UNIVERSITI PUTRA MALAYSIA

POTENTIAL USE OF SELECTED ANTAGONISTIC BACTERIA TO CONTROL FOOT ROT DISEASE OF BLACK PEPPER (Piper nigrum L.) IN SARAWAK, MALAYSIA

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master Science of Plant Pathology

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July 2014

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Black pepper (*Piper nigrum* L.) is one of high value export crops in Malaysia and this crop can easily been destroyedby pests and diseases. One of the most important diseases is foot rot disease which iscaused by *Phytophthora capsici.* Foot rot disease isthe most dangerous disease in black pepper and it is now become a major obstacle to the black pepper industry in Malaysia. Thus, the objectives of this study wereto survey the occurrence and distribution of foot rot disease in four major cultivation areas which are Sibu, Sarikei, Kapit and Bintulu in Sarawak, Malaysia. Secondly to isolate, identify and characterize *P. capsici* based on morphological and molecular methods. Lastly, to investigate the potential use of indigenous endophytic bacteria to control *P. capsici* under glasshouse condition. To achieve the first objective 13 survey areas in four major cultivating areas have been determined and conducted in between November 2010 to January 2011. Survey activity was conducted based on standard plant disease survey method. In study two, the causal pathogen and endophytic bacteria were isolated using standard isolation method. These isolates were then verified and characterized based on morphological and molecular characteristics. Finally, glasshouse study was conducted to assess the efficacy of potential endophytic bacteria to control *P. capsici.* Based on field survey data, infected plants normally exhibited symptoms of yellowingof leaves, leaves defoliationand wilting. High disease incidence and disease severity were recorded in all surveyed sites. The mean percentage for disease incidence anddisease severity were46.31 and 40.91%, respectively. *P. capsici* was successfully isolated using baiting method using rose bengal agar (RBA) and selective media, P_5ARPH.The morphological characteristics of *P. capsici* were globose oogonia with paragynous antheridia, chlamydospore, torulose hyphae, and lemon shape of sporangia with long pedicels. In colony morphology, *P. capsici* showed very thick mycelia and formed multiple like a rosewhen growthon PDA media. Pathogenicity test wasfurther confirmed that the isolated fungus was pathogenic to black pepper plants. It was then further confirmed by nested-PCR using specific primer pairs of PC-1/PC-2. Antagonistic bacteria wassuccessfully isolated and screened. The potential of biocontrol agents were found to be able to induce systemic resistance in plants as

well as showing biological control traits like producing antibiotic compounds which caused lysis to the phytopathogen cells. The use of antagonistic microorganisms should be preferable method because the biological control agents are internal colonizers and therefore more efficient to compete in the vascular systems. Thus, this will certainly deprive *P. capsici* in terms of nutrientsuptake and space for their proliferation. The three potential isolates known as BPA011, BPA040 and BPA025 were tested *in-vitro* showed high percentage of inhibition of radial growth (PIRG) which were81.40, 82.97 and 80.83%, respectively. These isolates were successfully identified using GC-FAME as *Burkholderia cepacia, B. cenocepacia* and *Bacillus alchalapilus*, respectively. Based on their colonization, establishment and localization ability in black pepper roots, *B. cepacia* and *B. cenocepacia* were selected for efficacy study in the glasshouse against *Phytopthora* foot rot in black pepper. Results revealed that they were able to suppress the growth of *P. capsici* compared to the control treatment. Besides delaying disease onset, they are also promoting the growth of the black pepper plant. Our study showed that disease incidence was significantly lower at 90 days after inoculation with *B. cenocepacia* (12.5%) and *B. cepacia* (18.75%). As expected positive control treatment presented the highest value of disease incidence (81.25%) at 90 days after inoculation. This indicated that *B. cepacia* and *B. cenocepacia* has a potential in controlling *Phytophthora* foot rot disease.

