CHAPTER 4

RESULTS AND DISCUSSIONS

1. Isolation and identification of wilt causing bacteria

Fourty-five bacterial isolates were obtained from water-soaked Pathumma rhizomes collected from the fields (Figure 12). On TZC media, red or pink colonies with white slime layer were formed. The strains were Gram negative, short-rod, facultative anaerobe, oxidase negative, catalase positive and non-motile. They were 0.5-1.0 μm width and 1.0-2.5 μm length (Figure 13-14). Most of all bacterial isolates had a mucus-like around colonies which is the key characteristics of wilt causing bacteria (Agrios, 1997). The TZC media consist of low amounts of glucose but these bacteria can produce high level of polysaccharides and thus resulting in plugging in xylem. Therefore, TZC medium is useful for primary isolation of wilt causing bacteria. This cultural characteristic was similar with *Ralstonia solanacearum* and *Pseudomonas*.

(A) (B)

Figure 12 (A) The collecting site of wilt disease in Bua Lai Pathumma garden, San Sai, Chiang Mai, Thailand . (B) The infected Pathumma rhizome

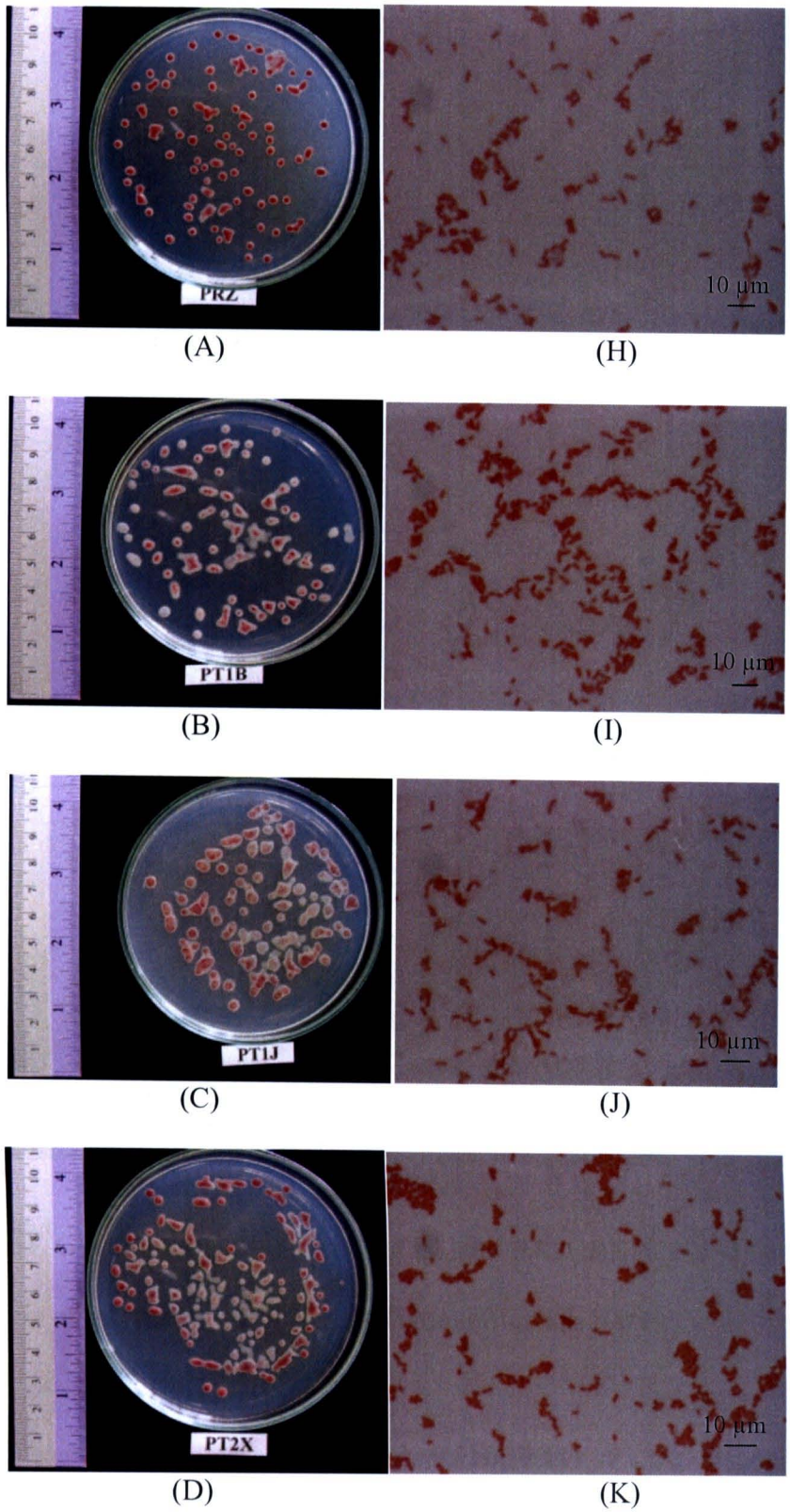

Figure 13 (A-D) Cultural characteristics of isolates PRZ, PT1B, PT1J and PT2X on TZC agar after 24 hours of incubation at 30°C. (H-K) Magnification 100x

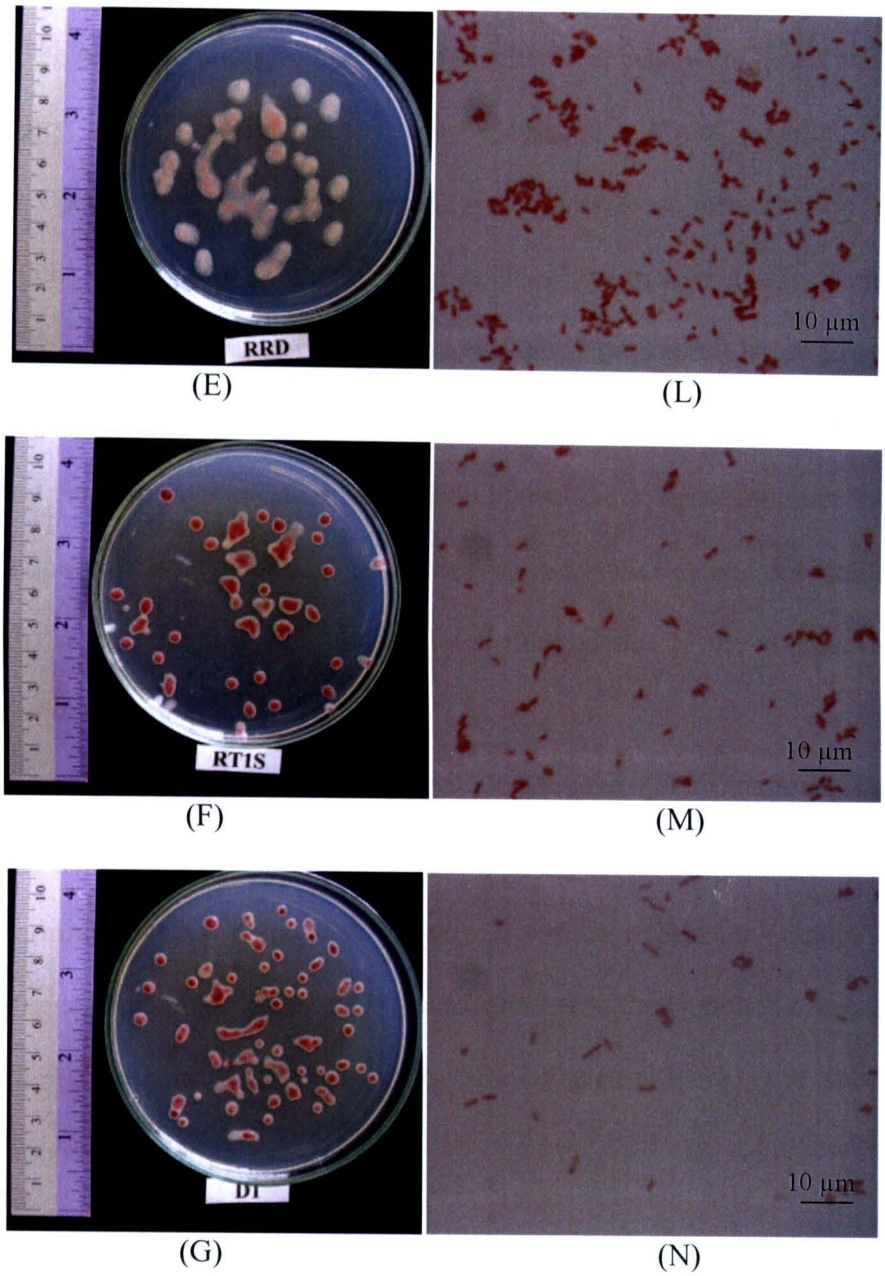

Figure 14 (E-G) Cultural characteristics of isolates RRD, RT1S and D1 on TZC agar after 24 hours of incubation at 30°C. (L-N) Magnification 100x

Ten bacterial isolates namely PRZ, PT1B, PT1J, PT2X, D1, RRD, RT1S, Rh1-1, Tu1-1 and Tu2-1, and 2 isolates of standard wilt causing bacteria namely R227 and R1512 provided from the Department of Agriculture, Ministry of

Agriculture and Cooperatives, were identified by both conventional and molecular methods. The results of morphological and biochemical tests were listed in Table 6, 7 and 8. Firstly, all wilt causing bacteria were classified as Gram negative, facultative anaerobes. They were then separated into 2 groups by lactose fermentation. Group 1, strains which were lactose fermentation positive were continually identified as *Enterobacter* and *Klebsiella*. Two genera could be distinguished by motility test that *Enterobacter* was motile whereas *Klebsiella* was non-motile. Group 2, strains which were lactose fermentation negative were then identified as Pseudomonas due to they could produce oxidase (Figure 15). However, All bacterial isolates could not be identified to species level by the conventional method due to the complexity of the genus *Enterbacter* and *Pseudomonas*.

The strains were comfirmed by 16S rRNA gene determination. The partial 16s RNA sequencing was used to identify the bacterial isolates to the species level and to determine whether there were clusters of similar organisms (Figure 16). The 16s rRNA gene sequences determined were deposited in the Genbank database. From database similarity and Neighbour-joining tree (Figure 17, 18), the species level identification was shown in Table 9.

Table 6 Biochemical characteristics of wilt causing bacteria : PRZ, PT1B, PT1J, PT2X, D1, RRD, RT1S, Tu1-1 and Rh1-1

Test	Strain													
	PRZ	PT1B	PT1J	PT2X	D1	RRD	RT1S	Tu1-1	Rh1-1	*Enterobacter aerogenes*	*Enterobacter asburiae*	*Enterobacter dissolvens*	*Enterobacter hormachei*	*Klebsiella varricola*
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase production	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	-	[+]
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-	-	-	+	-	d	+
Voges-Proskauer	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate (Simmons)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea hydrolysis	+	+	+	+	+	+	+	+	+	-	d	+	[+]	+
Phenylalanine deaminase (24h)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+	-	-	-	+
Motility	-	-	-	-	-	-	-	-	-	+	-	+	D	-
Gelatin hydrolysis, 22°C	+	+	+	+	+	+	+	+	+	-	-	-	-	+
D-Glucose, acid production	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 6 (continued)

Test	Strain													
	PRZ	PT1B	PT1J	PT2X	D1	RRD	RT1S	Tu1-1	Rh1-1	*Enterobacter aerogenes*	*Enterobacter asburiae*	*Enterobacter dissolvens*	*Enterobacter hormachei*	*Klebsiella varricola*
D-Glucose, gas production	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid production														
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	[+]	-
Glycerol	-	-	-	-	-	-	-	-	-	+	[-]	-	-	+
myo-Inositol	-	-	-	-	-	-	-	-	-	+	-	d	-	+
Lactose	+	+	+	+	+	+	+	+	+	+	[+]	d	-	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	-	+	+	d
Raffinose	+	+	+	+	+	+	+	+	+	+	d	+	-	+
L-Rhamnose	+	+	+	+	+	+	+	+	+	+	-	+	+	+
D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Symbols:
+ = 90% or more of strains are positive
- = 90% or more of strains are negative
d = 11-89% of strains are positive
D = Different reaction in different taxa (species of a genus or genera of a family)

Table 7 Biochemical characteristics of wilt causing bacteria : Tu2-1 and R1512

Test	Strain			
	Tu2-1	**R1512**	***Pseudomonas fluorescens***	***Pseudomonas putida***
Fluorescent, diffusible pigments	+	+	+	+
Growth at 41°C	-	-	-	-
Growth at 4°C	+	+	+	d
Oxidase reaction	+	+	+	+
Denitrification	-	-	d	-
Gelatin hydrolysis	-	+	+	-
Starch hydrolysis	-	-	-	-
Utilization of				
Glucose	+	+	+	+
Trehalose	-	+	+	-
β-Alanine	-	+	+	+
L-Arginine	+	+	+	+
Lactose fermentation	-	-	-	-
Nitrate used as a nitrate source	+	+	+	+

R1512 were standard wilt causing bacteria obtained from the Department of Agriculture, Ministry of Agriculture and Cooperatives

Symbols: + = 90% or more of strains are positive

- = 90% or more of strains are negative

d = 11-89% of strains are positive

D = Different reaction in different taxa (species of a genus or genera of a family)

Table 8 Biochemical characteristics of wilt causing bacteria: R227

Test	Strain	
	R227	***R. solanacearum***
Motility	-	-
Growth at 41°C	+	-
Colony pigmentation	-	-
Catalase	+	+
Oxidase	+	+
Citrate (Simmons)	+	+
Nitrate to gas	-	-
Nitrite test	+	+
Hydrolysis of		
Esculin	+	-
Gelatin	+	+
Starch	-	-
Lysine decarboxylase	+	-
Urease	-	+
Oxidative acid formed in		
OF base medium containing:		
Inositol	-	-
Mannitol	-	-
Sorbitol	+	-
L-Arabinose	-	-
Mannose	-	+
Dulcitol	-	-
Raffinose	-	-
Sucrose	+	+
Maltose	-	+
Cellobiose	-	-
Glucose	+	-
Galactose	-	-
Lactose	-	-
3% Ethanol	+	+
Glycerol	-	+
Fructose	+	+
Alkaline reaction in OF base medium	+	+
Assimilation of		
Glucose	+	+
D-Mannitol	+	-
Maltose	+	-
L-Arabinose	-	-
D-Mannose	+	-

R227 were standard wilt causing bacteria obtained from Department of Agriculture, Ministry of Agriculture and Cooperatives

Symbols: + = 90% or more of strains are positive

\- = 90% or more of strains are negative

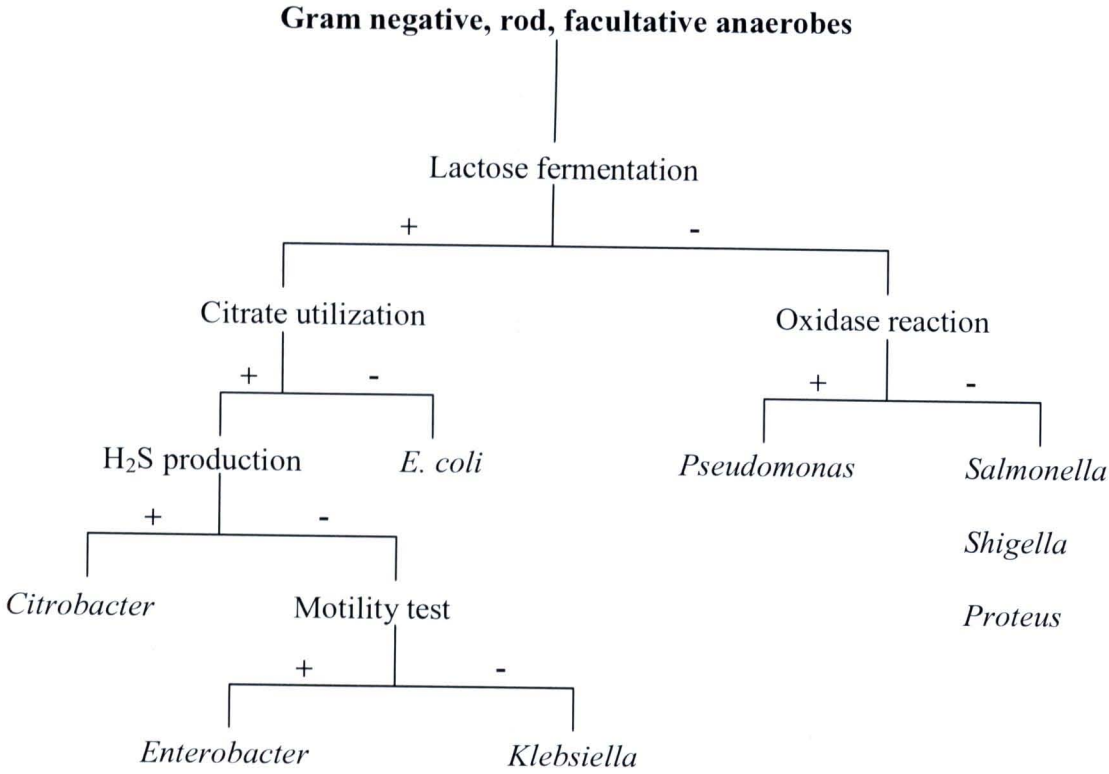

Figure 15 Scheme for differentiation of *Enterobacter*, *Klebsiella* and *Pseudomonas* from other Gram negative, rods

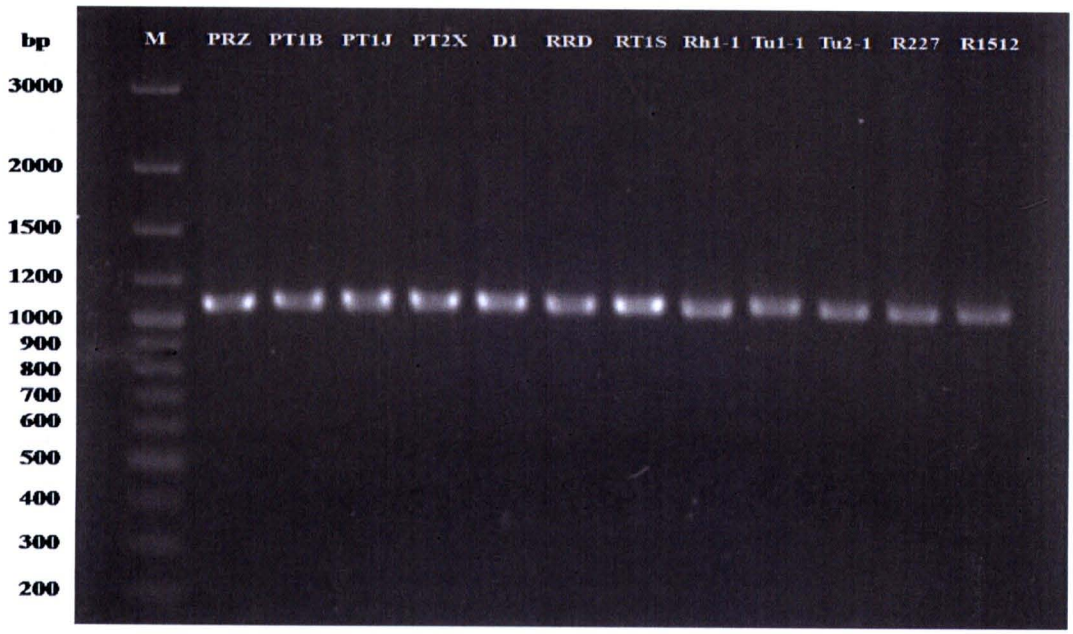

Figure 16 PCR products of wilt causing bacteria based on 16s rRNA gene; M = 100 bp plus

Table 9 Species identification of wilt causing bacteria based on biochemical and 16s rRNA gene sequence analysis

Isolate	Source	Species	Max identity (%)
PRZ	Pathumma Chiang Mai Pink	*Enterobacter hormachei*	99
PT1B	Pathumma Chiang Mai Pink	*Enterobacter dissolvens*	97
PT1J	Pathumma Chiang Mai Pink	*Enterobacter asburiae*	99
PT2X	Pathumma Chiang Mai Pink	*Enterobacter asburiae*	97
D1	Pathumma Chiang Mai Pink	*Enterobacter dissolvens*	95
RRD	Pathumma Chiang Mai Red	*Enterobacter asburiae*	99
RT1S	Pathumma Chiang Mai Red	*Enterobacter asburiae*	98
Rh1-1	Pathumma Chiang Mai Pink	*Enterobacter hormachei*	98
Tu1-1	Pathumma Chiang Mai Pink	*Klebsiella varricola*	99
Tu2-1	Pathumma Chiang Mai Pink	*Pseudomonas putida*	99
R227	Potato	*Ralstonia solanacearum*	98
R1512	Pathumma	*Pseudomonas fluorescens*	99

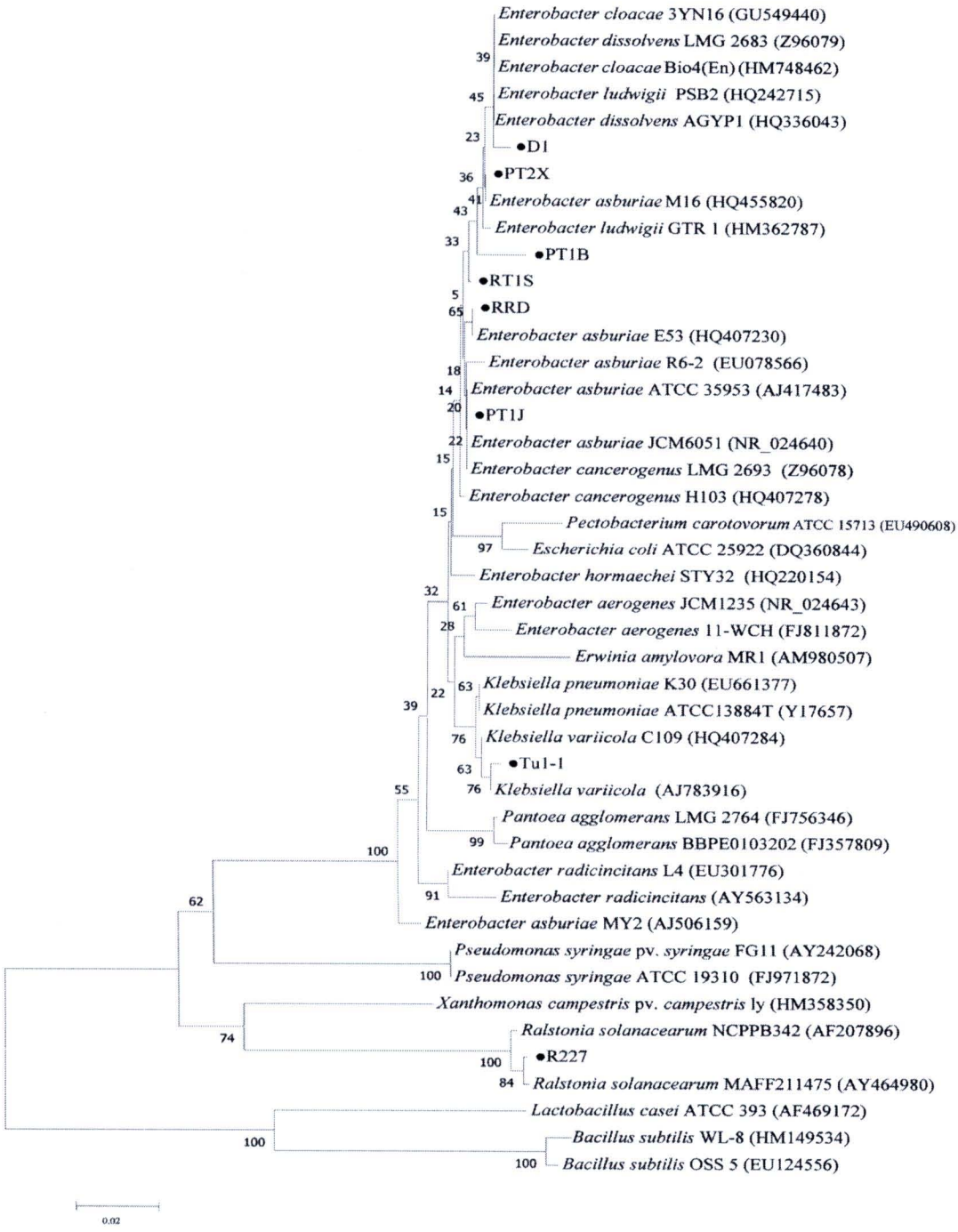

Figure 17 Neighbour-joining tree based on 16S rRNA gene sequences showing the position of the wilt bacterial isolates; PT11B, PT1J, PT2X, RRD, RT1S, D1, Tu1-1 and R227. The sequence of Bacillus subtilis WL-8 and OSS 5 were used as an outgroup. Bootstrap values were calculated from 1,000 re-samplings and the bar represents 0.02 showed substitution per nucleotide position. The GenBank accession numbers were in parentheses

