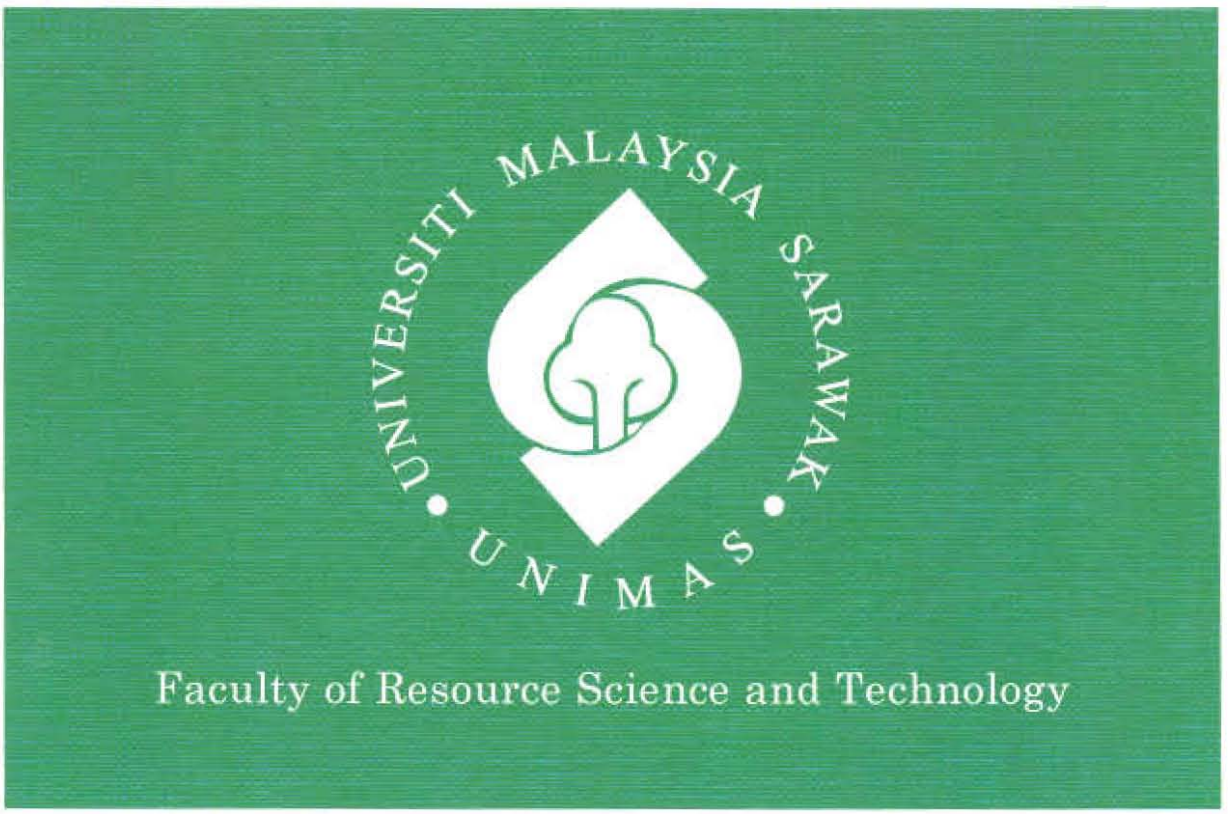

ISOLATION, IDENTIFICATION AND EVALUATION OF BACTERIAL FLORA IN PLANTATION SOILS AGAINST *PHYTOPHTHORA CAPSICI* OF BLACK PEPPER (*PIPER NIGRUM* L.)

Linda Nirwana Caroline

Bachelor of Science with Honours
(Resource Biotechnology)
2013

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LINDA NIRWANA CAROLINE

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

Supervisor: Assoc. Prof. Dr. Awang Ahmad Sallehin Awang Husaini

Co-supervisor: Dr. Samuel Lihan

Faculty of Research Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2013

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Declaration

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This declaration is made on the ...~~3rd~~ 3rd.....day of.....~~3~~ 3rd July..2012.

