UNIVERSITI PUTRA MALAYSIA

SCREENING OF ANTAGONISTIC RHIZOBACTERIA AND ENDOPHYTIC BACTERIA FROM PEPPER (PIPER NIGRUM) FOR CONTROLLING FUSARIUM OXYSPORUM

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SCREENING OF ANTAGONISTIC RHIZOBACTERIA AND ENDOPHYTIC BACTERIA FROM PEPPER (*Piper nigrum*) FOR CONTROLLING *Fusarium oxysporum*

By

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ABSTRACT

Bacteria isolated from soil of pepper rhizosphere, stem and root tissues of *Piper nigrum* were tested for their antagonistic effect against Fusarium oxysporum through in-vitro based on dual culture test. From the ten bacterial isolates selected for the test, nine of them which are BC1, BC2, BC3, BC4, BC5, BC6, BC7, BC8 and BC10 were identified. Evaluations of the mechanisms of antagonism were based on substrate competition (zone inhibition) and inhibitory effect on spore germination. The study demonstrated effective biological control by the rhizobacterial and endophytic bacterial isolates tested, thereby indicating the possibility of application of rhizobacteria and endophytic bacteria for control of soilborne diseases of *P. nigrum* and also other crops.

ABSTRAK

Bakteria yang diasingkan daripada tanah kawasan pengakaran, tisu batang dan akar pokok lada hitam, *Piper nigrum* telah diuji untuk menentukan kesan antagonistik terhadap kulat *Fusarium oxysporum* melalui ujian in-vitro dwi-kultur. Daripada sepuluh bakteria yang diasingkan itu, Sembilan daripadanya iaitu BC1, BC2, BC3, BC4, BC5, BC6, BC7, BC8 dan BC10 telah dikenalpasti sebagai bakteria yang berpotensi sebagai agen kawalan biologi untuk melawan *F. oxysporum*. Penilaian terhadap mekanisme antagonistik adalah dibuat berdasarkan persaingan ke atas substrat (perencatan zon pertumbuhan) serta kesan perencatan kepada percambahan spora. Kajian ini menunjukkan keupayaan kawalan biologi oleh bakteria endofitik dan rhizobakteria yang diuji, dengan itu menunjukkan kemungkinan bakteria-bakteria ini diaplikasikan untuk kawalan penyakit pokok lada hitam, *P. nigrum* serta tumbuhan lain.

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Thank you.

Wassalam.

APPROVAL

I certify that this project report entitled "Screening of Antagonistic Rhizobacteria and Endophytic Bacteria from Pepper (*Piper nigrum*) for Controlling *Fusarium oxysporum*" has been examined and approved as a partial fulfillment of the requirement for the degree of Bachelor of Science Bioindustry Science in the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Sarawak Campus.

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CHAPTER 1

INTRODUCTION

1.1 Background

Pepper (*Piper nigrum* L.) is a crop commonly known as black pepper and has been cultivated in Malaysia for more than a century. It is an important agricultural crop of which has once put Malaysia in the spice world as the largest pepper producer in the world (Bong and Saad, 1985). It is a native crop to the Western Ghats of Kerala State in India where it still occurs wild in the mountain areas. The crop reached South East Asia as early as 100 BC, brought by Hindu colonists migrating from India to Indonesia and other countries. In India, Indonesia and Malaysia, there is a long established tradition of commercial cultivation by smallholders. Early in the 19th Century, pepper was spread to Sarawak where nowadays 95% of the Malaysian crop is produced. In the 1990s, Sri Lanka and China overtook Malaysia in the crop's production, while Thailand and Vietnam also became important producers (Anunciado and de Waard, 1999).

Black pepper and white pepper are the two main dried commodities growers prepare from the fruits of *P. nigrum*. Their use for food flavouring was already known in classical Rome and Europe was an important importer of pepper as early as the 12th Century. About 80% of the pepper consumption is now concentrated in the industrially developed countries, where it is mainly used in domestic culinary and also used as flavourings in processed foods. Of secondary importance is the use of preserved immature green pepper or fresh green pepper fruits.

In the past decade, pepper has earned the country a total of more than $ 1 billion in foreign exchange from more than 300,000 tons of pepper exported during that period. However, in recent years Malaysia has lost its leading place in pepper production. Malaysia was being surpassed by Brazil and other pepper producers such as Indonesia and India. This was due to decrease of production, where from 1979 to 1984 there was a 54% or 9% per annum reduction in output of pepper (Bong and Saad, 1985).

Sarawak has introduced four black pepper varieties from India (Balancotta, Cheriakaniakadan, Kalluvally and Uthirancotta) and three from Indonesia (Bangka, Belantung and Djambi). Among the varieties being introduced, only those that are from Indonesia are of interest to Sarawakian farmers. Besides the seven varieties aforementioned, Sarawak herself has two varieties, namely Kuching and Sarikei. The former is widely planted throughout the State since it grows well, produce high yield and has superior quality. Despite the properties mentioned, the Kuching variety also favour to prolific multiplication of pests and the development of diseases (Kueh, 1985).

1.2 Problem Statement

Infection of diseases has been one of the factors that contribute to the decline of pepper cultivation in Malaysia (Bong and Saad, 1985). The diseases may include foot and crown rot caused by *Phytophthora capsici*, little sickle leaf disease caused by *Fusarium solani* and *F.moniliforme*, horse hair blight by *Marasmius equicrineis*, leaf rot by *Corticium solani*, leaf antrachnose by *Colletotrichum capsici*, *C.piperis*

and *Glomeria cingulata*, pink disease by *Corticium salmonicolor* and root rot by *Ganoderma lucidum* (Kueh, 1985).

