POTENTIAL OF ENDOPHYTIC BACTERIA FROM PAPAYA (*Carica papaya*) TO CONTROL *Colletotrichum gloeosporioides*

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by

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POTENSI BAKTERIA ENDOFIT DARIPADA BETIK (C*arica papaya*) BAGI MENGAWAL *Colletotrichum gloeosporioides*

oleh

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Tesis yang diserahkan untuk memenuhi keperluan bagi

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LIST OF SYMBOLS AND ABBREVIATIONS

bp	base pair (s)
cm	centimeter
DNA	deoxyribonucleic acid
g	gram
kb	kilobase pair (s)
L	litre
mg	milligram
rpm	revolution per minute
min	minute (s)
h	hour (s)
ml	mililitre
mM	milimolar
M	molar
° C	degree Celcius
PCR	polymerase chain reaction
rDNA	ribosomal deoxynucleic acid
pmol	picomolar
s	second (s)
U	unit (s)
µg	microgram

µl	microlitre
µm	micrometer

POTENSI BAKTERIA ENDOFIT DARIPADA C*arica papaya* BAGI MENGAWAL C*olletotrichum gloeosporioides*

ABSTRAK

Pendekatan semasa yang digunakan untuk mengawal penyakit buah betik terlalu bergantung kepada penggunaan bahan kimia yang boleh menjejaskan kesihatan manusia dan alam sekitar. Oleh itu, adalah sangat penting untuk mengenalpasti dan menghasilkan pengawal biologi yang mampu mengawal penyakit buah betik. Salah satu ancaman yang serius kepada penanaman betik adalah penyakit antraknos yang disebabkan oleh *Colletotrichum gloeosporioides*. Kajian ini dijalankan bertujuan untuk menyelidik potensi bakteria endofit daripada *Carica papaya* untuk bertindak sebagai agen kawalan biologi bagi mengawal antraknos secara *in vitro*. Sejumlah 47 dan 57 bakteria endofit telah dipencilkan daripada buah dan daun betik, masing-masing. Bakteria endofit telah dikenalpasti melalui ciri-ciri morfologi, fisiologi, Sistem Pengenalan BIOLOG, dan analisa jujukan 16S rDNA. Berdasarkan hasil kajian, dua pencilan F21 dan L63 telah dikenalpasti sebagai spesies *Bacillus*. Spesies *Bacillus* ini telah disaring untuk aktiviti antikulat terhadap *C. gloeosporioides* pencilan 1 dan 2 dan masing-masing menunjukkan kesan perencatan terhadap pathogen kulat. Berdasarkan ujian dua kultur, *Bacillus* sp. L63 berpotensi menjadi agen kawalan biologi yang berpotensi terhadap *C. gloeosporiroides* pencilan 1 dan 2 masing-masing dengan peratusan perencatan 47.05% dan 58.06%. Perubahan morfologi yang teruk telah dikenalpasti pada miselium kulat

apabila dirawat dengan *Bacillus* sp. L63. Kesimpulannya, *Bacillus* sp. L63 berpotensi untuk bertindak sebagai agen kawalan biologi terhadap patogen *C. gloeosporioides*.

POTENTIAL ENDOPHYTIC BACTERIA FROM *Carica papaya* TO CONTROL *Colletotrichum gloeosporioides*

ABSTRACT

Current approaches to control papaya disease rely on the use of chemicals fungicide which is detrimental to human health and environment. Thus, there is a need to find and develop biological means of controlling pathogens of papaya. One of the most serious threats to papaya crop is anthracnose disease caused by *Colletotrichum gloeosporioides*. This study was conducted to investigate the potential of endophytic bacteria from *Carica papaya* as biological control agents against anthracnose by *in vitro* means. A total of 47 and 57 bacterial endophytes were isolated from fruits and leaves of papaya, respectively. The endophytic bacteria were identified based on their morphological and physiological characteristics, BIOLOG Identification System, and 16S rDNA sequence analysis. Based on the results, both strains F21 and L63 were identified as *Bacillus* species. These *Bacillus* spp. successfully showed inhibitory effects against *C. gloeosporioides* isolates 1 and 2. Based on dual culture test, *Bacillus* sp. L63 has the potential to be biocontrol agent against *C. gloeosporiroides* isolates 1 and 2 with percentage of inhibition 47.05% and 58.06%, respectively. Severe morphological changes were observed on the fungal mycelia when treated with *Bacillus* sp. L63. In conclusion, *Bacillus* sp. L63 has the potential to serve as biological control agents against *C. gloeosporioides*.

CHAPTER 1 – Introduction

1.1 Background

Papaya (*Carica papaya* L.) is a native fruit of tropical America that spread and flourished in the tropics and subtropics. It is one of the most popular tropical fruits in Malaysia and is mostly exported to Hong Kong, Singapore, United Arab Emirates and Brunei apart from the huge domestic consumption (Rahman *et al*., 2008). A ripe papaya has yellowish green skin with orange yellow colored flesh, has a sweet taste, and is juicy in nature. The ripe fruit usually consumed as a fresh fruit (Alvarez and Nishijima, 1987), juice and unripe fruit commonly cooked as vegetable.

