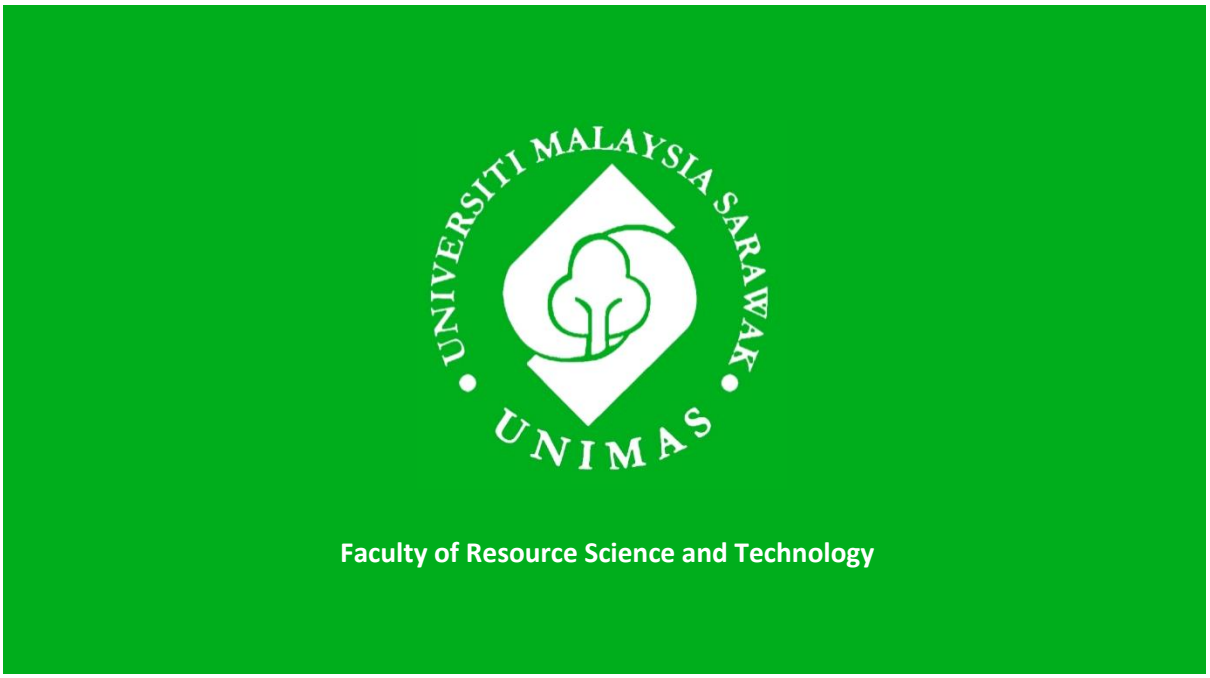

IN VIVO TEST AND ANALYSIS OF THE BACTERIA ISOLATES ON PLANT PATHOGENIC FUNGI

Ranina Alya Mohamad Radzi (38456)

Bachelor of Science with Honours
(Resource Biotechnology)
2015

UNIVERSITI MALAYSIA SARAWAK

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IN VIVO TEST AND ANALYSIS OF THE BACTERIA ISOLATES ON PLANT PATHOGENIC FUNGI

Ranina Alya Mohamad Radzi (38456)

This thesis is submitted in partial fulfillment of the requirement for the
Degree of Bachelor of Science with Honours
(Resource Biotechnology)

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Declaration

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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List of Abbreviations

P10	*Burkholderia unamae*
P11	*Enterobacter clocae*
ANOVA	Analysis of variance
°C	Celsius
cfu	colony-forming unit
cm	centimetre
g	gram
h	hour
ISR	Induced systemic resistance
mL	millilitre
mm	millimetre
nm	nanometre
NaClO	sodium hypochlorite
PGP	Plant growth promoting
R	Radial growth of pathogen
r	Radial growth of fungal colony
w/v	Weight per volume