The project focuses on *Fusarium oxysporum* which causes slow decline and wilting of the crop. Initially, pesticides were used to treat crop diseases. However, treating diseases calls for improvement since the use of pesticides itself cause problems: pesticides are injurious to the environment, not cost effective, induce generation of microbes resistant to pesticides (Francesco *et al*., 2008). Under such circumstances, biological control (biocontrol) using microbe is an alternative to treat diseases in crops. The project aims to obtain microbes from the pepper rhizopsphere that possess antagonistic property against *Fusarium oxysporum* through in-vitro screening, as an alternative for pesticides.

1.3 Objectives

The objectives of this study were:

1. To screen the rhizobacteria and endophytic bacteria for biological control of *Fusarium oxysporum*.
2. To determine whether isolated soil bacteria and endophytic bacteria possess suppressive properties towards *Fusarium oxysporum*.

CHAPTER 2

LITERATURE REVIEW

2.1 Black pepper (*Piper nigrum* L.)

Pepper belongs to the family *Piperaceae* which has about ten genera. *Piper* is a large genus in this family with over 1000 species of herbs, shrubs and climbers mainly found in the tropics of both hemispheres. The origin of the pepper family is not firmly determined. It is probably an independent, specialized and terminal off-shoot of direct Ranalian ancestry (Sim, 1985).

Pepper is native to the Western Ghats of Kerala State, India, where it still occurs wild in the mountains. Pepper reached South-East Asia as early as 100 BC, brought by Hindu colonists migrating from India to Indonesia and other countries. In about 1930, Japanese immigrants who had traveled through South-East Asia introduced the plant into Para State of Northern Brazil, where it became a major crop (Anunciado and de Waard, 1999).

Pepper which is commonly known as black pepper has been cultivated in Malaysia for more than a century. It is an important agricultural crop of which has once put Malaysia in the spice world as the largest pepper producer in the world. In the past decade, pepper has earned the country a total of more than $1 billion in foreign exchange from more than 300,000 tons of pepper exported during that period (Bong and Saad, 1985).

In recent years, Malaysia has lost its leading place in pepper production, being surpassed by the up and coming Brazil, and other pepper producers such as Indonesia and India. This was due to a marked decrease in production. From 1979 to 1984 there was a 54% or 9% per annum, reduction in output of pepper. This declining trend evoked great concern among the agricultural sector, especially in the state of Sarawak where pepper is the most important crop (Bong and Saad, 1985).

Since the late 70s the pepper industry in Malaysia had been constantly plagued by a number of major problems, notably the low price, high production costs and diseases (Bong and Saad, 1985).

2.2 Crop Diseases of *Piper nigrum*

Thirty-four species of vertebrate and invertebrate pests, eighteen fungi and one alga are associated with black pepper in Sarawak. Among the pests, root-knot nematodes, pepper weevil, green pepper bug, tinged bug and birds are of economic importance. The most important fungal diseases include black berry disease, foot rot, little sickle leaf disease, pink disease, thread blight and white root (Kueh, 1985).

The major disease of pepper cultivars in Malaysia and Indonesia is the foor-rot disease caused by Phytophthora palmivora MF4, a soilborne fungus thriving especially under warm and humid conditions. The disease may infect the leaves and roots, underground stem and root collar. Leaves especially the lower ones are infected through soil splash which results in the formation of black necrotic spots with typical fringed margins. The affected leaves will drop within a few days before the disease spreads out up to the stem, where the leaf drop contributes in building up

the soil inoculums. The symptoms of the leaf wilt are visible especially during the end of rainy season. They result from blocked water vessels in the stem and increasing water stress. Meanwhile, infected vines would die within days or weeks. A second significant disorder popular in the South East Asia is the slow wilt 'yellow disease' which occurs mainly in the Bangka. The symptoms of this disease include slow wilting and associated yellowing and drooping of leaves. The disorder has been identified as a combination of poor mineral nutrition and root invasion by *Rapholus* nematodes (Anunciado and de Waard, 1999).

2.3 Biocontrol

Biological control (or biocontrol) is the deliberate use of natural predators, antagonists, or competitor organisms to suppress a pest population, thereby making the pest less abundant and damaging than it would be in the absence of these organisms. Biocontrol of plant diseases can be achieved using a broad spectrum of organisms, from insects to fungi and bacteria (Cortes-Penagos *et al.*, 2007).

The use of biological control is not a new idea. Its conception was established between the late nineteenth and early twentieth centuries, as biological and ecological knowledge of the natural world was gradually accumulated. In successful biocontrol programs, the reduction of pest densities and recovery of adversely affected flora and fauna leads toward a system with better ecological balance and community structure. Following its establishment, successful natural enemies can provide enduring pest control; and they can replicate and disperse without continued human management (Cortes-Penagos *et al.*, 2007).

The implication of chemical fungicides in soil and water pollution and the evidence that fumigant methyl bromide is an ozone depletor has mandated the search for alternative approaches to disease control management. A promising strategy for the replacement of chemicals has been the implementation of biocontrol technology, used individually or as an Integrated Pest Management (IPM) component (Mao *et al.*, 1998).

The recent developments in the commercialization of biocontrol products have accelerated this approach. Biocontrol preparations of both fungi and bacteria have been applied to seeds, seedlings, and planting media in several ways to reduce tomato and pepper diseases in the field with various degrees of success (Mao *et al.*, 1998).

Two of the major biocontrol agents which reduce soilborne diseases of various crops include isolates of the bacterium *Burkholderia cepacia* and the fungus *Gliocladium virens* (Mao *et al.*, 1998).