Ripe papaya fruits that have soft in texture and high with nutrients usually prone to nematodes, bacteria, viruses as well as fungal infection which can bring heavy losses to yield. Anthracnose is the major postharvest disease caused mainly by *Colletotrichum* species that seriously causes decline in papaya production. *Colletotrichum gloeosporioides* is a pathogenic fungus that commonly affects papaya fruit (Alvarez and Nishijima, 1987) and this disease is one of the limiting factors for marketing of papaya and causes significant loss to the economy.

A recent approach used to control the disease is by using synthetic chemicals that also inflicts serious impact to human health and the environment. Hence, exploration of an alternative method to control this pathogenic fungus *in planta*, is imperative for

research. Endophytic bacteria are well known microorganisms that have been reported to inhibit growth of various phytopathogenic fungi (Wang *et al.*, 2009; Sundram *et al.*, 2011; Yin *et al.*, 2011). Thus, the main objective of this study is to use endophytic bacteria from *C. papaya* fruits and leaves as biological control agents (BCAs) against *C. gloeosporioides.*

1.2 Rationale of the investigation

Colletotrichum gloeosporioides is known can cause maximum loss to papaya crop especially during postharvest season**.** During ripening stage, the lesion will start appeared and caused severe damage to papaya fruits. Even though papaya fruits were gave a treatment during preharvest and postharvest by using chemical fungicides, but it still cannot control this disease. Besides, high usage of chemical fungicides on fruits will exposed high risks to human health as well as gave severe side effects on our environment. Moreover, the applications of efficient fungicides demand higher costs, lead to the development of resistance in target pathogen (Gamagae *et al.*, 2003; Rahman *et al.*, 2007), as well as caused soil and water contamination. As a result, in the past few years many studies have focused towards the development of BCAs which are ecologically safe and environmentally-friendly.

Several biological control agents have been successfully tested on several fruit during post harvest season such as apple, peach, citrus, pear, pome fruit, and cheery. Some bioagents such as Aspire (*Candida oleophila*), Biosave 100 (*Pseudomonas*

syringae), and Biocoat (*Candida saitoano*) have been commercialized in USA and Israel (Singh, 2010) which can help in managing some fruits disease. So, it is not possible to explore the biocontrol agent for papaya fruits so that postharvest disease of papaya can be control as well as delay the development of pathogens. In fact, it can help and give benefit to the global papaya fruits industries.

1.3 Objectives

The primary objective of this research is to screen for potential antagonistic endophytic bacteria isolated from *C. papaya* fruits and leaves to control growth of *C. gloeosporioides.* The aims of the study were:

1) To isolate, identify, and screen endophytic bacteria from papaya fruits and leaves.
2) To demonstrate antifungal activity of endophytic bacteria against *C. gloeosporioides in vitro*.

CHAPTER 2 – Literature Review

2.1 Papaya (*Carica papaya* L.)

Papaya (*Carica papaya* L.) is a member of the family *Caricaceae* (Berry *et al.*, 2004; Chukwuka *et al.*, 2010) and has about 48 species in the genus *Carica.* However, only *C. papaya* is well-known worldwide and edible (Khade *et al.*, 2010). Papaya is an important fruit crop with increasing demand in the national and international markets (Bautista-Banos *et al.*, 2002). In 2004, the most important papaya exporter was Mexico followed by Malaysia with 34.8% and 21.0% of the world export, respectively. Hawaii was the most important importer of papaya with 47.6% of the global import followed by China and Singapore with 11.9% and 9.3%, respectively. Malaysia was the major supplier of papaya for these two countries (Chan, 2009). Unfortunately, these few factors such as storage, transportation, and postharvest diseases cause the decline in quality and quantity of this valuable commodity (Gamagae *et al.*, 2004).

Carica papaya is a soft wooded perennial plant. It can be harvested within 5 to 6 months after flowering and their life span extends up to 5 years. Usually, the fruit size ranges from 7 to 30 cm long and the weight is about 250 to 3000 grams. Commercially, papaya tree will replanted every 2 to 3 years because after that tree becomes too tall for harvesting (Chan, 2009). The fruit is harvested when 80% of the skin color turns yellow. For long distance transportation, fruits were harvested at the color break stage or during the first appearance of yellow coloration (Berry *et al.*, 2004).

2.1.1 Diseases of papaya

There were few factors that bring about the spoilage of fruits such as temperature during handling, storage, transport, and distribution, the postharvest environment, the quality of product, treatments to control diseases, as well handling and packaging systems (Harvey, 1978). During harvesting, treatment, packaging, and transportation, pathogens can easily access the fruit through wounds (Spadaro and Gullino, 2004) which can cause substantial spoilage.