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ABSTRACT

In vivo screening and pot trial of two bacterial, *Burkholderia unamae* (P10) and *Enterobacter clocae* (P11), were conducted to investigate the plant growth promoting (PGP) activities and inhibition zone of mycelial growth. Both bacteria were capable of inhibiting three of plant pathogenic fungi, which are *Colletotrichum* sp., *Fusarium* sp. and *Phythophora* sp. to various degrees. The results primarily showed that the bacteria were effective and resulted in strong antifungal activity against the fungi. However, results shows that *Burkholderia unamae* P10 was the most effective bacterial, with the potential to suppress mycelial growth of *Colletotrichum* sp., *Fusarium* sp. and *Phythophora* sp. *in vivo*. Moreover, *Burkholderia unamae* (P10) exerted efficient antagonistic activity against the pathogens in a dual culture assay. *In vivo* suppression activity of selected bacteria was also analyzed in a pot trial with reference to their prominent *in vivo* antagonism efficacy. Induced systemic resistance in *Capsicum annum* against the plant pathogenic fungi was also observed under pot trial conditions. Bacteria *Enterobacter clocae* (P11) was found to be the most effective bacteria at suppressing *Capsicum annum* diseases caused by pathogenic fungi infection under pot trial. Moreover, both bacterial *Burkholderia unamae* (P10) and *Enterobacter cloacae* (P11) were identified as the good candidate for the development of biocontrol agent.

Keywords: *Burkholderia unamae* (P10), *Enterobacter cloacae* (P11), *Capsicum annum*, Induced systemic resistance (ISR), Plant growth promoting (PGP).

ABSTRAK

Pemeriksaan in vivo dan uji kaji pasu terhadap Burkholderia unamae (P10) dan Enterobacter clocae (P11) telah dijalankan untuk mengkaji aktiviti penggalakkan pertumbuhan tanaman (PGP) dan menyiasat zon perencatan tumbuhan kulat. Kedua-dua bakteria mampu menghalang pertumbuhan tiga kulat, iaitu Colletotrichum sp., Fusarium sp. dan Phythophora sp. sehingga beberapa darjah. Keputusan menunjukkan bahawa kedua-dua bakteria berkesan dan berjaya menghasilkan aktiviti anti-kulat yang kuat. Walaubagaimanapun, keputusan menunjukkan bahawa Burkholderia unamae (P10) adalah bakteria yang paling berkesan, dan berpotensi untuk menghalang pertumbuhan kulat Colletotrichum sp., Fusarium sp. dan Phythophora sp. dalam in vivo dan menunjukkan aktiviti antagonistik yang cekap terhadap kulat dalam uji kaji dua-kultur. Aktiviti perencatan secara in vivo juga dilakukan kepada bacteria terpilih dengan uji kaji pasu untuk melihat kesan antagonisik antara bakteria dan kulat. Penghalangan sistemik Capsicum annum terhadap pertumbuhan kulat dapat diperhatikan di bawah uji kaji pasu dimana Enterobacter clocae (P11) telah didapati menjadi bakteria yang paling berkesan untuk mengurangkan penyakit pada Capsicum annum. Sebagai keseluruhan,, kedua-dua bakteria Burkholderia unamae (P10) dan Enterobacter cloacae (P11) telah dikenal pasti sebagai agen biocontrol yang baik.

Kata kunci: *Burkholderia unamae* (P10), *Enterobacter cloacae* (P11), *Capsicum annum*, *Penghalangan sistemik, Aktiviti penggalakkan pertumbuhan tanaman*

1.0 INTRODUCTION

1.1 General Introduction

Few people may realize on the importance of the green plants towards everyone health. As the primary procedures in the ecosystem, green plants provide the energy and carbon which almost all of the living organisms dependent on. The growth and productivity of plants determines the food supply chain of animal populations, including the human population.

Almost 80% of all plant diseases are caused by fungi (Choudhary *et al.*, 2007). The most common pathogens that may cause disease towards plant are species of *Fusarium, Phytophthora, Pythium and Rhizotonia* (Giesler and Ziems, 2008). In agriculture and horticulture nowadays, nearly 200 different fungicides have been introduced (Kim and Hwang, 2004). But in present study shows that, the application of fungicides are insufficient to control disease of a crop plant (Choudhary *et al.*, 2007). Plus several studies show the potential health hazard for humans due to the production by pathogens of toxic metabolites in the affected sites.

