CHAPTER 3

MATERIALS AND METHODS

Materials

1. Media (Appendix A)

1.1 Bile esculin agar (Fluka®, Switzerland)

1.2 Chrome azurol S-modified Gaus No.1 (CAS-MGs-1) medium

1.3 Carbohydrate fermentation medium

1.4 Decarboxylase medium

1.5 King's B medium

1.6 Methyl red/Voges-Proskauer medium (MRVP) (Merck®, Germany)

1.7 Motility test medium

1.8 Nitrate medium

1.9 Nutrient medium

1.10 Phenylalanine deaminase medium

1.11 Starch agar

1.12 Simmons citrate agar (Merck®, Germany)

1.13 Triple sugar iron agar (TSI) (Merck®, Germany)

1.14 Triphenyltetrazoliumchloride (TZC) medium

1.15 Tryptic soy broth (TSB) (Merck®, Germany)

1.16 Tryptone medium

1.17 Urease test medium

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2. Chemical reagents for DNA isolation

2.1 Alkaline Sodium dodecyl sulphate (SDS) solution

2.2 Ethanol, absolute

2.3 Extraction buffer pH 8.0

2.4 Lysis buffer pH 8.0

2.5 Lysozyme, 10 mg/ml

2.6 Isopropanol

2.7 Potassium acetate, 5M

2.8 Proteinase K, 10 mg/ml

2.9 Sodium acetate, 3M

2.10 TES buffer pH 8.0

3. Chemical reagents for agarose gel electrophoresis (Appendix B)

3.1 Agarose gel 0.8 and 1%

3.2 Ethidium bromide, 1mg/ml

3.3 Loading dye

3.4 Marker 100 bp, λDNA/*Eco*130I (*Sty*I) (Fermentas, USA)

3.5 TAE buffer, 1X

4. Chemical reagent for siderophore detection (Appendix C)

4.1 Ferric perchlorate solution

4.2 Hydrochloric acid (HCl), 0.5M

4.3 Nitrite-molybdate reagent

4.4 Sodium hydroxide, 1M

6. Laboratory equipment

Table 2 Laboratory equipment

Equipment	Model	Manufacturer	Country
Autoclave	SS-325	Tomy	Japan
Balance, 2-digit precision	ARC120	Ohaus	USA
Balance, 2-digit precision	AB 304-S	Mettler Toledo	Switzerland
Centrifuge	Harrier 18/80	Sanyo	UK
	Mickro 200R	Hettich	Germany
Compound microscope	-	Olympus	USA
Electrophoresis gel system	EC320	Unitech	USA
Electrophoresis power supply	EC250-90	E-C Apparatus Corporation	USA
Freezer -20°C	SF-C991	Sanyo	Japan
Hot air oven	-	Heraeus	USA
Hot plate and magnetic stirrer	Cearastir	Clifton	UK
Incubator	-	Memmert	Germany
Lamina flow cabinet	TL2448	Holten LaminAir	Denmark
Microplate reader	Spectra MR	Dynex	USA
pH meter	713 pH meter	Metrohm	Switzerland
Spectrophotometer	Genesys20	Thermo spectronic	USA
Thermal cycler	GeneAmp® PCR System 9700	Applied Biosystem	USA
UV Transiluminator	Gene Flash	Syngene Bio Imaging	USA
Vortex mixer	VM-300	Gemmy Industrial Corporation	Taiwan
Water bath	TW12	Julabo	Germany

Methods

1. Isolation of wilt causing bacteria

The plant pathogenic bacteria were isolated from infected pathumma rhizomes obtained from Bua Lai Pathumma garden, Chiang Mai Province, Thailand. The bacteria were readily isolated from wilted rhizomes by streaking the plant ooze and exudates onto TZC agar prior to incubate at 30°C for 24-48 hours. The single colonies were transferred onto TZC agar to obtain pure culture. The bacteria were kept on TZC broth and maintained in 20% glycerol at -20°C for further studies. Some pathogenic bacterial isolates were obtained from stock culture.

2. Identification of wilt causing bacteria

The selected pathogenic bacteria were identified by both conventional and molecular method.