Biological control of *Fusarium* wilt diseases has become an increasingly popular disease management consideration in recent years, given its environmentally friendly nature and the discovery of novel mechanisms of plant protection associated with certain microorganisms (Weller *et al.*, 2002; Fravel *et al.*, 2003).

Fusarium wilt-suppressive soils have been reported in many regions of the world, and suppression has generally been shown to be due to biological factors (Alabouvette *et al.*, 1993).

2.4 *Fusarium sp.*

Fusarium is the largest genus of Tubercularіaceae which occurs either saprophytically or parasitically on many crop plants, fruits and vegetables. This genus causes serious wilting of the host plants. The mechanism of infection of *Fusarium* is through the invasion of mycelia on the vascular tissue which finally blocks the xylem vessels, adversely affects the translocation of water leading to wilting of plants. *Fusarium* also secretes toxic substance in the vessels of the host. which could also be the cause of wilting. *Fusarium* attack many plants. Some of the wilt-causing *Fusarium* species with the names of their hosts in parentheses, are *F. udum (on Cajanus cajan,* arhar*), F. lycopersicum (on Lycopersicon esculentum,* tomato*), F. lini (on Linum usitatissimum,* flax*), F. solani (on Solanum tuberosum,* potato*) and F. orthaceras* (on *Cicer arietinum*, gram) (Sharma, 1989).

Fusarium oxysporum f. sp. *lycopersici (FOL)* is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable production areas. The disease caused by this fungus is characterized by wilted plants, yellowed leaves, and minimal or absent crop yield. There may be a 30 to 40% yield loss in Florida alone (Mao et al., 1998).

Isolates of *Fusarium oxysporum*, colonizes vascular tissue, leading to the disruption of water translocation to the shoot. This disruption leads to typical wilt symptoms of foliage drooping, chlorosis, and eventually necrosis, as the leaves collapse around the pseudostem. As the disease progresses, the crown and pseudostem also collapse, resulting in plant death. An internal symptom of the disease is discolouration of the vascular tissue (Forsyth *et al.*, 2006).

The mycelia of *Fusarium* spp. consist of branched, septate, often colourless hyphae, which turn brown at maturity. The mycelium produces toxic secretion in the vessels, which will then ultimately blocks the water vessel, resulting in the wilting of the host plant (Sharma, 1989).

Fusarium spp. reproduce asexually through three kinds of asexual spores: macroconidia, microconidia and chlamydospores. Macroconidia are long, multicellular, crescent-shaped or sickle-shaped bodies produced in sporodochia. Both ends of macroconidia are pointed. In certain species, macroconidia developed separately form the sporodochia. The cell structures on which the macroconidia develop are called phialides. Meanwhile, microconidia are small bodies, usually unicellular but sometimes bi-celled, spherical or oval-shaped. They are produced from simple phialides or from branched or unbranched conidiophores. The microconidia are often held in small masses and resembles much to those of *Cephalosporium*, hence this stage is often referred to as *Cephalosporium*-stage of the *Fusarium*. Chlamydospores are round or oval, thick-walled, terminal or intercalary cells of the old hyphae. They are either developed singly or in chains. They are detached and germinate through germ tubes, if the condition is favourable. The chlamydospores are very durable and remain viable for a long time (Sharma, 1989).

CHAPTER 3

MATERIALS AND METHODS

3.1 Culture of *Fusarium* and Antagonistic Microbe

3.1.1 Soil Samples and Infected Roots and Leaves Collection

Soil samples of pepper rhizosphere and roots and leaves of pepper plants infected by *Fusarium oxysporum* were collected from the black pepper farm in UPMKB. The soils were then mixed together to form one composite sample. This was to ensure that large number of microbes will be obtained from the soil.

3.1.2 Isolation of Antagonistic Microbes from Soil

The microbes from the soil were isolated through soil dilution-plating technique of Johnson et al (1960). A sample of 10g soil will be placed in a graduated cylinder, and sterilized distilled water will be added to make up a total volume of 100ml. The suspension will be stirred and then poured into sterile 250ml Erlenmeyer flask and will then shaken thoroughly for 30 minutes. One milliliter of this suspension will be pipetted aseptically and dispensed in dilution test tubes with 9ml of sterilized distilled water. The series of dilution are of the following; 1:10, 1:100, 1:1,000 and 1:10,000. One milliliter of the desired solution (with 10^{-4} and 10^{-5} dilution factor) were transferred aseptically into Petri dishes containing Nutrient Agar (NA). Four Petri dishes were provided for each dilution. The Petri dishes containing the diluted solution were then incubated for 7 days. After incubation, a small portion of each bacterial colony was transferred into NA. The isolates were then incubated for 7 days.

3.1.3 Isolation of Antagonistic Microbes from Roots and Stems of *Piper nigrum*

The stems and roots from *Piper nigrum* were collected. The stems and roots were then rinsed with sterile water to remove fibres and any matter on the stem and root surface. Endosperms were cut off and were transferred into the conical flask and 100 ml of sterilized water were added. The conical flask containing the stems and roots were then placed on an orbital shaker and were shaken with 100 rpm overnight at room temperature. After that, serial dilutions (10^{-1} up to 10^{-4}) of suspension were made. A 0.5ml suspension from each diluted suspension is streaked on NA plates. The plates were then incubated at room temperature for 24 hours. Colonies were purified by doing subculture for single colony on medium plates. The bacterial isolates were then screened for their antagonistic potential against *Fusarium oxysporum* through dual culture.