As an initiative, biological control is one of the ways plant diseases to control disease as well as health hazard for human. Synthetic chemical are utilized widely because they often work very well for controlling fungi. However, fungicides are not generally the correct answer. Sometimes they cannot control fungi effectively for a variety of reasons

These pathogens may be cured or treated by using biological control agents. The goal of biological control is to deliberate use of living organisms to control fungi. As an example, microorganisms used as biological control agent are bacteria. Several studies have shown the efficacy of these bacteria in controlling pathogens of a plant. For examples, diazotrophs bacteria are known to counter the detrimental effects that follow with the onset of a pathogen attack. Previous study reported that diazotrophs are capable of synthesizing antibiotics and anti-fungal compounds (Dobbelaere *et al.*, 2003).

These bacteria produce bioactive metabolite that can inhibit the growth of the plant pathogenic fungi. Antibiotic-producing antagonists may be particularly effective against wound infections that occurred sometime prior to the application of the antagonist, as well as against pathogen that infect the commodity through latent infections (Bolland and Kuykendall, 1998).

In addition, several other bacterial strains are reported capable of inducing resistance in different plant species, whereas others show specificity, indicating specific recognition between bacteria and plants at the root surface. PGP or known as plant promoting growth can promote plant growth and reduce plant disease (Idriss *et al.*, 2002) It have been reported to benefits plant growth and improving plant health through various mechanisms. PGPB are commonly used as inoculate for improving the growth and yield of agricultural crops and offers an attractive way to replace chemical synthetic fertilizers (Saharan and Nehra, 2011).

In the present scenario, if the fungal pathogens affect the plant, the productivity of agricultural crops will decrease. The plant diseases need to be controlled to maintain the level of yield both quantitatively and qualitatively. For example, since *Capsicum annum* are a necessity in every household the consequences of plant disease may be more serious, and crop failure can damage local or national economies, and lead directly to famine and hardship. Other than that, farmers often rely heavily on the use of synthetic fungicides to control the plant diseases. However, the environmental problems caused by excessive use and misuse of synthetic fungicide have led to considerable changes in people's attitudes towards the use of synthetic pesticides in agriculture. Nevertheless, the environmental and commercial requirements for new fungicides become more demanding; it is increasingly difficult to discover new classes of compounds to justify the effort and the costs of development. In order to increase the public awareness with regards to the use of chemical fungicides, there is a need for better and safer compounds and to build-up resistant strains against targeted pathogens.

1.2 Objectives

i. To test and evaluate the bacteria antagonistic effect of selected bacteria on *Capsicum annum* plant pathogenic fungi.
ii. To evaluate the effects of plant growth promotion (PGP) and induced systemic resistance (ISR) effect on *Capsicum annum.*

2.0 LITERATURE REVIEW

2.1 Biological control

The term “biological control” was begun in connection to plant pathogens by Von Tubeuf in 1914 (Hajek, 2004). Biological control has commonly defined as the use of living organisms to suppress the population of a specific pest’s organism, making it less abundant or less damaging than it would otherwise be (Hajek, 2004).

According to Hajek (2004) the reason biological control is utilized are to develop the biological control methods after synthetic chemical pesticide application turned into the overwhelming strategy for pest control. Use of biological control grew due to practical needs to find solution to pest problems when chemical pesticides did not work or were not appropriate for controlling particular nuisances (Hajek, 2004). Biological controls leave no chemical residues and are usually quite host specific (Ji *et al*., 2013).

2.2 Modes of antagonism

The modes of antagonism are in competition, antibiosis and parasitism (Tronsmo, 1996). Competition occurs when there are demands by two or more microorganism and the application of biological control becomes more effective and successful (Tronsmo, 1996). Antibiotic substances are often based on results obtained from *in vitro* tests, in which the

antagonist inhibits the growth of the pathogen in culture (Bolland and Kuykendall, 1998). Similar results may be obtained also from *in vivo* tests indicating inhibitory activity for the same resource in excess of the immediate supply (Mukerji and Grag, 1998). For example, there is the need among the organism for nutrients, oxygen, space and others (Tronsmo, 1996). Antibiotic substances are often based on results obtained from in vitro tests, in which the antagonists inhibit the growth of the pathogen in culture (Boland and Kuykendall, 1998). According to Boland and Kuykendall's study in 1998, questions the sole involvement of the antibiotic substances pyrrolnitrin, produced by *Pseudomonas cepacia*, in the control of *Penicillium digitatum* on the lemon fruit. The development of antibiotic-resistance that isolates from *P. digitataum,* demonstrated that the pathogen was still inhibited by the antagonist (Boland and Kuykendall, 1998).

