ISOLATION AND SCREENING OF ANTAGONISTIC MICROORGANISMS FROM COMPOST AND THEIR POTENTIAL AS BIOLOGICAL CONTROL AGENTS AGAINST FOR BACTERIAL WILT OF TOMATO

By

PHUA CHOO KWAI HOE

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

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Specially Dedicated

To

My Family

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement of the degree of Master Science

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Bacterial wilt caused by *Ralstonia solanacearum* is the most widespread and destructive disease of tomato in Malaysia. In this study, isolation and screening of microorganisms antagonistic towards *R. solanacearum* from composts were carried out. The effect of these antagonists on germination and growth of tomato (variety MT 11) and their effectiveness in the control of bacterial wilt were evaluated under greenhouse trials.

Microorganisms were isolated from compost by using the serial dilution technique and antagonistic microorganisms were selected through the quick screening test. Results of the quick screening test showed that twelve isolates were antagonistic towards *R. solanacearum*. The antagonists were subsequently identified as *Pseudomonas aeruginosa, Pseudomonas fluorescens, Staphylococcus saphophyticus, Pseudomonas putida* biotype B*, Aspergillus* sp.*, Penicillum* sp.*,*

Trichoderma sp., *Brevibacterium casei* and three actinomycetes, which were designated as F 141, F 178 and F 179.

Antagonists were tested in dual culture assays, and for production of siderophore and volatile substances, to determine the possible mechanisms of antagonism. All the antagonists showed antibiosis against *R. solanacearum* in dual culture assays. In siderophore production test, all bacterial antagonists were antagonistic towards *R. solanacearum* on KB and KB+$FeCl_3$. Thus, indicating that these antagonists produced antibiotics and siderophores. Strains of *P. aeruginosa*, *P. fluorescens, S. saphophyticus,* actinomycetes F178 and F 179 also produced volatile substances which, inhibited the growth of the pathogen as shown in the tests for production of volatile substances.

In addition, *P. aeruginosa*, *P. fluorescens, S. saphophyticus* and *Aspergillus* sp. were also found to be phosphate solubilizers. These antagonists produced a clear zone on the phosphate agar plates. By using spectrophotometer and Salkowsky's reagent, the ability of antagonists to produce Indole-3-acetic acid (IAA) was carried out. All isolates except *S. saphophyticus* produced varying amounts of Indole-3-acetic acid (IAA).

Antagonists were bio-primed to seeds to evaluate their effect on seed germination. All antagonists except three fungal antagonists did not significantly reduce seed germination compared to the control. Fungal antagonists were therefore applied as soil drench while other antagonists were seed bio-primed in all the greenhouse experiments.

The effects of antagonists applied individually on the growth of tomato plants were carried in two trials. Seeds were bio-primed with bacterial and actinomycetous antagonists while fungal antagonists were applied by drenching fungal cultures (10^6 cfu/ml) at seedling stage. All antagonists except for *S. saphophyticus* significantly increased fresh and dry weights of tomato plants compared to the control in the first greenhouse trial. In the second greenhouse trial, *P. aeruginosa*, *P. fluorescens, S. saphophyticus* and *Aspergillus* sp. also increased fresh and dry weights of tomato plants when planted in sandy soils containing rock phosphate.

The effects of antagonists on the control of bacterial wilt were carried out in greenhouse trials. Results from application of antagonists individually to control bacterial wilt in greenhouse trial I showed that all antagonists gave significant reduction in bacterial wilt disease compared to the control. Application of *P. fluorescens* (B 12), *P. aeruginosa* (B 292) and *Trichoderma* sp. (F 196) individually and in combination to control bacterial wilt of tomato was carried out in greenhouse trial II. Results showed that combination treatments of B 292 and B 12; B 292 and F 196; B 12 and F 196 and B 292, B 12 and F 196 significantly reduced bacterial wilt compared to the individual treatments of antagonist. However, all the treatments were able to reduce the disease significantly compared to the control.

