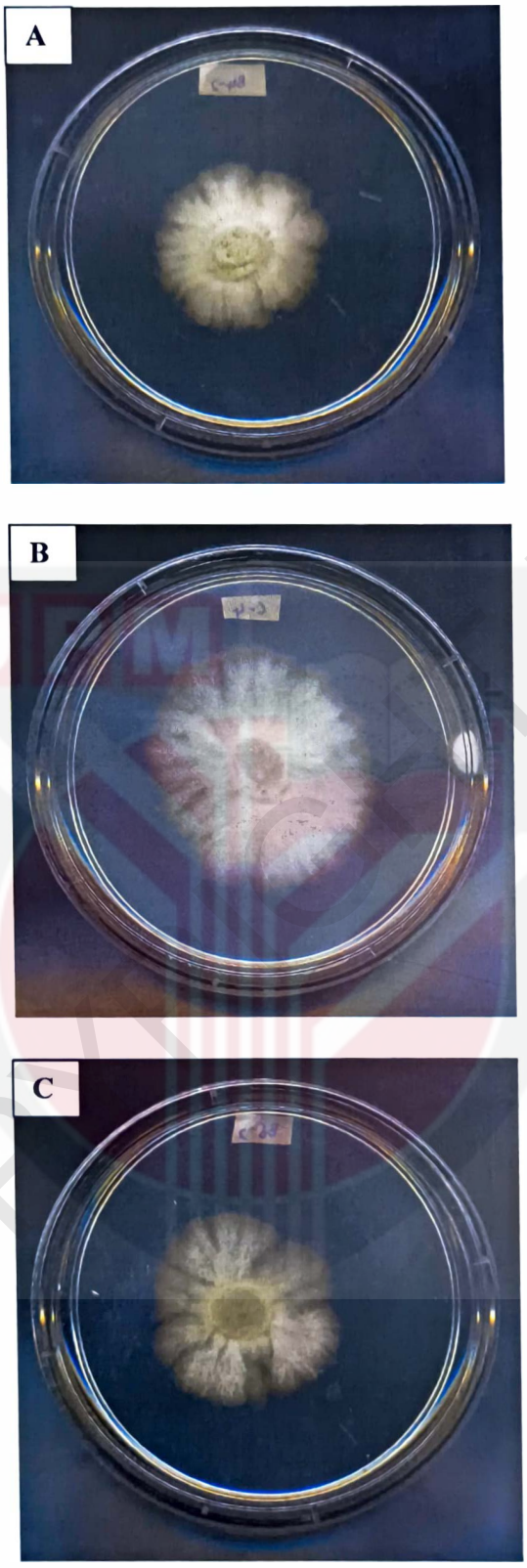

Figure 4.5: Suppression in diameter of fungal growth as treated with UPMKB4 (A) and UPMKB6 (C). Plate B served as a control

The effect of the antimicrobial activity was further study using compound microscope. The changes of the hyphal tips of *C. gloeosporioides* treated with UPMKB4 and UPMKB6 were observed at 100 magnifications. The hyphal tips of the plant pathogen become thickened and vacuolar after treated with UPMKB4 (Figure 4.6 (C) and (D) as compared with hyphae in the control plate (Figure 4.6 (A). Many swelling and vaculation occurred in this treated hyphae whereas in the control plate, normal hyphal walls were observed. Hyphae of *C. gloeosporioides* treated with UPMKB6 were shrunk as observed in Figure 4.6 (B). Moreover, the treated fungus also showed the reduction in the amount of spore produced.