Student's Declaration:

I LINDA NIRWANA CAROLINE (26746) . FSTS

------ (PLEASE INDICATE STUDENT'S NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled Isolation, identification and evaluation of bacterial flora in plantation soils against Phytophthora capsici of black pepper (Piper nigrum L.) is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

03/07/2013
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Supervisor's Declaration:

I Assoc. Prof. Dr Awang Ahmad Sallehin Awang Husaini (SUPERVISOR'S NAME) hereby certifies that the work entitled ----------(TITLE) was prepared by the above named student, and was submitted to the "FACULTY" as a * partial/full fulfillment for the conferment of bachelor of science with honour (resource biotechnology) (PLEASE INDICATE THE DEGREE), and the aforementioned work, to the best of my knowledge, is the said student's work.

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List of Abbreviations

PDA	Potato dextrose agar
NA	Nutrient agar
LB	Luria broth
mL	Milliliter
g	Gram
^0C	Degree celcius
cm^3	Centimeter cube
rpm	Revolution per minutes
w/v	Weight/Volume (concentration)
V	Volt
%	Percentage
X g	Times gravity
kb	Kilo base

List of Tables and Figures

Isolation, Identification and Evaluation of Bacterial Flora in Plantation Soils against *Phytophthora capsici* of Black Pepper (*Piper nigrum L.*)

Linda Nirwana Caroline

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Abstract

The purpose of this study was to isolate, identify and evaluate the bacterial flora in the plantation soils against *Phytophthora capsici* of the black pepper (*Piper nigrum* L.). The screening of the antagonistic bacterial isolates was done through dual plate method against *P.capsici*. Three antagonistic bacteria (P1, P10 and P11) were successfully isolated. All of the three antagonistic bacteria were characterized based on its colony morphology, gram staining, production of diffusible and volatile antibiotics and the microscopic observation of the abnormalities toward hyphae morphology of *P.capcisi*. The molecular identification was conducted using 16S rDNA primers conducted and PCR products of 1500 bp in molecular weight of sizes were obtained and sequenced. The antagonistic bacteria isolate, P1 could not be identified. Meanwhile, isolate P10 was established as *Burkhoderia unamae* and isolate P11 as *Enterobacter cloacae*. The result of the study showed that *B. unamae* is the most effective antagonistic strain as compared to the other two antagonistic strains.

Keywords: Biocontrol, *Phytophthora capsici*, *Piper nigrum* L., 16S rDNA

Abstrak

Tujuan kajian ini dijalankan adalah untuk mengasingkan, mengenalpasti dan menilai mikroflora bakteria yang terdapat di dalam tanah ladang yang berlainan untuk melawan Phytophthora capsici *yang menyerang lada hitam (*Piper nigrum *L.) Pengenalpastian untuk bakteria antagonistik yang melawan* P.capsici *dilakukan mengunakan kaedah dwi piring media. Tiga bakteria antagonistik (P1, P10, P11) telah berjaya diasingkan. Ciri-ciri ketiga-tiga bakteria antagonistik ini dikaji berdasarkan morfologi koloni, perwarnaan gram, penghasilan antibiotic meresap dan meruap dan pemerhatian terhadap ketidaknormalan morfologi hifa* P. capsici. *Identifikasi mengunakan kaedah biologi molekul dijalankan dengan mengunakan primer 16S rDNA dan produk PCR dengan berat molekul 1500 bp telah dihantar untuk penjujukan DNA. Strain bakteria P1 tidak dapat dikenalpasti secara kaedah molekul. Isolat P10 dikenalpasti sebagai* Burkhoderia unamae *dan P11 dikenalpasti sebagai* Enterobacter cloacae. *Hasil kajian menunjukkan* B. unamae *adalah strain bakteria antagonistik yang paling efektif berbanding dua strain bakteria antagonistik yang lain.*

Kata kunci: Kawalan biologi, Phytophthora capsici, Piper nigrum *L., 16S rDNA*

Introduction

The foot rot disease in black pepper (*Piper nigrum* L.) caused by *Phytophthora capsici* is a common disease among the other countries including Malaysia. *P. capsici* is a soil borne pathogen and attacks all parts and grows stages of the black pepper (Manohara *et al.*, 2004). The disease has been found in major black pepper plantation in Malaysia such as Sarawak.