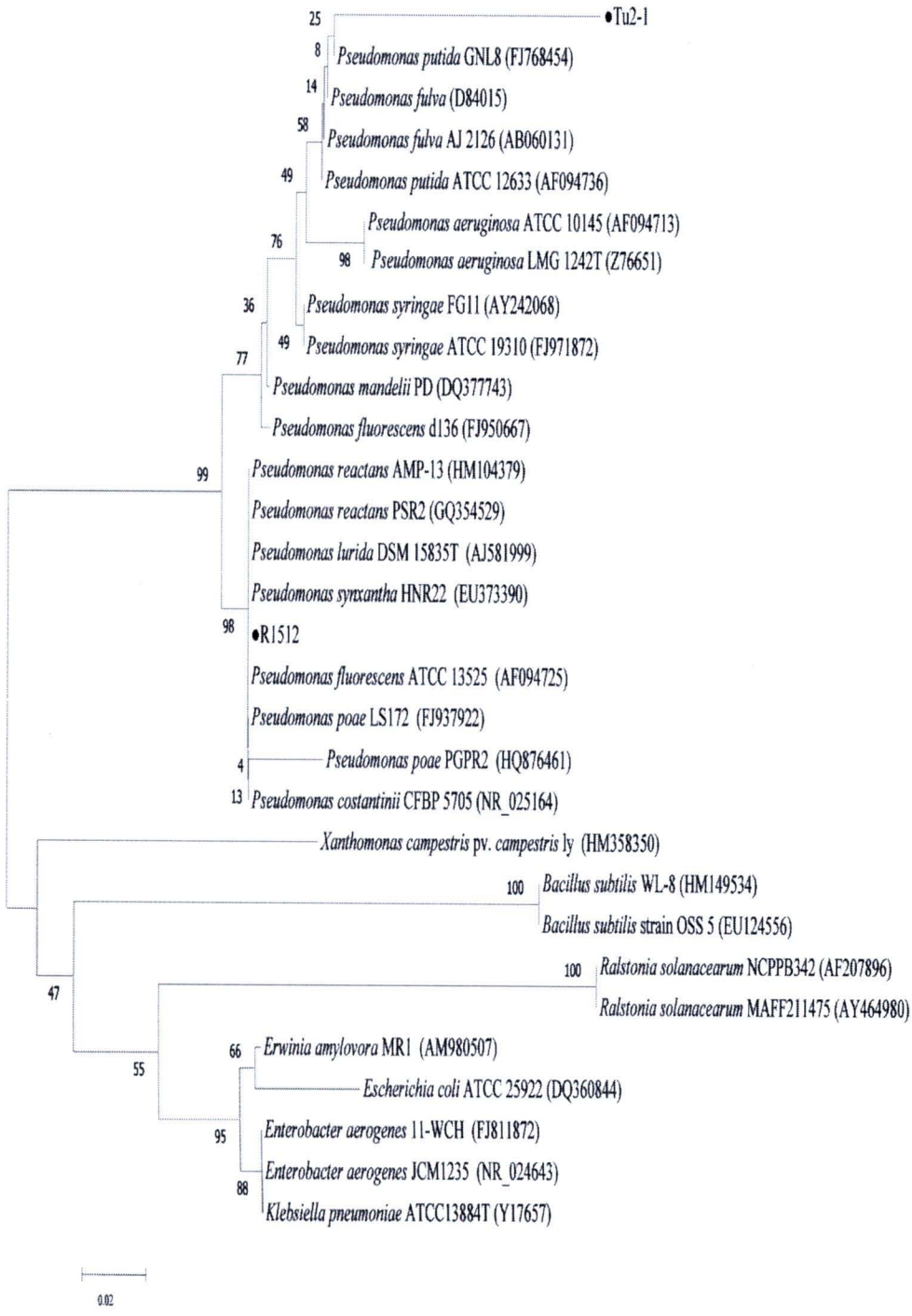

Figure 18 Neighbour-joining tree based on 16S rRNA gene sequences showing the position of the wilt bacterial isolates; Tu2-1 and R1512. The sequence of Bacillus subtilis Wl-8 and OSS 5 were used as an outgroup. Bootstrap values were calculated from 1,000 re-samplings and the bar represents 0.02 showed substitution per nucleotide position. The GenBank accession numbers were in parentheses

Formerly, we presumed that these bacterial strains isolated from infected pathumma rhizomes collected from Pathumma field were *R. solanacearum* but this research demonstrated that *Enterobacter* could be a causative agent of wilt disease in Pathumma. Four isolates of wilt causing bacteria were *Enterobacter asburiae*. On the basis of biochemical characterization, the results were quite different from type strain ATCC 35953 displayed in the Bergey's manual of determinative bacteriology (Holt *et al.*, 1994). The normal characters of the species were non-motile and Voges-Proskauer negative. Hoffmann *et al.* (2005) proposed that the valid description of *E. asburiae* needed to be emened that the Voges-Proskauer test was positive. Moreover, the key reactions used for differentiating *E. asburiae* from other species of the same genus consisted of a positive esculin test and lack of growth on dulcitol and putrescine.

The scientific documentation of *Enterobacter* infection was limited, however, some species were reported to cause the major diseases in crops. Onions were infected by *E. cloacae* that cause discolored and flaccid in bulbs. Similar to this research, Nishijima *et al.* (2004) reported that *E. cloacae* could cause rot disease in ginger rhizome. They attempted to isolate and collect *R. solanacearum* from infected plants in Hawaii but *E. cloacae* were repeatedly isolated along with the targeted bacteria. In Southeast Asia, Masyahit *et al.* (2009) also discovered that *E. cloacae* could cause soft rot disease on Dragon fruit (*Hylocereus* spp.) in Malaysia. The symptoms of the infected stem and fruits were yellowish to brownish soft. Moreover, *E. asburiae* and *Enterobacter* spp. could cause mulberry (*Morus alba*) wilt disease in China (Wang *et al.*, 2010). The causative agents were formerly presumed to be *R. solanacearum* but this study was able to distinguish between mulberry wilt caused by

Enterobacter and by *R. solanacearum*. The latter was characterized by flaccid wilted leaves without discoloration and defoliation. Consequently, the proper characterization of pathogenic bacteria was required for disease identification. The procedure in molecular identification was proved to be more accurate than the traditional methods.

2. Pathogenicity test of wilt causing bacteria

2.1 Infectivity of wilt causing bacteria in Pathumma plants

2.1.1 First experiment in 2007 during growing season

After inoculation of wilt causing bacteria in healthy Pathumma rhizomes, the wilt diseases showed leave discoloration and defoliation characteristics. In some cases, the rhizomes were not grown and showed wilt diseases (Figure 19). The disease incidences (DI) of direct and cross infection were 40-60% and 20-60%, respectively. The population numbers of almost wilt-bacterial isolates of direct and cross infection were increased by 11-34% and 2-35%, respectively (Table 10; Figure 20, 21).

Meanwhile, the inoculation of wilt causing bacteria in Pathumma pseudostems after 2 month of cultivation showed slightly wilt disease. The disease incidence was ranging from 20-50% which was slightly less than the inoculated rhizomes. The populations of bacterial cells were declined by 11-46% both in direct and cross infection (Table 11; Figure 22, 23). Accordingly, it might be concluded that the pathogenicity of wilt bacterial strains by rhizome infection was higher than pseudostem infection.

Most of all infected plants were not shown the apparent wilt disease and the disease incidence was low. The infected plants were smaller and grew slower than the control plant. The defect of this experiment was that the experimental area, the 7th floor building deck, had not a well enough water shield to protect experimental plants from rains. Therefore, pathogenic bacteria inoculated might be washed out from the planting pots.

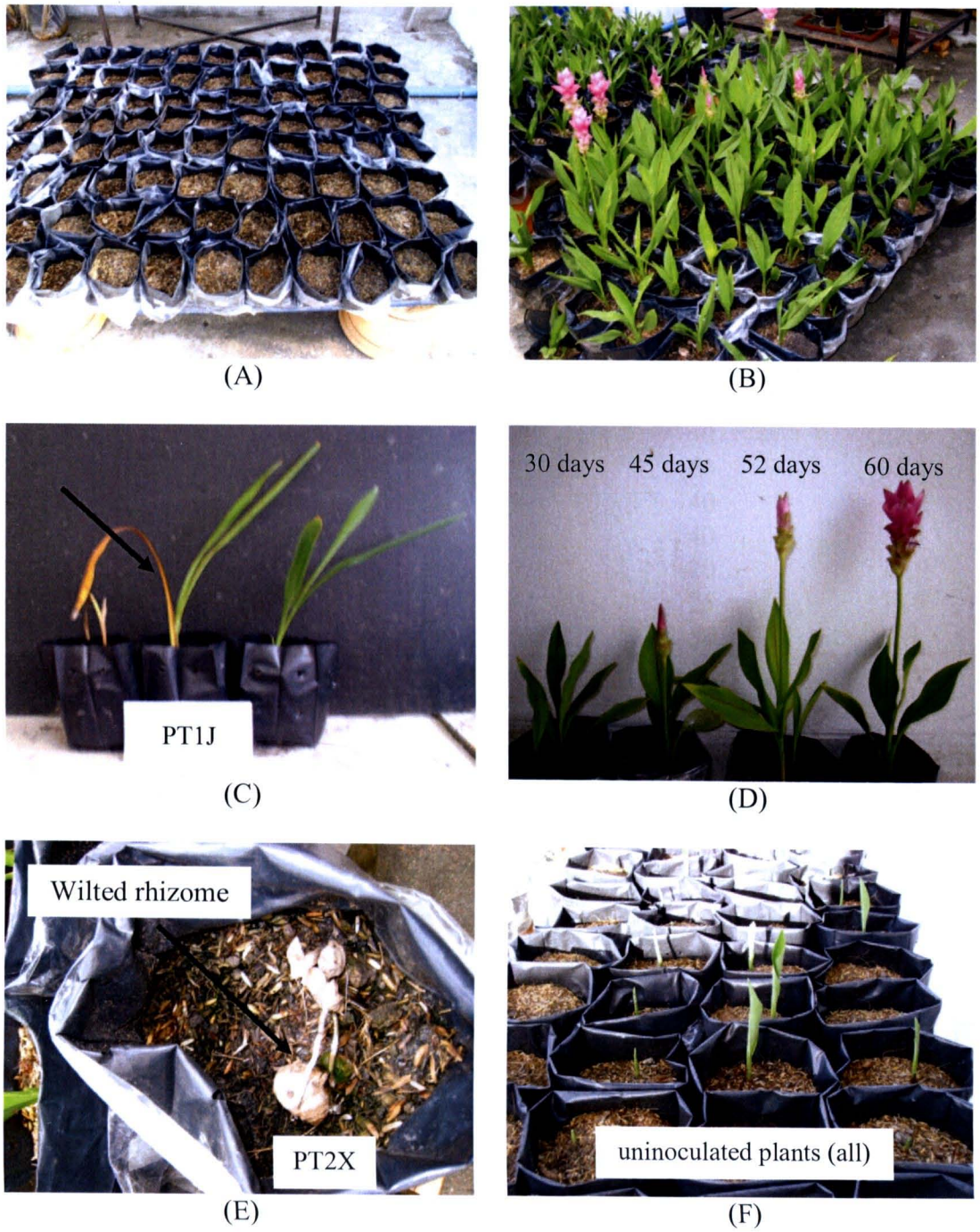

Figure 19 (A, B) The plantation of Pathumma. (C) Wilt disease appearances showing leave yellowing, defoliation (arrow) and incomplete growth. (D) Growth of uninoculated Pathumma plants different time during 2 months of cultivation. (E) The infected rhizome showing withered tuberous roots and undeveloped growth compared with (F) uninoculated plants

Table 10 Disease incidence (DI) and change of bacterial population in Pathumma cultivation when inoculated with wilt bacterial isolates on rhizomes. The experiments were conducted in 2007

Isolate	Direct infection		Cross infection	
	DI (%)	Bacterial population change (%)	DI (%)	Bacterial population change (%
PRZ	50	19.5	20	26.6
PT1B	50	15.0	25	21.0
PT1J	60	33.7	20	14.4
PT2X	40	12.6	40	22.6
RRD	50	11.2	60	35.1
RT1K	40	13.2	40	30.2
RT1S	50	17.9	40	26.1
RT2R	40	16.7	20	13.9
C4	50	34.6	25	-3.9
D1	50	18.9	50	2.1

- value displayed the decrease of cell number

Table 11 Disease incidence (DI) and change of bacterial population in Pathumma cultivation when inoculated with wilt bacterial isolates on pseudostems. The experiments were conducted in 2007

Isolate	Direct infection		Cross infection	
	DI (%)	Bacterial population change (%)	DI (%)	Bacterial population change (%)
PRZ	20	-30.6	40	-40.9
PT1B	20	-33.3	40	-30.3
PT1J	50	-16.2	25	-42.8
PT2X	20	-31.9	25	-30.0
RRD	20	-16.5	40	-44.1
RT1K	20	-11.3	20	-34.1
RT1S	20	-34.9	20	-34.0
RT2R	20	-35.7	20	-31.5
C4	25	-34.6	25	-46.3
D1	25	-11.1	40	-42.7

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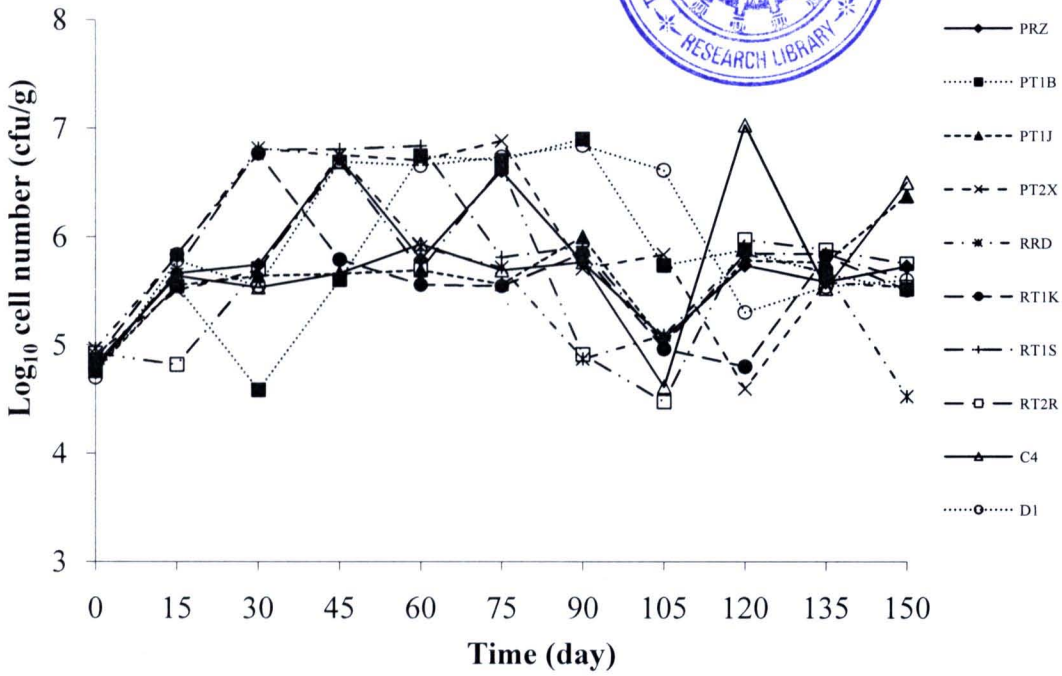


Figure 20 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma rhizomes at initial day of cultivation in direct infection

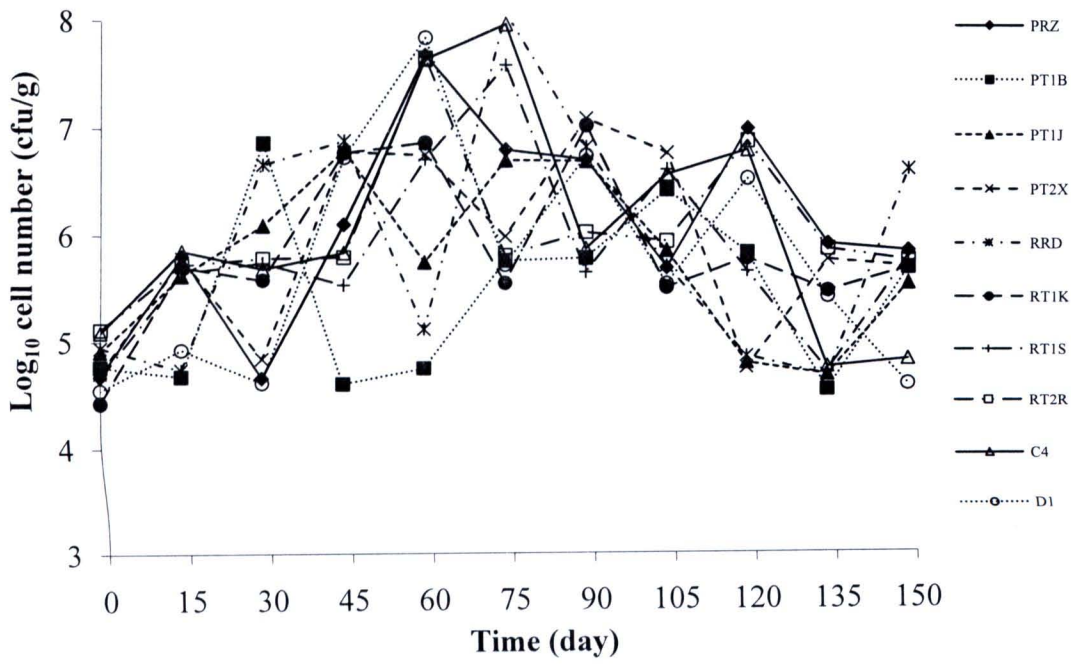


Figure 21 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma rhizomes at initial day of cultivation in cross infection

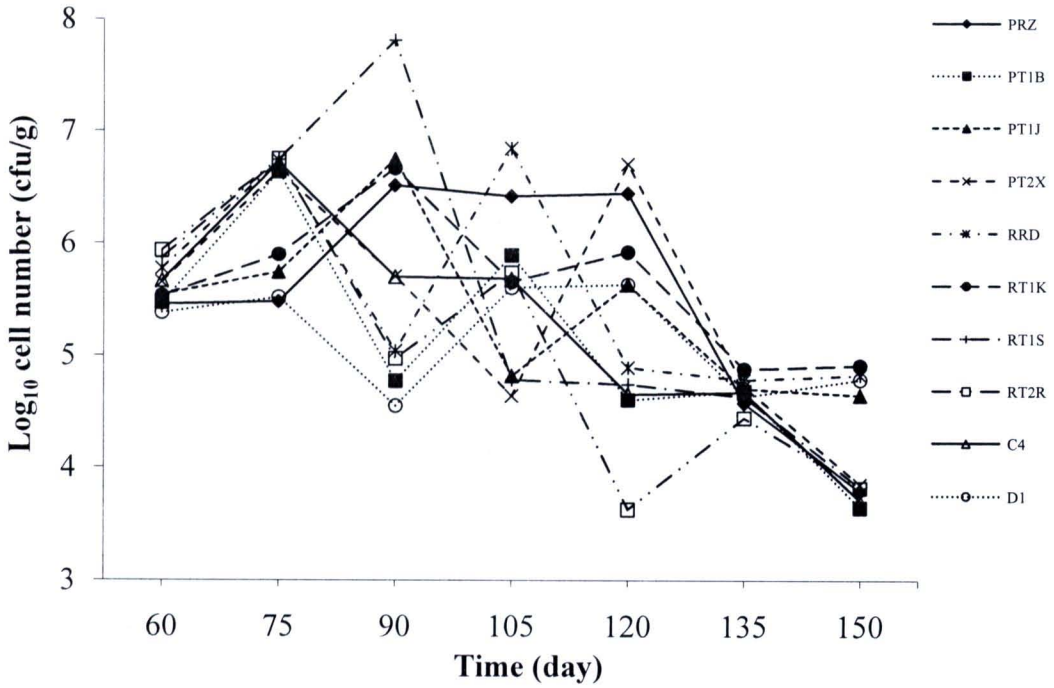


Figure 22 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma pseudostems at 30 day of cultivation in direct infection

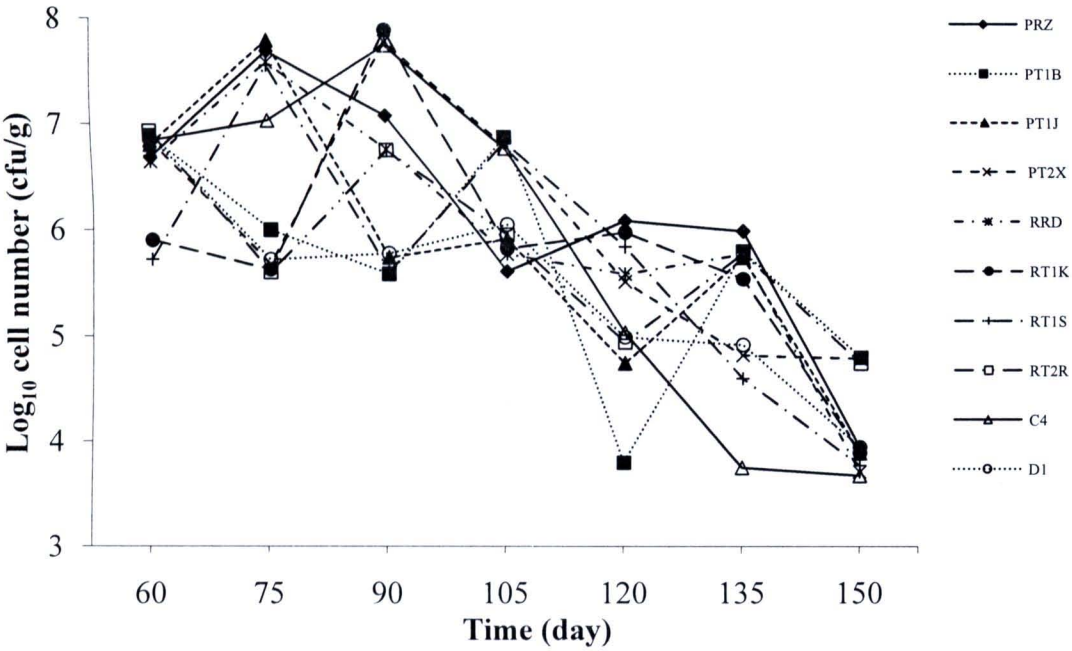


Figure 23 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma pseudostems at 30 day of cultivation in cross infection

2.1.2 Second experiment in 2008 during growing season

At the first experiment in 2007 was not be able to control cell wash out, the experiment was repeatedly performed in greenhouse where rain water was no more problem. The disease occurrence was also showed leave discoloration and defoliation (Figure 24). Some severely infected plants were collapsed and showed necrosis of pseudostems. From the cross section, the stem tissues were dark brown. Rhizomes were withered and decayed. The disease incidences (DI) and populations of wilt-bacterial isolates of direct and cross infection were different in individual strains (Table 12; Figure 25, 26).

At the same time, the inoculation of wilt causing bacteria in pseudostem showed slight wilt disease. The disease incidences were higher than rhizome inoculation. The pathogenic cell number in both direct and cross infection were mostly declined by 4-30% and 3-25%, respectively (Table 13; Figure 27, 28).

The appearance of wilt diseases was showed obviously in this experiment. Since the plantation was conducted in the greenhouse, the control of physical factor was easier. In addition, the feasible time of inoculation was importantly affected disease development. In this study, it was found that the inoculation in Pathumma rhizomes or at the initial day of incubation could encourage the disease development.