In conclusion, these isolates were not only antagonistic towards *R. solanacearum in vitro* but were also able to control the disease and enhanced the growth of tomato plants. Thus, these isolates show potential as plant growth-promoters and bio-control agent for bacterial wilt.

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains

ASINGAN DAN PENYARINGAN MIKROORGANISMA BERANTAGONIS DARI KOMPOS DAN POTENSINYA SEBAGAI AGEN KAWALAN BIOLOGI UNTUK PENYAKIT LAYU BAKTERIA PADA TOMATO

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Penyakit layu bakteria yang disebabkan oleh *Ralstonia solanacearum* merupakan penyakit tomato yang tersebar meluas dan paling memusnahkan di Malaysia. Asingan dan penyaringan mikroorganisma antagonis terhadap *R. solanacearum* dari kompos telah dijalankan. Dalam kajian ini, keberkesanan antagonis terhadap percambahan dan pertumbuhan pokok tomato (varieti MT 11) serta keberkesanannya dalam pengawalan penyakit layu bakteria telah diuji di rumah hijau.

Mikroogranisma telah diasingkan dari kompos dengan menggunakan teknik pencairan bersiri dan antagonis telah dipilih melalui ujian penyaringan ringkas. Keputusan perolehi dari ujian penyaringan ringkas menunjukkan dua belas asingan adalah berantagonis terhadap *R. solanacearum*. Antagonis ini dikenalpasti sebagai *Pseudomonas aeruginosa, Pseudomonas fluorescens, Staphylococcus saphophyticus, Pseudomonas putida* biotype B*, Aspergillus* sp.*, Penicillum* sp.*,*

Trichoderma sp., *Brevibacterium casei* dan tiga aktinomiset, dilabelkan sebagai F 141, F 178 and F 179.

Ujian dwi-kultur, ujian penghasilan siderofor dan penghasilan zat meruap dijalankan bagi menguji kemungkinan mekanisme antagonisme. Kesemua antagonis menunjukkan antibiosis terhadap *R. solanacearum* dalam ujian dwi-kultur. Dalam ujian penghasilan siderofor, kesemua bakteria antagonis menunjukkan antibiosis terhadap *R. solanacearum* pada media KB dan KB+$FeCl_3$. Ini menunjukkan semua antagonis menghasilkan antibiotik dan siderofor. *P. aeruginosa*, *P. fluorescens, S. saphophyticus,* aktinomiset F178 and F 179 juga menghasilkan zat meruap dalam ujian penghasilan zat meruap.

P. aeruginosa, *P. fluorescens, S. saphophyticus* and *Aspergillus* sp. juga merupakan pelarut fosfat. Antagonis ini menghasilkan zon jernih pada agar fosfat. Dengan menggunakan teknik spektrofotometer dan reagen Salkowsky, keupayaan antagonis menghasilkan asid indole-asetik (IAA) telah dijalankan. Semua antagonis kecuali *S. saphophyticus* menghasilkan IAA dalam pelbagai sukatan.

Keberkesanan antagonis terhadap percambahan biji benih telah diuji. Antagonis dirawat pada biji benih secara teknik bio-prim. Kesemua antagonis kecuali kulat tidak meningkatkan percambahan biji benih secara bererti berbanding dengan kawalan. Dari itu, antagonis jenis kulat digunakan terus ke tanah manakala antagonis lain dirawat ke biji benih secara bio-prim dalam semua eksperimen di rumah hijau.

Keberkesanan antagonis secara berasingan terhadap pertumbuhan anak benih tomato telah diuji dalam dua eksperimen di rumah hijau. Antagonis jenis bakteria dan aktinomiset dirawat ke biji secara bio-prim manakala antagonis jenis kulat dirawat pada tanah. Semua rawatan antagonis, kecuali *S. Saphophyticus*, meningkatkan berat basah dan kering anak benih secara bererti berbanding dengan pokok kawalan dalam eksperimen pertama. Dalam eksperimen kedua, *P. aeruginosa*, *P. fluorescens, S. saphophyticus* dan *Aspergillus* sp. juga menunjukkan peningkatan berat basah dan kering anak benih secara bererti berbanding kawalan apabila ditanam dalam tanah berpasir yang dicampur dengan fosfat batu.