3.1.4 Isolation of *F. oxysporum* from Infected Roots and Leaves

Fusarium oxysporum, the causal agent of black pepper crop wilting was isolated from infected roots and leaves. Infected roots and leaves from pepper plant were collected. Cuttings of these infected roots and leaves were then obtained. The cuttings were washed thoroughly with tap water followed by sterile distilled water. They were then dipped in 1.0 % Chlorox for 10 minutes and washed several times using sterile distilled water. Excess water adhering to the cuttings were then be removed by spreading them in a laminar airflow chamber (Anith *et al.*, 2003). These sterile cuttings were then be put on the PDA medium for culture purpose and incubated for 7 days.

3.2 Screening of Isolates against *F. oxysporum*

All 4 days old bacterial microbes isolated were individually tested for their antagonistic property against the 7 days old *Fusarium* sp. using the dual culture technique. Ten bacterial isolates and one Fusarium oxysporum alone as control were used in the experiment. The experiment was carried out in 8 replicates. These cultures were then incubated for 7 days at room temperature. After 7 days of incubation, the mycelial growth and the inhibition zone within each plate were measured. Percent inhibitions of radial growth (PIRG) were measured using the formula:

$$PIRG = (R1 - R2) / R1$$

Where, R1 is the diameter (cm) of colony growth of pathogen in control; R2 is the diameter (cm) of the pathogen in antagonist-tested plate (Noveriza and Quimio, 2004).

3.3 Spore Germination Test

Fresh culture of microbial isolate was diluted in sterilized water and the spores of *Fusarium oxysporum* were collected. Spore suspension of *F.oxysporum* was dropped on a PDA plate, followed by the addition of bacterial suspension. 4 replicates were used in the experiment. Plates were air dried in lamina airflow chamber to remove the excess water on agar plate surface. All plates were then left for 7 days at room 26±2°C. After 7 days, the spore germination of each plate was observed under light microscope.

3.4 Data Analysis

The experiment was carried out using the Complete Randomized Design (CRD). The data were transformed using Arc Sine Transformation and were then analyzed with analysis of variance (ANOVA) using general linear procedure of SAS 9.1. Duncan New Multiple Range Test was used in comparing the treatment means.

CHAPTER 4

RESULTS

4.1 Dual Culture Assay

From Table 1, it can be concluded that there were no significant difference among the different bacteria isolated from different sources (i.e stems, roots, and soil rhizosphere of *Piper nigrum*). In other words, we could say that almost all of the bacterial isolates being tested to suppress the growth of *F.oxysporum* in the experiment are potential biocontrol for *F.oxysporum*.

Table 1: The mean percentage of disease suppression of antagonistic microbes toward *Fusarium oxysporum*.

Treatments	Day						
	Percentage of Disease Suppression Toward *F.oxysporum* (%)[A]						
	1	2	3	4	5	6	7
C	0.68^{a}	-5.83^{a}	1.43^{a}	3.03^{b}	4.40^{c}	3.02^{b}	4.65^{b}
BC1	2.36^{a}	-5.03^{a}	13.37^{a}	20.74^{a}	20.84^{a}	24.38^{a}	21.63^{a}
BC2	-8.87^{a}	-9.81^{a}	0.62^{a}	7.22^{b}	12.93^{abc}	16.25^{a}	21.70^{a}
BC3	-0.29^{a}	-1.25^{a}	13.69^{a}	12.82^{ab}	16.13^{ab}	20.89^{a}	23.94^{a}
BC4	-3.53^{a}	-0.67^{a}	8.33^{a}	9.51^{ab}	12.98^{abc}	16.22^{a}	15.76^{a}
BC5	-3.02^{a}	-0.69^{a}	6.40^{a}	13.41^{ab}	16.10^{ab}	20.48^{a}	24.21^{a}
BC6	-38.13^{b}	8.36^{b}	-5.63^{b}	2.89^{b}	9.50^{bc}	15.48^{a}	16.41^{a}
BC7	-7.56^{a}	3.19^{a}	1.89^{a}	9.08^{ab}	18.23^{ab}	23.91^{a}	23.76^{a}
BC8	1.19^{a}	8.08^{a}	8.94^{a}	10.91^{ab}	15.85^{ab}	23.31^{a}	23.11^{a}
BC9	-9.05^{a}	2.21^{a}	1.49^{a}	2.37^{b}	4.30^{c}	2.34^{b}	5.38^{b}
BC10	-18.63^{ab}	0.75^{ab}	4.57^{ab}	9.67^{ab}	14.82^{abc}	14.12^{a}	16.39^{a}

Means within columns with the same alphabets are not significantly different ($p \leq 0.05$, DMNRT)

[A] Percent disease suppression was determined as [R1-R2/R1] × 100, where R1 is disease severity index in the Petri dishes inoculated with only *F.oxysporum* without bacterial antagonists, R2 is disease severity index in the Petri dishes inoculated with both the pathogen and bacterial antagonists.

"-" Negative values indicates negative interaction between the F.oxysporum and bacterial antagonists, where the value of R2 is greater than R1.