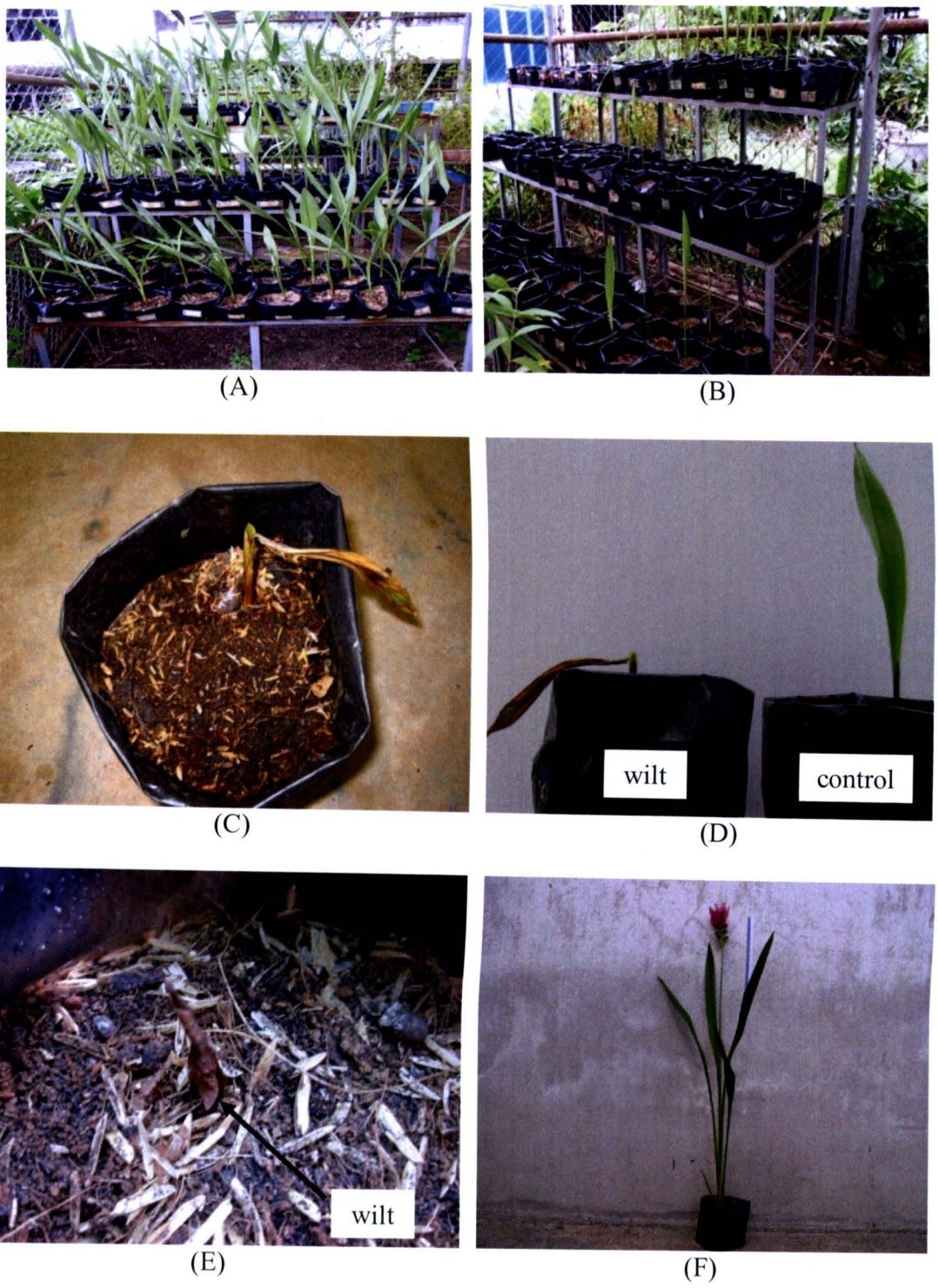


Figure 24 (A,B) The plantation of Pathumma in a greenhouse at the Department of Biology, Faculty of Science, Chiang Mai University. (C) Wilt disease of Pathumma appeared leave discoloration, defoliation and collapse compared with control (D), 1 month of cultivation. (E, arrow) Severe wilt symptom showed dried stems after inoculation for 3 days. (F) Control plant, 2 month of cultivation

Table 12 Disease incidence (DI) and change of bacterial population in Pathumma cultivation when inoculated with wilt bacterial isolates on rhizomes. The experiments were conducted in 2008

Isolate	Direct infection		Cross infection	
	DI (%)	Bacterial population change (%)	DI (%)	Bacterial population change (%)
PRZ	67	-13.6	67	-14.0
PT1B	67	2.9	33	-13.2
PT1J	33	2.8	0	0.5
PT2X	67	-1.3	0	-1.3
RRD	33	-22.2	33	-26.6
RT1K	0	-23.5	67	-16.4
RT1S	33	-2.6	33	-13.6
RT2R	0	-0.4	0	-14.2
C4	0	18.4	33	-14.2
D1	0	18.2	33	-14.3

- value displayed the decrease of cell number

Table 13 Disease incidence (DI) and change of bacterial population in Pathumma cultivation when inoculated with wilt bacterial isolates on pseudostems. The experiments were conducted in 2008

Isolate	Direct infection		Cross infection	
	DI (%)	Bacterial population change (%)	DI (%)	Bacterial population change (%)
PRZ	67	-13.0	0	-15.4
PT1B	33	-15.9	0	-19.2
PT1J	67	-25.6	0	13.0
PT2X	67	0.9	33	-13.3
RRD	33	-16.2	33	0
RT1K	33	-14.4	67	-14.5
RT1S	33	-31.8	0	-25.6
RT2R	0	-11.8	0	-3.7
C4	33	-14.4	33	-25.0
D1	67	-4.1	33	0.6

- value displayed the decrease of cell number

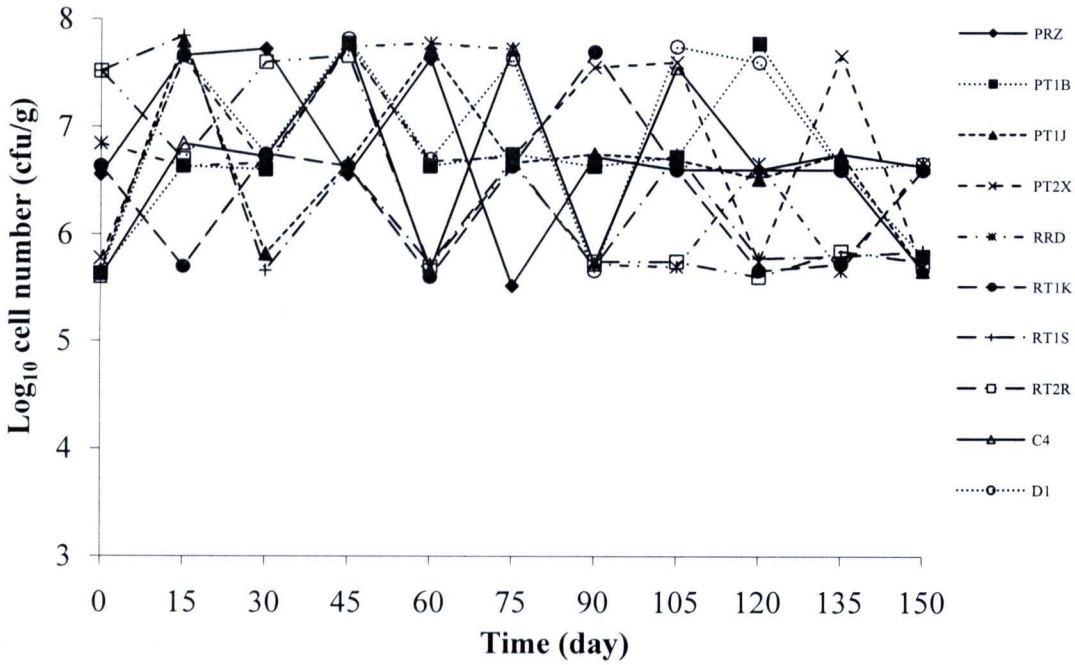


Figure 25 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma rhizomes at initial day of cultivation in direct infection

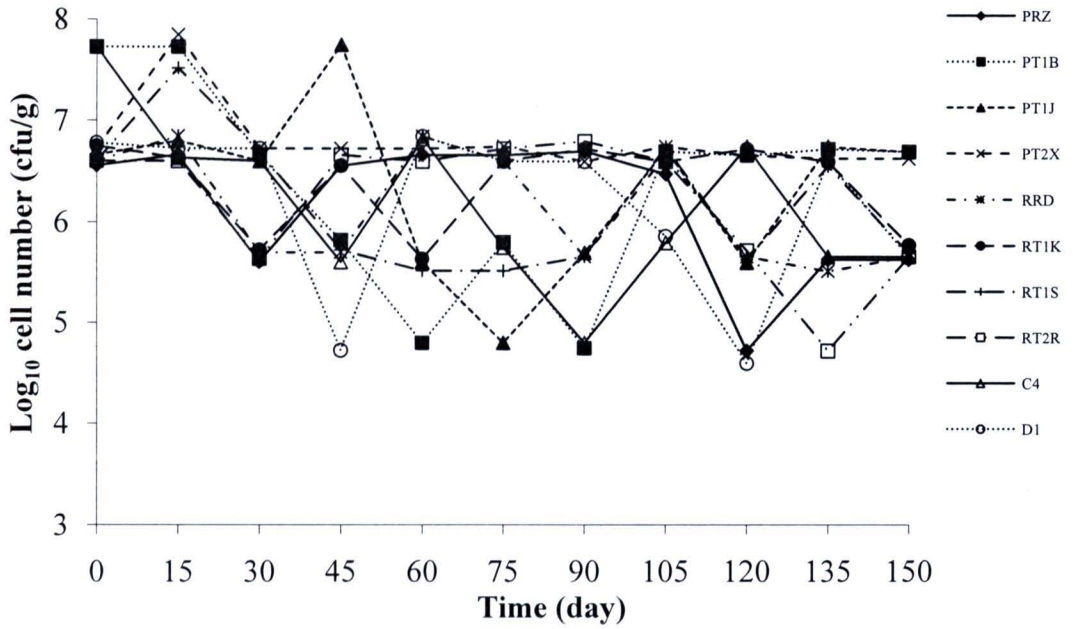


Figure 26 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma rhizomes at initial day of cultivation in cross infection

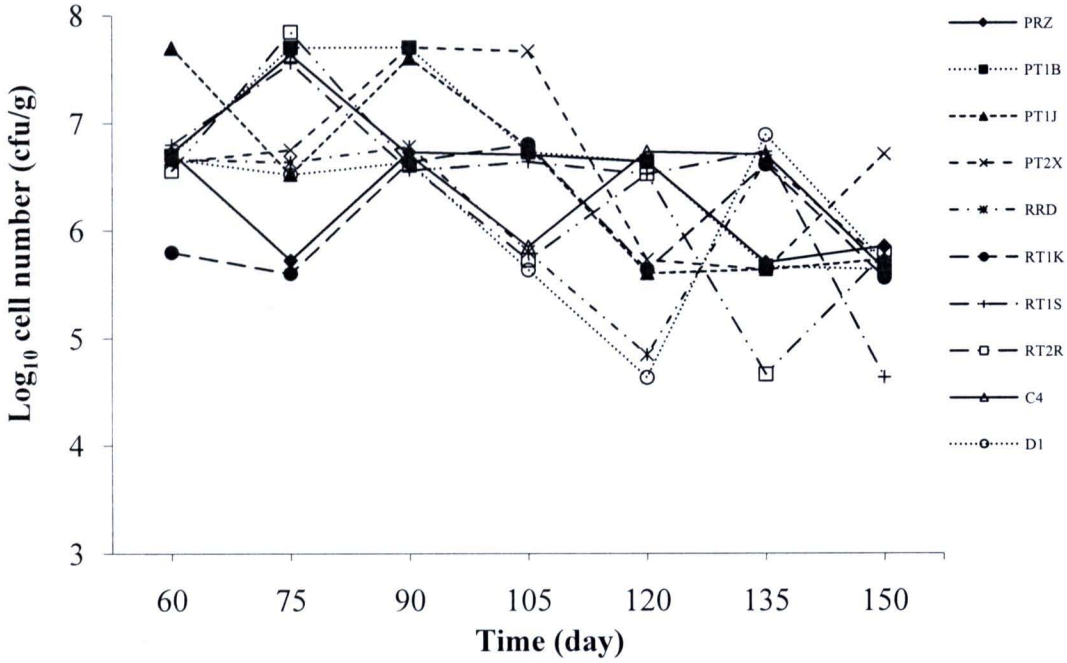


Figure 27 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma pseudostems at 30 days of cultivation in direct infection

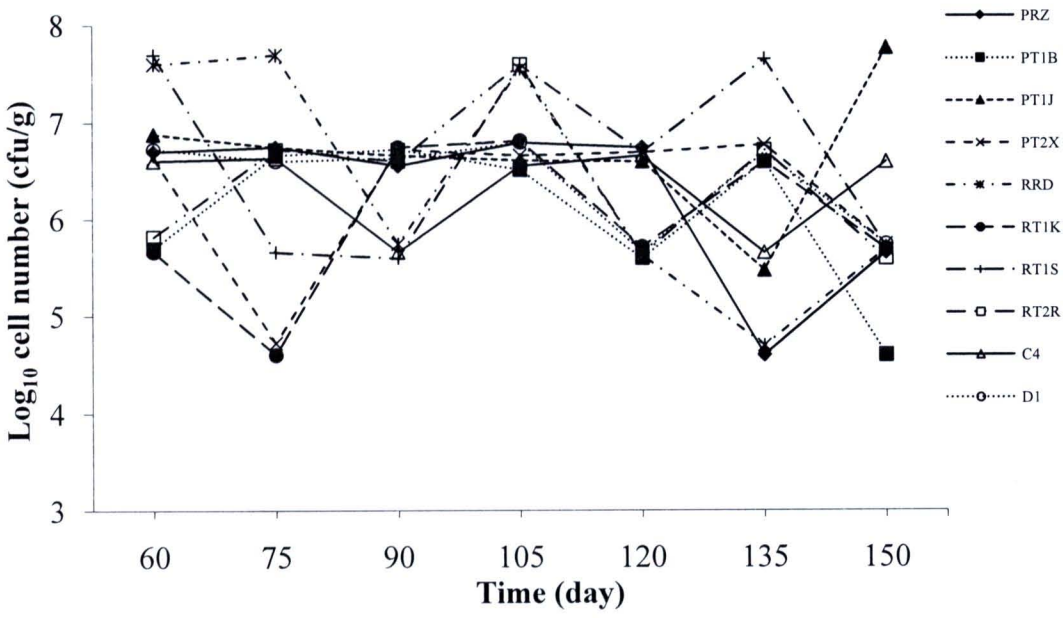


Figure 28 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma pseudostems at 30 days of cultivation in cross infection

2.1.3 Third experiment in 2009 during out of growing season

The experiment was established at the shelf providing 12hour-photoperiod fluorescent light (Figure 29) to evaluate the effects of wilt causing bacteria during out of growing season. All treatments were repeatedly carried out in both direct and cross infection. It was found that the wilt disease was not shown clearly compared with control. During the out of growing season, the plants were not normally grown. The seedlings were initially developed starting from April 2009 or growing season. Because the plants were not grown during the treatment period, thus, the wilt bacteria did not affect the plant. Coincidently, the dynamic of bacterial cells number was declined by 47% after 5 months of observation (Figure 30, 31).

Pathumma naturally blossom in rainy season that is not high season for travelling and the production quantity is not high enough for international markets. Thus, the farmers increase yields by growing pathumma in out of growing season. This study was focused on the effects of wilt bacteria on the growth of Pathumma plant and investigated the dynamic of bacterial population in this period. Consequently, the plantation should be improved that rhizomes should be properly incubated prior to culture in order to adapt with changed in physical factors and to grow completely.

Figure 29 The experimental pots were placed in the shelf with light provided

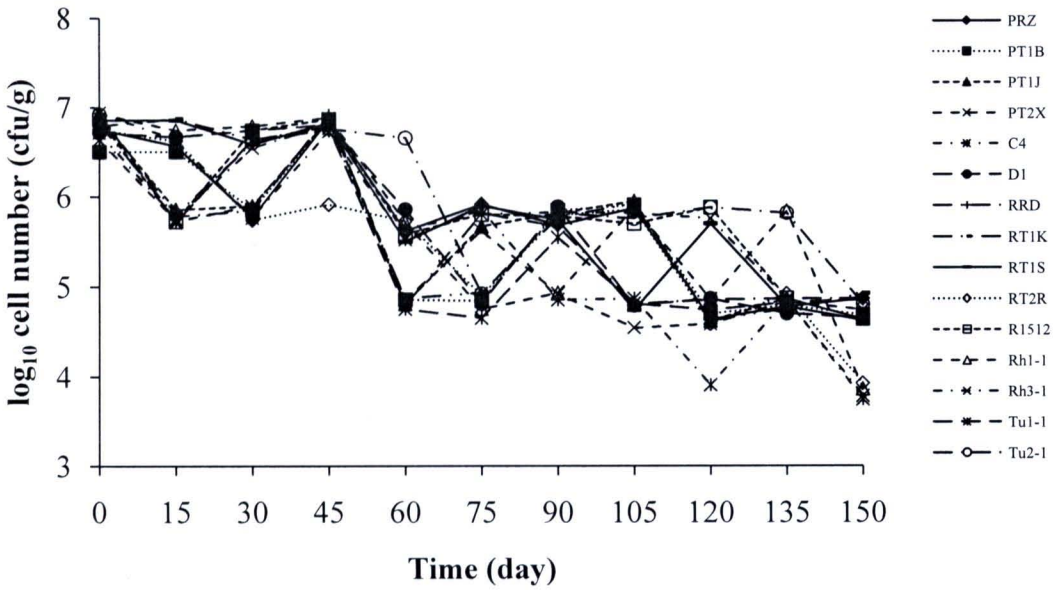


Figure 30 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma rhizomes at initial day of cultivation in direct infection

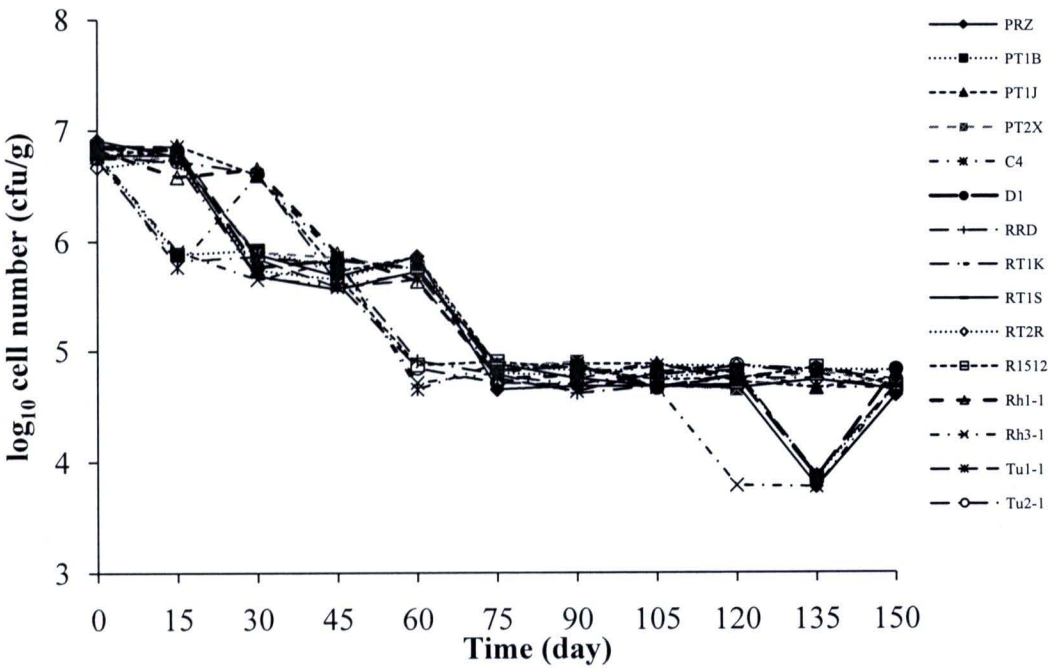


Figure 31 Viable cell count of wilt causing bacteria in the soil after inoculation on Pathumma rhizomes at initial day of cultivation in cross infection

2.1.4 Fourth experiment in 2009 during growing season

Twelve isolates of wilt causing bacteria were repeatedly examined in direct and cross infection using Pathumma Chiang Mai Snow white for cross infection. Wilt-bacterial isolates were cultured in 2 different medium including half composition of TZC medium and blended rhizome medium prior to test their pathogenicity in Pathumma rhizomes. It could be concluded from table 14 that both media were not influenced the severity of the disease. The disease incidences of PT1J and RRD were 33% whereas the other pathogenic bacteria were 0%. The populations of wilt-bacterial strains were declined by 1-27% except RRD. It might be that the isolate RRD was high disease severity, so the cell numbers of RRD was slight increased.

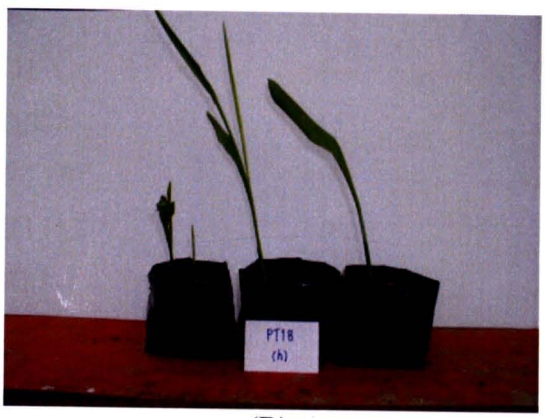
For pseudostem infection, all treatments had milder symptoms than the previous experiments. The disease appearances were slightly chlorosis and collapsed (Figure 32, 33). The disease incidences of the majority of wilt causing bacteria were 33%. The cell amounts of all strains were reduced by 2-28% (Table 15).

(A)

(B)

Figure 32 (A) The plantation of Pathumma in the greenhouse. (B) control plant showing healthy pseudostem and flower after 2 months of cultivation

(A)



(B)

(C)

(D)

(E)

Figure 33 Examples of Pathumma plants showing wilt symptoms by wilt-bacterial strains; (A) RT1S, (B) PT1B, (C) PT1J, (D) R1512 and (E) RRD after inoculation on 1 month-old pseudostems

Table 14 Disease incidence (DI) and change of bacterial population in Pathumma cultivation when inoculated with wilt bacterial isolates on rhizomes. The experiments were conducted in 2009

Isolate	Half TZC medium		Blended rhizome medium	
	DI (%)	Bacterial population change (%)	DI (%)	bacterial population change (%)
PRZ	0	-27.1	0	-14.6
PT1B	0	-14.4	0	-14.1
PT1J	0	-13.1	33	-27.2
PT2X	0	-28.8	0	-12.0
C4	0	-26.4	0	-16.2
D1	0	-16.2	0	-14.7
RRD	33	1.1	33	-25.8
RT1S	0	-24.7	0	-2.4
R1512	0	-17.0	0	-27.9
Rh1-1	0	-26.6	0	-26.1
Rh3-1	0	-26.8	0	-3.1
Tu1-1	0	-16.5	0	-0.8

- value displayed the decrease of cell number

Table 15 Disease incidence (DI) and change of bacterial population in Pathumma cultivation when inoculated with wilt bacterial isolates on pseudostems. The experiments were conducted in 2009

Isolate	Direct infection		Cross infection	
	% DI	%bacterial population change	% DI	%bacterial population change
PRZ	0	-27.6	0	-26.4
PT1B	0	-17.3	33.3	-27.7
PT1J	33	-3.7	33.3	-14.8
PT2X	33	-27.1	0	-28.8
C4	0	-13.0	0	-18.2
D1	0	-28.8	0	-15.5
RRD	33	-14.3	33.3	-16.9
RT1S	33	-28.8	33.3	-13.9
R1512	33	-18.6	33.3	-26.4
Rh1-1	33	-26.9	0	-1.9
Rh3-1	0	-28.8	0	-26.8
Tu1-1	0	-28.5	0	-15.2

- value displayed the decrease of cell number

2.1.5 Fifth experiment in 2010 during out of growing season

The experiment was repeatedly conducted during out of growing season. The rhizomes were incubated at 30°C for 15 days prior to cultivate. The seedlings were initially developed in February. The symptoms of wilt disease were slightly leave discoloration and dark brown stems (Figure 34). The disease incidence was increased whereas the populations of pathogenic bacteria were declined by 27-30% (Table 16). This experiment revealed that the incubation of rhizomes before cultivation could encourage the growth of plants during out of season. The results of bacterial cell

numbers indicated that the survival rate of pathogenic bacteria in this experiment was higher that the previous study in 2009 during out of season.