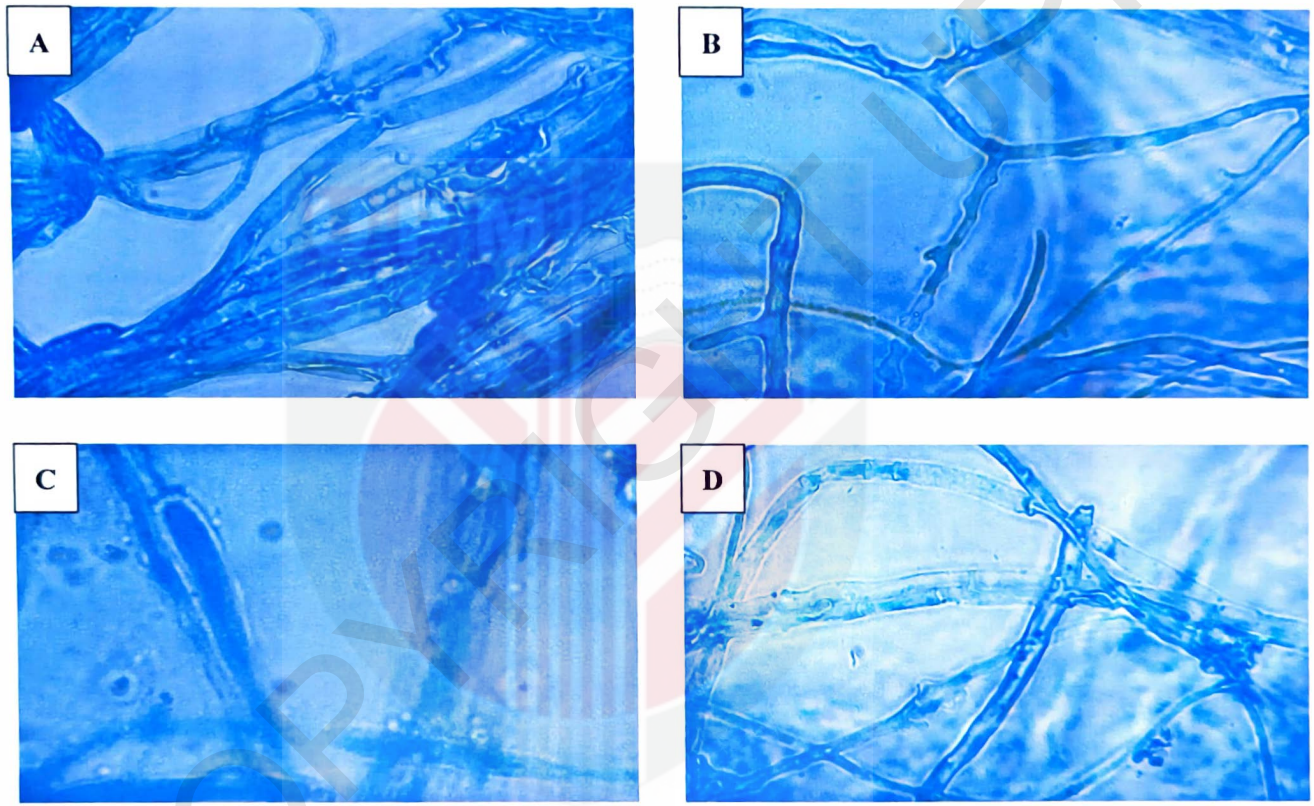

Figure 4.6: Observation of hyphal abnormalities of *C. gloeosporioides* at 400 magnifications as treated with UPMKB4 and UPMKB6. Normal hyphae from control treatment (A). Shrinkage of hyphae affected by UPMKB6 (B). Occurrence of vacuoles and swelling in hyphae affected by UPMKB4 (C and D)

4.5 Spore Germination Test

In this study all eight antagonistic bacteria isolates namely UPMKB1, UPMKB2, UPMKB3, UPMKB4, UPMKB6, UPMKB7, UPMKB9, and UPMKB10 were tested to determine the ability of the isolate to inhibit the spore of *C. gloeosporioides* from germinated. Spore treated with UPMKB1, UPMKB2, UPMKB3, UPMKB4, UPMKB6, UPMKB7 and UPMKB9 showed clear inhibition zone of spore around the filter paper disc placed at the center of the petri dish. These results showed that these bacteria can significantly inhibit the spore germination after seven days of incubation with respect to the control.