Keberkesanan antagonis dalam pengawalan penyakit layu bakteria telah diuji dalam rumah hijau. Keputusan dalam ujian rumah hijau pertama menunjukkan penurunan penyakit layu bakteria yang lebih rendah secara bererti bagi pokok yang dirawat dengan antagonis berbanding dengan pokok kawalan. Dalam ujian rumah hijau kedua, *P. fluorescens* (B 12), *P. aeruginosa* (B 292) dan *Trichoderma* sp. (F 196) dirawat secara berasingan atau kombinasi untuk pengawalan penyakit layu bakteria. Keputusan menununjukkan pokok yang dirawat dengan kombinasi antagonis iaitu B 292 dan B 12; B 292 dan F 196; B 12 dan F 196 serta B 292, B 12 dan F 196 memberi kawalan penyakit layu bakteria lebih berkesan secara bererti apabila dibandingkan dengan pokok yang dirawat dengan antagonis secara berasingan. Walaubagaimanapun, kesemua rawatan memberi pengawalan penyakit yang bererti bila dibandingkan dengan kawalan.

Kesimpulannya, asingan antagonis ini bukan sahaja berantagonis terhadap *R. solanacearum in vitro* tetapi juga berkesan mengawal penyakit layu bakteria serta

meningkatkan pertumbuhan pokok tomato. Maka, antagonis ini berpontensi sebagai pengalak pertumbuhan dan agen kawalan biologi dalam pengawalan penyakit layu bakteria.

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I certify that an Examination Committee met on date of viva to conduct the final examination of Phua Choo Kwai Hoe on her Master of Agricultural Science thesis entitle "Isolation and screening of antagonistic microorganism from compost and their potential as biological control agent for bacterial wilt of tomato" in the accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follow:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that is has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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PHUA CHOO KWAI HOE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIA	= Actinomycetes isolation agar
ANOVA	= Analysis of Variance
cfu	= Colony forming unit
CPG	= Casamino acid peptone glucose
DI	= Disease incidence
DSI	= Disease severity index
FAMA	= Federal Agriculture and Marketing Authority
IAA	= Indole-1-acetic acid
KB	= King's B
MARDI	= Malaysian Agricultural Research and Development
MEB	= Malt extract broth
NA	= Nutrient agar
O.D.	= Optical density
P	= Phosphorus
PGPR	= Plant Growth-Promoting Rhizobacteria
pH	= Hydrogen ion concentration
TYB	= Tryptic soy broth
TZC	= Tetrazolium chloride

CHAPTER I

INTRODUCTION

Tomato is an economically important vegetable in Malaysia. A total of 470 hectares in Peninsular Malaysia is under tomato cultivation (Dept.Agric., 1999) and 16,742 metric tons of tomatoes were exported in year 2002 while 22,792 metric tons tomatoes are estimated to be exported in year 2005 (FAMA, 2002). Bacterial wilt affects a wide range of host plants (Abdullah, 1992). More than 50 families of plants have been recorded as hosts (Hayward, 1995). Bacterial wilt is also the most widespread and destructive disease of tomato in Malaysia (Mah, 1987). *Ralstonia solanacearum* is the causal agent of the disease, which is characterized by the sudden wilting of susceptible plants, without yellowing of leaves. Milky exudates are apparent if the end of a cut stem is placed in water (Kelman, 1953).

The disease is particularly destructive in the lowlands, limiting tomato production (Graham *et al.*, 1977). High temperature enhances disease development (Buddenhagen and Kelman, 1964). When temperature increase to 30 - 35^0C, bacterial wilt on host plants such as tomato also increase (Hayward, 1991). The disease can bring about almost total destruction during the rainy season (Persley *et al.*, 1986).