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POTENSI PENGGUNAAN BAKTERIA ANTAGONIS TERPILIH UNTUK MENGAWAL PENYAKIT REPUT KAKI LADA HITAM (*Pipernigrum* L.) DI SARAWAK, MALAYSIA

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Lada hitam (*Piper nigrum* L.) adalah salah satu tanaman ekspot bernilai tinggi di Malaysia dan tanaman ini boleh dengan mudah dimusnahkan oleh perosak dan penyakit. Salah satu penyakit yang paling penting ialah penyakit reput kaki yang disebabkan oleh *Phytophthora capsici*. Penyakit reput kaki adalah penyakit yang paling berbahaya pada lada hitam dan ianya kini menjadi halangan utama kepada industry lada hitam di Malaysia. Olehitu, objektif kajian ini adalah untuk meninjau kejadian dan taburan penyakit reput kaki di empat kawasan penanaman iaitu Sibu, Sarikei, Kapit dan Bintulu di Sarawak, Malaysia. Kedua untuk memencil, mengenalpasti dan mencirikan *P.* capsici berdasarkan kaedah morfologi dan molekular. Akhir sekali adalah untuk mengenalpasti potensi penggunaan bacteria endofit untuk mengawal *P. capsici* di dalam kondisi rumah kaca. Untuk mencapai objektif pertama, 13 kawasan tinjauan di empat kawasan penanaman utama telah ditentukan dan dijalankan di antara November 2010 hingga Januari 2011. Aktiviti tinjauan telah dijalankan berdasarkan kaedah kajian piawai penyakit tumbuhan. Dalam kajian kedua, pathogen penyebab dan bacteria endofit telah dipencilkan menggunakan kaedah pemencilan piawai. Seterusnya, pengasingan tersebut disahkan dan dicirikan berdasarkan ciri-ciri morfologi dan molekul. Akhir sekali, kajian dalam rumah kaca dijalankan untuk mengatahui keberkesanan endofit bakteria yang berpotensi dalam mengawal *P. capsici.* Berdasarkan data kajian, tanaman terjangkit biasanya menunjukkan gejala daun kekuningan, daun keguguran dan kelayuan. Kejadian penyakit dan keterukan penyakit yang tinggi telah dicatatkan di semua kawasan tinjauan. Min peratusan bagi kejadian penyakit dan keterukan penyakit adalah masing-masing 46.31 dan 40.91%. *P. capsici* telah berjaya dipencilkan dengan menggunakan kaedah umpanan pada agar bengal (RBA) dan media terpilih, P_5ARPH. Ciri-ciri morfologi *P. capsici* adalah oogonia bundar dengan “antheridia paragynous”, “chlamydospore”, hifa“torulose”, dan “sporangia” bentuk lemon dengan “pedicels” panjang. Morfologi koloni *P. capsici* menunjukkan “mycelia” sangat tebal dan membentuk lapisan berganda seperti mawar apabila tumbuh pada media PDA. Selanjutnya, ujian penjangkitan telah mengesahkan kulat terpencil adalah patogenik kepada pokok lada hitam. Ia kemudiannya disahkan oleh ujian

"nested-PCR" menggunakan pasangan primer spesifik, PC-1/PC-2. Bakteria antagonis telah Berjaya dipencil dan disaring. Agen kawalan biologi yang berpotensi didapati dapat merangsang system pertahanan dalam tumbuhan dan juga menunjukkan ciri-ciri kawalan biologi seperti menghasilkan sebatian antibiotik yang menyebabkan lisis pada sel-sel fitopatogen. Penggunaan mikroorganisma antagonis merupakan kaedah terbaik kerana agen kawalan biologi adalah pengkoloni dalaman dan oleh yang demikian ianya lebih cekap untuk bersaing dalam system vaskular. Oleh sebab itu, ini sudah tentu akan menghalangi *P. capsici* dari segi pengambilan nutrient dan ruang untuk percambahan. Tiga pencilan berpotensi dikenali sebagai BPA011, BPA040 dan BPA025 telah diuji secara *in-vitro* menunjukkan peratusan yang tinggi perencatan pertumbuhan jejari (PIRG) yang mana masing-masing 81.40, 82.97 dan 80.83%. Pencilan ini telah berjaya dikenal pasti menggunakan "GC-FAME" masing-masing sebagai *Burkholderia cepacia*, *B. cenocepacia* dan *Bacillus alchalapilus*. Berdasarkan kepada pengkolonian, penubuhan dan keupayaan penyetempatan mereka di akar lada hitam, *B. cepacia* dan *B. cenocepacia* telah dipilih untuk kajian keberkesanan dalam rumah kaca terhadap *Phytopthora* reput kaki di lada hitam. Keputusan menunjukkan bahawa mereka dapat menyekat pertumbuhan *P. capsici* berbanding rawatan kawalan. Selain daripada melambatkan perbentukan penyakit, mereka juga menggalakkan pertumbuhan lada hitam. Kajian kami menunjukkan bahawa kejadian penyakit adalah jauh lebih rendah pada hari ke 90 selepas diinokulasi dengan *B. cenocepacia* (12.5%) dan *B. cepacia* (18.75%). Seperti yang dijangkakan, rawatan kawalan positif dibentangkan nilai tertinggi kejadian penyakit (81.25%) pada hari ke 90 selepas diinokulasi. Ini menunjukkan *B. cepacia* dan *B. cenocepacia* mempunyai potensi dalam mengawal penyakit *Phytophthora* reput kaki.