The means of spore germination inhibition of *C. gloeosporioides* were expressed in Table 4.4 and Figure 4.7. In this study, the highest inhibition rate was 3.82 cm, obtained from the plate of fungus treated with UPMKB3. However, the lowest inhibition rate was 0.00, which was obtained from the UPMKB10 plate and lead to 100% germination of the spore. Comparison between isolates tested showed no significant different at $p<0.05$ among them as observed at UPMKB1, UPMKB2, UPMKB3, UPMKB4, UPMKB6, UPMKB7 and UPMKB9 plates. The highest inhibition rate of spore germination treated with UPMKB3 lead to the lowest germination of spore in the plate, which was 45.43%. There was no significant different in percentage of germinated spore among UPMKB1, UPMKB2, UPMKB3, UPMKB4, UPMKB6, UPMKB7, and UPMKB9.

Table 4.4: Effect of antagonistic bacteria on the spore germination of *C. glocosporioides* as expressed by clearing zone

Antagonistic bacteria	Mean of clearing zone (cm)	Germinated spore (%)
Control	0.000[b]	100.00[b]
UPMKB1	1.870[ab]	73.29[a]
UPMKB2	2.903[a]	58.52[a]
UPMKB3	3.820[a]	45.43[a]
UPMKB4	2.453[a]	64.95[a]
UPMKB6	2.937[a]	58.05[a]
UPMKB7	2.710[a]	61.29[a]
UPMKB9	2.937[a]	57.95[a]
UPMKB10	0.000[b]	100.00[b]

Means in a column with the same letter(s) do not significantly different at $p<0.05$ according to Tukey

Figure 4.7: Inhibition of spore germination of *C. gloeosporioides* as shown by the development of clearing zone around filter paper disc

4.6 Fruit Detached Assay

Fruit detached assay was carried out to determine the potential of the antagonistic bacteria in controlling the infection of *C. gloeosporioides* during storage condition. In this study three treatments were carried out to evaluate the potential of selected antagonistic bacteria based on the *in-vitro* test. Results of this study were expressed in the Table 4.5. The visible disease severity between the treatments was shown in Figure 4.8. Results of this study showed that T3 (combination of UPMKB4 and UPMKB6) significantly suppressed ($p<0.05$) the development of infection compared to the control treatment with disease severity index of 53%. On the other hand, there is no significant different at $p<0.05$ for T2 (UPMKB4). Based on disease severity rating, T1 (control) showed very severe disease infection followed by T2 (severe infection) and T3 (moderate infection).

Table 4.5: Effect of antagonistic bacteria on disease severity at the papaya fruits' surface

Treatment	Disease Severity Index (%)	Disease Severity
T1 (Spore suspension + distilled water)	81.00[a]	Very severe infection
T2 (Spore suspension + UPMKB4)	75.00[ab]	Severe infection
T3 (Spore suspension + UPMKB4 + UPMKB6)	53.00[b]	Moderate infection

The mean with the same alphabet is not significant at $p< 0.05$

Figure 4.8: Effect of antagonistic bacteria towards anthracnose disease in papaya as observed after five days of inoculation. Very severe infection was observed at the surface of papaya fruits in T1 treatment (A). Severe in T2 treatment T2 (B) and moderate infection in T3 (C)

CHAPTER 5

DISCUSSION

In the present study, eight accessions of bio-control agents from infected papaya were obtained from the infected surface of papaya fruit. The isolated bacteria were known as UPMKB1, UPMKB2, UPMKB3, UPMKB4, UPMKB5, UPMKB6, UPMKB7, UPMKB8, UPMKB9, UPMKB10, UPMKB11, UPMKB12, UPMKB13, UPMKB14 and UPMKB15. Isolation of potential bacteria from infected papaya fruit using liquid assay was a simple and effective technique. The liquid assay and agar plate media are commonly used for isolation of antagonistic bacteria from infected plant. Higher number of isolated bacteria proven that the diversity of the bacteria is affected by environmental complexity and availability of the nutrition.