Direct parasitism is well documented in biological control by *Trichoderma spp.* of soil-borne and foliar diseases (Elad, 1995). According to Wisniewski *et al.* (1991), studies in vitro attachment between a cells and yeast against *P. guilliermondii* towards the mycelium of *Botrytis cinerea*. This may involve lectin-type binding, which was blocked by exposure to compounds that affect protein integrity and respiration. Wisniewski *et al.* (1991), reported that *P. guiliermondii* exhibited high activity of β-1, 3-glucanase, which may be associated with its attachment and degradation of the cell walls of pathogen.

2.3 Plant Growth Promoting (PGP) bacteria

There is a huge assortment of literature describing the potential use of plant associated bacteria, the plant growth-promoting (PGP) bacteria as agents stimulating plant growth, plant health and managing soil (Ji *et al*., 2013). PGP can competitively colonizing plant root, promote plant growth, and reduce plant diseases (Ji *et al*., 2013). Competition for an ecological niche/substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens are generally recognized mechanism of biocontrol mediated by PGP (Choudary *et al*., 2007). PGP can suppress diseases through antagonism between bacteria and soil-borne pathogens, as well as by inducing a systemic resistance in the plant against both the root and foliage pathogen (Kloepper *et al.*, 2004).

For examples, in a previous study done by Liu *et al.* (2006), a nitrogen fixing bacteria, *Bacillus megaterium* was isolated from maize which did not showed any antifungal activity. In another recent report, was isolated from banana plant. It was concluded that even-though *Bacillus megaterium* is not a nitrogen fixing bacterium, it can be efficiently used in a bio-organic fertilizer against plant pathogenic fungi (Zhang *et al.*, 2011). In other hand, a present study shows that endophytic bacteria were isolated from rice cultivates in Korea. It were then identified and characterized for their functional traits with plant promoting growth (Ji *et al.*, 2013).

2.4 Induced Systemic Resistance (ISR)

Induced systemic resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli. Plant defences are preconditioned by prior infection or treatment that results in resistance against subsequent challenge by a pathogen are forms of induced resistance (Choudhary *et al.*, 2007). ISR which can be differentiated and offering total protection on the basis and the nature of elicit and regulatory pathways involved. ISR is induced by plant growth-promoting (PGP), of which best characterized are strains that belong to genus Bacillus that cause no visible damage to the plant's system (Ray, 2007). Many effective biocontrol PGP elicit ISR also reacted faster and more strongly to pathogen attack by inducing defence mechanism (Choudhary *et al.*, 2007).

2.6 Plant Pathogenic Fungi

Plant diseases are caused by several of fungi; this will result in a significant lost in agricultural crops (Suprapta, 2012). According to Agrios (2005), reported that more than 10, 000 species of fungi are able to cause diseases towards agricultural crops. The pathogenic fungi are the main threat towards food production and food storage (Arguelles-Arias *et al.*, 2009). Post-harvest food spoilage also represents a potential health hazard for humans due to the production by pathogenic fungi (Arguelles-Arias *et al.*, 2009).

2.6.1 *Colletotrichum* sp.

Colletotrichum is the causal agent of anthracnose and other diseases on leaves, stems and fruits of various plant species, including several essential crops (Yang *et al.*, 2009). For example, a previous studies done by Rojas *et al.* (2010), a *Colletotrichum* species associated with cacao plant such as *Colletotrichum tropical* and *Colletotrichum ignotum*, frequently as asymptomatic while *Colletotrichum theobromicola* is associated with foliar and fruit anthracnose lesions of cacao.

2.6.2 *Fusarium* sp.

According to Champeil *et al*. (2004), an extensive review has been studied that Fusarium sp. may produce mycotoxin production. With this, it have been proven by Mesterhazy (2003) that Fusarium sp. have often looked as a fungicides which damaging the agricultural crop. Fusarium sp. is reported the most isolated fungi in soil that act as decomposers and decaying the plant nutrients (Summerbell, 2003). One of the examples that, cause by Fusarium sp. is Fusarium Head Blight (FHB) on wheat or barley plant (McMullen *et al.*, 2008).