2.1 Conventional method

Morphological characterization, macroscopic and microscopic, was observed and described, e.g. colony growth pattern, pigment production and spore formation. Biochemical characterization was tested including catalase, lysine decarboxylase, phenylalanine deaminase, oxidase, motility, Methyl red and Voges-Proskauer test; hydrolysis of gelatin, urea and esculin; production of indole and hydrogen sulfide ; assimilation of citrate; acid production from carbohydrates including arabinose, cellobiose, dulcitol, glycerol, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, sorbitol, sucrose and xylose (Holt *et al.*, 1994; Forbes *et al.*, 2002).

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2.2 Molecular identification

Total genomic DNA of each bacterial isolate was extracted from fresh culture following Dellaporta *et al.* (1983) with some modifications as the following: 1 ml of overnight culture were centrifuged to obtain pelltet cells prior to mixed with 220 µl of lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0), incubated at 95°C for 10 minutes in water bath and cooled down on ice for 5 minutes. Then, 80 µl of freshly lysozyme solution (50 mg/ml in 10 mM Tris-HCl pH 8.0) was added before incubating at 37°C for 30 minutes. Afterwards, 400 µl of extraction buffer (500 mM NaCl, 100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 10 mM mercaptoethanol) were added and mixed. After addition of 40 µl of 20% sodium dodecyl sulphate (SDS) and mixing, the homogenate was incubated at 65°C for 10 minutes. Then, 250 µl of 5M potassium acetate was mixed in. The sample was incubated for 20 minutes on ice and centrifuged at 12,000 rpm for 20 minutes. Four-hundred µl of supernatant was transferred to a fresh tube and the pellet was discarded. Nucleic acids were precipitated twice, once with 240 µl of isopropanol and one with 40 µl of 3M sodium acetate and 1,000 µl of ethanol. After the final centrifugation, the pellet was washed with 70% ethanol and air-dried, then the pellet was dissolved in 100 µl sterile water. Genomic DNAs were maintained at -20°C for further studies.

A nearly complete 16S rRNA gene (~1.5 kb) was amplified using the universal primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Frank *et al.*, 2008), and 8F (5′-TGCGGATCCAGAGTTTGATCCTGGCTCAG-3′) and 1045R (5′-AATCTGCAGACGACAGCCATGCAGCACCT-3′) (Khemaleelakul *et al.*, 2002).

Amplification was carried out in a 50 µl reaction volume. The PCR conditions were performed as the following

Initial denaturation	94°C	for	1	minute	
Denaturation	94°C	for	1	minute	35 cycles
Annealing	58°C	for	2	minutes	
Extension	72°C	for	2	minutes	
Final extension	72°C	for	7	minutes	

PCR products were purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Catalog no. DF100) following the manufacturer's protocol. The purified PCR products were sent to sequence at First base company, Malaysia using primer 27F and 1492R, and 8F and 1045R as sequencing primers. The identities of nucleotide sequences of the 16S rRNA gene obtained were subjected to BLAST analysis (Altschul *et al.*, 1990) with the NCBI database (http://www.ncbi.nlm.nih.gov). Nucleotide sequences were edited using BioEdit software and aligned using ClustalW program (Thompson *et al.*, 1994). Neighbor-joining method (NJ) was used in the construction of a phylogenetic tree using the program in MEGA4 (Tamura *et al.*, 2007). The topological analysis was performed with 1000 bootstrap replicates.

3. Pathogenicity test of wilt causing bacteria

To determine the pathogenicity of bacterial isolates, whole plant inoculations in the greenhouse and laboratory assays were performed. The disease incidence (DI) was calculated by the following equation (Masyahit *et al.*, 2009).

$$DI = \frac{\text{Number of infected plant units}}{\text{Total number of inoculated plant units}} \times 100$$

3.1 Infectivity of wilt causing bacteria in Pathumma plants

The pathogenicity tests were performed in 4 experiments at growing season during 2007-2010 and 2 experiments at out of season during 2009-2010. The rhizomes were newly purchased in each experiment per each year. The isolates of wilt causing bacteria conducted in 2007-2009 were selected based on morphology on TZC plate showing large slime surrounded colonies, and diversity of infected cultivar rhizomes. For year 2010, the isolates of wilt causing bacteria were selected based on the severity of pathogenicity in previous study (2007-2010).