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The thesis submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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ANOVA	Analysis of variance
AMF	Arbuscularmychorrhizal fungi
bp	Base pair
cfu	Colony forming unit
cm	Centimeter
CRD	Completely randomized design
^0C	Degree celsius
DAI	Day after inoculation
DNA	Deoxyribonucleic acid
DI	Disease incidence
DS	Disease severity
DNMRT	Duncan new multiple range test
G	Gram
H_2O_2	Hydrogen peroxidase
Kb	Kilo-base pair
µL	Micro liter
µm	Micrometer
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Milimolar
M	Mol
NA	Nutrient agar
NB	Nutrient broth
%	Percent
PIRG	Percentage inhibition of radial growth
PO	Peroxidase
PCR	Polymerase chain reaction
SDW	Sterilize distilled water
Taq	Thermos aquaticus
TPC	Total phenolic content
V/V	Volume/ volume
PFR	Phytophthora foot rot
ISR	Induced systemic resistance
PGPR	Plant growth promoting rhizobacteria
SAR	Systemic acquired resistance

CHAPTER 1

INTRODUCTION

Black pepper (*Piper nigrum* L.) the king of spices is a traditional, historic spice which has been used as a spice since 4th B.C and one of the most important commodities in Malaysia (Anon, 2003). In Malaysia, pepper is available as black pepper or white pepper. The difference between these two types of pepper is in the way it is processed. Black pepper is prepared by drying mature berries of pepper under the sun for about 3 to 10 days, while white pepper is produced by rotting the ripe or nearly ripe berries in running water in order to remove the pulp and pericarp before the drying process begins (Zahara Merican, 1985). Up to 80% of pepper is processed as black pepper while the remaining 20% is processed as white pepper. The quality and price of white pepper are higher than that of black pepper, but the production still remains low. In 2012, Malaysia annual total export of black pepper was 11,094 tons and white pepper was 3,106 tons. In 2012, the annual total export was less than 2011 where the total export of black pepper was 8,516 tons, while white pepper was 2,017 tons (IPC, 2012).

Malaysia was the largest pepper producing country in the world. However, after 1980, Malaysia lost its top position to India and Indonesia (Azmil, 1993). Currently, Malaysia is ranked sixth in terms of world pepper production (IPC, 2012). Vietnam is the first ranked (120,000 metrics tones), followed by Indonesia (75,000 metrics tones), India (43,000 metrics tones), Brazil (33,500 metrics tones), Republic of China (28,000 metrics tones), Malaysia (26,000 metrics tones), Sri Lanka (17,370 metrics tones), Thailand (4,000 metrics tones), Madagascar (4,000 metrics tones) and others (9,720 metrics tones). Pepper's contribution to the local socio-economy is substantial. Around 45,000 farming families and more than 115,000 workers are involved in the pepper industry. This crop generates about a third of Sarawak's agriculture export earnings and Sarawak is the main export producing state in Malaysia.

However, the production of this crop is on the decline trend because of pests and disease infestation. Foot rot disease is one of the major diseases in black pepper crop production in Malaysia.*Phytophthora* foot rot disease (PFR) has reduced the yield of black pepper at ranges from 20 to 80% (Bong and Saad, 1985). *Phytophthora* is a very important soil borne fungus which causes many diseases on roots, leaves and fruits of many important crops in most tropical countries.*Phytophthora* foot rot is considered the most devastating disease of black pepper, has been reported to cause an annual crop loss of 5-10% (Kueh, 1990) and up to 95% for individual farmers (Manohara *et al*. 2004).*Fusarium* slow wilt disease and blackberry disease are also anotherimportant diseases which are affecting pepper crop in Sarawak. The drastic drop in the black pepper production in Malaysia has been mainly due to pronounced mortality of vines from the dreaded disease called foot rot caused by *Phytophthora capsici* as explained by Tsao and Alizedeh (1988).
In Malaysia, *Phytophthora* foot rot pathogen was firstly isolated by Holiday and Mowat (1963) in the infected underground stem of a black pepper vine. Later, they