These bacteria were screened to determine to best candidate to control anthracnose disease in papaya. Result of the present study showed that there are potential antagonistic bacteria to control anthracnose disease in papaya, caused by *C. gloeosporioides*. The most efficacious bio-control agents identified in this present study were UPMKB4 and UPMKB6. Unfortunately, in the present study, identification of the isolated bacteria could not be carried out because of the lack of instruments and facilities to identify them.

However, according to Rahman *et al.*, (2007) most of potential bacteria isolated was identified as *Bukholderia cepacia* and *Pseudomonas aeruginosa* by the BIOLOG system. These bacteria are suitable for bio-pesticides, plant growth promoting bacteria and for bioremediation (Sigee, 1993). There is close association between *B.*

cepacia and crop plant surface as *B. cepacia* have been found as a plant associated bacterium from soil and inner tissue of crops, such as maize and wheat (Balandreau *et al.*, 2001). Commercial products containing *B. cepacia* are available in the market for control of many soil-borne pathogens (Fravel *et al.*, 1998).

The potential antagonistic bacteria obtained from dual culture assay test were UPMKB1, UPMKB2, UPMKB3, UPMKB4, UPMKN6, UPMKB7, UPMKB9, and UPMKB10. The inhibition zone was observed between the pathogen and bacteria. The inhibition zone could be due to the effect of diffusible inhibitory substances produced by the bacteria, which suppressed the growth of *C. gloeosporioides*. The presence and size of the zone of inhibition have been used as evidence of the production of antibiotics by the bacteria (Rothrock and Gottlieb, 1981, Jackson *et al.*, 1991, Crawford *et al.*, 1993). Another possibility is that the bacterial isolates depleted the nutrient in the agar surrounding them and thereby inhibited the growth of *C. gloeoporioides*.

Alvindia and Natsuaki (2008) ware reported that there is an advantage of using antagonistic bacteria isolated from the same habitat as that in which they are effective for bio-control. Antagonistic bacteria isolated from the surface of infected papaya fruits significantly control the anthracnose disease. Knowledge of the mechanism of antagonism may be used to improve the action of bio-control agents (Spurr, 1981). Antagonistic bacteria may have more than one mode of action. Exploiting all modes of action will increase the efficacy of the antagonistic bacteria. Potential antagonistic bacteria are able in reducing the activities of *C. gloeosporioides* by production of extracellular antibiotics and direct contact as

observed *in-vitro*. Several reports indicate that many pathogens are sensitive to *Bacillus* and *Pseudomonas* strain or their antibiotic metabolites (Phae and Shoda, 1990). Taking into account, the important effects of metabolites on growth of pathogens and the inflicted damage on pathogens when antagonistic bacteria were in contact with them, these mechanisms seem to be complementary giving the antagonist a competitive edge in the natural substrate.

In the present study, mycelia growth test was used to study the relationship between the diameter of mycelia growth and antagonistic bacteria. The isolated bacteria namely UPMKB4 and UPMKB6 showed high mycelia inhibitory towards *C. gloeosporioides*. These potential antagonistic bacteria are able to produce substances such as antibiotic *in-vitro*. This substance is responsible to inhibit the mycelia growth of *C. gloeosporioides*. The low mycelia inhibitory effect by UMPKB1, UPMKB2, UPMKB3, UPMKB7, UPMKB9, and UPMKB10 was probably due to the less interaction of these bacteria against the fungus.

The inhibition of mycelia growth in this study could be due to the toxic chemicals released by the antagonistic bacteria. Thus, UPMKB4 and UPMKB6 were thought to release more toxic chemicals that may penetrate into the cell of *C. gloeoprorioides* and inhibit its activities. According to *Rahman et al.*, (1989), *B. cepacia*, which is one of the potential antagonistic bacteria manage to reduce the severity of anthracnose disease known to produce a wide range of secondary metabolites such as pyrrolnitrin, phenazine and cepabactin, which influence the growth and morphology of fungal mycelium. Pyrrolnitrin is a broad-spectrum antifungal antibiotic. Antibiotic

production by *B. cepacia* has been suggested to be the mode of disease control (Roitman *et al.*, 1990; Hwang *et al.*, 2002).