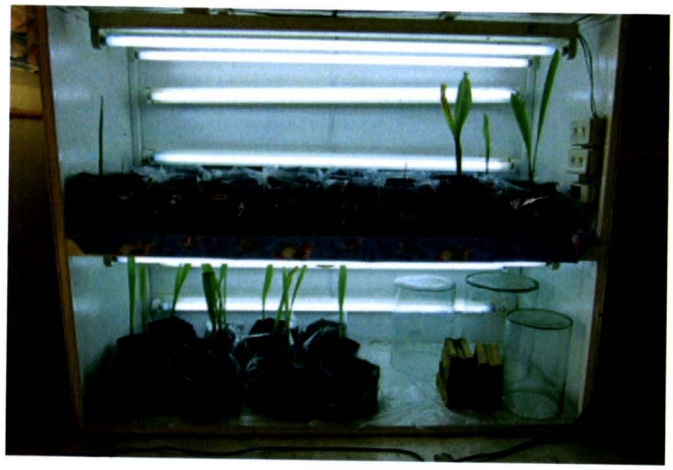


Figure 34 The experimental pots were placed in the shelf with light provided

Table 16 Disease incidence (DI) and change of bacterial population in Pathumma cultivation when inoculated with wilt bacterial isolates on rhizomes. The experiments were conducted in 2010 during out of season

Isolate	**DI (%)**	**Bacterial population change (%)**
PT1B	33.3	-30.3
PT1J	66.6	-29.1
PT2X	0	-31.3
RRD	66.6	-30.5
RT1S	0	-30.4
R227	0	-27.3

- value displayed the decrease of cell number

2.1.6 Sixth experiment in 2010 during growing season

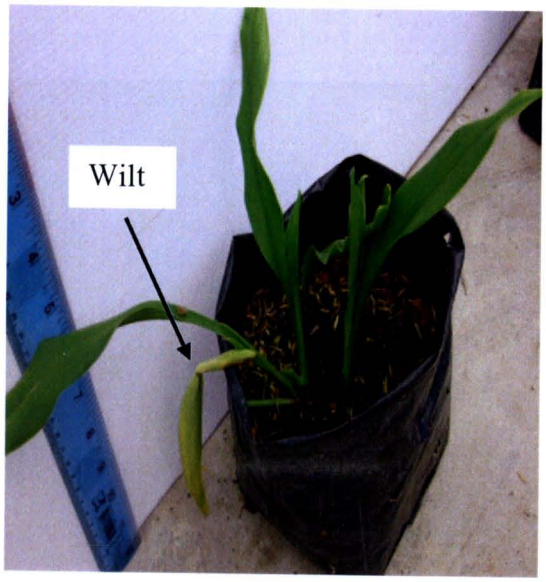
The pathogenicity test was investigated by using packed cell of wilt-bacterial strains. The disease appearances showed chlorosis and necrosis of pseudostems

(Figure 35, 36). Some planting pots were not found seedlings or growth development. The disease incidences of individual isolates were about 33%. The cell numbers of wilt causing bacteria were declined by 26-28% (Table 17). This trial had focused on the proper type and quantity of inoculums for pathogenicity test. The results indicated that the packed cell was not suitable for inoculation. From the results of infectivity of 1 month-old pseudostems, the disease incidences of all wilt-bacterial strains were 33% (Table 18; Figure 37). It was interesting that the disease incidences were decreased compared with the previous study in 2008 during growing season that the disease incidences were 33-67%. It could be concluded that the virulence of pathogenic strain was loss after subculture.

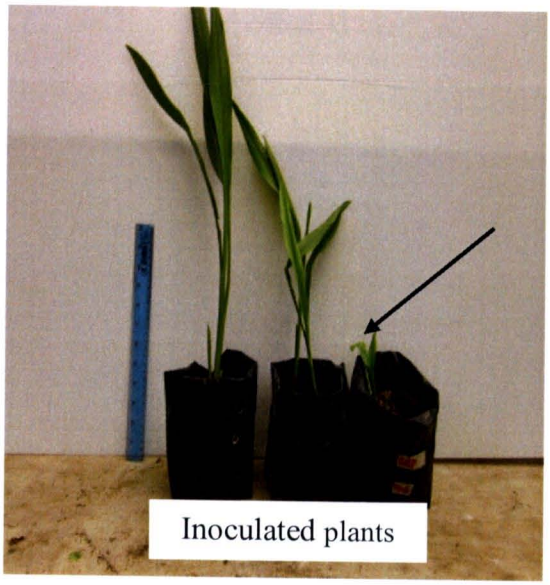
When the wilt-bacterial mixtures were inoculated into Pathumma rhizomes and pseudostems. The disease occurrences displayed severely symptoms that the seedlings were not occurred and pseudostems were evidently wilted, discolored and defoliated. This study proposed that the mixture of wilt-bacterial strains could enhance the ability to attack host plants.

Figure 35 Uninoculated plant after 2 months of cultivation

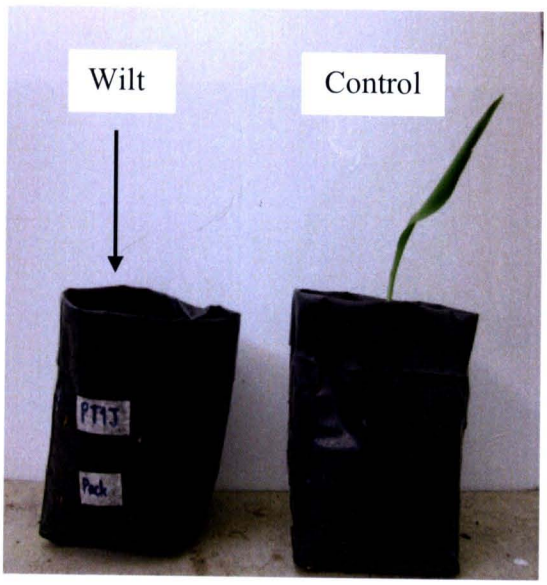
(A)



(B)



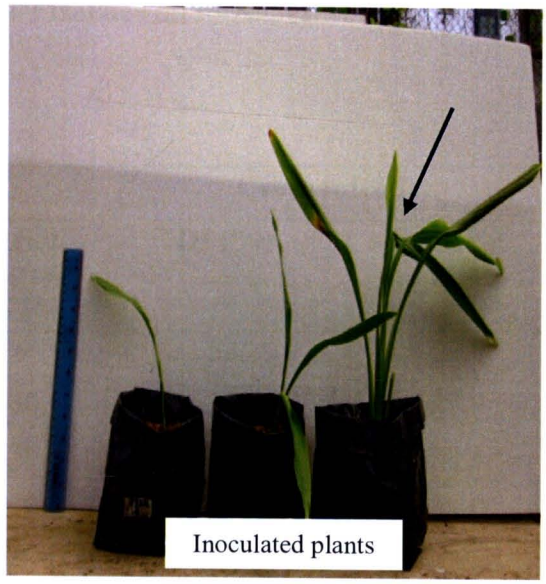
(C)



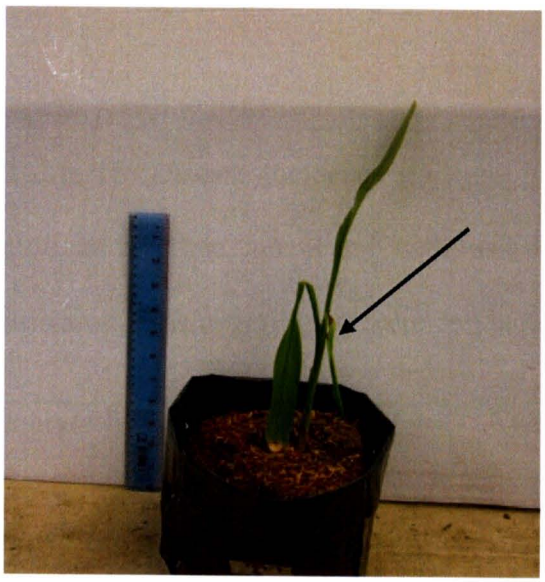
(D)

Figure 36 (A, B, C) The wilt symptoms showing leave folding and chlorosis after inoculation of wilt causing bacteria on rhizomes at initial day of cultivation. (D) Seedling was not occurred compared with control pot

(A)



(B)



(C)

(D)

Figure 37 (A-D) The wilt symptoms showing leave discoloration and defoliation after inoculation of various wilt-bacterial strains in 1 month-old pseudostems

Table 17 Disease incidence (DI) and change of bacterial population in Pathumma cultivation when inoculated with wilt bacterial isolates on rhizomes. The experiments were conducted in 2010

Isolate	Direct infection		Cross infection	
	DI (%)	Bacterial population change (%)	DI (%)	Bacterial population change (%)
PT1J	33	-27.4	33	-27.5
PT2X	33	-26.9	33	-27.9
RRD	33	-26.9	66	-28.5
RT1S	33	-27.9	33	-28.0
R227	33	-27.9	0	-27.2
Rh1-1	0	-28.2	0	-25.8
Tu1-1	0	-28.9	0	-28.5

- value displayed the decrease of cell number

Table 18 Disease incidence (DI) and change of bacterial population in Pathumma cultivation when inoculated with wilt bacterial isolates on pseudostems in direct infection. The experiments were conducted in 2010

Isolate	DI (%)	Bacterial population change (%)
PT1J	33	-26.2
PT2X	33	-26.9
RRD	33	-27.2
RT1S	33	-27.8
R227	33	-27.0
Rh1-1	33	-25.6
Tu1-1	33	-26.5

- value displayed the decrease of cell number

Table 19 Summary of the disease incidence (DI) and change of bacterial population in all experiments

year	Rhizome inoculation				Pseudostem inoculation			
	Direct infection		Cross infection		Direct infection		Cross infection	
	DI (%)	Bacterial population change (%)	DI (%)	Bacterial population change (%)	DI (%)	Bacterial population change (%)	DI (%)	Bacterial population change (%)
Growing season								
2007	50-60	12-34	20-60	2-35	20-50	(-) 11-35	20-40	(-) 30-46
2008	33-67	(-) 2-23	33-67	(-) 1-23	33-67	(-) 4-31	33-67	(-) 13-25
2009	33	(-) 1-28	-	-	33	(-) 3-28	33	(-) 2-28
2010	33	(-)28-28	33-66	(-)25-27	33	(-)25-27	-	-
Out of growing season								
2009	0	27-47	0	27-47	-	-	-	-
2010	-	-	-	-	33-67	(-)27-30	-	-

Symbols; - = treatment was not conducted

(-) = display the decrease of cell number

2.2 Infectivity of wilt causing bacteria in laboratory bioassays

2.2.1 Experiment 1: pathogenicity in cutting Pathumma

Five isolates of wilt causing bacteria including PT1J, PT2X, RRD, RT1S and R227 were tested for the ability to cause disease in cutting Pathumma placing in Erlenmeyer flask. After the Pathumma cuttings were put into bacterial suspension, slightly wilt symptom appeared at day 7. Only stem tissues inoculated with PT2X showed necrosis and browning. Leaves turned yellow or discolored and wilt at 14-21 days post inoculation but other wilt-bacterial strains and control were slightly discolored due to lack of nutrients (Figure 38, 39).

This result was not shown in obviously wilt symptom. In contrast with the report from Wang *et al.* (2010), the shoots of mulberry were apparently showed wilt symptoms after inoculated with *E. asburiae* R2-2 and R6-2. At 17 days post inoculation, the wilted leaves fell off and turned yellow, and eventually died at 21 days after inoculation. Moreover, many researchers usually test the pathogenicity in other parts of plant. Nishijima *et al.* (2004) reported that the disease incidence and disease severity of *E. cloacae* treatment were high percentage when tested on tissue-cultured ginger plantlets in test tubes. In addition, mild symptoms were observed on mature ginger rhizome slices.

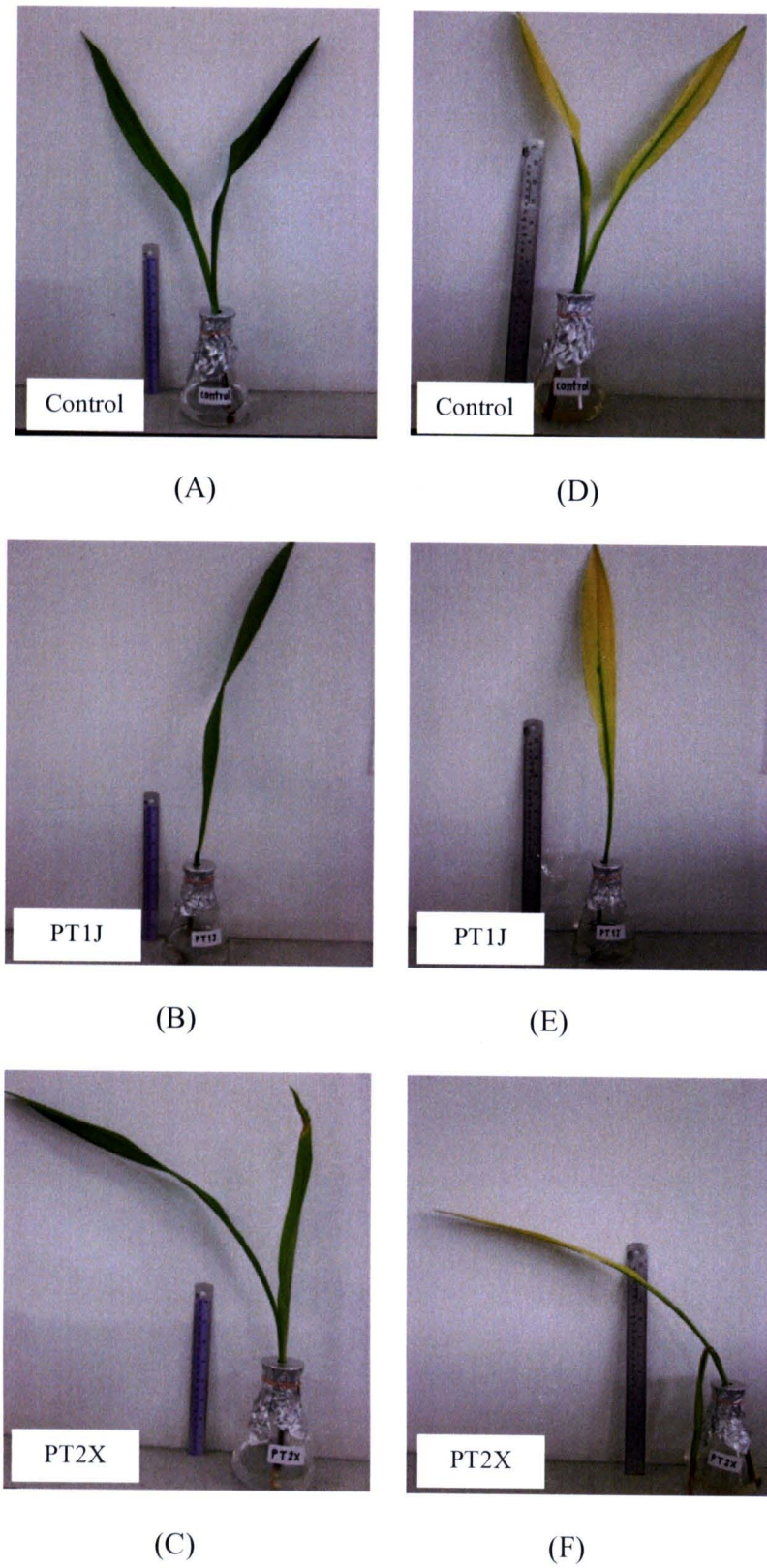


Figure 38 (A-C) The pathogenicity bioassay in Erlenmeyer flask of control, PT1J and PT2X at initial day of inoculation and (D-F) 21 days after incubation

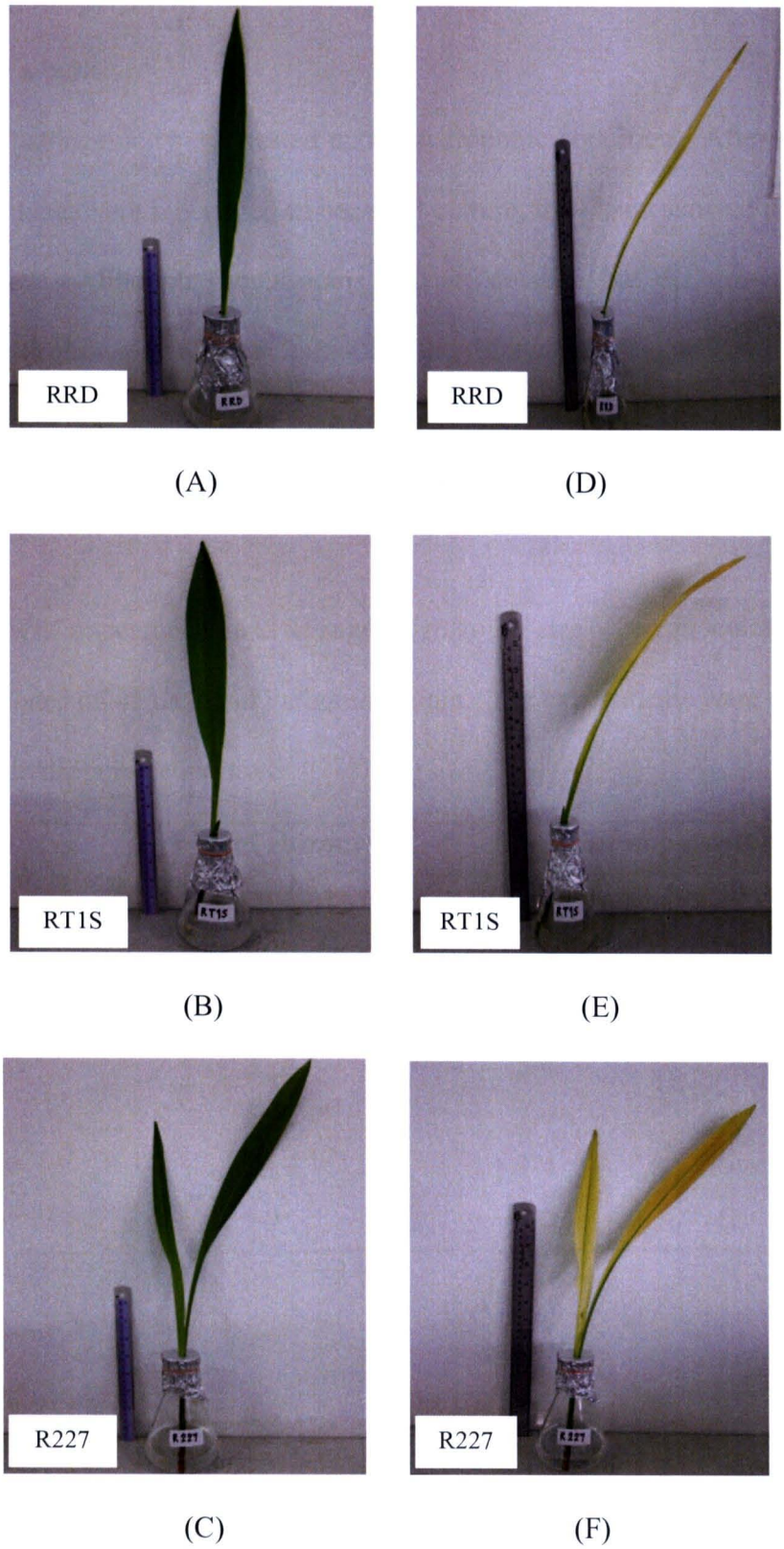


Figure 39 (A-C) The pathgenicity bioassay in Erlenmeyer flask of RRD, RT1S and R227 at initial day of inoculation and (D-F) 21 days after incubation

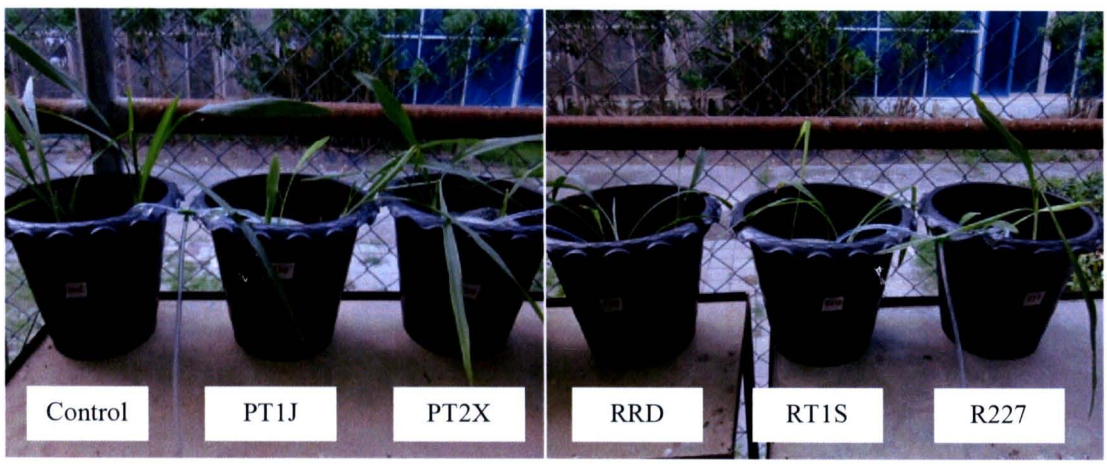
2.2.2 Experiment 2: pathogenecity in Pathumma growing under hydroponic condition

The pathogenicity was tested under hydroponic condition. After 45 days old Pathumma plants were inoculated in bacterial culture, the plants showed discoloration and collapsed. Although pseudostems did not develop but the growth could be measured with rhizomes. It was found that the rhizomes inoculated with pathogenic bacteria were wither and smaller than control. The control plants demonstrated the extended root development (Table 20; Figure 40, 41, 42).

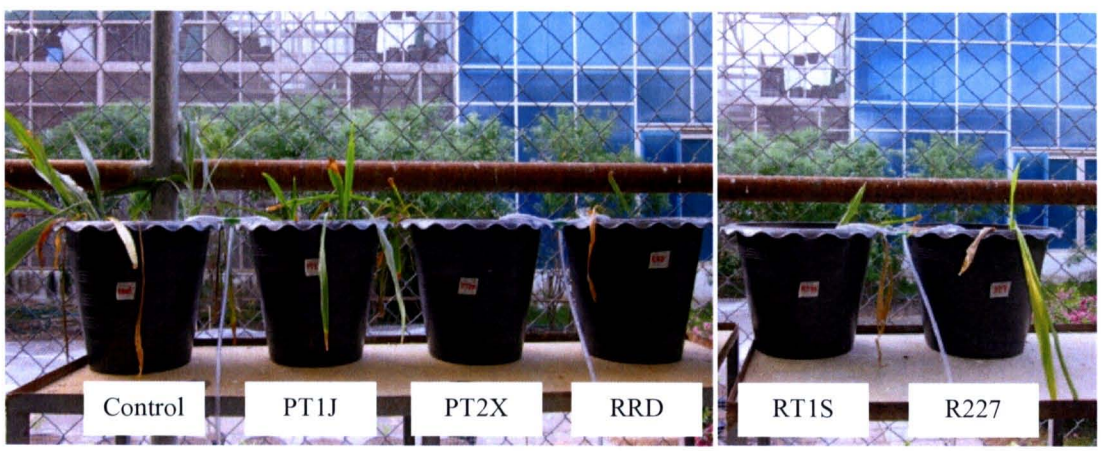
Table 20 Wilt appearances and change in rhizome size when inoculated with wilt bacterial isolates on 45 days old Pathumma plant. The experiments were conducted in 2010 under hydroponic condition

Isolate	Wilt of rhizome (%)	Rhizome size (cm)	
		Initial day	30 days after incubation
PT1J	66.6	3-4	5
PT2X	66.6	4-5	8-9
RRD	33.3	3-4	3-6
RT1S	66.6	3-4	4
R227	33.3	3-4	3-9
Control	0	4-5	7-9

Figure 40 The pathogenicity of five wilt-bacterial strains were tested under hydroponic condition. The experimental pots were placed in greenhouse



(A)



(B)

Figure 41 The treatment pots at (A) initial day of inoculation and (B) 30 days after incubation

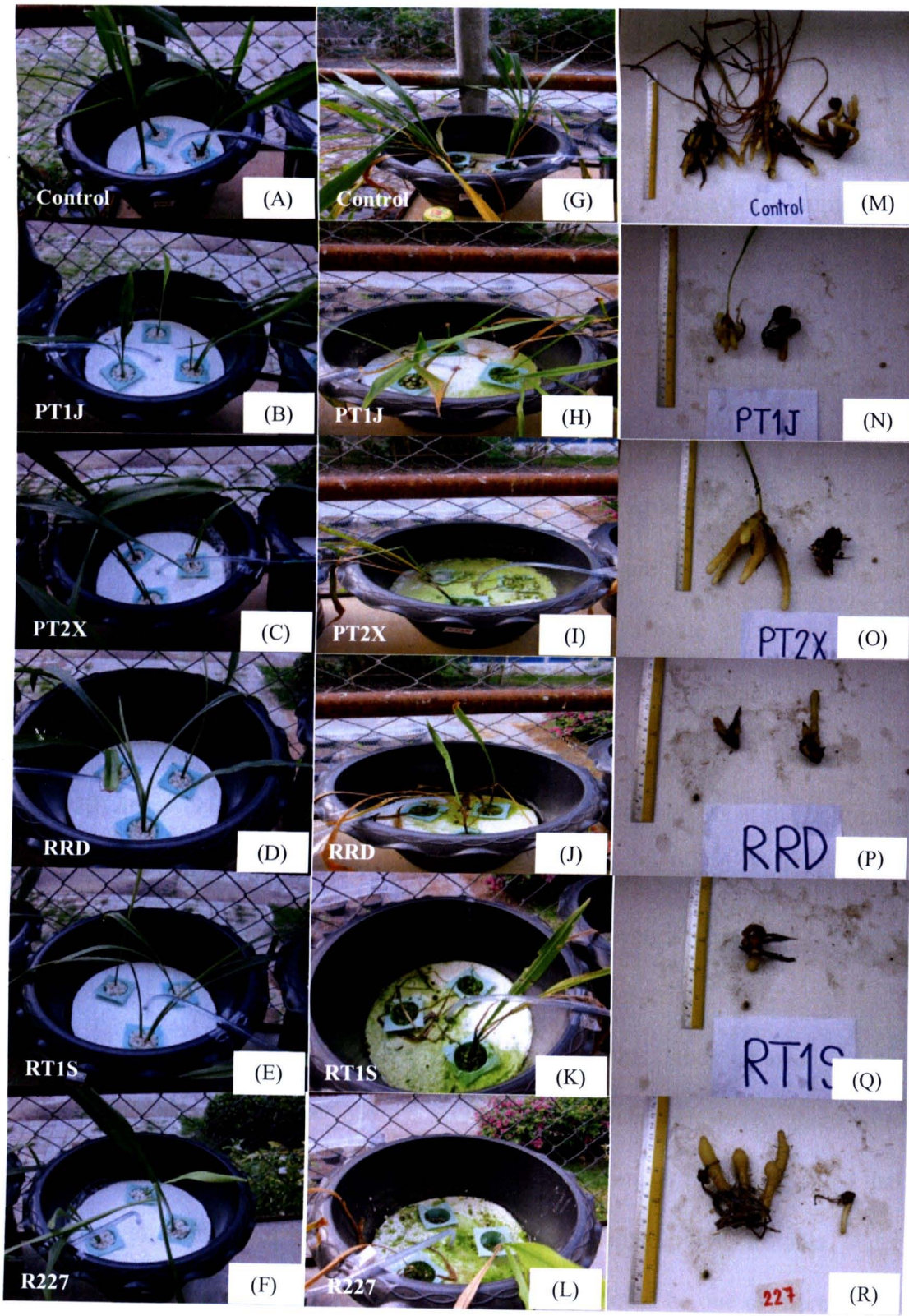


Figure 42 The treatment pots at (A-F) initial day of inoculation and (G-L) 30 days after incubation. (M-R) The infected rhizomes after 30 day of incubation

From the results of all experiments, it was found that some wilt causing bacteria isolated from wilted Pathumma rhizomes had a capacity to cause wilt disease. On the basis of Koch's postulation, the wilt-bacterial isolates had to be re-tested for their pathogenicity in host plants to confirm their virulence. The wilt symptoms in this study were leave discoloration or chlorosis, defoliation and tissue necrosis, and rhizome wither. These results were in accordance with many researches. Bishop (1990) reported that *E. clocae* could cause internal decay of onions. The symptoms of bulbs were discolored (brown to black) and flaccid. Nishijima *et al.* (2004) demonstarted that the ginger strains of *E. cloacae* produced basal stem and root rot, with foliar chlorosis and necrosis in tissue-cultured ginger plantlets, and had discolored and spongy tissue in both mature ginger rhizome slices and whole segments. Masyahit *et al.* (2009) revealed that the *in vitro* pathogenicity test resulted in yellowish to brownish soft watery symptoms on infected stems and fruits. Wang *et al.* (2010) discovered that leaf wilt symptoms began on older leaves at the bottom of the plants and then spread to the younger leaves on the top. Leaves of infected plant became withered and dried, turned dark brown and eventually dropped. Xylem tissues in these plants were moist and discolored with brown stripe.