3.1.1 First experiment in 2007 during growing season

Ten isolates of wilt causing bacteria including PRZ, PT1B, PT1J, PT2X, RRD, RT1K, RT1S, RT2R, C4 and D1 were inoculated into rhizomes and pseudostems of Pathumma, Chiang Mai Pink and Chiang Mai Red cultivars. The plants were re-infected both with the bacteria isolated from similar cultivar and cross strain reinfection. The rhizomes were planted in 15cm-diameter plastic bags containing sterile soil mixture prepared from commercial soil, chaff and coir at the ratio of 3:1:1. To prepare bacterial inoculum, bacteria were cultured in TZC medium and incubated at 30°C for 24 hours. The bacterial suspension was then adjusted to $1x10^7$-$1x10^8$ cfu/ml. Rhizomes and pseudostems of Pathumma were wounded prior to drenched with 10 ml of bacterial inoculums. The plants were maintained at the 7^{th} floor deck of Science Building 2, Faculty of Science, Chiang Mai University during growing season. There were 10 replicates per treatment (one treatment made from one bacterial isolate). The occurrence of wilt disease was observed and the percentage of

the wilted plants was evaluated. The pathogenic bacteria were re-isolated from rhizomes and soils while the viable cell counts were performed every 15 days for 5 months. To determine the number of live bacterial cells in the soil mix, core samples from each infested bag were taken using a spatula. One gram of soil samples was added into a test tube containing 9 ml of sterile distilled water. The suspension was agitated and serial diluted prior to spread on TZC agar plate containing 100 μg/ml cycloheximide. Plates were incubated at 30°C for 48 hours and colony forming units were counted. The bacterial population data were transformed to $\log_{10}$ number value (Huang and Allen, 2000).

3.1.2 Second experiment in 2008 during growing season

Ten isolates of wilt causing bacteria including PRZ, PT1B, PT1J, PT2X, RRD, RT1K, RT1S, RT2R, C4 and D1 were inoculated into rhizomes and pseudostems of Pathumma, Chiang Mai Pink and Chiang Mai Red cultivars. The plants were re-infected both with the bacteria isolated from similar cultivar and cross strain reinfection. Rhizomes were wounded and dipped into bacterial suspension overnight before putting into the soil. The plantation and observation were performed as described in section 3.1.1 with some modifications that plants were placed in greenhouse of Department of Biology, Faculty of Science with ambient temperature and watered daily. There were 3 replicates per treatment.

3.1.3 Third experiment in 2009 during out of growing season

Fifteen isolates of wilt causing bacteria including PRZ, PT1B, PT1J, PT2X, RRD, RT1K, RT1S, RT2R, C4, D1, Rh1-1, Rh3-1, Tu1-1, Tu2-1 and R1512 were inoculated into rhizomes of Pathumma, Chiang Mai Pink and Chiang Mai Red cultivars. The plants were re-infected both with the bacteria isolated from similar

cultivar and cross strain reinfection. Rhizomes were wounded and dipped in bacterial suspension overnight before put into the soil. The plants were placed on the shelf provided with 12hours-photoperiod fluorescent light. There were 3 replicates per treatment.

3.1.4 Fourth experiment in 2009 during growing season

Twelve isolates of wilt causing bacteria including PRZ, PT1B, PT1J, PT2X, RRD, RT1S, C4, D1, Rh1-1, Rh3-1, Tu1-1 and R1512 were inoculated into rhizomes with only direct infection. The bacterial isolates were cultured in 2 different mediums that are blended rhizome medium and a half composition of TZC medium prior to pour into the wounded rhizomes. Additionally, the pathogenicity was tested in pseudostems both direct and cross infection using Pathumma Chiang Mai Pink and Snow white cultivars. The experiment was conducted in greenhouse. There were 3 replicates per treatment.

3.1.5 Fifth experiment in 2010 during out of growing season

Six isolates of wilt causing bacteria including PT1B, PT1J, PT2X, RRD, RT1S and R227 were inoculated into rhizomes with only direct infection. The rhizomes were incubated at 30°C for 15 days prior to culture. There were 3 replicates per treatment.

3.1.6 Sixth experiment in 2010 during growing season

Seven isolates of wilt causing bacteria including PT1J, PT2X, RRD, RT1S, Rh1-1, Tu1-1 and R227 were inoculated into rhizomes and pseudostems. For rhizome inoculation, treatment1: bacterial isolates were centrifuged to obtain packed cell prior to infect the rhizome and treatment2: rhizomes were wounded and dipped into bacterial suspension overnight before putting into the soil which mixed with bacterial

packed cells. In addition, the mixed culture of 7 pathogenic bacteria was inoculated into rhizomes and pseudostems. There were 3 replicates per treatment.