The mycelia malformation observed in this study was probably due to the toxic effect of the chemical released by the antagonistic bacteria. The vacuolar appearance of the mycelium also probably may be due to the antibiotic or other metabolites produced by the bacteria, which may penetrate and caused protoplasmic dissolution (Cook, 1985).

For spore germination test, UPMKB1, UPMKB2, UPMKB3, UPMKB4, UPMKB6, UPMKB7 and UPMKB9 showed clear inhibition zone around the filter paper disc placed in the center of the petri dish. Antibiotic substance produced by potential antagonistic bacteria may also responsible to inhibit the spore germination of *C. gloeopsorioides* (Rahman *et al.*, 2007).

The failure of the spore of *C. gloeopsorioides* to germinate in 24 hours exposure indicated that chemical substance produced by antagonistic bacteria is fingicidal to the spore of the tested fungus. Suppression of spore germination of several fungi by *B. cepacia* has been reported (Phae *et al.*, 1990; Droby *et al.*, 1998). Cook (1985) in his report also stated that the use of antagonistic bacteria as bio-control agents has been discussed and is extremely promising in reducing the spore of the pathogen from germinated.

Potential antagonistic bacteria may reduce the lesion development on detached papaya fruit when applied prior to inoculating fruit with the pathogen. The results

from the present study showed that the application of selected antagonistic bacteria was able to reduce the severity of anthracnose disease in papaya. The visible disease severity was reduce when combining two antagonistic bacteria, UPMKB4 and UPMKB6 (moderate infection) in the application compared to the single bacteria, UPMKB4 (severe infection).

Based on the above results, UPMKB4 and UPMKB6 were used to study the association with C. *gloeopsorioides* under storage condition. Application of single bio-control agent showed no significant different in controlling anthracnose disease in papaya. This could be due to limited ability of bio-control agent that may lead to less effectiveness to suppress the anthracnose disease. Many factors play an important role in the process of antifungal production and consequently affect the antagonistic activity of the bacterial species. Carbon compounds constitute the major requirement for growth as they enter in different metabolic process resulting in the production of primary and secondary metabolites including antifungal compounds.

On the other hands combination of two or more bio-control agents showed more satisfactory result in the present study. Disease severity of papaya fruits could be reduced significantly and this could be due to synergistic effect of their interaction. Stockwell *et al.*, (1997) reported that *T. harzianum* is effective in controlling the plant diseases by produced a growth regulating factor. The regulating factor may increase the rate of germination and stem dry weight of the crops. However, inoculation of a mixture of antagonistic bacteria has been more effective, rather than a high population of single bacteria.

Strains of *B. cepacia* are widely studied for postharvest control in anthracnose disease in papaya (Rahman *et al.*, 2007). However, when used alone, the bio-control efficacy of *B. cepacia* was not as effective as fungicides. Therefore, from a practical view, combining the UPMKB4 with more than one strain of potential antagonistic bacteria was a much more effective strategy to control anthracnose disease in papaya. Moreover, the capability of antagonist to control a range of pathogens at low concentrations is an advantage in bio-control activities (Sigee, 1993).

Kim *et al.*, (2008) reported that the control effects of the antagonists could be variable depending on the strains of target fungal species in the field test. Environmental conditions also could affect the antagonistic effects of the selected isolates in the field. Therefore, it is desirable that *in-vitro* tests for selection of prospective isolates for the biological control should be accomplished with several isolates of target pathogens prior to field tests.

Luz, (2000) reported that inoculation of wheat with *Fusarium graminearum* can be significantly dismissed by *Bacillus megatherium* and *B. subtili.* The disease was showed the incidence and severity up to 50 and 67%, respectively. The variation in antifungal activity may reflect differences in the site of action or the ability of fungi to detoxify the metabolites. It may have also been due to the inactivation of the antibiotics in the environment and application method. Treatments with *Streptomyces* also reduce *Fusarium* head blight severity. In addition to antibiotics production by *Streptomyces*, Bochow and Fritzche (1991) reported that the effectiveness of *Streptomyces* isolates against *Phytophthora* infections was due to the induction of

host resistance by the antagonists. *Streptomyces* spp. has been implicated as effective biological control agents.