BC1, BC6 and BC9 = Bacteria isolated from stem of *Piper nigrum*
BC3, BC5, BC7 and BC8 = Bacteria isolated from root tissue of *Piper nigrum*
BC2, BC4 and BC10 = Bacteria isolated from soil rhizosphere of *Piper nigrum*

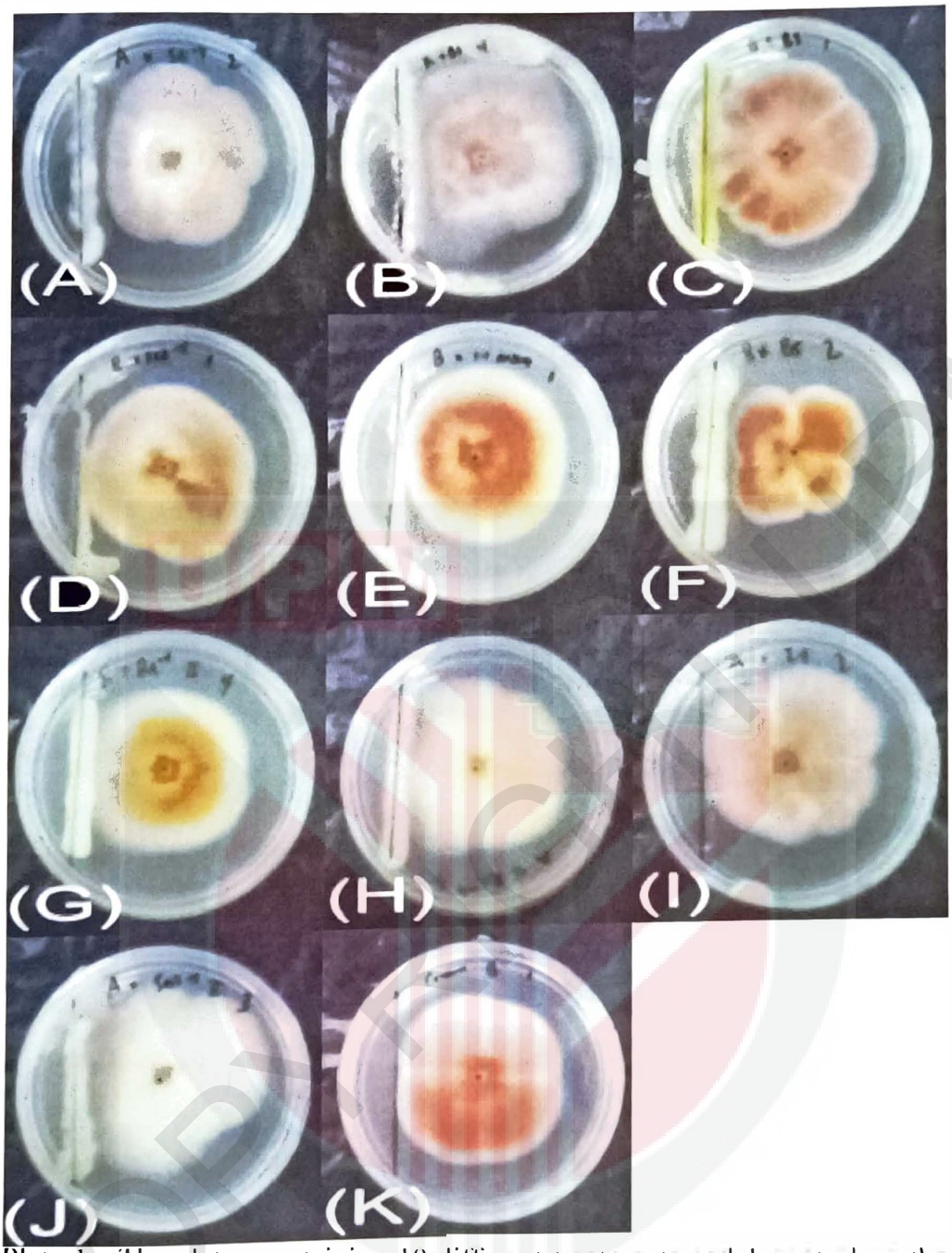

Plate 1: The plates containing 10 different treatments and 1 control on the 7th day after incubation. [A-BC1, B-BC2, C-BC3, D-BC4, E-BC5, F-BC6, G-BC7, H-BC8, I-BC9, J-BC10, K-Control]

The first three days of incubation after the dual culture assay was carried out show consistent percentage of inhibition toward *F.oxysporum*. Starting from Day 4 until Day 7, it was found that there were increases in percentage of inhibition toward *F.oxysporum*.

On Day 4, it was found that BC2, BC6 and BC9 give no significant effect to the growth of the pathogen. These treatments were similar to that of the C where there were no significant effects toward the *F.oxysporum*.

According to the table, starting from Day 5 until Day 7 it was becoming more obvious that all treatments have significant effects in inhibiting the growth of the pathogen. There is an exception for BC9 as for the three days, it acts similar to the C where it has no significant difference.

4.2 Mycelium Growth Assessment for *F.oxysporum*

After 7 days of inoculation, it was found that the *Fusarium oxysporum* being tested were of the slow growing strain. As demonstrated in Figure 1, the *F.oxysporum* was unable to fully colonize the plates after being incubated for 7 days. The assessment was then extended until the tenth day.

All 8 replicates of the *F.oxysporum* observed from the assessment gave the same result. The fungi were red in colour for the whole time. On the second day, the presence of white coloured part was observed. This white part continues to grow vigorously throughout the 10 day period.