The pathogenicity test in the greenhouse in this study showed milder symptoms compared with the infected Pathumma in fields. However, there were various factors affecting the severity of pathogens. Nishijima *et al.* (2005) stated that the failure to cause disease symptoms on ginger plants grown in pots under greenhouse conditions when inoculated with *E. cloacae* could be one or more of the following factors: (i) the young plants were not at a susceptible maturity (ii) temperature and relative humidity conditions were not optimal for infection (iii)

aeration and low soil moisture were not conducive to infection. Nevertheless, many wilt causing bacteria had latent period which did not show visible symptoms including *E. asburiae*, *E. cloacae* and *R. solanacearum* (Bishop, 1990; Masyahit *et al.*, 2009; Huang and Allen, 2000). Breukers *et al.* (2006) explained that one pathogens had been introduced in such chains, they could survive in the tissues or on the surface of propagation material from one production cycle to the next. Thereby, they had an opportunity to multiply and disperse, which might eventually result in an epidemic.

A major problem in controlling diseases in production chains occured when infected entities remain asymptomatic, while being capable of transmitting the disease. In addition, the pathogenicity test should be examined as soon as possible due to the loss of some virulent factors or involving gene (Agrios, 1997). The initial bacterial populations for pathogenicity also play important roles for wilt symptoms. Huang and Allen (2000) reported that the appearance of wilt symptoms reliably correlated with *R. solanacearum* population level in the potato stem. Whenever plants had a disease incidence more than 50%, they detect at least 10^8 cfu/cm stem immediately above the cotyledon.

According to the results of pathogenicity tests, the wilt-bacterial isolates including *E. asburiae* PT1J, *E. asburiae* PT2X, *E. asburiae* RRD, *E. asburiae* RT1S, *E. dissolvens* D1 and *E. hormachei* Rh1-1 could be an important wilt causing bacteria in Pathumma. In addition, the mixture of these pathogenic bacteria had high percentage of disease incidence. Hence it could be concluded that Pathumma plants could be attacked by various strains in natural fields. These bacteria had a synergistic effect that enhance their ability to overwhelm host. Moreover, the standard

pathogenic bacteria including *R. solanacearum* R227 and *Pseudomonas fluorescens* R1512 could not showed the severely wilt disease. It was possible that these bacterial strains were not specific to Pathumma. Further testing of these promising bacteria for host range capacity would provide useful information to prevent wilt disease.

3. Study of bacterial adhesion in plant tissue under compound light and electron microscope

E. asburiae PT1J was selected to investigate the adhesion in plant tissues due to its high percentage of disease incidence and apparent wilt symptoms.

3.1 Infectivity of wilt causing bacteria in Pathumma

After 7-10 days of bacterial inoculation, wilt disease showed early leave discoloration and defoliation. The infected plants grew slowly compared to the control plants. The bacterial strain might enter the vascular system via opening wounds, rapidly multiply, adhere and block xylems. The stems which showed early appearance of wilt symptoms were collected to observe under compound and electron microscopes. For easier sample preparation, the infected stems should be collected before the late of wilt disease.

3.2 Study of bacterial adhesion in plant tissue under light compound and scanning electron microscope

The light micrographs showed the cell parenchyma in xylems and vascular bundles (Figure 43). It could not show bacterial adhesion apparently due to the light contrast of visible plane between plant cells and bacteria. In addition, the changes of plant cell structures between control and infected plants were not different.

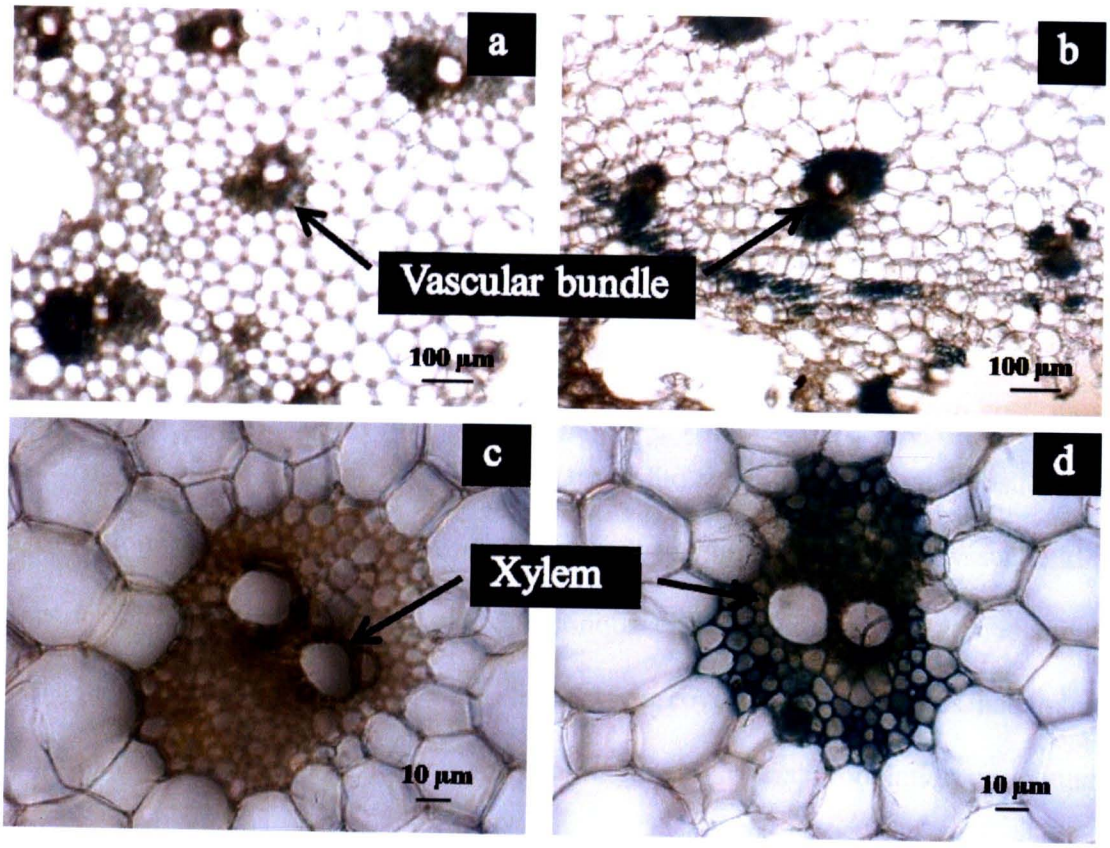


Figure 43 Light micrographs of cell parenchyma and vascular bundles of (a,c) control plant and (b,d) infected plant. Magnifications: (a,b) 40x, (c,d) 100x

To deeply understand the adhesion of the bacteria to the plant cell, morphological study was performed using electron microscopic technique. Figure 44 showed scanning electron micrographs of healthy and infected plant pseudostem tissues. Some parts of infected stem were shrunken (Figure 44-B) compared with the healthy stem. This study indicated that the infected plant had structural changes. However, little was known about the early events in the development of the symptoms and which factors contributed to the systemic colonization of host plants by the bacteria. It may caused by the host plant defense mechanisms or some compounds from bacteria. Figure 45 (B, D, F) showed adhesion and colonization of bacteria in

vascular bundles and parenchyma. The results suggested that bacteria entered the plant through wounds or natural openings and invaded the parenchyma and vascular bundles especially xylems resulting in the structural changes of plant tissues and dehydration. The bacteria obviously adhered to the vascular bundles but how they adhere and multiply in vascular bundles had not yet understood. In general, plant pathogens must first attach host surfaces before penetrate and colonize in host, most bacteria had pili or fimbriae which is relevant to the attachment of host cell surfaces. In addition, *Enterobacter asburiae* PT1J produced high level of polysaccharides outside the cell envelope since these glutinous or mucilaginous substances might assist bacterial cell to attach host surfaces. Moreover, polysaccharides had many important physiological functions for maintaining bacterial population in natural habitats, for example, protection of bacterial cells from desiccation and some defense mechanisms from plant host. Accordingly, fimbriae and polysaccharides might play important role in bacterial adhesion and multiplication in plant cells.

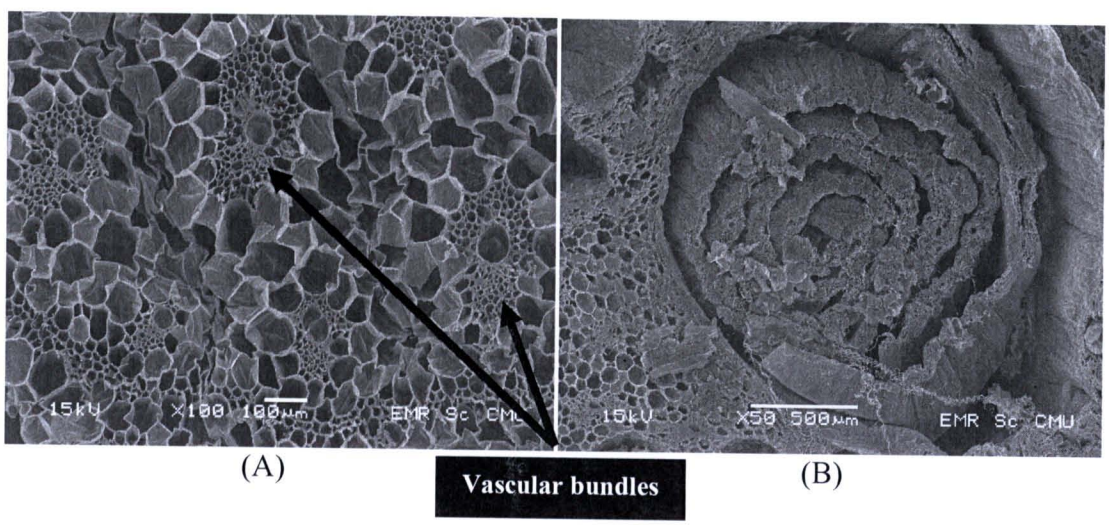


Figure 44 Scanning electron micrograph of pseudostem tissue: (A) Control plant and (B) Infected plant. Magnification (A) 100x, (B) 50x

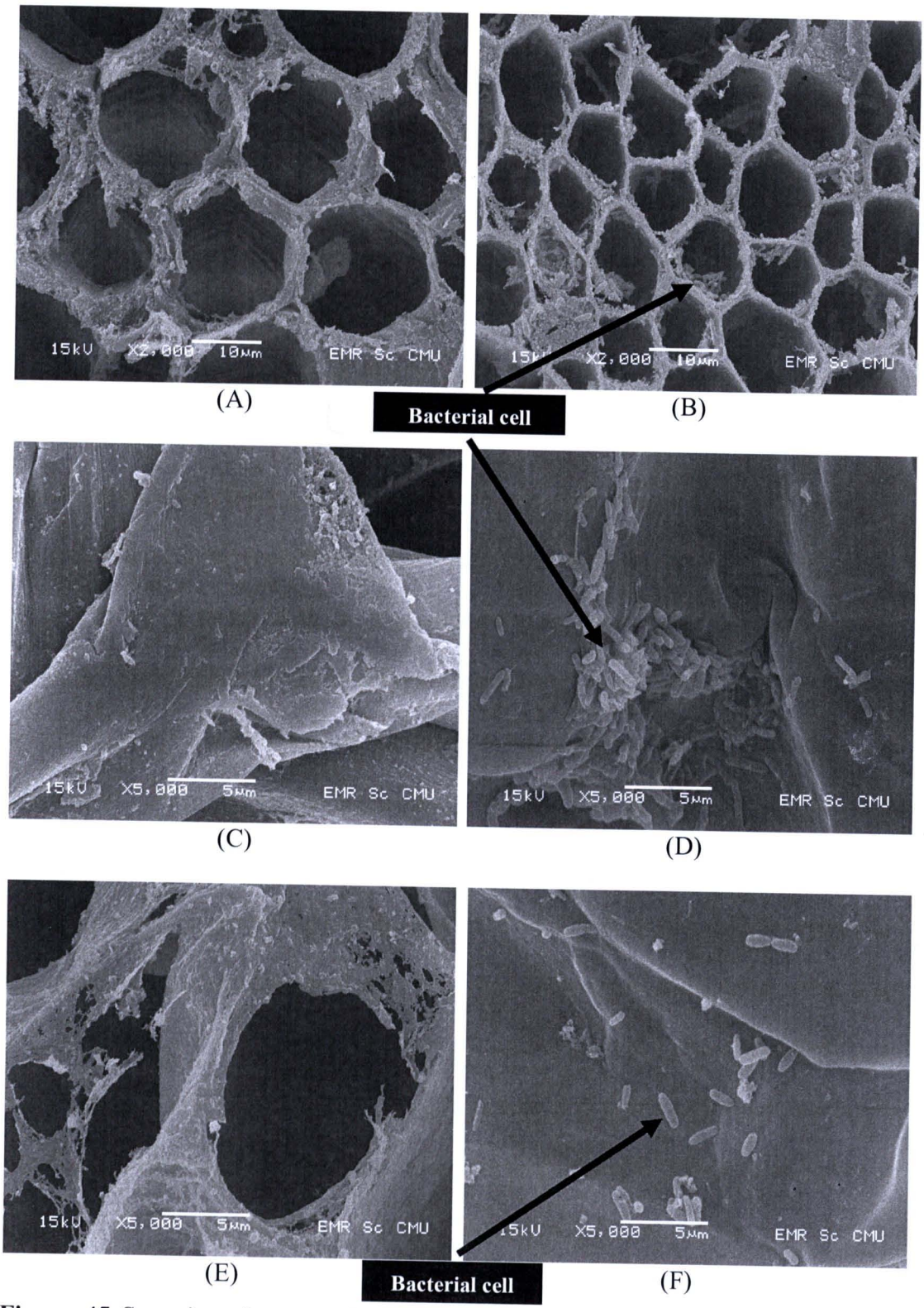


Figure 45 Scanning electron micrograph of pseudostem tissue. (A, C, E) Control plant and (B, D, F) Infected plant showing bacterial adhesion. Magnification (A, B) 2,000x, (C, D, E, F) 5,000x

There are no reports about the adhesion and colonization of *Enterobacter* in *Curcuma* plants. The previous works demonstrated colonization or morphological changes solely in *R. solanacearum* (Wydra and Beri, 2006). The pathogenic bacteria enter the plant vascular tissues through wounded roots or natural openings, which occur after the emergence of secondary roots. It progresses into the water conducting vessels. The adhesion of bacteria in host tissues causes browning of the xylem, foliar epinasty and wilt (Wydra and Beri, 2006). Grimault *et al.* (1995) also found that root invasion was not a limiting factor for bacterial multiplication when a large numbers of bacteria thrived in tomato stems in absence of wilt symptoms and indicated that plant-bacterial interactions were involved in reduction of bacterial propagation. Huang and Allen (2000) revealed that besides extracellular polysaccharide, wall-degrading enzymes; endoglucanase and polygalacturonases; were necessary for bacterial colonization and full virulence of *R. solanacearum* on tomato plants. In addition, this report also demonstrated that in the early stage of the disease, polygalacturonases might express at the maximum level while the bacterial cell number was low. Wydra and Beri (2006) showed that homogalacturonan I and arabinogalactan protein in xylem cell walls of tomato could react with *R. solanacearum* and caused cell damages in plant tissues. They also suggested that skin of the plant cell or the wall participated in adhesion. Thus, to study how bacteria adhere and multiply in vascular bundles would assist researchers to determine more precisely the roles of bacteria in plant and electron microscopic technique was a useful tool for study on plant-bacterial interaction especially in the small structural plant tissues where the location of adhesion was difficult to observe under compound microscope.

4. Survival of wilt causing bacteria in soil without host

To evaluate the persistence of wilt-bacterial strains in natural soil, the isolates PRZ, PT1B, PT1J, PT2X, RRD, RT1K, RT1S, RT2R, C4, D1, Rh1-1, Rh3-1, Tu1-1, Tu2-1 and R1512 were tested the survival in soil without Pathumma plants. The viable cell numbers of these bacteria in soil mix were recorded every 30 day after inoculation. The amounts of pathogenic cells were declined by approximately 34-57% after 1 year of inoculation (Figure 46). The amounts of wilt-bacterial strains were significantly separated into 4 groups (group a, b, c and d) at initial month of incubation and 3 groups (group a, b and c) at month 12 after incubation. The survival cells of individual strain appeared in the soil were almost not significantly different (Table 21). In addition, the result was indicated that these wilt-bacterial isolates could still alive more than 1 year. Reports on survival of *Enterobacter* in soil without host were scarce. Many researchers usually investigated other wilt bacteria in planting soil particularly *R. solanacearum* (Huang and Allen, 2000). Breukers *et al.* (2006) reported that *R. solanacearum* had a survival period in potato planting soil up to 2 years. It was interesting that the survival of pathogenic bacteria was related with latent period. When Pathumma plants are harvested or rested, the wilt bacteria remain in natural soil and re-infected the plants in the next growing seasons. According to the Pathumma producer's observation, the Pathumma fields must be left for 3-4 years to ensure that the pathogenic bacteria are not persisted. Therefore, assessment the numbers of wilt causing bacteria in natural environment is necessary.

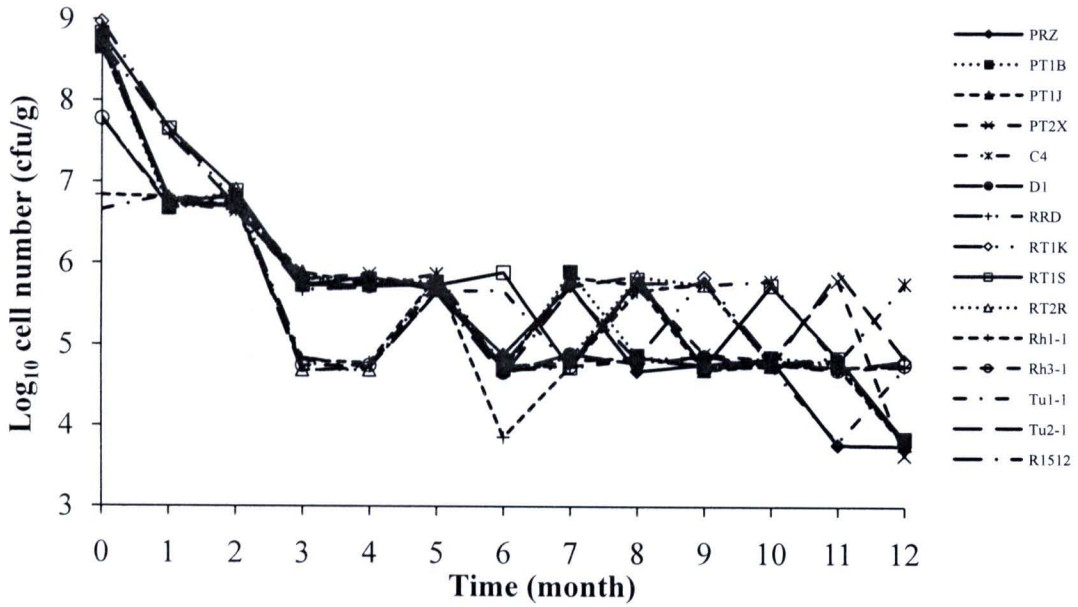


Figure 46 Average populations of wilt causing bacteria in soil mix after 12 months of cultivation

Table 21 Population and survival rate of wilt causing bacteria after 12 months of cultivation

Isolate	Populations (cfu/g)		Survival rate (%)
	Month 0	Month12	
PRZ	5.5×10^8 cd	5.7×10^3 a	42.6 ab
PT1B	4.5×10^8 c	7.0×10^3 ab	44.3 abc
PT1J	4.5×10^8 cd	6.0×10^3 a	43.5 abc
PT2X	4.3×10^8 cd	4.3×10^3 a	41.9 a
C4	6.0×10^8 cd	5.7×10^5 c	58.1 def
D1	6.3×10^8 cd	6.2×10^3 a	43.0 abc
RRD	5.1×10^8 cd	5.3×10^4 bc	54.1 cde
RT1K	9.3×10^8 d	6.3×10^4 c	53.4 bcde
RT1S	6.7×10^8 cd	6.7×10^3 ab	43.3 abc
RT2R	7.3×10^8 cd	6.0×10^3 a	42.5 ab
Rh1-1	6.8×10^6 a	5.5×10^4 c	69.5 fg
Rh3-1	6.0×10^7 b	5.7×10^4 c	61.2 efg
Tu1-1	4.5×10^6 a	5.2×10^4 bc	70.9 g
Tu2-1	6.2×10^7 b	6.8×10^4 c	62.1 efg
R1512	6.0×10^7 b	6.5×10^3 ab	49.1 abdd

Means followed by the same letter (a, b, c, d, e, f, g) within a column are not significantly different as determined by the Tukey's Test (P=0.05)

5. Isolation and screening of antagonistic bacteria

One hundred and two bacterial isolates were obtained from seven soil samples collected from different sites in Thailand (Table 22). Among 102 bacterial isolates, 55 isolates were Gram positive rods and 47 isolates were Gram-negative rods. After testing their ability to inhibit growth of the pathogenic bacteria including *E. hormachei* PRZ, *E. asburiae* PT1J, PT2X, RRD RT1S and *E. dissolvens* D1, only four isolates, namely SP15, SP38, SP46 and SP58 which were isolated from soils collected from Sankumpang hot spring, Srinakarin Dam and Bangsai Arts and crafrs centre, displayed a high ability to make clear inhibitory zone of 8-25 mm in diameter after incubation at 30°C for 48 hours (Table 23; Figure 47). The four bacterial antagonists showed different degrees of antagonism when studied *in vitro* for their antagonistic activity against the pathogen. There was a slight difference between five strains of pathogenic bacteria. The results indicated that the microorganisms isolated from soils had high capacity to suppress growth of wilt-bacterial strains. This conclusion was supported by the fact that plant growth promoting rhizobacteria (PGPR) competitively colonized plant roots and stimulated plant growth and/or reduce the incidence of plant disease (Haas and Défago, 2005). In addition, Aliye *et al.* (2008) isolated *Bacillus subtilis* PFMRI from a potato field where there was no report of bacterial wilt disease. This antagonist was also effective in suppressing the growth and perpetuation of the pathogen both *in vitro* and in *vivo*. Thus, the absence of disease in the field from which the antagonists were isolated could be feasible. The potential bacterial antagonists which shown strong degree of inhibition were maintained and further *in vivo* evaluated in the greenhouse.