All experiments of pathogenicity tests by wilt causing bacteria were concluded in table 3.

Table 3 Summary of the experiments of pathogenicity tests

Treatment	Year					
	Growing season				Out of growing season	
	2007	2008	2009	2010	2009	2010
Pathogenic isolates						
PRZ	✓	✓	✓	-	✓	-
PT1B	✓	✓	✓	-	✓	✓
PT1J	✓	✓	✓	✓	✓	✓
PT2X	✓	✓	✓	✓	✓	✓
RRD	✓	✓	✓	✓	✓	✓
RT1K	✓	✓	-	-	✓	
RT1S	✓	✓	✓	✓	✓	✓
RT2R	✓	✓	-	-	✓	-
C4	✓	✓	✓	-	✓	-
D1	✓	✓	✓	-	✓	-
Rh1-1	-	-	✓	✓	✓	-
Rh3-1	-	-	✓	-	✓	-
Tu1-1	-	-	✓	✓	✓	-
Tu2-1	-	-	-	-	✓	-
R1512	-	-	✓	-	✓	-
R227	-	-	-	✓	-	✓
Time of inoculation to						
Rhizome (day 1)	✓	✓	✓	✓	✓	✓
Pseudostem (day 30)	✓	✓	✓	✓	-	-
Cultivar infection						
Direct infection	✓	✓	✓	✓	✓	✓
Cross infection	✓	✓	✓	-	✓	-

Symbols; ✓ = the treatment was performed

\- = the treatment was not performed

3.2 Infectivity of wilt causing bacteria in laboratory bioassays

For the bioassay, the pathogenicity test was performed in 2 experiments. Five isolates of wilt causing bacteria including PT1J, PT2X, RRD, RT1S and R227 were examined.

Experiment 1: The healthy shoots of Pathumma, 30 cm long, were cut and placed into Erlenmeyer flasks containing 100 ml of bacterial suspension ($1x10^7$ cfu/ml). These were compared with sterile distilled water as negative control. The occurrence of wilt symptom was investigated.

Experiment 2: The rhizomes were inoculated with pathogenic bacteria under hydroponic condition. The wounded rhizomes were cultured in the pots containing 100 ml of plant-growth media without soil and agitated by oxygen power supply. Concurrently, the bacterial inoculums approximately of $1x10^7$ cfu/ml were poured into the pots. The observation of plant growth was evaluated.

4. Study of bacterial adhesion in plant tissue under compound light and electron microscopes

4.1 Compound light microscope

To study plant tissue changes in the stems of inoculated Pathumma plants, the wilted plants were removed from the pots after 7-10 days of inoculation. The roots and the tops of the plants were cut off, and the remaining stems were washed under running water. The stems were cross sectioned and stained with lactophenol cotton blue prior to observe under a compound light microscope.

4.2 Electron microscope

The plant stems were cross section about 3-4 cm of length prior to prepare for scanning electron microscopic assay. Briefly, a portion of stems was fixed with 2.5% glutaraldehyde at 4°C overnight. After fixation, the cells were covered with 1% osmium tetroxide (OsO_4) in 0.1 M phosphate buffer, incubated at 4°C for 2 hours, washed three times in phosphate buffer. The stems were subsequently dehydrated in ethanol, critical point-dried, mounted on scanning electron micrograph stubs, sputter-coated with gold and observed by a scanning electron microscope JSM5910LV (JEOL Ltd., Japan) at Science and Technology Service Center, Faculty of Science, Chiang Mai University.

5. Survival of wilt causing bacteria in soil without host

Fifteen isolates of pathogenic bacteria including PRZ, PT1B, PT1J, PT2X, RRD, RT1K, RT1S, RT2R, C4, D1, Rh1-1, Rh3-1, Tu1-1, Tu2-1 and R1512 were tested for the capacity to survive in soil without host. The bacterial suspension containing approximately $1x10^7$ - $1x10^8$ cfu/ml were poured into soil in plastic bags prepared as described in section 3.1.1. Plastic bags were placed in the greenhouse. The experiment was carried out with three replications (bags). The viable cell count was performed in order to calculate survival percentage of wilt causing bacteria. One gram of samples was drawn every 30 day after inoculation for a duration of 1 year. The bacterial population was assessed by a dilution plate method. The viable cell of pathogenic bacteria were analyzed by analysis of variance (ANOVA) using SPSS version 14. Means were compared using the Tukey's test (Huang and Allen, 2000).