Three general diseases that usually infect papaya are fruit surface rots, stem-end rots, and internal infections (Alvarez and Nishijima, 1987). Several other diseases of papaya reported in Australia include crown rot caused by *Erwinia papaya,* bacterial leaf spot caused by *Pseudomonas carica papayae,* and black spot caused by *Asperisporium caricae* (Persley and Vawdrey, 2009). A papaya dieback disease caused by *Erwinia mallotivora* is one of the major threats in Malaysia that causes greasy, water soaked lesions and spots on leaves, as well as foliar and angular lesions (Amin *et al*., 2011). A study in South Western Nigeria found that *Rhizopus nigricans, Colletotrichum lunata, C. capsici* and *Fusarium moniliforme* were the pathogenic fungi which caused postharvest diseases of marketed pawpaw fruit (*C. papaya* L.) (Baiyewu *et al.*, 2007). In Brazil, postharvest disease of *C. papaya* usually caused by *C. gloeosporioides* (Peres *et al.*, 2002).

2.1.2 Postharvest diseases of papaya

There are two different categories of diseases which are preharvest and postharvest diseases. Preharvest diseases are those, where the pathogen affects the developing fruits that are still in the field while postharvest diseases occur when pathogen damage matured fruits during storage (Freeman *et al.*, 1998). Most of the severe infections caused by pathogens commonly occur during pre-harvest and symptoms may appear only in postharvest stages (Capdeville *et al.*, 2007a). It is very important to apply preharvest management practices because it can decrease direct losses of papaya fruit and also to increase the effectiveness of postharvest treatment. A study reported that fungi responsible for losses of papaya in Oyo State, south western Nigeria was *Aspergillus flavus, A. niger, Rhizopus nigricans, Fusarium* sp. and *Mucor* sp. (Chukwuka *et al.*, 2010). Papaya anthracnose is an important postharvest disease caused mainly by *C. gloeosporioides* in tropical countries (Paull *et al.*, 1997). The main pathogen affecting papaya production in Mexico is *C. gloeosporioides* (Bautista-Banos *et al.*, 2003b). Numerous types of postharvest diseases affecting papaya are enlisted (Table 2.1).

Table 2.1: Major postharvest diseases of papaya caused by pathogenic fungi (Rahman *et al.*, 2008).

Pathogens	Postharvest diseases
Colletotrichum gloeosporioides, C. capsici	anthracnose
Phomopsis sp.	Phomopsis rot
Stemphylium lycopersici	Stemphylium rot
Fusarium sp.	Fusarium fruit rot
Botryodiplodia theobromae, Phomopsis sp.	stem-end rot
Rhizopus stolonifer	Rhizopus rot

2.2 The genus *Colletotrichum*

The genus *Colletotrichum* (teleomorph *Glomerella*) is generally the cause of preharvest and postharvest diseases in the tropics. The capability of this pathogen to cause latent infections makes them as one of the major postharvest pathogens. Out of 80 'species' in genus *Glomerella*, only 20 'species' were reported in genus *Colletotrichum* anamorphs (Sutton, 1992).

Colletotrichum is known as detrimental pathogen on numerous plants (Esquerre-Tugaye *et al.*, 1992). Some of the *Colletotrichum* species cause severe postharvest diseases of perennial plants, ornamental plants and annual crop (Agrios, 2005). These pathogens usually caused damage to plant organs such as leaves, fruits, stems, roots, and flowers (Bailey *et al.*, 1992). Various species of *Colletotrichum* were reported to cause postharvest disease on various plants. For example, *C. lindemuthianum* was reported to cause postharvest disease of bean (*Phaseolus vulgaris*) and it is the major bean disease

in Ontario (Tu, 1992). Another species of *Colletotrichum* is *C. graminicola* which caused postharvest disease of maize (*Zea mays*) and sorghum (*Sorghum bicolor*) (Nicholson, 1992). *Colletotrichum acutatum* causing strawberry postharvest disease in Israel and the fungal pathogen usually infect fruit, stolons and crowns of strawberry (Freeman and Katan, 1997). Another report of *Colletotrichum* species caused postharvest disease is *C. gloeosporioides* that infect guava (*Psidium guajava*) fruit in Ibadan, South Nigeria (Amusa *et al.*, 2005). *Colletotrichum gloeosporioides* also reported to cause postharvest disease on other crops such as pomegranate, mango, cashew, custard apple, acid lime, and papaya (Lakshmi *et al.*, 2011).

Colletotrichum gloeosporioides isolated from *C. papaya* lesions have orange pustules, brown, smooth, and septate hyphae, cylindrical conidia with obtuse ends, hyaline, aseptate, uninucleate, and up to 10-15 µm x 3-5 µm in size. The conidia grow on the conidiophores in the acervuli. Setae are dark brown in color, straight to slightly curved in shape, have two to three septa, swollen at the base, and are tapering towards the apex (Rahman *et al.*, 2008). A study by Tarnowski and Ploetz (2010) reported that *C. gloeosporioides* isolated from infected papaya in South Florida produced sunken lesions with dark gray centers and pink or gray sporulation.

2.3 Infection process of *Colletotrichum*

The process of infection and colonization of *Colletotrichum* species in plants tissues involve several stages. The infection process starts when conidia attach to host

surface of a plant. After the attachment, conidia or ascospores will germinate and differentiated to form appressoria. Pathogen activates the pathogenicity factors and penetrates into the host cells and after that infection hyphae start to develop and colonize the plant tissues. Successful colonization by the pathogen will develop decay symptoms in the host tissues (Bailey *et al.*, 1992). In most host-pathogen interactions, penetration process is accomplished within 12 to 24 hrs after the germination of spores in the host (Koller, 1991). In the case of papaya, the completion of penetration process by *C. gloeosporioides* takes three to four days (Chau and Alvarez, 1983). The penetration process by some pathogens need enzymes to dissolve the cuticle of the host (Bailey *et al.*, 1992).