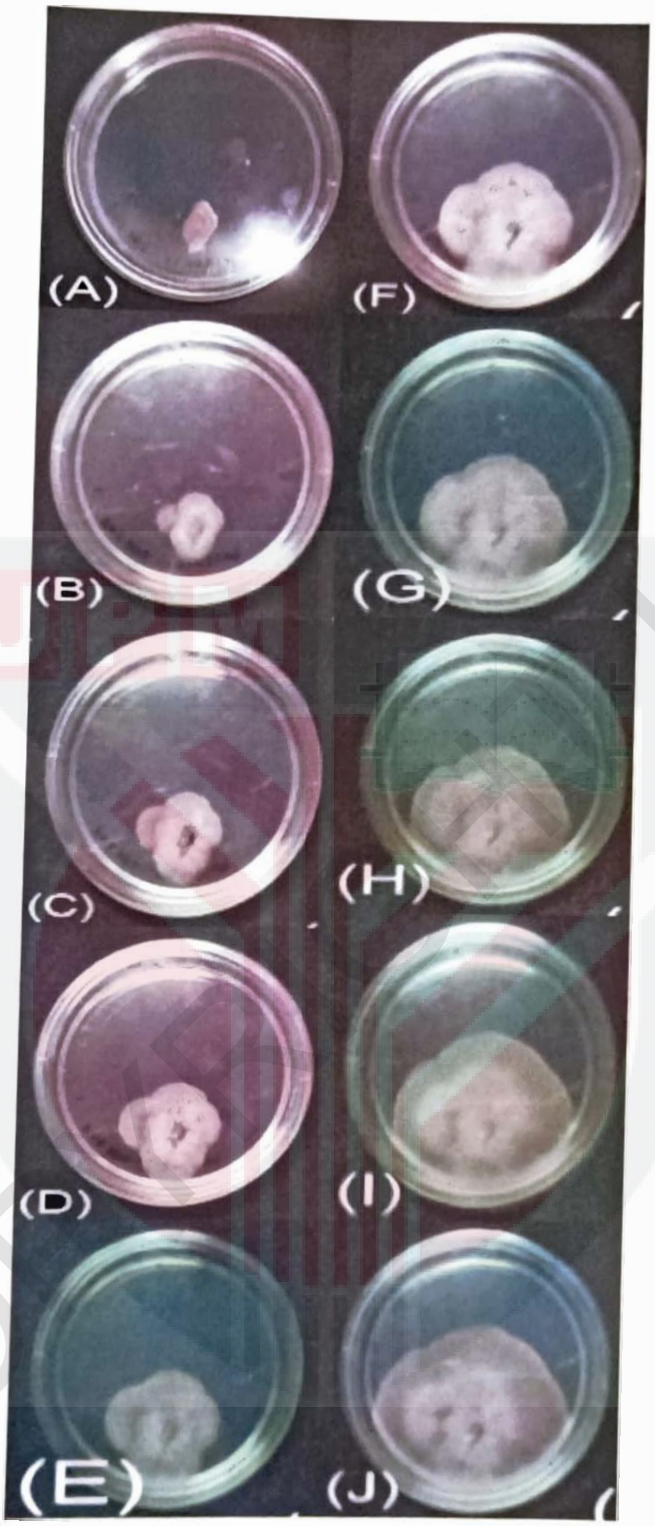

Plate 2: Mycelial growth stages of *F.oxysporum* on PDA within 10 days of incubation. [A-Day 1, B-Day 2, C-Day 3, D-Day 4, E-Day 5, F-Day 6, G-Day 7, H-Day 8, I-Day 9, J-Day 10]

Table 2: The means for mycelium radius of *F.oxysporum* for 10 days of incubation.

Day	Mean mycelium radius (cm)
1	0.64
2	0.94
3	1.28
4	1.50
5	1.80
6	1.95
7	2.30
8	2.56
9	2.81
10	3.09

Table 2 shows the mycelium radius of *F.oxysporum* on PDA within 10 days of incubation. The mean mycelium radius of *F.oxysporum* at the tenth day after inoculation on PDA was 3.09 cm. It was found that the fungi being tested were of the slow growing strain, as it did not fully colonize the Petri dish, despite being incubated for 10 days to allow optimum growth.

4.3 Spore Germination Test

Since there were no significant differences between the bacteria being tested for their antagonistic property against *F.oxysporum*, only 5 isolates and a control were being tested for the test. The isolates were chosen randomly.