6. Isolation and screening of antagonistic bacteria

Soil samples from several part of Thailand were collected for bacterial isolation using tryptic soy broth (TSB). One gram of each soil samples was put into 5 ml of TSB and incubated at 30, 37 and 45°C for 24-48 hours. To obtain pure isolate, the cultures were streaked on tryptic soy agar (TSA) and incubated at 30, 37 and 45°C for 24-48 hours. Colonies of different shapes were randomly picked from agar plates. Each pure isolate was maintained at 4°C and -20°C in 20% glycerol for further studies.

An agar disc diffusion method was performed to determine the ability of each bacterial isolate to inhibit growth of the selected wilt causing bacteria. Each bacterial isolate was cultivated in TSB at 30°C for 48 hours. The culture broth was centrifuged at 6000 rpm for 10 minutes to obtain a supernatant. Concomitantly, the wilt causing bacteria were grown in TZC broth at 30°C for 12 hours (Optical Density at 660 nm were 0.5 corresponding to approximately $1x10^6 - 1x10^7$ cfu/ml) prior to swabbing onto TZC agar plates. A paper disc, 6 mm in diameter, was dipped into each supernatant obtained before placing onto surface of the plate prepared above. The TSB medium was used as a negative control. All plates were incubated at 30°C for 24-48 hours, prior to observe the appearance of clear inhibition zones. Inhibition of wilt causing bacteria was assessed by measuring the radius in millimeters of the inhibition zone. Accordingly, those bacterial isolates that showed positive *in vitro* inhibitory activity during preliminary screening were selected as "antagonistic bacteria" (Aliye *et al.*, 2008). The potential bacterial antagonists that demonstrated strong degree of inhibition were maintained and further evaluated.

7. Identification of antagonistic bacteria

The isolate SP15, SP38, SP46 and SP58 were identified by both conventional and molecular methods.

7.1 Conventional method

Antagonistic bacterial strains were tentatively identified according to traditional morphological criteria including colony growth pattern, pigment production, spore formation. Biochemical characterization were tested including catalase, lysine decarboxylase, phenylalanine deaminase, oxidase, motility, Methyl red and Voges-Proskauer test; hydrolysis of gelatin, casein and starch; production of indole, lecithinase and gas from glucose; growth in TSB containing 2, 5, 7 and 10% NaCl; growth at 30, 40, 50 and 55°C; utilization of citrate, adonitol, alanine, arginine, mannitol, rhamnose, ribose, sucrose, trehalose and xylose (Holt *et al.*, 1994; Forbes *et al.*, 2002).

7.2 Molecular method

Genomic DNA of the isolate SP15 was extracted by the method of Martin-Platero *et al.* (2007) as the following: 1 ml of overnight culture was harvested and resuspened in 100 µl of TES buffer (10% w/v sucrose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mg/ml freshly made lysozyme and 40 µg/ml RNaseA) for 30 minutes at 37°C. The protoplast cells were immediately lysed by adding 600 µl of lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 10mM NaCl and 1% w/v SDS) for 15 minutes at room temperature. The lysate were treated with 10 µl of proteinase K, 10 mg/ml for 15 minute at 37°C. After incubation at 80°C for 5 minutes and cooling down to room temperature for 5-10 minutes, 200 µl of sodium acetate (3 M, pH 5.2) were added, chilled on ice for 15 minutes and centrifuged. The supernatant was taken

to a new tube and 600 µl of isopropanol were added to precipitate the DNA. Finally, genomic DNA was dissolved in distilled water. Genomic DNA was maintained at -20°C for further studies.

The extraction of genomic DNAs of the isolate SP38, SP46 and SP58 was followed Dellaporta *et al.* method as described in section 2.2.

PCR was done with the universal 16s rRNA primer pair 8F and 1045R. The PCR conditions were conducted as described in section 2.2.

8. Optimization of inhibitory substance produced by antagonistic bacteria

Four antagonistic bacteria including SP15, SP38, SP46 and SP58 were selected for production optimization. To study the medium optimization process, various carbon and nitrogen sources were supplemented to the basal production medium. The efficacy of the optimized medium was established on the basis of zone of inhibition (ZOI) size (Banga *et al.*, 2008).