The management of this disease usually relies on the chemical such as fungicides which are very toxic to human and environment, costly and labor intensive. Examples of the chemical used are metalaxyl, bordeaux and copper oxychloride (Dinu *et al.*, 2007).

In order to minimize the usage of toxic chemicals, other alternative method was introduced which is known as biological control method. Biological control method is an environmental friendly method in managing the foot rot disease.

Root-free soil usually rich with bacteria species. In a gram of soil, there are billions of bacteria. Most of the bacteria live 10 cm from the soils surface where the organic matter presence.

Thus, this study investigated the antagonist bacterial flora in different plantation soils against *P. capsici.*

The objectives of this research study were as follow:

i. To isolate the bacterial flora from three different types of plantation soils.

ii. To compare the inhibition efficiency among the bacterial flora isolated from different plantation soils against *P. capsici* of the black pepper.

iii. To identify the antagonists bacterial flora species against *P. capsici* by using molecular method.

iv. To study the production of diffusible antibiotics, production of volatile antibiotics and study on hypal morphology.

(Shashidhara, 2007) in pepper plantation. At the end of 1952, there was a serious outbreak due to foot rot disease cause by *P. capsici* which threated to destroy the black pepper industry (Det, 2008). This caused a depressed price and reduced the black pepper production. But in 1959, rising in black pepper price cause an extensive new planting (Det, 2008).

Managing the disease cause by *P. capsici* has many challenges which include long term-survival of oospore in the soil, spread of the pathogen by water channel and a lack of commercially acceptable resistant cultivars (Granke *et al.*, 2011).

1.3 Chemical methods against *Phytophthora capsici*

Plant pathogens are among the most serious causing agents to agricultural product. Several different strategies are currently being employed to manage and control plant pathogen. The foot rot disease caused by *P. capsici* spreads very rapidly but the symptoms of the disease take some time to develop (Shashidhara, 2007). This makes it really difficult to control the disease.

The farmers often rely heavily to the application of chemical methods to reduce the infection of *P. capsici* toward the black pepper. Once the disease is detected, the fungicide will be sprayed surrounding the infected vine include the healthy vines to prevent further spread of the disease to those vines. The common fungicide uses are metalaxyl, Bordeaux mixture and copper oxychloride (Dinu *et al*, 2007).

Another chemical method that was used in Sarawak is root infusion technique (Wong, 2004). Root infusion technique increase the level of phosphate in the vine and root in which can help to increase tolerant toward *P. capsici* (Wong, 2004).

The frequently usage of fungicide and chemical substance have been a subject of public concern due to potential harmful effects on the environmental, unsafe to the human and undesirable effect on non-target organisms (Heydari & Pessaraklii, 2010). Besides, chemical method is quite expensive (Sang *et al.*, 2010).

1.4 Biological control against *Phytophthora capsici*

Since, chemical method in agricultural is dangerous, it is important to find another ways to develop of non-chemical alternative methods to control the growth of *P. capsici*.

One of the ways is through biological control method. Biological control method refer to the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens (Pal & Gardener, 2006). The organisms that suppress the pest or pathogen are referred to as Biological Control Agent or known as BCA (Heydari & Pessaraklii, 2010).

Nevertheless, biological control may not able to completely eliminate the pathogen (Kamil *et al.*, 2004). But, biological control methods with the antagonistic microorganism have been introduced for better environmental management, not harmful to human and cost effective. Besides, they cannot work as rapidly as chemical methods and may provide only a partial level of control (Kamil *et al.*, 2004).

Many researches have been conducted regarding the biological control toward the *P. capsici*. In 2004, Noveriza and Quimio had isolated the soil mycoflora of black pepper rhizosphere against *P. capsici*. They discovered that *Aspergillus* genera can produce antibiotics which can inhibit the pathogen apparently whereas, *Tricoderma* were able to penetrate through the hyphae of the pathogen.