Table 22 Soil samples used for bacterial isolation

No.	Soil source	Collecting place	Amount of isolated bacteria
1	Hot spring	Sankumpang hot spring, Chiang Mai	19
2	Turmeric	Home garden, Lampang	2
3	Ginger	Home garden, Lampang	8
4	Rose	Home garden, Lampang	8
5	Rain tree	Srinakarin Dam, Karnchanaburi	15
6	Red ginger	Home garden, Krabi	9
7	Grass	Ang Kaew reservoir, Chiang Mai	11
8	Pathumma	Bua Lai Garden, Chiang Mai	21
9	Banyan	Bangsai Arts and crafts centre of H.M. Queen Silikit of Thailand, Ayuthdaya	10

Table 23 Inhibition of four antagonistic bacteria against growth of wilt causing bacteria

Bacterial Isolate	Clear zone of growth inhibition (mm)					
	***E. hormachei* PRZ**	***E. dissolvens* PT1B**	***E. asburiae* PT1J**	***E. asburiae* D1**	***E. asburiae* RRD**	***E. asburiae* RT1S**
SP15	13.0 ± 1.0	13.7 ± 2.1	14.3 ± 0.6	13.3 ± 0.6	13.3 ± 0.6	13.3 ± 1.2
SP38	16.7 ± 2.9	22.3 ± 4.0	19.7 ± 2.1	19.0 ± 1.7	18.0 ± 1.7	18.3 ± 3.5
SP46	14.7 ± 3.5	21.0 ± 1.7	24.0 ± 1.7	23.3 ± 2.9	19.0 ± 1.7	17.7 ± 2.5
SP58	15.3 ± 4.0	16.0 ± 0.0	18.0 ± 2.0	20.0 ± 0.0	25.0 ± 0.0	19.7 ± 3.5

Results are shown as mean ± standard error

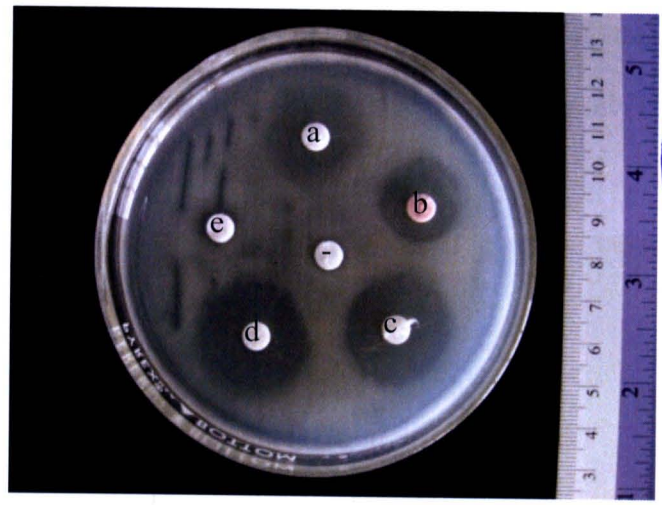


Figure 47 Clear zone of PT1J growth inhibition by antagonistic bacterial isolates (a) SP15, (b) SP38, (c) SP46, (d) SP58, (e) negative antagonistic strain, (-) TSB

6. Identification of the antagonistic bacteria SP15, SP38, SP46 and SP58

The antagonistic bacterial isolate SP15 was Gram-positive, rod shaped with ellipsoidal endospores located at the cell terminal, oxidase negative, catalase positive and non-motile. It was 05.-1.5 μm width and 1.0-3.0 μm length (Figure 48). The strains SP38, SP46 and SP58 were Gram negative, short-rod shaped, facultative anaerobe, oxidase positive and motile. They were 0.5-1.0 μm width and 1.0-2.5 μm length (Figure 48). The results of morphological and biochemical tests were listed in table 24 and 25. Three bacterial isolates including SP38, SP46 and SP58 were classified as *Pseudomonas* since they produced oxidase and could not ferment lactose. However, they could not be identified to species level by the conventional method due to the complexity of the genus *Pseudomonas*. All strains were repeatedly comfirmed by 16S rRNA gene determination. The partial 16s RNA sequencing was used to identify the antagonistic bacterial isolates to the species level and to determine whether there were clusters of similar organisms (Figure 49). The 16s rRNA gene

sequences determined were deposited in the Genbank database. From database similarity and Neighbour-joining tree (Figure 50), the species level identification was shown in table 26.

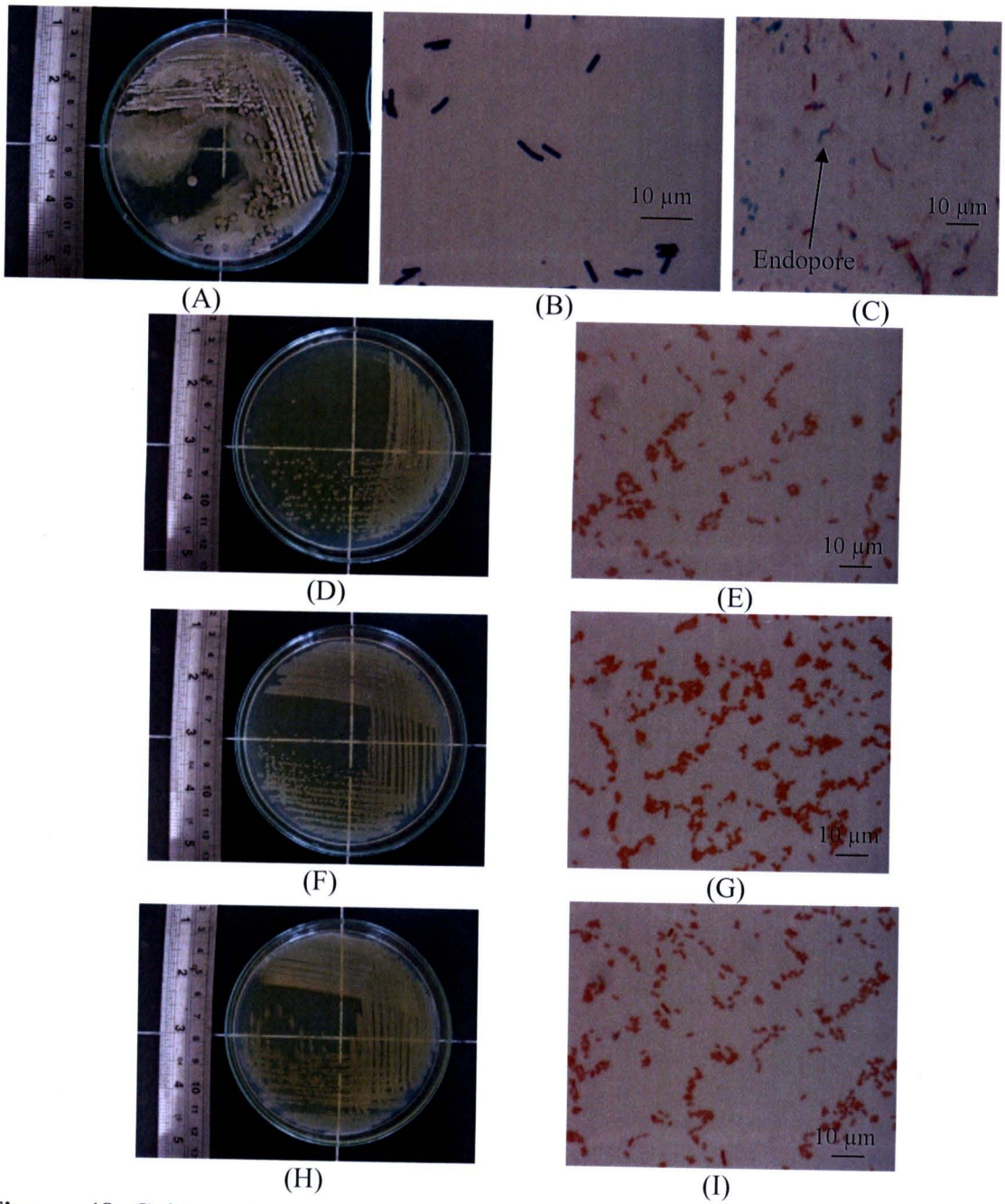


Figure 48 Culture characteristics on TSA and characteristics under compound microscope of (A-C) SP15, (D, E) SP38, (F, G) SP46 and (H, I) SP58. Magnification; (B, C, E, G, I) 100x

5. Materials

5.1 Beaker 50, 100, 250 and 500 ml

5.2 Bottle 250, 500 and 1,000 ml (Duran®)

5.3 Centrifuge tube 15 and 50 ml

5.4 Chaff

5.5 Coir

5.6 Cuvette

5.7 Cylinder 100 and 1,000 ml

5.8 Durham tube

5.10 Erlenmeyer flask 250, 500 and 1,000 ml

5.12 Glass bottle 15 and 30 ml

5.13 Glass slide

5.14 Microcentrifuge tube 1.5 ml

5.15 Micropipette 0.5-10.0, 10-100, 20-200, 50-200 and 100-1,000 µl

5.16 Microplate, 96-wells (Nunc™, Denmark)

5.17 Paper disc diameter 6 mm (Macherey-Nagel, Germany)

5.18 Petri dish

5.19 Pipette 1, 5 and 10 ml

5.20 Pipette tip

5.21 Plastic bag, 15-cm diameter

5.22 Razor blade

5.23 Spatula

5.24 Test tube 13x100 and 16x150 mm

5.25 TLC sheet, silica gel 60 F_{254} (Merck®, Germany)

Table 24 Biochemical characteristics of the isolate SP15

Characteristics	SP15	*Bacillus subtilis*
Catalase	+	+
Anaerobic growth	-	-
Voges-Psoskauer	+	+
pH in V-P broth		
<6	-	d
>7	-	-
Acid from		
D-Glucose	+	+
D-Xylose	+	+
D-Mannitol	+	+
Gas from glucose	-	-
Hydrolysis of		
Casein	+	+
Gelatin	+	+
Starch	+	+
Utilization of citrate	+	+
Deamination of phenylalanine	-	-
Nitrate reduced to nitrite	+	+
Formation of indole	-	-
Growth at pH		
6.8, nutrient broth	+	+
5.7	+	+
Growth in NaCl		
2%	+	+
5%	+	+
7%	+	+
10%	-	ND
Growth at		
30°C	+	+
40°C	+	+
50°C	+	d
55°C	-	-

Symbols: + = 90% or more of strains are positive
- = 90% or more of strains are negative
d = 11-89% 0f strains are positive
ND = no data available

Table 25 Biochemical characteristics of the isolate SP38, SP46 and SP58

Characteristic	**SP38**	**SP46**	**SP58**	***Pseudomonas aeruginosa***	***Pseudomonas mosselii***
Fluorescent, diffusible pigments	+	+	+	+	+
Growth at 41°C	-	+	+	+	-
Growth at 4°C	-	-	-	-	d
Oxidase reaction	+	+	+	+	+
Denitrification	+	-	-	+	d
Gelatin hydrolysis	+	+	+	+	d
Starch hydrolysis	-	-	-	-	-
Utilization of					
Glucose	-	+	+	+	-
Trehalose	-	+	+	-	-
β-Alanine	-	+	+	+	+
L-Arginine	-	-	-	+	+
D-Xylose	+	+	+	d	d
D-Ribose	+	+	+	d	d
L-Rhamnose	+	+	+	d	d
Adonitol	+	+	+	d	d
Sucrose	+	+	+	d	d
Nitrate used as a nitrate source	+	+	+	+	+
Lactose fermentation	-	-	-	-	-

Symbols: + = 90% or more of strains are positive
- = 90% or more of strains are negative
d = 11-89% 0f strains are positive

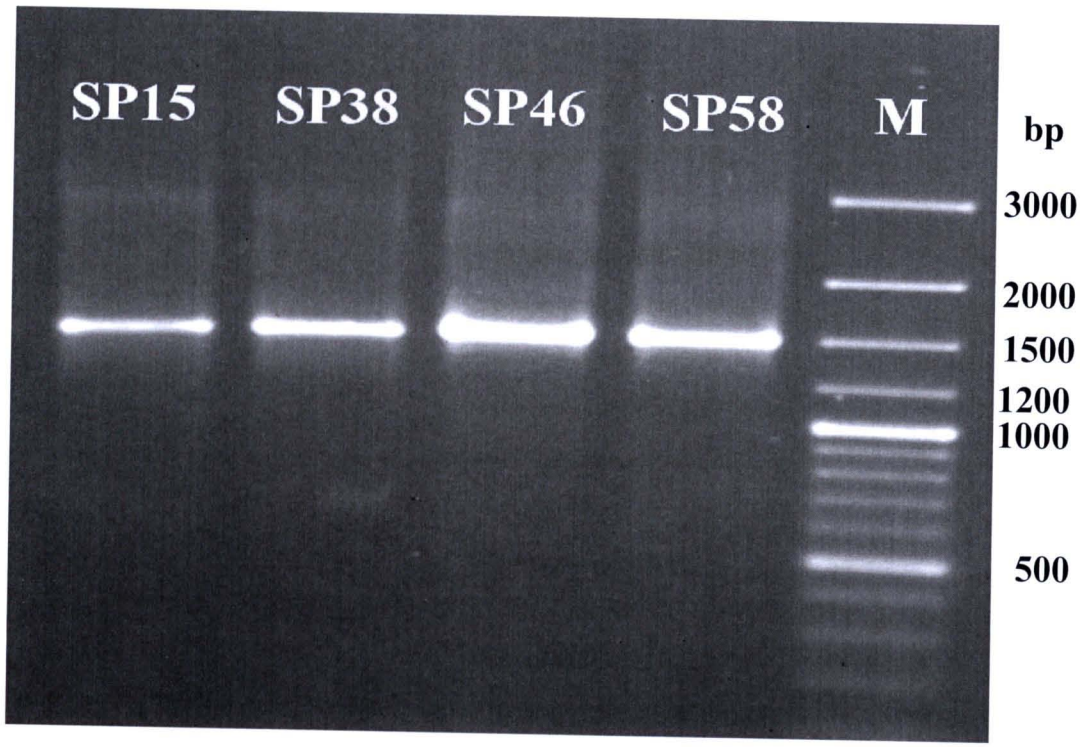


Figure 49 16S rRNA gene PCR products of antagonistic bacteria, M = 100 bp plus

Table 26 Species identification of antagonistic bacteria based on biochemical and 16S rRNA sequence analysis

Isolate	Source	Species	Max identity (%)
SP15	Hot spring, Chiang Mai	*Bacillus subtilis*	99
SP38	Rain tree soil, Karnchanaburi	*Pseudomonas mosselii*	97
SP46	Rain tree soil, Karnchanaburi	*Pseudomonas mosselii*	97
SP58	Banyan tree soil, Ayuthdaya	*Pseudomonas aeruginosa*	99

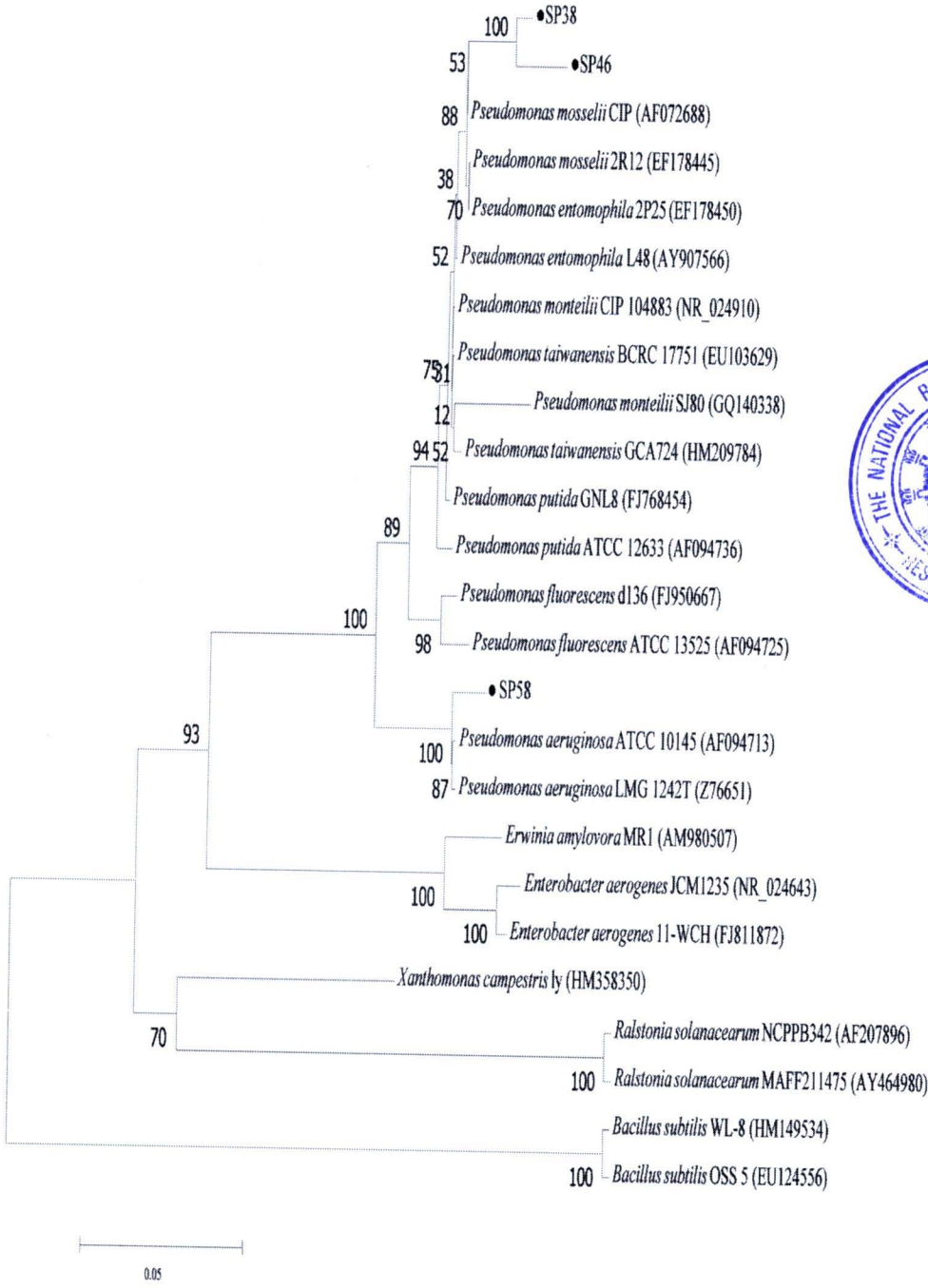


Figure 50 Neighbour-joining tree based on 16S rRNA gene sequences showing the position of the antagonistic bacterial isolates including SP38, SP46 and SP58. The sequence of Bacillus subtilis WL-8 and OSS 5 were used as an outgroup. Bootstrap values were calculated from 1,000 re-samplings and the bar represents 0.02 showed substitution per nucleotide position. The GenBank accession numbers were in parentheses

The isolation of bacteria from within rhizosphere of many crop was essential for successful identification of potential biocontrol agents. Among the bacterial biocontrol agents, fluorescent *Pseudomonas* spp. had emerged as the largest and potentially promising group of rhizobacteria involved in the biocontrol of plant diseases. These bacteria were ideally suited as soil inoculums because of their rapid and aggressive colonization of the root (Das *et al.*, 2009). In addition, *Bacillus* spp. had been studied extensively for many years. The use of *Bacillus* and *Pseudomonas* as biocontrol agents was in agreement with other observations (Guo *et al.*, 2004; Lee *et al.*, 2006; Abdallah *et al.*, 2008; Plodpai *et al.*, 2008; Das *et al.*, 2009). For instance, fluorescent *Pseudomonas* J3 and *Bacillus* BB11 had ability to control bacterial wilt of tomato caused by *R. solanacearum*. *B. licheniformis* and *Ps. Corrugate* P94 could inhibit growth *of Botrytis cinerea*, the grey mold of tomato. *B. amyloliquefaciens* could use as potential biological control agents against *Sclerotinia sclerotiorum*. *Bacillus* sp. and *B. megaterium* reduced anthracnose, *Cercospora* leaf spot and root rot of chilli spur pepper. Also, *Ps. chlororaphis SRB127* could effectively control charcoal rot of sorghum caused by *Macrophomina phaeseolina*.

However, *Bacillus* and *Pseudomonas* usually inhibited some fungal and bacterial pathogens. The scientific documentation of these bacteria in *Enterobacter* control was little. This study seemed to be the first report showing that *B. subtilis*, *Ps. mosselii* and *Ps. aeruginosa* antagonism to *Enterobacter* in Pathumma.

It was inclined that these antagonistic isolates had high potential for Pathumma bioprotection or as part of integrated disease management. However, additional testing was still needed to prove efficacy under bacterial mechanisms.

7. Optimization of antagonistic substances from antagonistic bacteria

A study on the production of antimicrobial substances usually involves a search for optimal media. This is achieved by a systemic study of the suitability of a large number of carbon and nitrogen sources, pH media and temperature of incubation.

7.1 Effect of carbon sources

The effect of carbon sources on antibacterial activity production was determined using TSB medium which glucose was substituted with various sources. The antibiotic activity was reported as the zone of inhibition (ZOI). The results of these supplementations were shown in table 27, 28. The best type and concentration of carbon sources were selected under the highest value of antimicrobial activity that was significantly different. If the highest value of ZOI was not significantly different, the suitable source was selected from not only low cost but also more commercially available.

The suitable carbon source and carbon source concentration of *B. subtilis* SP15 was 0.5% (w/v) glucose since it had not only highest value of ZOI but also were significantly different from other sources and concentration.

Sucrose was the optimal carbon source for *Ps. mosellii* SP38, the ZOI value was not significantly different from maltose and galactose but this carbon source was selected due to the highest value of ZOI and more available.

In *Ps. mosellii* SP46 optimization, various carbon sources and carbon source concentration were not significantly different, glucose were selected because it had high value of ZOI.

In *Ps. aeruginosa* SP58 optimization, various carbon sources were significantly divided into 2 groups, sucrose was in the group which had high ZOI value and it had the highest ZOI value. Therefore, sucrose was the best carbon source for *Ps. aeruginosa* SP58. For sucrose concentration, it was found that the ZOI value was high when 0.5-2.5% (w/v) sucrose were used. The 0.5% (w/v) sucrose was selected because it was the minimum concentration and not significantly different from 1.0, 1.5, 2.0 and 2.5% (w/v).

In conclusion, the highest antimicrobial activity production of *B. subtilis* SP15, *Ps. mosselii* SP38, *Ps. mosselii* SP46 and *Ps. aeruginosa* SP58 were obtained in media containing 0.5% glucose, 0.5% sucrose, 0.5% glucose and 0.5% sucrose (w/v), respectively.

It is interesting to note from optimization results that these antagonists required a simple carbon source such as glucose and sucrose at low concentration. This result was associated with the findings of Dikin *et al.* (2007) and Gouveia *et al.* (2001) that glucose and sucrose were the best carbon sources for antimicrobial substance production. This evaluation was conducted in *Burkholderia multivorans*, *Microbacterum testaceum* and *Streptomyces clavurigerus*. On the contrary, Tabbene and Slimene (2009) revealed that lactose influenced both cell growth and antibacterial activity production of *B. subtilis* B38. The antibacterial activity was 2- to 4-fold higher in the modified culture medium than in TSB medium under the same conditions. Moreover, they discovered that glycerol and sucrose reduced antibacterial activity when compared with TSB medium without glucose (negative control).