Control measures of bacterial wilt includes using resistant cultivars, chemicals and crop rotation (Hayward and Hartman, 1994; Hayward, 1991; Kelman 1953; Ho, 1988). The use of resistant varieties is currently the most effective, popular and

an easy means of control. However, it is difficult to obtain cultivars with stable resistance under conditions of high temperature and humidity in the tropical lowlands (Hayward, 1991; Krausz and Thurstan, 1975; French and De Lindo, 1982). Resistant plants became susceptible when grown under high temperature (Mew and Ho, 1977).

Several biological control agents have been reported to reduce the incidence of bacterial wilt (Apiras and Angela, 1986; Hartman *et al.,* 1993; Hsu *et al.*, 1993b and Abdullah *et al.*, 2003). Previous studies showed that fluorescent pseudomonads could be successfully used to suppress plant pathogens of many crops in the greenhouse and field (Guo *et al.*, 2003; Sharma and Nowak, 1998; Maurhofer *et al.*, 1992; Loper, 1988; Hultberg *et al.*, 2000; Abdullah *et al.*, 2003). *Trichoderma* sp. has been found to be effective in the control of many diseases (Jinantana and Sariah, 1998; Bin *et al.*, 1991; Latunde-dada, 1993; Paulitz *et al.*, 1990, Ahmed *et al.*, 1999; Sariah and Chan, 1999). The results of these studies have led to increased interest in the use of biological control agents for the control of bacterial wilt.

Some antagonists have been found to enhance growth of plants (Glick, 1995; Gamalero *et al.*, 2002) and some of them were rhizobacteria. Plant growth enhancement could be through production of plant growth regulators (Benizri *et al.*, 1998) such as indol acetic acid (IAA) or through their ability to solubilize phosphates (Illmer *et al.*, 1995; Rodriguez and Fraga, 1999; Nautiyal, 1999).

In previous studies, microbial antagonists have been isolated from compost (Kwok *et al.*, 1987; Kuter *et al.*, 1983; Nelson and Hoitink, 1982; Kleyn and

Wetzler, 1981; Hoitink and Fahy, 1986). Seed treatment with fluorescent pseudomonads has shown increased seed germination, growth and yield of chickpea and soybean (Kumar and Dube, 1992). Seed priming has been used as a delivery system for application of antagonists (Callan *et al.,* 1991; Abdullah and Lee, 2003). It also enhanced seed germination and vigor through the addition of moisture (McDonald, 2000; Bennett and Waters, 1987; Sung and Chang, 1993). This study was therefore carried out with the following objectives:

1. To isolate microorganisms from compost and to screen them for antagonism against *Ralstonia solanacearum*
2. To determine the effect of antagonists on germination and growth of tomato
3. To evaluate the effectiveness of antagonists individually and in combination for the control of bacterial wilt of tomato in the greenhouse

CHAPTER II

LITERATURE REVIEW

Tomato (*L.esculentum(L.)Mill*)

Tomato belongs to the family Solanaceae and genus *Lycopersicon*. It is a perennial that is grown as an annual crop in temperate regions (Rubatzky, 1997). Tomato originated from the dry coastal desert of Peru (Tomato, 2001).

Tomato became popular in the 18th century when it was introduced into America from Europe (Tomato, 2001; Jones, 1999). Second to potato, tomato is the most widely grown *solanaceous* vegetable. Based on statistics from the Food and Agricultural Organization (FAO), in 1994 worldwide tomato production (fresh market and processing) was approximately 2.8 million hectares (Jones, 1999). Tomato is an economically important vegetable in Malaysia. In 1999, a total of 470 hectares is under tomatoes cultivation in Peninsular Malaysia (Dept. Agric., 1999). In 2002, a total of 16,742 metric tons tomatoes were exported while 22,792 metric tons are estimate to be exported in the year 2005 (FAMA, 2002). In the year of 2002, households used 72,616 metric tons of tomatoes, and in 2005, it is estimated to increase to 103,159 metric tons with greater consumption from factories (FAMA, 2002).