According to Akgul and Mirik (2008), all the strains of *Bacillus megaterium* and their combinations are effective in controlling the *P. capsici* in black pepper. Apart from that, *Trichoderma harzianum* (Manohara *et al.*, 2004; Mohammed *et al.*, 2009), *Pseudomonas* sp. and *Bacillus* sp. (Shashidhara, 2007) also showed antagonistic characteristic against *P. capsici*.

There are a few different mode of action of the biological control agent to fight against pathogens. The modes of action include; the inhibition of the pathogen by antimicrobial compounds (antibiosis), competition for iron through the production of siderophores, competition for colonization sites and nutrients supplied by seeds and roots exudates, degradation of pathogenicity factors of the pathogen such as toxins and production of the extracellular cell wall degrading enzymes such and chitinase and glucanase which can lyse the pathogen cell walls (Whipps, 2001).

2.0 Materials and Methods

2.1 *Phytophthora capsici* sample

The *P. capsici* was obtained from the Malaysian Pepper Board of Sarawak. The *P. capsici* was cultured in the PDA media at the room temperature.

2.2 Plantation soil samples

The plantation soil samples was collected from three different plantations which are from a small black pepper plantation in Penrissen, palm oil plantation in Kota Samarahan and pineapple plantation near Asajaya. The soil samples were placed in a Falcon tube aseptically.

2.3 Bacteria isolation by serial dilution method

The bacterial flora from the plantation soils were isolated through serial dilution method based on Noveriza and Quimio (2004). A volume of 10 g of the soil sample from the black pepper plantation was placed in a graduated cylinder containing 90 ml of distilled water. The suspension was stirred and poured into the 250 ml Erlenmeyer flask. The suspension was agitated for 30 minutes to obtain a good separation of the particles. Approximately, 1 ml of the suspension was pipetted aseptically into the dilution test tube containing 9 ml of sterilized distilled water to get a 10^{-2} dilution. To obtain a solution of 10^{-3}, 1 ml of the 10^{-2} solution was pipetted aseptically in to 9 ml of sterile distilled water. The dilution was done until 10^{-6}. 1 ml from each of the dilutions (10^{-1} to 10^{-6}) was pipetted on the NA and was spread by using spread plate method. The same steps were repeated and applied for

pineapple and palm oil plantation soils, respectively. The petri dishes were incubated at 37^0C for overnight.

2.4 Isolation of pure bacteria culture

The isolated bacteria were further subcultured to the fresh media for several times to obtain the pure isolates bacteria by streaking the bacterial culture onto the fresh NA media.

2.5 Screening for antagonists bacteria against *Phytophthora capsici in vitro*

The bacteria floras from three different plantation soils were screened for antagonism against *P. capsici* by using dual plate assay (Kerr, 1999; Akgul and Mirik, 2008). Each of the pure bacterial culture from three different plantation soils were streaked at one side of the 4 cm line of the petri dish containing PDA media. After the incubation at room temperature for 2 days, *P. capsici* disk was placed at the other side of the same petri dish. Three replicates were used for each bacterial strain. The controls also have three replicates for each bacteria strains and *P. capsici*. All of the plates were incubated at room temperature for six days. When the growth of the control of *P. capsici* reaches more than 2 cm in diameter, the radial growth of the *P. capsici* in the test plate were measured. The antagonistic activity was evaluated by measuring the distance growth between *P. capsici* and the bacterial strains. Percentage of inhibition over control was calculated according to the equation given by Vincent (1972) cited in Shashidhara *et al.* (2008).

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Percent inhibition
C= Growth in control
T = Growth in treatment

2.6 Characterization of the antagonistic bacteria

2. 6.1 Identification of bacteria's colony morphology and gram staining

The bacteria were further purified by streaking each single colony onto a new NA media by comparing the colony morphology such as shape, color and surface.