Table 27 (continued)

Antagonistic isolate	Carbon source	Clear zone of inhibition (mm)				
		PT1J	PT2X	RRD	RT1S	mean
SP58	glucose	20.7±0.6	20.7±1.2	21.7±0.6	19.3±0.6	20.6 a,b
	sucrose	22.3±4.6	24.0±1.7	28.3±2.9	23.0±1.7	24.4 b
	maltose	16.7±2.9	22.3±1.2	28.7±1.2	28.7±1.2	24.1 b
	galactose	16.7±2.9	25.7±1.2	26.7±2.9	26.5±2.1	23.9 b
	fructose	17.0±1.0	17.0±0.0	22.3±0.6	17.0±1.0	18.3 a
	lactose	19.3±0.6	19.7±0.6	25.7±1.2	19.3±0.6	21.0 a,b
	mannitol	18.3±1.5	19.0±1.7	23.0±1.7	20.0±2.0	20.1 a,b
	sorbitol	18.7±1.2	19.0±1.0	23.7±1.2	22.7±0.6	21.0 a,b

Results are shown as mean ± standard error

Means followed by the same letter (a, b, c and d) within a column are not significantly different as determined by the Tukey's Test (P=0.05)

Means were compared in each antagonistic isolate

Table 28 Effect of carbon source concentration on production of antibacterial substances by the antagonistic bacteria to inhibit growth of wilt causing bacteria after incubation at 30°C for 48 hours. The carbon source used for SP15, SP38, SP46 and SP58 was glucose, sucrose, glucose and sucrose, respectively

Antagonistic isolate	Carbon source (%)	Clear zone of inhibition (mm)				
		PT1J	PT2X	RRD	RT1S	mean
SP15	0	10.0±0.0	9.7±0.6	9.7±0.6	10.0±0.0	9.8 a
	0.1	10.7±0.6	11.3±0.6	12.7±0.6	12.0±0.0	11.7 b
	0.5	13.3±0.6	13.3±0.6	13.7±0.6	13.3±0.6	13.4 c
	1.0	13.0±0.0	12.3±0.6	13.0±1.0	13.7±0.6	13.0 c
	1.5	11.7±0.6	12.0±1.0	12.0±1.0	11.7±0.6	11.8 b
	2.0	11.7±0.6	11.6±0.6	10.7±0.6	11.3±0.6	11.3 b
	2.5	11.3±0.6	11.3±0.6	11.0±0.0	11.3±0.6	11.3 b
SP38	0	21.6 ±0.6	20.0±1.7	16.7±0.6	24.7±0.6	20.8 a
	0.1	23.6±0.6	20.0±1.0	18.7±1.2	27.3±1.2	22.4 a
	0.5	25.3±1.2	20.3±2.5	23.3±0.6	21.3±1.2	22.6 a
	1.0	21.6±0.6	20.7±0.6	24.7±0.6	22.0±0.0	22.3 a
	1.5	21.0±0.0	19.3±1.2	24.7±0.6	21.7±1.5	21.7 a
	2.0	21.3±0.6	19.7±2.9	23.7±0.6	21.7±2.9	21.6 a
	2.5	22.0±.1.0	20.0±2.0	24.3±0.6	22.7±1.2	22.3 a
SP46	0	18.6±0.6	20.7±0.6	17.7±0.6	18.3±0.6	18.8 a
	0.1	18.3±0.6	20.7±0.6	17.3±2.1	19.7±1.5	19.0 a
	0.5	21.0±1.0	20.7±0.6	18.3±3.2	20.7±1.2	20.2 a
	1.0	21.0±1.0	20.7±0.6	19.0±3.6	23.0±1.0	20.9 a
	1.5	20.3±0.6	20.7±0.6	18.7±2.1	23.3±0.6	20.8 a
	2.0	20.6±1.5	21.3±0.6	16.7±1.2	22.7±1.5	20.3 a
	2.5	22.3±0.6	22.7±0.6	17.7±1.2	22.7±2.5	21.3 a

Table 28 (continued)

Antagonistic isolate	Carbon source (%)	Clear zone of inhibition (mm)				
		PT1J	PT2X	RRD	RT1S	mean
SP58	0	8.7±1.2	12.7±0.6	22.0±1.0	13.7±2.3	14.3 a
	0.1	14.7±0.6	13.0±1.7	23.3±2.9	13.0±2.6	16.0 a
	0.5	20.3±2.9	18.3±0.6	24.0±1.7	17.3±1.2	18.5 a,b
	1.0	18.3±2.5	17.7±0.6	25.0±0.0	19.7±1.5	18.7 a,b
	1.5	18.7±1.2	17.7±0.6	22.7±0.6	20.7±1.5	18.6 a,b
	2.0	16.3±1.2	20.0±1.7	24.7±1.2	19.3±1.2	18.6 a,b
	2.5	23.7±1.2	20.3±0.6	25.7±1.2	21.7±0.6	21.6 b

Results are shown as mean ± standard error

Means followed by the same letter (a, b and c) within a column are not significantly different as determined by the Tukey's Test (P=0.05)

Means were compared in each antagonistic isolate

7.2 Effect of nitrogen sources

Among seven types of nitrogen sources including peptone, ammonium dihidrogen phosphate, ammonium nitrate, corn flour, corn steep liquor, tryptone and yeast extract, peptone demonstrated the highest activity to inhibit growth of the pathogenic bacteria for all antagonistic isolates (Table 29, 30).

From the result of effect of nitrogen sources on production optimization, it was found that all antagonists did not produce inhibiting substances when ammonium dihidrogen phosphate, ammonium nitrate, corn flour and corn steep liquor were used. While the ZOI value of each antagonist was not significantly different when peptone, tryptone and yeast extract was used.

B. subtilis SP15 used peptone as the best nitrogen source due to the highest value of ZOI. For peptone concentration, the concentration at 2 and 2.5% (w/v) was higher and significantly different from other concentration, 2% (w/v) peptone was thus selected because it was the minimum concentration.

Peptone was selected for the suitable nitrogen source for *Ps. mosselii* SP38 since it was the highest ZOI value. As well as *B. subtilis* SP15, 2% (w/v) peptone was the best concentration for *Ps. mosselii* SP38.

In *Ps. mosselii* SP46 optimization, peptone was the best nitrogen source due to the highest value of ZOI. Peptone concentration was significantly separated into 3 groups. At the concentration of 1.5% (w/v), peptone had the highest ZOI value for this bacterial strain.

In *Ps. aeruginosa* SP58 optimization, peptone was the best nitrogen source due to its highest ZOI value. Peptone concentration was significantly separated into 3 groups. The high ZOI value was obtained when 1.5-2.5% (w/v) of peptone were used; 1.5% (w/v) was selected because it was the minimum concentration.

As a result from zone of inhibition, the organic nitrogen sources including yeast extract, tryptone and peptone had advantage more than inorganic sources as they also had trace mineral and ions that could enhance the production of enzymes (Francis *et al.*, 2003). Similar studies had been carried out by other workers, Gouveia *et al.* (2001) presented that production of clavulanic acid which was produced by *Streptomyces clavuligerus* were enhanced by using yeast extract and peptone in medium. Coincidentally, Schmidt *et al.* (2007) concluded that peptone as important supplement to enhance the production of antifungal metabolites was shown by *Bacillus subtilis* in concentration 0.25% to inhibit *Eutypa lata*, causal agent of dieback

of gravepine. In contrast with Tabbene *et al.* (2009) observation, ammonium succinate was proved to be an excellent nitrogen source for antibacterial activity production. The effectiveness of these inorganic nitrogen sources may be due in part to their ability to serve as direct sources of amino acids for the synthesis of the antimicrobial metabolites and/or might be implicated as antibiotic precursors.

Table 29 Effect of 2% (w/v) nitrogen sources on production of antibacterial substances by the antagonistic bacteria to inhibit growth of wilt causing bacteria after incubation at 30°C for 48 hours

Antagonistic isolate	**Nitrogen source**	**Clear zone of inhibition (mm)**				
		PT1J	**PT2X**	**RRD**	**RT1S**	**mean**
SP15	peptone	15.3±0.6	15.7±0.6	16.3±0.6	17.3±0.6	19.9 b
	$NH_4(H_2PO_4)$	0±0	0±0	0±0	0±0	0 a
	NH_4NO_3	0±0	0±0	0±0	0±0	0 a
	corn flour	0±0	0±0	0±0	0±0	0 a
	corn steep	0±0	0±0	0±0	0±0	0 a
	tryptone	14.3±0.6	14±0	16.7±0.6	16.3±2.3	18.7 b
	yeast extract	13.7±0.6	13.7±0.6	15.3±0.6	14.3±0.6	17.3 b

Table 29 (continued)

Antagonistic isolate	Nitrogen source	Clear zone of inhibition (mm)				
		PT1J	PT2X	RRD	RT1S	mean
SP38	peptone	25.3±0.6	21.3±0.6	28.3±0.6	26.7±0.6	25.4 b
	$NH_4(H_2PO_4)$	0±0	0±0	0±0	0±0	0 a
	NH_4NO_3	0±0	0±0	0±0	0±0	0 a
	corn flour	0±0	0±0	0±0	0±0	0 a
	corn steep	0±0	0±0	0±0	0±0	0 a
	tryptone	21.3±1.2	20.3±1.5	24.7±2.5	25.7±0.6	23.0 b
	yeast extract	22.7±1.2	19.3±0.6	25.3±0.6	26±0	23.0 b
SP46	peptone	22.0±1.0	20.7±2.1	22.0±2.6	21.3±1.2	21.2 c
	$NH_4(H_2PO_4)$	0±0	0±0	0±0	0±0	0 a
	NH_4NO_3	0±0	0±0	0±0	0±0	0 a
	corn flour	0±0	0±0	0±0	0±0	0 a
	corn steep	0±0	0±0	0±0	0±0	0 a
	tryptone	20.3±0.6	18.3±1.5	18.3±1.5	20.3±1.2	19.3 b
	yeast extract	21.6±0.6	19.0±2.6	19.7±1.2	19.0±0.0	19.8 b
SP58	peptone	24.3±0.6	19.7±0.6	26.3±0.6	25.7±4.0	24.0 b
	$NH_4(H_2PO_4)$	0±0	0±0	0±0	0±0	0 a
	NH_4NO_3	0±0	0±0	0±0	0±0	0 a
	corn flour	0±0	0±0	0±0	0±0	0 a
	corn steep	0±0	0±0	0±0	0±0	0 a
	tryptone	24.7±0.6	21.7±0.6	24.3±0.6	27.3±2.5	24.5 b
	yeast extract	25.7±0.6	26.3±0.6	26.7±0.6	26.7±2.9	26.3 b

Results are shown as mean ± standard error

Means followed by the same letter (a and b) within a column are not significantly different as determined by the Tukey's Test (P=0.05)

Means were compared in each antagonistic isolate

Table 30 Effect of peptone concentration on production of antibacterial substances by the antagonistic bacteria to inhibit growth of wilt causing bacteria after incubation at 30°C for 48 hours.

Antagonistic isolate	Peptone (%)	Clear zone of inhibition (mm)				
		PT1J	PT2X	RRD	RT1S	mean
SP15	0	0±0	0±0	0±0	0±0	0 a
	0.1	7.7±0.6	7.7±0.6	7.7±1.2	7.7±0.6	7.7 b
	0.5	7.7±0.6	8.0±0.0	7.3±0.6	7.0±0.0	7.5 b
	1.0	10.3±0.6	9.7±0.6	10.3±0.6	9.7±0.6	10 c
	1.5	11.3±0.6	11.3±0.6	12.3±0.6	13.0±1.0	12 d
	2.0	14.7±0.6	14.7±0.6	15.3±0.6	14.0±1.0	14.7 e
	2.5	14.7±0.6	14.3±0.6	15.7±0.6	15.3±0.6	15 e
SP38	0	0±0	0±0	0±0	0±0	0 a
	0.1	15.3±2.1	10.7±1.2	14.0±1.0	13.7±1.2	13.4 b
	0.5	18.7±1.2	16.3±0.6	19.7±1.5	18.3±2.9	18.3 c
	1.0	21.7±0.6	18.3±2.3	20.0±1.0	20.3±0.6	20.1 d
	1.5	22.7±1.2	19.0±1.0	21.7±1.5	20.3±2.9	20.9 d
	2.0	25.0±0.0	23.7±0.6	24.3±0.6	23.7±3.2	24.2 e
	2.5	24.3±0.6	22.7±0.6	24.7±0.6	23.0±2.0	23.7 e
SP46	0	7.7±0.6	7.3±0.6	0±0	0±0	3.8 a
	0.1	7.7±0.6	7.3±0.6	0±0	0±0	3.8 a
	0.5	13.3±1.2	14.0±0.0	19.3±1.2	14.3±0.6	15.3 b
	1.0	14.0±1.0	17.0±0.0	21.3±1.2	16.0±1.7	17.1 b,c
	1.5	22.7±2.3	22.0±1.0	24.3±0.6	22.0±0.0	22.8 c
	2.0	18.3±1.2	18.0±1.0	23.3±0.6	16.7±0.6	19.1 b,c
	2.5	19.0±0.0	19.7±2.1	24.0±0.0	18.0±1.0	20.2 b,c

Table 30 (continued)

Antagonistic isolate	Peptone (%)	Clear zone of inhibition (mm)				
		PT1J	PT2X	RRD	RT1S	mean
SP58	0	0±0	0±0	0±0	0±0	0 a
	0.1	15.7±0.6	17±0	13.3±0.6	12.3±0.6	14.6 b
	0.5	18.7±0.6	18.3±0.6	18.0±0.0	21.3±1.2	19.1 c
	1.0	17.3±0.6	18.3±0.6	19.3±0.6	21.7±0.6	19.2 c
	1.5	20.3±0.6	19.7±0.6	22.7±0.6	22.3±1.2	21.3 c,d
	2.0	20.0±1.0	20.3±0.6	24.3±1.2	22.3±0.6	21.8 c,d
	2.5	22.7±0.6	22.7±0.6	23.3±0.6	22.7±0.6	22.8 d

Results are shown as mean ± standard error

Means followed by the same letter (a, b, c, d and e) within a column are not significantly different as determined by the Tukey's Test (P=0.05)

Means were compared in each antagonistic isolate

7.3 Media pH

The suitable pH of media for antibacterial production *Bacillus subtilis* SP15 was pH 8. From optimazation results, the ZOI value of media pH 8, 9, 10 and 11 had significantly higher than other media pH. The media pH 8 was selected for cultivation because it was near neutral pH which was suitable for bacterial growth and easy to prepare.

In *Ps. mosselii* SP38 optimization, the media pH 4, 5, 6, 7, 8 and 9 were significantly different from media pH 10 and 11. The media pH 7 was selected for cultivation because it was neutral pH which was suitable for bacterial growth and easy to prepare.

In *Ps. mosselii* SP46 optimization, the media pH 5, 6, 7, 8, 9, 10 and 11 were significantly classified in the same group. In similar with *Ps. mosselii* SP38, the media pH 7 was selected for cultivation because it was neutral pH which was suitable for bacterial growth and easy to prepare.

For *Ps. aeruginosa* SP58, the inhibiting substances were not produced when media pH 4 and 11 were used. The media pH 5, 6, 7, 8, 9 and 10 were significantly classified in the same group, the media pH 7 was selected for cultivation because it was neutral pH which was suitable for bacterial growth and easy to prepare.

According to the results, it was found that the inhibition zone produced by antagonists were moderately different at various pH value. Almost antagonistic bacteria had high antimicrobial substance production at neutral pH ranging from 6-8. This result was similar with report of Chen *et al.* (2002) that *Bacillus* sp. were cultured in media pH 7.5 for high activity of elastase. Their experiment was evaluated by fractional factorial design.

Table 31 Effect of initial media pH of modified TSB medium on production of antibacterial substances by the antagonistic bacteria to inhibit growth of wilt causing bacteria after incubation at 30°C for 48 hours

Antagonistic isolate	pH	Clear zone of inhibition (mm)				
		PT1J	PT2X	RRD	RT1S	mean
SP15	4	0±0	0±0	0±0	0±0	0 a
	5	9.7±0.6	9.7±0.6	9.3±0.6	9.0±0.0	9.4 b
	6	10.7±0.6	10.3±0.6	10.0±1.0	10.3±0.6	10.3 b
	7	11±0.0	12.3±0.6	11.0±0.0	12.3±0.6	11.7 c
	8	12.0±1.0	13.3±0.6	13.0±0.0	13.3±0.6	12.9 c,d
	9	12±1.7	13.3±0.6	13.3±0.6	14.3±0.6	13.3 d
	10	12.7±0.6	14.3±0.6	14.3±0.6	14.7±0.6	14 d
	11	0±0	0±0	0±0	0±0	0 a
SP38	4	0±0	0±0	0±0	0±0	0 a
	5	22.3±0.6	20.3±2.5	23.0±0.0	22.7±2.5	22.1 b
	6	21.3±3.1	20.7±2.1	22.3±1.2	22.0±1.0	21.6 b
	7	22.7±1.2	20.3±0.6	21.7±1.2	21.7±0.6	21.6 b
	8	19.7±2.1	18.0±1.0	22.0±1.0	21.3±2.1	20.3 b
	9	20.0±1.7	20.7±0.6	21.3±3.5	22.3±2.5	21.1 b
	10	0±0	0±0	0±0	0±0	0 a
	11	0±0	0±0	0±0	0±0	0 a
SP46	4	0±0	0±0	0±0	0±0	0 a
	5	22.0±0.0	19.3±1.2	20.0±0.0	22.7±1.2	21 b
	6	20.0±2.0	18.3±1.5	18.3±1.5	21.3±1.2	19.5 b
	7	19.7±2.1	19.6±1.5	20.7±1.2	21.7±1.5	20.4 b
	8	19.7±2.5	18.3±0.6	20.7±1.2	21.7±1.5	20.1 b
	9	18.0±1.7	18.7±2.1	22.0±0.0	20.3±1.5	19.8 b
	10	17.0±0.0	19.0±1.0	21.7±0.6	19.7±0.6	19.3 b
	11	20.0±2.0	18.7±2.1	21.7±1.2	20.0±0.0	20.1 b

Table 31 (continued)

Antagonistic isolate	pH	Clear zone of inhibition (mm)				
		PT1J	PT2X	RRD	RT1S	mean
SP58	4	0±0	0±0	0±0	0±0	0 a
	5	26.3±1.2	21.7±1.2	25.7±1.2	23.7±1.2	24.3 b
	6	23.7±1.5	21.0±1.0	25.7±1.2	21.7±1.5	23 b
	7	21.0±1.0	21.3±1.5	25.7±1.2	21.0±1.7	22.3 b
	8	20.7±1.2	20.7±0.6	26.3±1.2	20.7±0.6	22.1 b
	9	23.3±1.5	20.7±0.6	27.0±1.7	20.7±1.2	22.9 b
	10	20.7±2.1	21.3±1.5	0±0	22±2	16 b
	11	0±0	0±0	0±0	0±0	0 a

Results are shown as mean ± standard error

Means followed by the same letter (a, b, c and d) within a column are not significantly different as determined by the Tukey's Test (P=0.05)

Means were compared in each antagonistic isolate

7.4 Effect of incubating temperature

The effects of incubating temperature on antimicrobial substance production were shown in table 32. The optimum temperature for cultivation of *B. subtilis* SP15 was 30°C, whereas *Ps. mosselii* SP38, *Ps. mosselii* SP46 and *Ps. aeruginosa* SP58 was 25°C. However, the antimicrobial substance production at 25, 30 and 37°C were slightly different at P<0.05. This result might be indicated that all antagonistic bacteria might be cultured and produced inhibiting substances at temperature range around room temperature. Temperature is one of a crucial factor concerned in biological control due to the bioproduct required a simple physical factor to store. This study was in accordance with El-Sersy *et al.*, (2010) that the highest

antimicrobial protein was produced by *B. licheniformis* at 30°C in suppression of *Staphylococcus aureus* growth.

Table 32 Effect of temperature on production of antibacterial substances by the antagonistic bacteria to inhibit growth of wilt causing bacteria after cultivation in modified TSB medium for 48 hours

Antagonistic isolate	Temperature (°C)	Clear zone of inhibition (mm)				
		PT1J	PT2X	RRD	RT1S	mean
SP15	25	8.7±0.6	10.3±0.6	9.0±0.0	8.0±0.0	9.0 a
	30	17.3±0.6	13.0±1.0	14.7±0.6	16.3±0.6	15.3 b
	37	15.7±0.6	12.3±0.6	13.7±1.2	14.7±0.6	14.1 b
	45	15.7±0.6	11.3±0.6	13.7±0.6	12.0±1.0	13.2 b
SP38	25	22.7±0.6	23.7±0.6	23.0±1.7	23.7±1.2	23.3 c
	30	21.7±0.6	22.0±2.0	21.3±1.2	22.3±0.6	21.8 b,c
	37	21.0±1.7	23.7±1.2	20.3±1.5	19.7±0.6	21.2 b
	45	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0 a
SP46	25	20.7±0.6	24.3±0.6	23.7±1.2	23.7±1.2	23.1 b
	30	20.3±0.6	24.0±1.0	22.0±1.7	22.7±0.6	22.3 b
	37	22.3±0.6	23.3±0.6	20.7±1.2	21.7±0.6	22.0 b
	45	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0 a
SP58	25	22.3±0.6	24.7±0.6	24.0±1.0	23.3±0.6	23.6 c
	30	22.3±0.6	24.3±1.2	24.7±0.6	22.3±0.6	23.4 c
	37	20.7±1.2	21.3±1.2	23.7±1.2	20.7±0.6	21.6 b
	45	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0 a

Results are shown as mean ± standard error

Means followed by the same letter (a, b and c) within a column are not significantly different as determined by the Tukey's Test (P=0.05)

Means were compared in each antagonistic isolate

The results of optimization production were concluded in table 33. Interestingly, it was found that the antibacterial activity had risen if the bacterial cell growth was increased. It was reported that variation in the composition of the culture medium could affect both the bacterial yield and antibacterial compounds (Tabbene *et al.*, 2009).

Table 33 Summary results of antimicrobial product optimization

Antagonistic isolate	Optimum compositions and conditions					
	C-source	C-source (%)	N-source	N-source (%)	pH	Temperature (°C)
SP15	glucose	0.5	peptone	2.0	8	30
SP38	sucrose	0.5	peptone	2.0	7	25
SP46	glucose	0.5	peptone	1.5	7	25
SP58	sucrose	0.5	peptone	1.5	7	25

The information of nutritional requirements of biocontrol agents will help researchers to introduce them to appropriate pathosystems. The production of antimicrobial compounds by microbial cells is strongly influenced by the composition of the medium, which is in turn determined by the carbon sources, nitrogen sources and by culture conditions such as pH medium, incubation temperature, and agitation rate. Therefore, optimization of these parameters is necessary for the overproduction of these antimicrobial products (Tabbene *et al.*, 2009). However, the C:N ratio of the medium used to produce antagonists for greenhouse trials had little effects on the efficacy of the antagonists strain in greenhouse study (Schisler *et al.*, 2004). Further

studies will be focused on the characterization of the different antibacterial compounds and their applicability in bioprocess.

8. Study on microbial substances of antagonistic bacteria

8.1 Siderophore production

Four isolates of antagonistic bacteria were preliminary screened for siderophore production on MGs-1 medium containing CAS. All antagonists were grown on CAS agar and yellow or pink halo formed around the colonies (Figure 51). The culture supernatants were further categorized for hydroxamate or catechol compounds by using specific chemical tests. The range of hydroxamate and catecholate production was 5.0-49.5 μM and 1.6-3.5 μM, respectively (Table 34). The ability of antagonistic bacteria to produce siderophore was widely distributed between antagonistic isolates. Moreover, the 2-fold concentrated broth culture of each antagonistic bacteria were tested, the ultra-filtrates of bacterial supernatants were demonstrated the higher level of siderophore concentration ranging from 10.0-70.8 μM and 1.9-4.2 μM, respectively. This result concluded that the ultrafiltration technique was very beneficial for metabolite concentration. This experiment was examined with 96 well-microplate due to it was easier than test tube in term of the time and amount of substances.