Development of appressoria is an important stage in the process of infection by the fungus. Penetration process by the pathogen typically happens after formation of an appressorium (Bailey *et al.*, 1992). During penetration, the appressoria of infection hyphae from *C. gloeosporioides* form different shapes depending on the host (Brown, 1975). Development of appressoria is surrounded by mucilaginous material and this spore matrix structure performs a significant role in protecting appressoria from extreme environment such as cold, heat or drought as it is in adhesion (Bailey *et al.*, 1992). Development of appressoria help the fungus penetrated through epidermal cells (Agrios, 2005). Appressoria and infection pegs are common entry mode for penetration of papaya cuticle (Chau and Alvarez, 1983). Penetration of infection peg into the epidermal cells is control by high turgor pressure. This pressure is created by the glycerol from the

appressoria and glycerol in appressoria walls is protected by melanin from leaking out (Agrios, 2005).

There are few possible modes of penetration by *Colletotrichum* such as through wounds, stomata, and direct penetration of cuticular barrier (Bailey *et al.*, 1992; Isaac, 1992). Direct penetration of plant cuticle is the most common penetration spot by the fungus (Bailey *et al.*, 1992). *Colletotrichum graminicola* is a stalk-rot fungus that was reported has the ability to infect and colonize maize stalks through direct penetration method (Venard and Vaillancourt, 2007). A study of an unidentified *Colletotrichum* species was reported to penetrate the leaves of cowpea (*Vigna unguiculata*) tissue through stomata (Latunde-Dada *et al.*, 1999). Another examples were about *C. musae* which is known as wound anthracnose pathogen of banana (Chillet *et al.*, 2007) and *C. gloeosporioides* infected mango fruit also through wound (Dinh *et al.*, 2003).

2.4 Anthracnose of papaya

The word anthracnose is originated from a Greek word which means ulcer-like lesions that infected several hosts (Lucas *et al.*, 1985). Anthracnose is commonly characterized by very dark, sunken lesions, containing spore. These characteristics are derived from a Greek word meaning ‘coal’ (Isaac, 1992). Anthracnose caused by *C. gloeosporioides* (Penz.) Sacc, is a major postharvest disease that affects many tropical and subtropical fruits including papaya (Prusky, 1996). It was reported that atypical postharvest losses may due to surface shipments (5 to 30%) and air shipments (10-40%).

About 1-93% of postharvest losses were caused by some diseases such as stem end rots, fruit surface rots and internal fruit infections and the infections depends on handling and packaging procedures (Alvarez and Nishijima, 1987). In fact, 90-98% postharvest disease inflicted loss in papaya are caused by *C. gloeosporioides* (Rahman *et al.*, 2008).

Colletotrichum gloeosporioides is fungal phytopathogen that causes postharvest disease in papaya and the infection effect caused extreme postharvest losses to the papaya growers (Tapia-Tussell *et al.*, 2008). It happens to be the major postharvest disease of papaya in Sri Lanka (Gamagae *et al.*, 2003), Serbia (Zivkovic *et al.*, 2010), Hawaii, and several other tropical countries (Paull *et al.*, 1997). *Colletotrichum gloeosporioides* also causes postharvest diseases of other fruits such as avocado, almond (Freeman *et al.*, 1996), peach, pecan (Bernstein *et al.*, 1995), mango (Sangeetha and Rawal, 2008), pear, apple, and sour cherry (Zivkovic *et al.*, 2010).

However, other species of *Colletotrichum* also caused postharvest disease of papaya such as *C. capcisi*, *C. dematium* and *C. truncatum*. There was a report in South Florida about *C. capsici* which caused postharvest disease on papaya (Tarnowski and Ploetz, 2010). Another report in Yucatan, Mexico found that *C. capsici* and *C. gloeosporioides* were caused postharvest disease of papaya fruits (Tapia-Tussell *et al.*, 2008). A study on infected papaya fruits cvs. Red lady and Tainung No. 2 - F1 hybrid in Trinidad found that 21% species were belong to *C. truncatum* and 79% species were belong to *C. gloeosporioides* (Rampersad, 2011). Instead of *C. gloeosporioides*, *C.*

dematium which have hyaline conidia with sunken dark spots, also reported to cause postharvest disease of papaya in Maradol, Yucatan (Basulto *et al.*, 2011).

2.4.1 Symptoms of infection

Postharvest disease attacks all host plant parts at all stages of growth. The symptoms are most detectable on leaves and ripe fruits. Common symptoms that appear on fruits are dark, sunken, circular lesions that produce mucilaginous, and pink to orange conidial masses. The lesions unite under severe disease pressure (Zivkovic *et al.*, 2010). Symptoms of infection by *Colletotrichum* species are clearly characterized by sunken necrotic tissue and orange conidial masses (Bailey *et al.*, 1992).