The gram staining was conducted for the each of the antagonistic bacteria. A few drops of water were placed on a clean slide. A loopful of bacteria P1 was smeared onto the clean slide. The slide was air dried and fixed by passing through the Bunsen flame for several times. The slide was then flooded with crystal violet solution for 1 minute. The smear was washed with tap water until no more stain removed. The smear is then flooded with iodine solution for 1 minute and washed again with tape water. The smear was decolorized by washing with 95% ethanol until there is no stain fading and then was washed with tap water. The smear is then counterstain by flooded with safranin solution for 10-20 seconds. The slide was washed with tap water, blot dried. The same step was repeated for bacterial isolates P10 and P11, respectively. All of the grams staining of the bacteria samples were examined under the compound microscope.

2.6.2 Production of diffusible antibiotics

The PDA plates were covered with a cellophane membrane in the center and inoculated with 20 µl of each of the antagonistic bacteria P1, P10 and P11 suspension. After incubation for 24 h at 25°C, the membrane with the grown bacterial isolate was removed and a 10 mm disk of a pure culture of *P. capsici* was inoculated in the middle. Plates were further incubated at 25°C for 7 days and the growth of the *P. capsici* was measured. Controls were run with mocked inoculated PDA containing plates on the cellophane membrane (replacing the bacterial suspension by sterile distilled water) and further inoculated with *P. capsici*. Each experiment considering a single bacterial isolate was run in triplicates. Results were expressed as means of % inhibition + S.D. of growth of pathogen in the presence and absence of any antagonist bacterial isolate (Ann, 2012).

2.6.3 Production of volatile antibiotics

Approximately, 100 µl of each of the antagonistic bacterial sample P1, P10 and P11 suspensions were placed at the center of one half petri dish containing NA medium and a 10 mm disk of four days old pure culture of *P. capsici* was placed at the center of another Petri dish containing PDA medium. Both half plates were placed face to face preventing any physical contact between the *P. capsici* and the bacterial suspension, and were sealed to isolate the inside atmosphere and to prevent loss of volatiles formed. Plates were incubated at 25°C for 7 days and the growth of the fungal pathogen was measured and compared to controls developed in the absence of the bioantagonist (mocked inoculation with an 8 mm disk of PDA). Each experiment considering a single bacterial isolate was run in triplicate. Results obtained were expressed as means of % inhibition + S.D. of the growth of pathogen in the presence and absence of any bacterial isolate (Ann, 2012).

2.6.4 Study on hypal morphology

From the screening plate of each of the antagonist bacteria sample against *P. capsici*, approximately 0.5 cm^3 of the hypae at the side that near the bacteria streak was cut out and examined under the microscope for any abnormalities as compared to the control. The control was prepared in the absence of antagonist bacteria.

2.7 Molecular identification of the bacterial flora

Molecular identification of the antagonistic bacterial isolates involved the DNA extraction, PCR amplification, gel electrophoresis, PCR purification and 16S rDNA sequencing.

2.7.1 DNA extraction

The overnight bacteria cultures from single colony of the each of antagonistic bacteria P1, P10 and P11 were prepared by inoculating the pure bacteria isolated in 10 ml of LB broth for 24 hours at 30^0C. One and a half of each of the overnight culture was transferred into Eppendorf tube (1.5 ml microcentrifuge) and centrifuged at 13 000 rpm for 30 seconds. This step was repeated for five times. The supernatant was removed and the cell pellet was resuspended in 567 µl TE buffer. 30µl of the 10% (w/v) SDS and 3µl 20mg/ml Proteinase K was added in to give a final concentration of 100µg/ml Proteinase K in 0.5 % SDS solution. 100µl of 5M NaCl solution was added in and mix well. 80µl of the CTAB/NaCl was added into the mixture. The mixture was mixed well and was incubated for 10 minutes in a water bath set at 65^0C. After 10 minutes, an equal volume of Phenol/Chloroform/Isoamyl alcohol (25:24:1) was added into the mixture. The mixture was vortexed briefly and centrifuged for 5 minutes. The supernatant was carefully transferred into a new centrifuge tubes. The aqueous DNA layer was re-extracted once

again using Chloroform/Isoamyl alcohol (24:1) and centrifuged again for 5 minutes. The supernatant was transferred into a new eppy tube and added with 0.6 volume of isopropanol to precipitate the nucleic acid and centrifuged again for 2 minutes to get the DNA pellet. The supernatant was removed and the DNA pellet was washed with 200µl of 70% ethanol and the pellet was air dried. The dried DNA pellet was dissolved in 100µl of TE buffer and was store at 4^0C until further use.