CHAPTER 6

CONCLUSION

These experiments were conducted in order to know the effect of antagonistic bacteria in reducing the anthracnose disease in papaya. *C. gloeosporioides* has been subjected to dual culture assay test, mycelia growth test and spore germination test. Fruit detached assay also was carried out in order to determine the disease severity when treated with potential antagonistic bacteria.

The effect of the antagonistic bacteria in suppressing *C. gloeosporioides* was observed. The application of antagonistic bacteria has significant different in mycelia growth, spore germination and fruit detached assay action compared to the control treatment. Eight potential antagonistic bacteria were selected. UPMKB4 and UPMKB6 showed significant suppression of the diameter of *C. gloeosporioides* in mycelia growth test. UPMKB1, UPMKB2, UPMKB3, UPMKB4, UPMKB6, UPMKB7, and UPMKB9 could inhibit the spore from germinated. There is no significant different among them when observed at $p<0.05$. Fruit detached assay showed clear reduction in disease severity when treated with antagonistic bacteria. Moderate severity was obtained compared to very severe disease in control treatment.

Therefore, *in-vitro* activities of selected antagonistic bacteria against *C. gloeoporioides* of papaya in this study suggested that the antagonistic bacteria can be an effective biological control agent. The use of antagonistic bacteria also may be an economical as well as environmental friendly to suppress the disease.

To improve this study, the potential antagonistic bacteria should be identified so that the specific metabolites of the bacteria can be determined. Moreover, several authors have also reported that results of dual culture assay could be contrast with the *in-vivo* performance (Baker, 1968). This is due to the different environment condition between the laboratory and field test. This is supported by Jubina and Girija (1998), when their studies on already established nursery plants of black pepper revealed that one of the *Bacilli* isolates showing poor inhibition of the fungal pathogen in the dual culture, exhibited the highest disease suppression in the *in-vivo* biological control assay.

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APPENDICES

Figure A1: Slices of infected papaya fruit surface

Figure A2: Shaking process in obtaining the potential antagonistic bacteria from infected papaya fruit

Figure A3: Shaking process for inoculation of bacteria

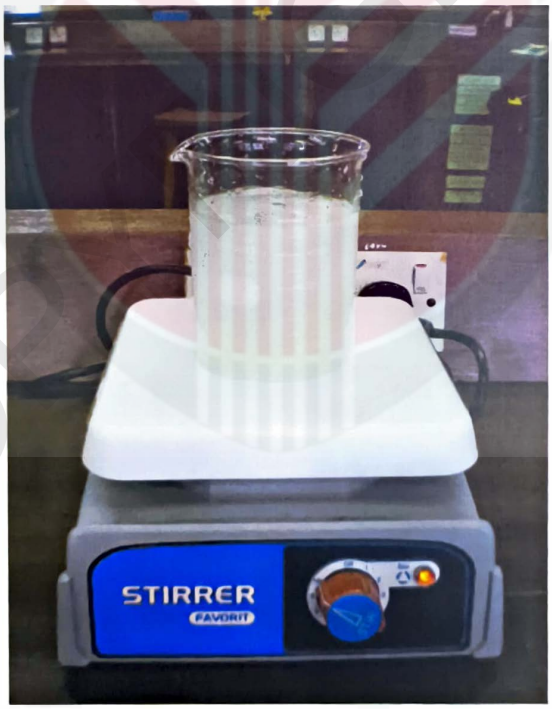

Figure A4: Preparation of spore suspension using magnetic stirrer

PUBLICATION OF THE PROJECT UNDERTAKING

This is to certify that I have no objection to publish the project entitled **"Isolation and Screening of Potential Antagonistic Bacteria Against *Colletotrichum gloeosporioides*, The Causal Agent of Anthracnose Disease in Papaya"** by the supervisor in a joint authorship. However, it has to be evaluated by the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Sarawak Campus and published in the form approved by the Faculty.

Nur Aimi Binti Radi

Date: 5/5/2009