There are two types of lesions on papaya fruit caused by *C. gloeosporioides* which are typical and atypical lesions. Typical lesions usually form round, water soaked, and a sunken spot with pinkish-orange areas that are formed by conidial masses. These conidial masses cover up the lesion center and produce concentric ring patterns. Atypical lesions form when infected plant parts are covered with brownish-black conidial masses (Tapia-Tussell *et al.*, 2008). There are three types of symptoms that appeared when papaya fruit infected by *C. gloeosporioides*. The first symptom is typical anthracnose lesions. The second symptom is chocolate spots or also known as reddish brown lesion that usually appeared when fruit ripened. The third symptom is stem end rot that commonly occur after harvest on fruit stems (Dickman and Alvarez, 1983). A study by Rahman *et al*. (2008) stated that symptoms of infection on wounded and unwounded

inoculated papaya show different types of lesions. Wounded inoculated papaya showed small, round water-soaked lesions, and the lesions become circular and slightly sunken and coated with dense white mycelium. Unwounded inoculated papaya shows small water-soaked, round sunken lesions that are translucent, and form light brown margins.

2.4.2 Postharvest disease management

There are a few methods that are used to control postharvest diseases of papaya such as hot water treatment (Alvarez and Nishijima, 1987), UV-C, gamma irradiation (Cia *et al.*, 2007), preharvest spraying, and dipping of fruits in fungicides (Bautista-Banos *et al.*, 2003a). Chemical fungicides that are primarily used to control anthracnose include trizoles and benzimidazoles (Coates and Johnson, 1997). Chlorothalonil or mancozeb were the greatest preharvest control of papaya six to eight month after planting (Alvarez and Nishijima, 1987). Unfortunately, fungicides that are present on the fruits are highly toxic and harmful to human health.

There are reports of biological means of controls of the postharvest disease caused by *C. gloeosporioides*. Previous study shows that stem and leaf extracts of papaya have a potential to reduce anthracnose infection of papaya and mango (Bautista-Banos *et al.*, 2002). Chitosan is a nontoxic compound derived from crab or prawn shells after deacetylation of chitin, is another treatment used to control the disease. The application of chitosan on papaya fruit gives an effective effect to control *C. gloeosporioides*. Besides that, chitosan helps to slow the metabolic activity and thus

delay ripening and senescence process of the papaya fruits (Hewajulige *et al.*, 2009). Used of chitosan alone to control *C. gloeosporioides* that infect papaya fruit gave the greatest effect. The application of chitosan coating on papaya fruit acted as a barrier by limiting fungal germ tube to penetrate the fruit (Bautista-Banos *et al.*, 2003b).

Spray treatment or postharvest dip with the combination between 2% sodium bicarbonate and biological agent, *Candida oleophila* also can be used to control *C. gloeosporioides* (Gamagae *et al.*, 2004). Similar study was observed when *Burkholderia cepacia* B23 was combined with 0.75% chitosan and 3% calcium chloride to control *C. gloeosporioides* (Rahman *et al.*, 2009).

2.5 Endophytes

The word endophyte indicate "in the plant" (endon = within, phyton = plant) (Schulz and Boyle, 2006). Microbial endophytes can be defined as active colonizers of aboveground tissues by performing long-term associations with their host without causing any damage (Bacon and Hinton, 2002). The most common microorganisms that existed as endophytes are fungi and bacteria (Strobel and Daisy, 2003). Endophytic bacteria is a bacteria which live in healthy plant tissues without causing any symptoms or injuries to the host (Bacon and Hinton, 2006).

There were three possibilities where endophytic bacteria start their colonization in plant tissues. It may be from the plant surface, begin from seed and pass on by migrating to the next generation of plants, or they originated from root and transported to the shoot through vascular system (Mano and Morisaki, 2008). Endophytic bacteria are usually found to be localized in the stem, root, fruit, leaf and tuber tissues of horticultural, agricultural, and forest plants (Sturz *et al.*, 2000). Endophytic bacteria also enter the plant tissues through wound, natural openings, and sometimes by using hydrolytic enzymes such as pectinase and cellulase (Hallmann *et al.*, 1997). Bacterial endophytes actively colonized plant tissues such as the roots, leaves, stems, fruits, and inflorescences (Bacon and Hinton, 2006).

Studies on the roots of banana plants showed that roots harbour the highest endophytic bacterial species (67.5 %) compared with the cortex (22.7 %) and central cylinder (9.8 %) (Pocasangre *et al.*, 2000). Studies on endophytic colonization of *Vitis vinifera* by *Burkholderia* sp. indicated that primary roots, secondary roots, lateral roots, and root tips have the highest bacterial concentrations (Compant *et al.*, 2005). Endophytic bacteria from *Bacillus* spp. have been isolated from few plants such as sunflower (Forchetti *et al.*, 2007), cotton (Reva *et al.*, 2002) and citrus plant (Araujo *et al.*, 2001).