2.7.2 Agarose Gel Electrophoresis (AGE) analysis of the genomic DNA

A volume of 1 µL of the loading dye was pipetted onto the parafilm. Then, 4 µl of the genomic DNA was added into the loading dye and pipetted up and down. The genomic DNA containing the loading dye was loaded into the well and 3µL of the DNA ladder was loaded at the first well of the agarose gel. The gel electrophoresis was operated at 100 V power supply for 37 minutes. The agarose gel was observed under the UV illuminator.

2.7.3 PCR amplification of 16S rDNA

PCR mastermix.

All of the PCR components (except the DNA template, forward primer and reversed primer) was mixed together in an Eppendorf tube in multiple of 3 since there are three number of samples need to be run.

PCR master mix (Table 1.2) was then pipetted in total volumes of 21 µl into each PCR tube. Then 1 µl of each of the forward (pA) and reversed primer (pH) were added into each PCR tube followed by 3 µl of the respective DNA template of bacteria samples. The 16S rDNA primer sequences were used are listed in the Table 1.1.

Table 1.1. Primer sequences of the universal primer pA and pH.

Primers	Sequences
pA	5' AGA GTT TGA TCC TGG CTC AG 3'
pH	5' AAG GAC GTG ATC CAG CCG CA 3'

Table 1.2. PCR mastermix.

Component	Volume (µl)
PCR buffer	2.5
Taq DNA polymerase	0.5
MgCl	2.0
dNTPs	1.0
Distilled water	15
Forward primer (pA)	1.0
Reversed primer (pH)	1.0
DNA template	3.0
Total	26

The thermal cycler machine was set with the following PCR cycles (Table 1.3).

Table 1.3. PCR parameter

Step	Temperature	Time	No. of Cycles
Initial denaturation	96^0C	5 minutes	1 cycle
Denaturation	96^0C	30 seconds	30 cycles
Annealing	55^0C	1 minutes	
Elongation	72^0C	1 minutes	
Final elongation	72^0C	7 minutes	1 cycle
Temporary storage temperature	4^0C	∞	

The amplified PCR product were kept then at -20^0C until further analysis.

2.7.3 Agarose Gel Electrophoresis (AGE) analysis of PCR products

Approximately, 4 µl of PCR products was loaded into the agarose well and 3µL of the DNA ladder was loaded at the first well of the agarose gel. The gel electrophoresis was

operated at 120 V power supply for 37 minutes. The agarose gel was observed under the UV illuminator.

2.7.4 PCR purification

The PCR product purification was conducted by using PCR Clean-Up System by Promega. The protocol is based on the PCR Clean-up System Quick Protocol. An equal volume of Membrane Binding Solution was added to the PCR amplification. Then, SV Minicolumn was inserted into the Collection Tube. The prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute. Then, the Collection Tube was centrifuged at 16,000 X *g* for 1 minute. The flowthrough was discarded and the Minicolumn was reinserted into the Collection Tube. 700 µl of Membrane Wash Solution was added in and centrifuged at 16,000 X *g* for 1 minute. The flowthrough was discarded and the Minicolumn was reinserted into the Collection Tube. The step was repeated again by using 500 µl of Membrane Wash Solution and centrifuged again at 16,000 X *g* for 5 minutes. The Collection Tube was emptied and the column assembly was centrifuged again for 1 minute. The Minicolumn was transferred carefully to a clean 1.5 ml microcentrifuge tube. 50 µl of Nuclease-Free Water was added to the Minicolumn and incubated at room temperature for 1 minute. The microcentrifuge tube was then centrifuged again at 16,000 X g for 1 minute. The Mini column was discarded and the DNA was stored at -20^{0}C.

2.7.5 16S rDNA sequencing and analysis

16S rDNA sequencing analysis was performed by using BLAST analysis and the bacterial isolates identification were established according to the closest sequences match.