2.6.3 *Phytophthora* sp.

Phytophthora sp. is one of the most abundant of soil fungi which can infect both via the roots and air, causing roots and the basal part of the stem to rot and it wounds the stem cortex (Del Rio *et al.*, 2003). Some of *Phytophthora* sp. are also found on trees, such as pistachio, almond, and the exact species is *Phytophthora parsiana* (Mostowfizadeh-Ghalamfarsa et al., 2008). Previous study also shows that in United State, *Phytophtora ramorum* has been found to cause oak death (Werres *et al.*, 2001). *Phytophthora* sp. also is the most serious problem that could be seen in cultivating *Capsicum annum* (Akihiri et al., 1992).

2.7 *Burkholderia unamae* P10 and *Enterobacter cloacae* P11 as biological control agents

Burkholderia and *Enterobacter* are two different and dynamic genuses, it containing pathogenic species and also species that form complex structures interactions with plants (Angus *et al.*, 2014). The opportunistic *Burkholderia* pathogens are known to promote plant growth and even fix nitrogen. Most of the species could be utilized for promotion of growth and development of the plants. It is also have the ability to produce bioactive compounds such as hydrolytic enzymes and antifungal. Their biological and metabolic properties have been exploited for biological control of fungal pathogens and diseases in plants as well as for plant growth promotion (Kim and Hwang, 2004).

2.8 *Capsicum annum*

Chilli or red pepper is the dried ripe fruits of *Capsicum annum* of the family Solanaceae and the leading vegetable-cum-spice of India (Farooqi *et al*., 2005). The very hot, peppery variety, with high capsaicin content, is used as spices and seasoning; also in medicine (Seidemann, 2005). Few decades ago, the production of chilli has gradually decreased due to the infection of various diseases such as anthracnose caused by *Colletotrichum capsici* and fusarium wilt caused by *Fusarium oxysporum* (Bokaew *et al*., 2011). These diseases are very widespread, affecting a large number of agricultural plants (Farooqi *et al*., 2005).

3.0 Materials and Methods

3.1 Media Preparation

3.1.1 Potato Dextrose Agar (PDA) media

Potato Dextrose Agar (PDA) powder was weighed and mixed with distilled water inside a 500 ml Schott bottle. The mixture was stirred using magnetic stirrer. Next, the media was autoclaved at the temperature at 121 °C. While autoclaving, the cap of the bottle is loosen and sealed with aluminum foil to prevent contamination. Then, the media were poured onto a Petri plate and kept in the refrigerator.

3.1.2 V8 agar

V8 concentrate were defrosted. A volume of 500 ml of V8 juice was prepared by weighing and mixing the V8 juice with Difco agar inside a 500 ml Schott bottle. The mixture was stirred using magnetic stirrer and the media was autoclaved at the temperature at 121 °C. The cap of the bottle is loosen and sealed with aluminum foil to prevent from contamination before it being autoclaved. Media were then poured onto a Petri plate and kept in the refrigerator.

3.1.3 Nutrient Agar media

Nutrient agar (NA) powder was weighed and mixed with distilled water inside a 500 ml Schott bottle. The mixture was stirred using magnetic stirrer. Next, the media was

autoclaved with the temperature at 121 °C. The cap of the bottle is loosen and sealed with aluminium foil to prevent from contamination before it being autoclaved. Then, the media were poured onto a Petri plate and kept in the refrigerator.

3.1.4 Water agar media

Difco agar powder was weighed and mixed with distilled water inside a 500 ml Schott bottle. The mixture was stirred using magnetic stirrer. Next, the media was autoclaved with the temperature at 121 °C and soon dropped. The cap of the bottle is loosen and sealed with aluminium foil to prevent from contamination before it being autoclaved. Then, the media were poured onto a Petri plate and kept in the refrigerator.

3.1.5 Nutrient Broth media

Nutrient Broth (NB) powder have been weighed and mixed with distilled water inside a 250 ml of Erlemenyer flask. The mixture was stirred using magnetic stirrer. Then the media were poured in a bijou bottles equally. The media was autoclaved with starting temperature at 121 °C and soon dropped. The cap of the bottle is loosen and sealed with aluminium foil to prevent contamination before it being autoclaved.