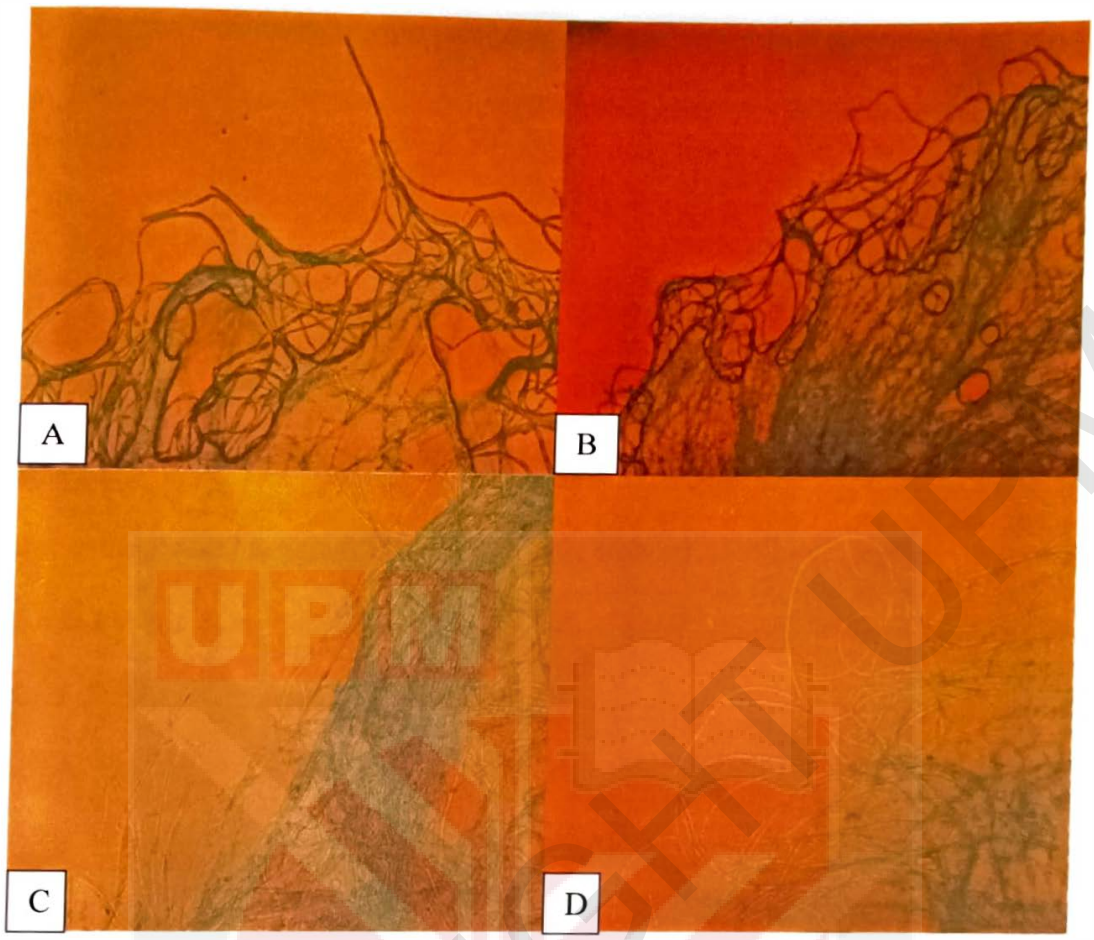

Figure 1: *Fusarium oxysporum* under light compound microscope after spore germination test.[A- BC1, B-BC1, B-BC6, D-Control].

Table 3: The presence of spores in Spore Germination Test after 7 days of incubation.

Treatment	Presence of spores
BC1	-
BC2	-
BC3	-
BC5	-
BC7	-
Control	-

"-" indicates no spores were observed under the light microscope / no sporulation occurred.

From the experiment, no spores, microconidia or macroconidia were found from the observation under light compound microscope. There were only heavy masses of reddish-white mycelia present under the microscope.

CHAPTER 5

DISCUSSIONS

5.1 Dual Culture Assay

The aim of the study was the screening and selection of rhizobacteria and endophytic bacteria inoquous to *P. nigrum* with antagonistic activity against *F. oxysporum*. The development of a proper *in vitro* screening system that provides repeatable and reliable results that could be obtained in shorter periods of time is considered as an important initial step for isolation of efficient bacterial antagonists for plant disease management (Anith *et al.*, 2003).

The approach was hence to screen the bacterial isolates for their *in vitro* biocontrol activity against *F. oxysporum*. The bacterial isolates used were initially isolated from the rhizosphere of *P. nigrum* and also the stem and root tissues of *P.nigrum*. As it has been observed earlier that the rhizosphere environment may be helpful in selecting the effective antagonistic strain of bacteria in the same environment as where they will be used eventually (Weller, 1988; Edward *et al.*, 1994; Landa *et al.*, 1997; Marilley and Aragno, 1999). Moreover, isolating the bacteria from within the the rhizosphere and the plant tissues of the target crop is essential for successful identification of potential biocontrol agents among the bacterial isolates (Williams and Asher, 1996).

It was clearly known that the *in vitro* screening method being applied (the dual culture assay) for the purpose have certain limitations in that the results to be obtained would not equally expressed under gnotobiotic (axenic) and *in vivo*

condition[s] (Inam-ul-Haq *et al.*, 2003). However, the in vitro assays conducted in the stud[y] were used to screen and select the bacterial isolates having potential to suppress the growth of *F. oxysporum*.

Microbial antagonists can involve biological control mechanisms in the control of soil-borne plant pathogens. These include competition for space and nutrient sources and production of anti-microbial substances, as suggested by Suparman *et al.* (2000). The inhibition of hyphal growth of fungal isolate in dual cultures suggests the involvement of antibiotics and/or other antifungal substances by the bacterial isolates (Kaur *et al.*, 2007). The percentage of in vitro mycelia growth inhibition by the bacterial isolates against *F. oxysporum* varied from day to day within the 7 days of incubation (Table 1). On the 7th day, most of the bacterial isolates were found to possess the inhibitory characteristics toward *F. oxysporum*. BC9 showed only mild activity or no activity at all. This suggests that the mode of action exerted and/or the type of antifungal metabolite produced by the isolates may vary and may also be taxonomically different from each other (Williams and Asher, 1996).

For the bacterial isolates that caused noticeable inhibition toward fungal growth in the dual culture assays, the inhibition zones formed were of such sizes that there were less or no physical contact with the pathogens. This occurred because that the bacterial isolates could be producing certain antifungal metabolites (AFMs), as suggested by Montealegre *et al.* (2003). Based on current studies, the involvement of antifungal compounds produced by bacteria in the inhibition of fungal growth was confirmed by the ability of cell-free culture filtrates of bacteria to inhibit *in-vitro* conidia germination and hyphal growth of *F. oxysporum* (Kaur, 2007). This

would also happen as the PDA medium used for the dual culture assay is rich in nutrients, competition might be excluded as the mode of action for these isolates (Landa *et al.*, 1997).

However, there were also bacterial isolates that do not effectively suppress the growth of the pathogen, but instead the pathogen continues to grow up to the extent the mycelia touches the bacteria i.e there was a contact between the bacteria and the fungal mycelia. This suggests that the bacterial isolates do produce AFMs or other suppressive substances, but the amount or dosage of disease suppression were low. This idea was supported by what was proposed by Johnson (1994), which mentioned that the amount of disease suppression obtained with a biocontrol agent depends on the density of the agent, the density of the pathogen, how efficiently individual units of the agents render units of the pathogen ineffective, and the proportion of the pathogen population that is potentially affected by the agent.