Endophytic bacteria were usually isolated from several monocotyledonous and dicotyledonous plants. For examples, *Bacillus endophyticus* was isolated from cotton

plants (*Gossypium* sp.) (Reva *et al.*, 2002) and several species of endophytic bacteria such as *Enterobacter agglomerans, Klebsiella terrigena, Pseudomonas corrugate, P. fluorescens, P. marginalis, Pseudomonas* spp., and *Vibrio* sp. were isolated from stem of maize (*Zea mays* L.) (Fisher *et al.*, 1992). Other study reported that endophytic bacteria such as *Clavibacter*, *Cellulomonas*, *Curtobacterium*, and *Microbacterium* were isolated from agronomic crops such as soybean, sorghum, wheat, and corn; and prairie plants for examples alfalfa, grass, and weed (Zinniel *et al.*, 2002).

Six genera of endophytic bacteria which belong to *Pseudomonas* spp., *Bacillus* spp., *Micrococcus* spp., *Serratia*, *Arthrobacter* spp., and *Curtobacterium* sp., have been isolated from black pepper (*Piper nigrum* L.) stem and root tissues (Aravind *et al.*, 2009). Another example is *Pseudomonas putida* strain MGP1 was isolated from papaya fruit (*C. papaya*) (Shi *et al.*, 2010). Other species such as *Alcaligenes* sp., *Methylobacterium* spp., *B. pumilus*, *B. cereus*, *Burkholderia cepacia*, E*nterobacter cloacae*, *Pantoe agglomerans*, *Nocardia* sp., *Streptomyces* sp., *Curtobacterium flaccumfaciens,* and *Xanthomonas campestris* were the population of endophytes that was isolated from citrus plants (Araujo *et al.*, 2002).

To date, endophytes play a significant role in several potential applications. Endophytic bacteria can improve phytoremediation of an organic contaminant. Several endophytic bacteria isolated from poplar tree tissues such as root, shoot, and leaf showed that they have the potential to degrade benzene, toluene, ethylbenzene, and xylene

compounds (Moore *et al.*, 2006). Other study stated that engineered endophytic *B. cepacia* can improve contaminant degradation and ultimately reduce evapotranspiration to the environment (Barac *et al.*, 2004). Besides that, they also can promote growth of the plant. The *gus*-tagged *Pantoe agglomerans* isolated from deepwater rice seeds indicated that this bacterium can produce indole acetic acid which leads to root elongation and proliferation of the host plant (Verma *et al.*, 2001).

Endophytes also play a role to control plant diseases because they are able to produce various natural products. Endophytic bacteria have the ability to protect their host by secreting natural compounds against insect (Zhang *et al.*, 2011) , virus (Harish *et al.*, 2009), pathogens (Sturz and Matheson, 1996; Sharma and Nowak, 1998) and nematodes (Mekete *et al.*, 2009). The ability of bacterial endophytes to control plant diseases make them as a successful candidates as biological control agent. For example, *Streptomyces aureofaciens* CMUAc130 was reported to produce antifungal compounds, 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin. This antifungal element could inhibit the growth of damping-off pathogen, *Fusarium oxysporum* and *Colletotrichum musae* (Taechowisan *et al.*, 2005). Endophytic *B. subtilis* strains EPCO 16 and EPCO 102 and *Pseudomonas fluorescens* (Pf1) with the combination of chitin could reduced the invasion of aphid against cotton plant (Rajendran *et al.*, 2011). Another study stated that the application of endophytic *B. subtilis* E1R-j against *Gaeumannomyces graminis* var. *tritici* (*Ggt*) which infected the wheat plant caused some effects such as retardation of the *Ggt* infection and colonization in root tissues and the hyphae of *Ggt* appeared swollen, ruptures, and shriveled (Liu *et al.*, 2009).

The present and future trends in research show that biological control agents are effective strategy to inhibit major postharvest decays of fruit. A variety of microbial antagonists can act as a biological control of diseases and pests of crops which are eco-friendly in comparison to the chemical pesticides and fungicides. Microbial antagonists are being studied broadly against several different plant diseases and they are important component of integrated plant disease management (Palaniyandi *et al.*, 2011). Potential oncogenic risks of fungicides, possibilities of fungicide to develop resistance against pathogens, and risk aspects to human health and environment encourage researchers to develop the alternative control method which is by using antagonistic microbes (Wilson and Wisniewski, 1989).

Antagonistic microbes can compete with the pathogens for nutrient availability, inhibit the growth of pathogen by producing toxins or antibiotics, and/or diminish the pathogen population through hyperparasitisms (Zivkovic *et al.*, 2010). The effectiveness of antagonist is achievable by applying them on the fruit surface before the pathogen reach the infection site (Capdeville *et al.*, 2007a). The effectiveness of endophytes towards crop growth, yield enhancement, and potential BCA depends on their abilities to colonize plant tissues, promote root growth, and have a natural association with the host (Chen *et al.*, 1995).

Endophytic *Bacillus amyloliquefaciens* strain GA1 which produced chlorotetaine as a dipeptide antibiotic can be a potential BCA as well as promote plant growth

(Arguelles-Arias *et al.*, 2009). *Bacillus vallismortis* strain ZZ185 which was isolated from Broadleaf Holly (*Ilex latifolia*) plant was reported to inhibit plant pathogens by producing Bacillomycin D bioactive compounds (Zhao *et al.*, 2010). Endophytic *Bacillus subtilis* strain EDR4 produces an antifungal protein that inhibits the growth of take-all disease in wheat caused by *Gaeumannomyces graminis* var. *tritici* (Liu *et al.*, 2010). Besides that, endophytic *Pseudomonas viridiflava* which was isolated from grass tissues was found to produce two novel ecomycins. These compounds were known as an inhibitor against two human pathogens, *Cryptococcus neoformans* and *Candida albicans* (Ryan *et al.*, 2008).