placed this isolate under the *P. palmivora* group (Holiday and Mowat, 1963). However, further work was conducted by Kueh and Kuthubutheen in 1985 and they revealed that the *Phytophthora* isolated from infected black pepper vines from Sarawak are similar to *P. capsici* in terms of their morphology, growth and pathogenicity properties. Thus, from these findings, the causal pathogen of foot rot disease of black pepper in Malaysia was identified as *Phytophthora capsici* Leonian. However, they provided no molecular evidences to further support their findings. Since then, not much research regarding foot rot disease has been published in Malaysia, although the disease is still infecting the black pepper plants in the field.

Recently, a group of researchers from Malaysian Pepper Board (MPB) and Universiti Putra Malaysia (UPM) conducted a research on the etiology and epidemiology for the development of control measures to control the PFR disease. Several new issues wereraised up such as ineffective isolation method, rapid and sensitive molecular identification method and ineffective control measures for foot rot disease in the orchards have limited their researchs. *Phytophthora* is one of the most difficult plant pathogens to be isolated. To date, many attempts have been made in Malaysia to isolate the pathogen from the infected roots, leaves and soil, but these attempts were not successful due to insufficient information about the need of suitable media for isolation process. As reported in the literature, the isolation of this pathogen had relied upon on the use of selective medium with special types of carbon sources. Utilization of old types of selective medium such as PVP, PPP and NVP were less successful. Thus, new selective medium for *Phytophthora* sp. is urgently required.

In recent years, the increase in the use of potentially hazardous fungicides (in agriculture) has been subject of growing concern of both environmentalists and public health authorities. Today, integration of several methods has been subject of extensive research for management of the disease. Integration of chemicals and biological agents for managing soil borne diseases have been considered as a novel approach, as it requires low amounts of chemicals, thereby reducing cost of control as well as pollution hazards, with minimal interference of biological equilibrium (Papavizas, 1973).

Biological control has the potential for a much more specific effect on the pathogen and a much more limited effect on the environment (Sigee, 1993). Utilization of antagonistic bacteria as biological control agents have been successfully implemented in controlling many plant pathogens. Many studies on the exploration of beneficial organisms have been carried out such as *Pseudomonas fluorescens* which is one of the best examples used for the control of *Fusarium* wilt of tomato. Similarly, *P. fluorescens* is found to be an effective biocontrol agent against the *Phytophthora* disease in black pepper (Diby *et al*., 2005a, 2005b). Several fungi, bacteria and actinomycetes are found to be antagonists to various species of *Phytophthora.*

Since the pathogen is soil borne, indigenous antagonistic microorganisms play a major role in keeping the population of pathogen at low levels. Although work on screening of some of the commercially biocontrol agents for the control of *P. capsici*

is available, but there are no available reports on isolation, identification and studies on the antagonistic ability of indigenous biocontrol agents. Some of endophytic microorganisms such as *Serratia* sp., *Burkholderia* sp., *Pseudomonas* sp., and *Bacillus* sp. have been found to be able to induce systemic resistance in plants (Kloepper *et al*., 1992, Dorwoth and Callan, 1996) and show biological traits like antibiotic activity and lysis. They have been isolated from a wide range of hosts, including wild and cultivated crops, such as woody plants, banana (Pan *et al*., 1997), oil palm (Zaiton *et al*., 2008; Bivi *et al*., 2010) and grasses. Endophytic microorganisms are therefore a relatively new field of study in biological disease control. As an ecological approach, it enhances the natural complexity and diversity of the plant environment, providing greater biological balance and stability. The use of antagonistic bacteria, microorganisms should be preferable to other biological control agents as they are internal colonizers, and therefore capable to compete within the vascular systems, inhibiting *P. capsici* from space for its proliferation. Furthermore, the resistance conferred by biological preparations is not heritable and may even be irreproducible under different environmental settings. To obviate these shortcomings, control is preferably achieved within the host plant through the enhancement or induction of its disease resistance. This research is therefore undertaken with the following objectives:

1) To assess the occurrence and distribution of *Phytophthora* foot rot disease in major black pepper cultivation areas in Sarawak.
2) To isolate, identify and characterize *P. capsici* based on morphological and molecular methods.
3) To investigate potential indigenous endophytic bacteria to control *P. capsici* under glasshouse condition.

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PUBLICATION

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