8.1 Carbon sources

Each antagonistic bacterial isolate was grown at 30°C in Erlenmeyer flask containing 50 ml of TSB or modified TSB which glucose had been substituted with sucrose, maltose, fructose, galactose, lactose, manitol or sorbitol. After 48 hours of incubation, the culture were centrifuged to collect supernatant for further agar disc diffusion method against four isolates of wilt causing bacteria i.e. PT1J, PT2X, RRD and RT1S. The modified TSB that substituted glucose with various carbon sources was used as negative control. The carbon source with the highest inhibiting activity was selected for further evaluation of the optimal concentration; 0.1, 0.5, 1.0, 1.5, 2.0 or 2.5 % (w/v).

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8.2 Nitrogen sources

Each antagonistic bacterial isolate was grown at 30°C in 50 ml of TSB or modified TSB which peptone was substituted with yeast extract, tryptone, corn flour, corn steep liquor, ammonium dihydrogenphosphate ($NH_4(H_2PO_4)$) or ammonium nitrate (NH_4NO_3). The modified TSB was negative control. The nitrogen source with the highest activity was selected for determination of the optimal concentration; 0.1, 0.5, 1.0, 1.5, 2.0 or 2.5 % (w/v). Culture were centrifuged to obtain supernatant and tested for growth inhibition of pathogenic bacteria.

8.3 Media pH

To determine the optimal media pH of culture medium, each antagonistic isolate was grown in 50 ml modified TSB with the pH adjusted to 4, 5, 6, 7, 8, 9, 10 or 11. Culture supernatants were evaluated for their ability to inhibit growth of wilt causing bacteria. The modified TSB with various pH were used as negative control.

8.4 Temperature

The tested temperature was 25, 30, 37, or 45°C. Each antagonistic bacteria was cultured in 50 ml of modified TSB. After 48 hours of incubation, each culture broth was centrifuged to obtain a supernatant which was further investigated its activity by an agar disc diffusion method. The modified TSB with the optimal carbon source, nitrogen source and media pH was used as negative control. After selecting the best medium for optimum cell growth and antibacterial activity, the producer strain was subsequently cultured in the optimal medium.

All data were analyzed using SPSS software version 14 for Windows. Statistical significance was evaluated using Tukey's test and $P<0.05$ was considered as significant difference.

9. Study on substances produced by antagonistic bacteria

9.1 Siderophore production

9.1.1 Screening of siderophore production

Production of siderophores was preliminary examined according to modified universal chrome azurol S (CAS) assay method (Pérez-Miranda *et al.*, 2007). Siderophore production was tested on modified Gaus No.1 medium (MGs) containing CAS (Appendix A) (Khamna *et al.*, 2009). Pure isolate of SP15, SP38, SP46 and SP58 were stabbed on CAS-agar plates using sterile toothpicks and incubated at 30°C for 72 hours in the dark. Bacterial colonies surrounded with orange, purple or yellow zones were considered as siderophore-producing isolates (Chaiharn *et al.*, 2009). The bacterial isolates which changed medium color were secondarily detected for sideophore type and concentration.

9.1.2 Detection of chemical nature of siderophore

The positive isolates were cultured in MGs-1 broth and incubated at 30°C with agitation rate of 150 rpm for 48 hours. Throughout the incubation, the population density was monitored turbimetrically at 660 nm. When the culture reached a stationary phase, samples were taken. The cells were removed from growth media by centrifugation at 6,000 rpm, 4°C for 10 minutes. The supernatants were concentrated by ultra-filtration technique prior to detect the nature of siderophores using chemical assays. Types of siderophore evaluated in this study were catecholates and hydroxamates.

9.1.2.1 Catecholates

Catecholate-type detection was performed by Arnow's method (Arnow, 1937) with some modifications. A mixture comprising 50 µl of culture

supernatant, 50 µl of 0.5 M HCl and 50 µl nitrite-molybdate reagent (Appendix C) was conducted in 96-well microplate. When formation of yellow color occured, 50 µl of 1 M NaOH were added. The plates were continually incubated at room temperature for 5 minutes to allow full reaction. The absorbance of developed red pigmentation was measured at 500 nm by a microplate reader using the uninoculated media mixed with reagent as a blank. The level of catecholate produced was estimated against a standard 2,3-dihydroxybenzoic acid (Khamna *et al.*, 2009).