Other studies stated that yeast *Cryptococcus magnus* is very effective in the control of *C. gloeosporioides* in papaya by suppressing the development of anthracnose (Capdeville *et al.*, 2007b). Endophytic bacterial strain H-6, which was identified as *Burkholderia* sp. strongly inhibited the growth of six phytopathogenic fungi such as *Fusarium graminearum, Sclerotinia libertiana, Phytophthora capsici, Rhizoctonia solani, Sclerotinia scleroliorum,* and *Fusarium oxysporum* (Sesame fusarium wilt) (Wang *et al*., 2010). *Burkholderia cepacia* isolated from fruit surfaces of papaya produce pyrrolnitrin, that is effective in controlling papaya postharvest disease caused by *C. gloeosporioides* (Kadir *et al.*, 2008).

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CHAPTER 3 - Materials and Methods

3.1 Workflow of research

The flow chart shows the workflow to find potential bacterial endophyte against *C. gloeosporioides* (Figure 3.1).

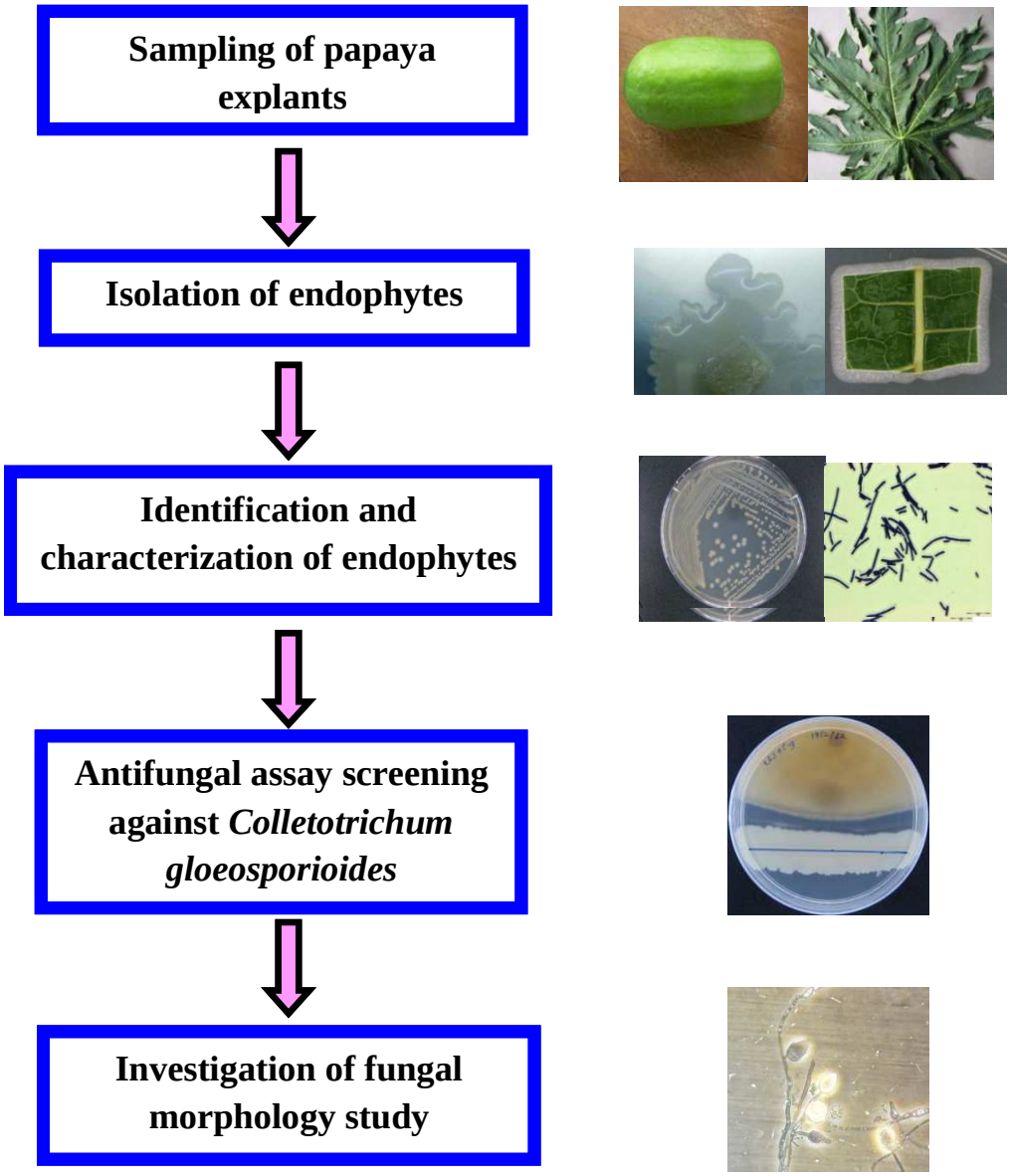


Figure 3.1: Experimental work flow followed in this investigation for isolation and characterization of antifungal endophytic bacteria from *C. papaya* fruits and leaves.