In the dual culture assay, it was found that there were a number of plates that show brownish colour on the fungal mycelia of the pathogen. This suggests that the fungal mycelia might have been inhibited not only by antibiosis but also by other antifungal metabolites such as siderophores, hydrogen ions and gaseous products including ethylene, hydrogen cyanide and ammonia (Williams and Asher, 1996; Kumar *et al.*, 2002; Saravanan *et al.*, 2004). Furthermore, it also suggests that the efficacy of a given biological control agent mostly results, not only from a single mechanism but could also be from a combination of different modes of actions (Alabouvette *et al.*, 1993).

5.2 Spore Germination Test

The spore germination test conducted used only five isolates (BC1, BC2, BC3, BC5, and BC7) that were virtually observed to be potentially antagonistic against the pathogen among the ten bacterial isolates used for the dual culture assay conducted earlier. The test, which should be done in 1 to 2 days were prolonged up to 7 days, due to the consideration that the *F. oxysporum* being tested was of the slow growing strain.

Based on the observation being made under the compound microscope, it was found that there were no spores detected. There were no macroconidia, microconidia and chlamydospores found from the observation made (Sharma, 1989). Instead, only fungal mycelia were observed under the microscope. This would probably explain that the five bacterial isolates being used for the test must have produced substances that are similar to that of the *Bulkholderia cepacia*, a bacterial species being widely used as biocontrol agent for controlling many crop diseases. According to Chen and Wu (1999) and Larkin and Fravel (1998), the *B.cepacia* produces pyrollnitrin and other volatile compounds which were able to inhibit a wide range of pathogen including *Fusarium spp*. Its antibiotic ability is what makes the *B. cepacia* plays an important role for controlling plant diseases.

5.3 Identification of Biocontrol

The five bacterial isolates chosen for the test were BC1, BC2, BC3, BC5 and BC7. The BC1 isolate was obtained from the stem tissue of *P. nigrum*. The BC3, BC5 and BC7 were isolated from the root tissue of *P. nigrum* meanwhile BC2 was isolated from the rhizosphere of *P. nigrum*. The bacterial isolates obtained from stem tissues

and root tissues are the endophytic bacteria while the isolates obtained from the *P.nigrum* rhizosphere is known as the rhizobacteria.

Endophytic bacteria have been found in virtually every plant studied, where they colonize the internal tissues of their host plant and can form a range of different relationships including symbiotic, mutualistic, commensalistic and trophobiotic. Most endophytes appear to originate from the rhizosphere or phyllosphere; however, some may be transmitted through the seed. Endophytic bacteria can promote plant growth and yield and can act as biocontrol agents (Ryan *et al.*, 2007). Meanwhile, rhizobacteria live on plant roots or reside in the rhizosphere, a soil zone spanning a few millimeters around roots, where they feed on plant juices (Hardin, 1998).

Identification of the bacterial isolates was not carried out as there were no equipments made available especially for the purpose. However, further identification of these isolates could be made possible through PCR method, as suggested by Widjojoatmodjo *et al.* (1995) and Fouad *et al.* (2002).

Based on a study done by Aravind *et al.* (2008), the endophytic bacteria isolated from the roots and stems of *P.nigrum* are *Pseudomonas spp, Serratia spp., Bacillus spp., Arthrobacter spp., Micrococcus spp.*, and *Curtobacterium sp.* A different study done by Idris *et al.* (2007) suggested that the bacterial isolates obtained from the rhizosphere are members of the Genus *Bacillus* including *B. cereus, B. subtilis, B. circulans, B. lichenformis and B. stearothermophilus, Bulkholderia cepacia*, and *Chromobacterium violaceum.*

As being described earlier, it was known that the dual culture assay has its own limitation in the sense that the results obtained through dual culture assay would not express the biocontrol efficiencies equally to the test being done under gnotobic (axenic) and *in vivo* conditions (Inam-ul-Haq *et al.*, 2003). Under these circumstances, further testing in the field to observe the disease suppression of bacterial isolates toward *F. oxysporum* slow decline and wilt of *P. nigrum* under greenhouse conditions would be very useful to further identify the potential bacterial strains (Idris *et al.*, 2007).

CHAPTER 6

CONCLUSION

Based on this study, 90% of the 10 bacterial isolates from the rhizosphere, stem and root tissues of *P. nigrum* inhibited the growth of *F. oxysporum* in dual cultures. Nine bacterial isolates which are BC1, BC2, BC3, BC4, BC5, BC6, BC7, BC8, and BC10 were found to have the potential as biological control agents against *F. oxysporum* since these isolates show significant effects and positive percentage of inhibition toward the pathogenic fungi. BC9 was found out to be unsuccessful in controlling the growth of the pathogen as the result shown in Table 1 indicates that the BC9 isolate does not give any significant difference from day 5 until day 7 towards the fungal isolate.

Results from the dual culture assay and the morphology based on observation indicated that the selected bacterial isolates could be taxonomically different. Thus, there is a possibility of using these isolates to control other *Fusarium* isolates which are pathogenic to *P. nigrum* and other crops. Nevertheless, the potential bacterial isolates should first be tested in the field for their biocontrol efficacy under field conditions. Further investigations could be conducted to determine the modes of action of the promising bacterial isolates.

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PUBLICATION OF THE PROJECT UNDERTAKING

This is to certify that I have no objection to publish the project entitled "Screening of Antoganistic Rhizobacteria and Endophytic Bacteria from Pepper (*Piper nigrum*) for Controlling *Fusarium oxysporum*" 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.

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