9.1.2.2 Hydroxamates

Hydroxamate-type siderophore detection was performed by iron-perchlorate assay (Csaky, 1948) with some modifications. Thirty microliter of culture supernatant was added to 150 µl of ferric perchlorate solution (Appendix C) and incubated at room temperature for 5 minutes. The presence of a hydroxamate-type siderophore was shown by the development of an orange-red color. Amount of hydroxamates was quantitated by measuring absorbance at 480 nm using a microplate reader. The uninoculated medium mixed with reagent was used as a blank. The level of hydroxamate produced was estimated against a standard deferoxamine mesylate (Khamna *et al.*, 2009).

Results from catecholate and hydroxamate chemical assays were statically analyzed by ANOVA using SPSS software version 14 for Windows. Means were compared using Tukey's test at $P<0.05$.

9.2 Phenazine production

The antagonistic bacterial isolates SP15, SP38, SP46 and SP58 were screened for the production of phenazine by thin layer chromatography (TLC) (Perneel *et al.*,

2007). Each isolate was streaked onto King's B (KB) agar plate. Fully-grown KB plates were washed twice with sterile water to remove bacterial cells. Agar was excised in small pieces and pooled in Erlenmeyer flask. Twelve milliliters of chloroform was added and the suspension was shaking incubated at 37°C for 2 hours while shaking. Subsequently, an equal volume of 0.1 M NaOH was added to the chloroform fraction. Phenazine compound shifted to the aqueous phase. Chroloform fraction was air-dried and dissolved in methanol. For TLC analysis, the methanol extract was spotted carefully on TLC plates (silica gel 60 F_{254}). The silica plates were conducted in a solvent mixture containing toluene/acetone (3:1 v/v) and detected under UV light for phenazine production.

10. *In vivo* study on growth inhibition of wilt causing bacteria in Pathumma

The antagonistic bacterial isolates SP15, SP38, SP46 and SP58 were evaluated the ability to control bacterial wilt under greenhouse trials. Four antagonists were mixed cultivated in 100 ml of modified TSB obtained from earlier study. The cultures were incubated at 30°C for 24 hours. To prepare wilt-bacterial inoculums, each pathogenic bacterial isolate was grown in 100 ml of TZC medium at 30°C for 24 hours. The concentration of antagonistic mixed-culture and wilt causing bacteria were approximately $1x10^7$ - $1x10^8$ cfu/ml. The soil mixtures were prepared as described in section 3.1.1. Three experiments were performed (Thongwai and Kunopakarn, 2007).

Experiment 1: Both antagonistic and pathogenic bacteria were co-applied to Pathumma rhizomes and to soil in pots before cultivation.

Experiment 2: Both antagonistic and pathogenic bacteria were co-applied to shooting pathummas.

Experiment 3: Pathogenic bacteria were applied to rhizomes and to soil before pathumma cultivation while the mixed culture of antagonistic bacteria were added to the plant pots after shooting.

The experiments were conducted in 2008, 2009 and 2010 in greenhouse at the Department of Biology, Faculty of Science, Chiang Mai University. The mixture pattern of antagonists and pathogenic bacteria were designed differently in each year.

10.1 Year 2008

The antagonistic bacteria were mix-cultured as shown in Table 4. The treatment designs were demonstrated in Table 5.

10.2 Year 2009

The experiments were duplicate performed. The wood vinegar was additionally used to inhibit growth of wilt causing bacteria (Table 5).

10.3 Year 2010

The wilt-bacterial isolate R227 was additionally determined (Table 5). There were 3 replicates per treatment.

Table 4 The mixture of antagonistic bacteria at the ratio of 1:1

Mixture	Antagonistic bacteria used			
	SP15	**SP38**	**SP46**	**SP58**
A	✓	✓	✓	-
B	✓	✓	-	✓
C	✓	-	✓	✓
D	-	✓	✓	✓
E	✓	✓	✓	✓

Table 5 Design of treatment using mixture of antagonistic bacteria to suppress growth of wilt causing bacteria