3.2 Pathogenic strains used in the study

Two pathogenic fungi of *Colletotrichum gloeosporioides* isolates 1 and 2 were obtained from Plant Pathology Lab 117, School of Biological, Universiti Sains Malaysia (USM), Pulau Pinang. The strains were cultured for four days on Potato Dextrose Agar (PDA) (Conda) plates at 30°C. The culture was transferred and grown in universal bottle contain PDA medium and stored in a refrigerator at 4°C, as stock culture.

3.3 Plant materials

Healthy fruits and leaves of *C. papaya* L. (Sekaki) were collected from an orchard in Ladang Mengkuang, located at Bukit Mertajam, Pulau Pinang (Figure 3.2). Upon arrival in the laboratory, the fruits and leaves were immediately washed under running tap water.

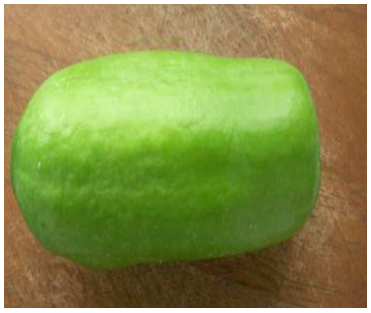
(a)  (b) 

Figure 3.2: Healthy *C. papaya*. (a) fruit and (b) leaf.

Surface sterilization of the intact explants were done aseptically in a laminar flow hood with 70 % ethanol, followed by 5 % sodium hypochlorite, and finally rinsed with sterile distilled water, before leaving them to air dry.

3.4 Isolation of endophytes

The isolation of endophytic bacteria was done as described by Araujo *et al*. (2002) and Yin *et al*. (2011) with some modifications. The plant materials were cut into small pieces (approximately 1 x 1 cm) with a sterile knife and then surface disinfected with 80 % ethanol for 10 min, sterile distilled water for 10 min, 5 % sodium hypochlorite for 6 min, sterile distilled water for 10 min, 80 % ethanol for 10 min and finally with two rinses in sterile distilled water for 10 min. The effectiveness of surface sterilization of samples were checked by plating 1 ml aliquots of water from the final wash, onto Nutrient Agar (NA) (Merck) and further incubated at 27°C for 2 - 4 days, to check for visible growth of bacterial colonies.

The plant tissues were placed on NA and incubated at 27°C for 1 - 3 days to allow growth of endophytic bacteria. After three days of incubation period, the endophytic bacteria were seen emerging from the edges of plant tissues onto the NA medium. Well grown endophytic bacteria were streaked onto fresh NA plates and were further purified by repeatedly streaking until pure culture was obtained. Pure cultures were cultivated in 5 ml of Nutrient Broth (NB) (Merck) with constant shaking at 37°C

overnight. The cultures were suspended in 80 % glycerol solution and stored at -80°C for future use.

3.5 Identification of endophytes

The identification of endophytic bacteria was based on morphological, biochemical characterizations, and 16S rDNA gene sequence as described in the sections below.

3.5.1 Gram staining

Gram staining was carried out to observe the cell morphology as describe by Pommerville (2011) with some modification. Pure culture of endophytic bacteria were streaked on NA and incubated at 37°C for 15 h. Small droplet of sterile distilled water was applied to the center of the slide. Freshly grown endophytic bacteria was picked with a toothpick and smeared into the water droplet. The smeared bacterial endophytes were allowed to dry slowly through the Bunsen burner. The dried smear was stained with crystal violet, left for 1 min and rinsed with water, followed by staining with iodine solution for 1 min and again rinsed with water. The smear was decolorized by using acetone for 10 s and immediately washed with water. The slide was air dried and observed under a light microscope (Olympus, BX51) under 20X magnification.

3.5.2 Morphological characterization

Colony morphologies of bacterial endophytes were determined by observation of the shape, elevation, margin, color, appearance and texture of the single colonies (Bauman, 2006).

3.5.3 Identification and biochemical characterization by phenotyping (BIOLOG GEN III MicroPlate System)

The identification and biochemical characterization of bacterial endophytes were carried out with BIOLOG GEN III Microbial Identification System (Focus Biotech) following the manufacturer's instructions. Data analysis was carried out using Omnilog Data Collection software version 2.3.

The endophytic bacterium was cultured on a general growth medium, NA for 15 h. Then, a colony of pure culture (approximately 3 mm diameter) was picked from the surface of the NA by using sterile cotton swab. The bacterium was released into the Inoculation Fluid B (IF-B) by rubbing the swab tip against the tube containing IF-B. Any cell clumps were crashed against the tube wall. The inoculation fluid (IF-B) was gently inverted upside-down a few times to obtain a uniform cell suspension. The transmittance of cell density (%T) was read using Biolog Turbidimeter (Focus Biotech) with a target cell density in the range of 93-98%.