Antagonist mixture	Wilt causing bacteria used					
	PT1J	**PT2X**	**D1**	**RRD**	**RT1S**	**R227**
Year 2008						
A	1A	-	2A	3A	4A	-
B	1B	-	2B	3B	4B	-
C	1C	-	2C	3C	4C	-
D	1D	-	2D	3D	4D	-
E	1E	-	2E	3E	4E	-
W	-	-	-	-	-	-
Year 2009						
A	1A	2A	-	3A	4A	-
B	1B	2B	-	3B	4B	-
C	1C	2C	-	3C	4C	-
D	1D	2D	-	3D	4D	-
E	1E	2E	-	3E	4E	-
W	1W	2W	-	3W	4W	-
Year 2010						
A	1A	2A	-	3A	4A	5A
B	1B	2B	-	3B	4B	5B
C	1C	2C	-	3C	4C	5C
D	1D	2D	-	3D	4D	5D
E	1E	2E	-	3E	4E	5E
W	1W	2W	-	3W	4W	5W

Symbols; W = wood vinegar

\- = the treatment was not performed

All experiments were examined the amount of both antagonistic bacteria and wilt causing bacteria using serial dilution method and spread on TSA and TZC agar plates. The number of total bacteria and wilt causing bacteria were counted in TSA and TZC plate, respectively, with morphologically characteristics observation. Consequently, the numbers of antagonistic bacteria were calculated by subtracting the number of total bacteria with the numbers of wilt causing bacteria. The occurrences of wilt disease and growth of plants were assessed. The viable cell count of bacteria and growth plant evaluation were performed every 15 days for 5 months. All treatments were done three times.

11. Designation of mixed culture products

The culture of antagonistic bacterial isolates SP15, SP38, SP46 and SP58 were formulated with various carriers. Formulations of microbial mass were conducted 2 forms including liquid and dry formulation products.

11.1 Liquid formulation products

The antagonistic bacteria were primary screened for the optimal carrier by inoculation in 100 ml of various designed medium including

- 2% (w/v) potato starch
- 2% (w/v) potato starch + 2% (w/v) glucose
- 5% (w/v) potato starch + 2% (w/v) glucose
- soybean whey, neat
- 50% (v/v) soybean whey
- 2% (v/v) molasses
- 5% (v/v) molasses

- 2% (v/v) molasses + 2% (w/v) soybean flour

The inoculated medium were incubated at room temperature for 48 hours. The growth of antagonistic bacteria was evaluated by streaked on TSA and turbidity observation. The medium which had high mass production were selected for secondary screening. The antagonists were individually cultured in 500 ml of selected medium and incubated at room temperature for 7 days. The population of bacteria was examined by dilution plate method prior to spread on TSA. From this experiment, the best medium was selected upon the maximum number of viable bacterial cell. Ultimately, the antagonistic bacteria were grown in 1,000 ml of medium and incubated at room temperature for 90 days. The viable cell count was examined every 15 day.

11.2 Dry formulation products

Three carrier materials including chaff, coir and soil were evaluated for their ability to support growth of antagonistic bacteria. The isolate SP15, SP38, SP46 and SP58 were cultured on TSB and incubated at 30°C for 24 hours. The cultures were centrifuged at 6,000 rpm for 10 minutes to separate bacterial cells. The pellets were mixed with sterile distilled water. The individual or mixture of four antagonistic cell suspension containing $1x10^8$ cfu/ml was added into 100 g of the carrier materials and mixed well under sterile condition. The materials were incubated at room temperature for 60 days. The viable cell count was assessed by the serial dilution method prior to spread onto TSA.

Summary of methods in this research

Wilt causing bacteria

1) Isolation
2) Identification
   a) Conventional method
   b) Molecular method
3) Pathogenicity test
   a) Infectivity in pathumma plants
      i) Growing season
         2007
         2008
         2009
         2010
      ii) Out of growing season
         2009
         2010
   b) Infectivity in laboratory
      i) Shooting cuts in flask
      ii) Hydroponic condition
4) Adhesion in plant tissue
   a) Compound light microscope
   b) Electron microscope
5) Survival in soil without host

Antagonistic bacteria

1) Isolation and screening
2) Identification
   a) Molecular method
   b) Molecular method
3) Optimization of inhibitory substance
   a) Carbon source
   b) Nitrogen source
   c) Media pH
   d) Temperature
4) Study on inhibitory substance
   a) Siderophore
   b) Phenazine
5) *in vivo* study on growth inhibition of wilt bacteria
      2008
      2009
      2010
6) Designation of mixed products
   a) Liquid formulation
   b) Dry formulation