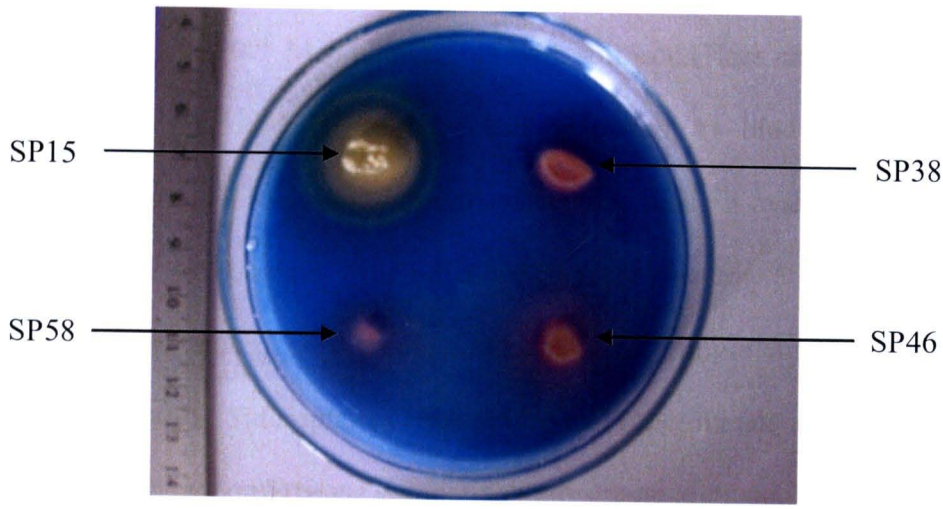


Figure 51 MGs-1-CAS agar plate assay showing the yellow or pink halo after incubation at 30°C for 3 days

Table 34 Siderophore production by antagonistic bacteria in MGs-1 medium incubated at 30°C for 2 days

Isolate	Hydroxamate concentration (μM)		Catecholate concentration (μM)	
	Culture broth	Concentrated culture broth	Culture broth	Concentrated culture broth
SP15	5.00±2.12 a	10.00±3.68 a	1.64±0.50 a	1.90±0.39 a
SP38	27.12±5.68 b	41.00±3.37 b	3.50±0.37 c	4.24±0.52 c
SP46	45.87±6.12 c	63.87±6.57 c	1.75±0.19 a	2.25±0.32 a,b
SP58	49.50±7.64 c	70.75±4.49 d	2.69±0.93 b	2.71±0.42 b

Results are shown as mean ± standard error

Means followed by the same letter (a, b and c) within a column are not significantly different as determined by the Tukey's Test (P=0.05)

In aerated, neutral or alkaline soils, Fe^{3+} is poorly soluble; the total soluble Fe^{3+} represent about $1x10^{-10}$ M at equilibrium with soil iron (Haas and Défago, 2005). Siderophores are produced by various soil microbes to bind Fe^{3+} from the environment, transport it back to the microbial cell and make it available for growth. Soil bacteria have to compete with other rhizosphere bacteria and fungi for iron supply and therefore siderophore production may be very important for their growth (Khamna *et al.*, 2009). Siderophores chelate iron and other metals and contributes to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats. Siderophores may directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria (Maleki *et al.* 2010).

Results from the qualitative and quantitative estimations of siderophore by *B. subtilis* SP15, *Ps. mosselii* SP38, *Ps. mosselii* SP46 and *Ps. aeruginosa* SP58 showed that they were a powerful producer of siderophores. Several published data have reported the ability of *Bacillus* spp. and *Pseudomonas* spp. to produce siderophore. Ahmadzadeh and Sharifi Teharani (2009) demonstrated that siderophore from fluorescent pseudomonads could promote the ability to inhibit *Rhizoctonia solani* in common bean. Jagadeesh *et al.* (2001) reported that fluorescent *Pseudomonas* which had an activity against *R. solanaceraum* causing bacterial wilt disease in tomato could produce siderophore. Chaiharn *et al.* (2009) discovered that *B. firmus* D 4.1 and *Ps. aureofaciens* AR1 could produce only hydroxamate type approximately 100 μM. These strains also use as potential biological control agent for rice fungal pathogens in Thailand. Nevertheless, most biocontrol strains of *Pseudomonas* spp. with a proven

effect in plant bioasssays produce one or several antibiotic compound that are unrelated to typical siderophores.

From the results of the siderophore characterization, although bacteria grew well in culture medium but some antagonistic strains secreted trace amounts of siderophore, however, they were still considered positive siderophore-producing isolates. Siderophore are the secondary metabolite, a microorganism will produce secondary metabolite when its growth rate decreases. For this reason, siderophore concentration is not correlated with bacterial turbidity. In addition, *Bacillus subtilis* SP15 showed a large positive reaction on CAS agar but it produced a small amounts of hydroxamate and catecholate. It is suggested that the detection in liquid media should be used to confirm siderophore production. The sensitivity of chemical reaction method was higher than the CAS agar plate assay in term of the low accumulation of siderophores (Pérez-Miranda *et al.*, 2007).

The resulting siderophores postulate hypothesis revealed that plant growth promoting bacteria could exert their plant growth promotion activity by depriving of iron. For example, under greenhouse conditions, *Ps. putida* strain B10 could suppress *Fusarium* wilt and take-all disease, this suppression was lost when the soil was amended with iron, which repressed siderophore production in *Ps. putida* (Haas and Défago, 2005).

8.2 Phenazine production

The antagonistic bacterial isolates were preliminary screened for phenazine production by pigment detection under UV light at 254 nm using KB plates. Among four antagonists, only three strains *Ps. mosselii* SP38, *Ps. mosselii* SP46 and *Ps.*

aeruginosa SP58 showed fluorescent UV absorbance (Figure 52). The only positive pigment-producing isolates including *Ps. mosselii* SP38, *Ps. mosselii* SP46 and *Ps. aeruginosa* SP58 were then tested for the production of phenazines by TLC analysis. The chromatographic of antagonistic substances were compared with standard compounds. It was found that both the antagonistic and reference compounds behaved similarly (Figure 53). Analysis of silica plate under UV light demonstrated a 2-hydroxyphenazine spot (R_f = 0.39) for substances from *Ps. mosselii* SP46 and *Ps. aeruginosa* SP58. The phenazine-1-carboximide (PCA) spot (R_f = 0.82) were displayed only in standard compound. Meanwhile, *Ps. mosselii* SP38 and standard compound showed the spot at R_f = 0.65. Therefore, it was found that only antagonistic bacteria *Ps. mosselii* SP38, *Ps. mosselii* SP46 and *Ps. aeruginosa* SP58 could produce phenazine derivatives. However, the production and characterization of phenazine compounds should be further studied using HPLC, mass spectrometry or NMR techniques.

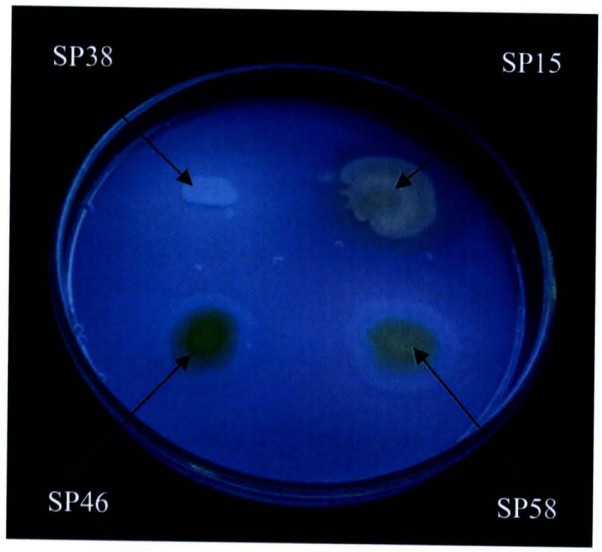


Figure 52 The antagonistic culture on KB plate under UV light showing fluorescent light around colonies (SP38, SP46, SP58).

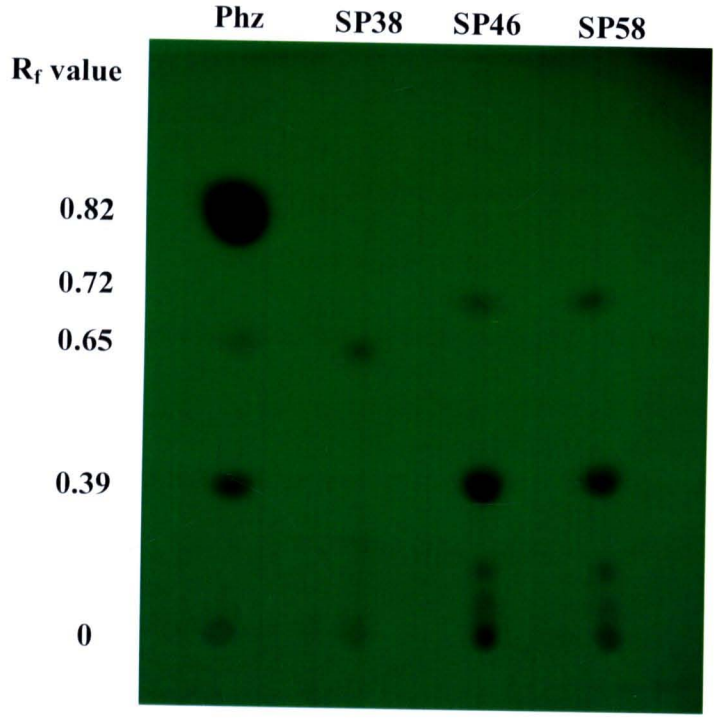


Figure 53 Silica plate chromatogram showing the spot of standard phenazine (Phz) and antagonistic bacterial extracts under UV light at 254 nm

Earlier studies of phenazine producing pseudomonads had been carried out by other workers. Rane *et al.* (2007) revealed that PCA produced by *Ps. aeruginosa* was a strong and broad range antifungal metabolite. The PCA was purified and crystalline PCA was characterized by HPLC. Rosales *et al.* (1995) demonstrated that *Pseudomonas aeruginosa* In-b-109 and In-b-784 had antimicrobial activity against *Rhizoctonia solani*, the rice sheath blight pathogen. These antibacterial substances were PCA that detected by TLC assay. Perneel *et al.* (2007) reported that *Ps. mosselii* CMR5c and CMR12a isolated from cocoyam rhizosphere could produce PCA, whereas, *Pseudomonas aeruginosa* PNA1 produced phenazine-1carboxamide (PCN). In addition, *Ps. chlororaphis* PCL1391 as a biocontrol agent of tomato root rot caused

by *Fusarium oxysporum*, displayed the ability to produce PCN (Chin-A-Wong *et al.*, 1998).

The mode of action of phenazines is partly understood. Phenazines, which are analogues of flavin coenzymes, inhibit electron transport and are known to have various pharmacological effects on animal cells. If phenazines mobilize iron in soils, they could be considered as auxiliary siderophores and this might explain the greater ecological fitness of the phenazine producers. Althogh it might be assumed that antibiotics help the microbial producers to define their ecological niches against antibiotic-sensitive competitors in the rhizosphere, there is little experimental evidence to support this assumption.

Accordingly, four antagonistic bacterial isolates had the ability to produce high antibacterial compounds, siderophore or phenazine. This implied that the antagonists had high potential to be used as an agent for *in vivo* bioprotection of Pathumma plant. However, more detailed investigation was required to demonstrate the potential of these organisms for the biocontrol of pathogenic bacteria and in plant growth promotion which might be useful in agricultural fields in the future. It might be possible to produce the inhibitory compound by chemical process, genetically modified microorganisms or super-producer organisms. The compound or its analog would be used as pesticides.

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

9.1 Greenhouse study in 2008

The mixed culture of antagonistic bacteria were evaluated under greenhouse conditions for efficacy to control Pathumma wilt in three experiments. There was no

mixed cultures of antagonists completely protected the plants against wilt causing bacteria, although the disease incidence of all mixture was low. Plants treated with antagonistic bacteria showed a reduced number of wilt bacterial cells in soil. The treated Pathumma plants were illustrated in figure 54, 55.

The application of antagonists following inoculation with bacterial-wilt pathogens in Pathumma's rhizomes and soil in pots at initial day of cultivation had increased the healthy of Pathumma plants. The average disease incidence of antagonists was 0% except treatment 1A (Table 35). The viable cell number of antagonistic bacteria increased by 3% on average while pathogenic bacteria PT1J, D1, RRD and RT1S declined by 8, 10, 32 and 31%, respectively (Figure 56-59).

When antagonists were co-applied with pathogens on shooting pseudostems and soil, the antagonists declined by 4.4% on average while pathogenic bacteria PT1J, D1, RRD and RT1S declined by 2, 19, 35 and 32%, respectively.

Similar results were recorded when the pathogens were applied to cultivated Pathumma plants, while the antagonists were introduced to the shooting pseudostems. The cell number of antagonists declined by 3% on average while pathogenic bacteria PT1J, D1, RRD and RT1S declined by 38, 54, 38 and 34%, respectively.

In addition, when the mixed culture of antagonistic bacteria were only inoculated into the Pathumma pots, it was found that the plants were not shown wilt symptoms. According to the results of the disease incidence and the survival of antagonistic and pathogenic bacteria, it was indicated that the mixed antagonists could suppress growth of wilt causing bacteria.

Table 35 The disease incidence (%) of plants after the application of antagonistic bacteria in Pathumma pot conducted in 2008

Treatment	Disease incidence (%)		
	Experiment 1	**Experiment 2**	**Experiment 3**
1A	66	0	33
1B	0	0	0
1C	0	0	0
1D	0	0	0
1E	0	0	0
2A	0	0	0
2B	0	0	0
2C	0	0	0
2D	0	0	0
2E	0	33	0
3A	0	0	0
3B	0	0	0
3C	0	0	0
3D	0	0	0
3E	0	0	0
4A	0	0	0
4B	0	0	0
4C	0	0	0
4D	0	0	0
4E	0	0	0
Control pathogen	60	50	60

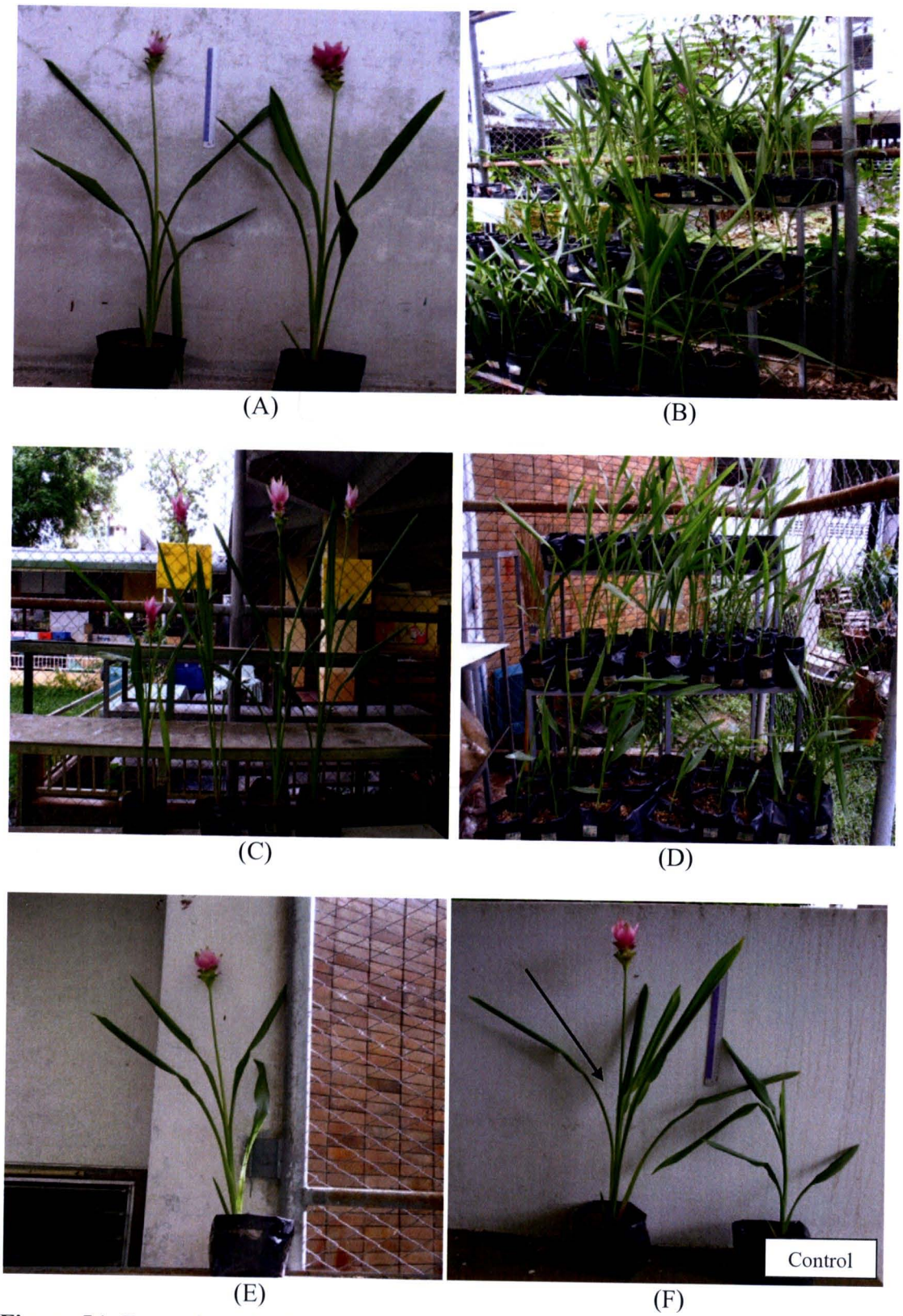


Figure 54 Examples of (A) uninoculated and (B-E, F arrow)treated plant inoculated with mixed antagonists showing healthy plants compared with (F) disease plant control

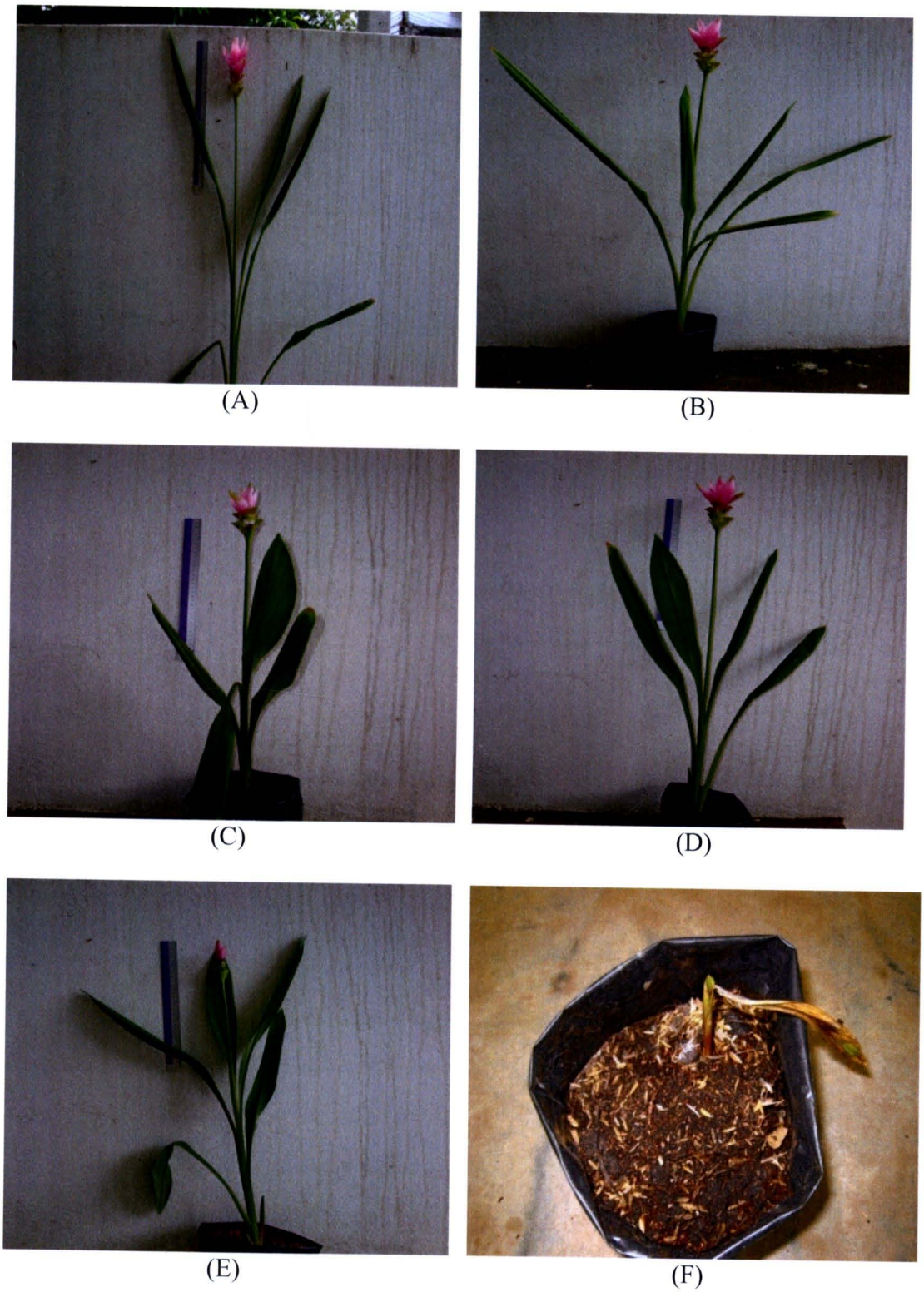


Figure 55 Examples of treated plant inoculated with mixed antagonists in various experiments; (A) 1B, (B) 2B, (C) 3B, (D) 3C, (E) 4C. (F) Disease plant control showing wilt symptoms after inoculated with pathogenic bacteria in 1 month-old plant

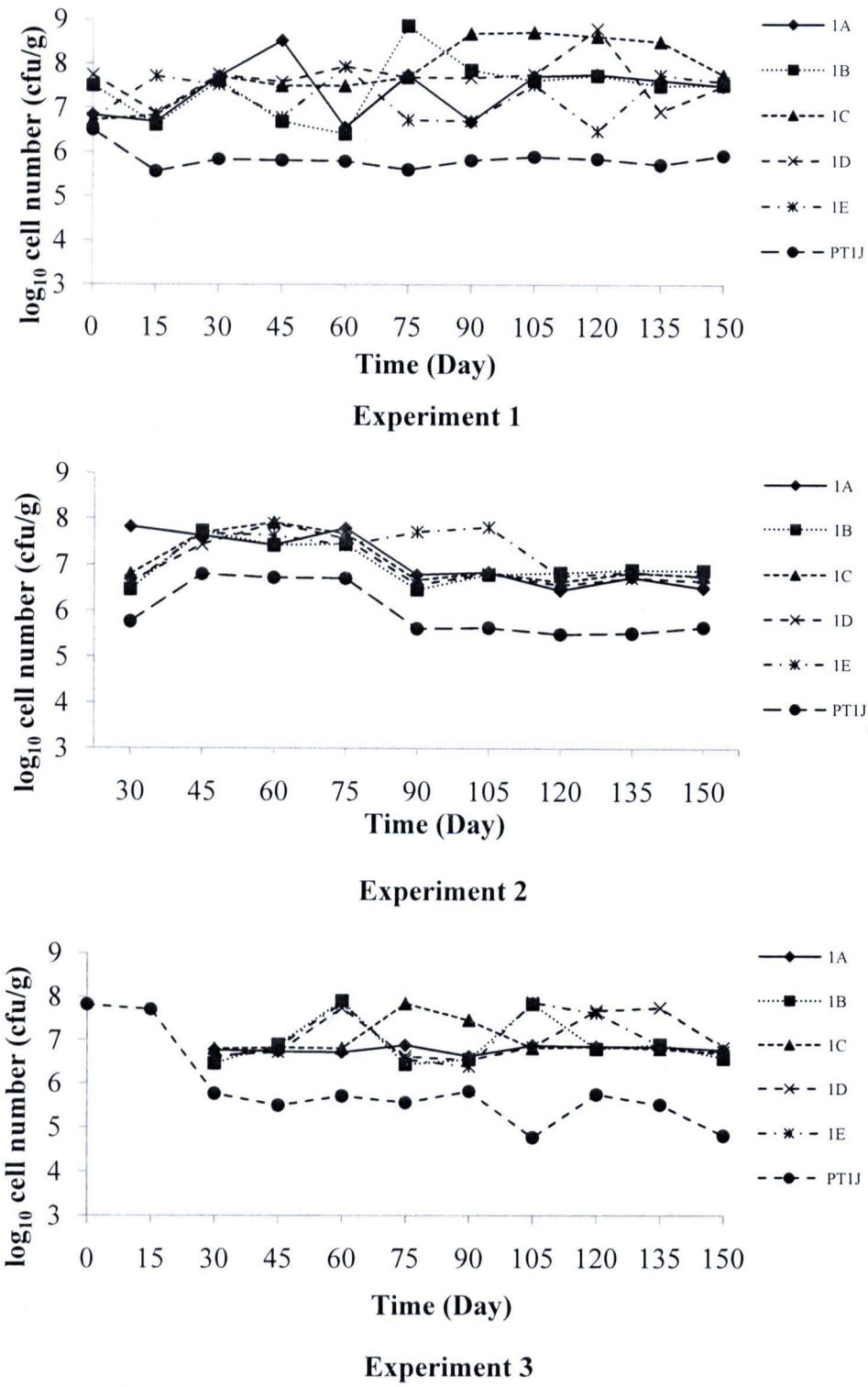


Figure 56 Viable cell count of mixed antagonists and pathogenic bacteria PT1J in soil. Three experiments were applied to Pathumma plants

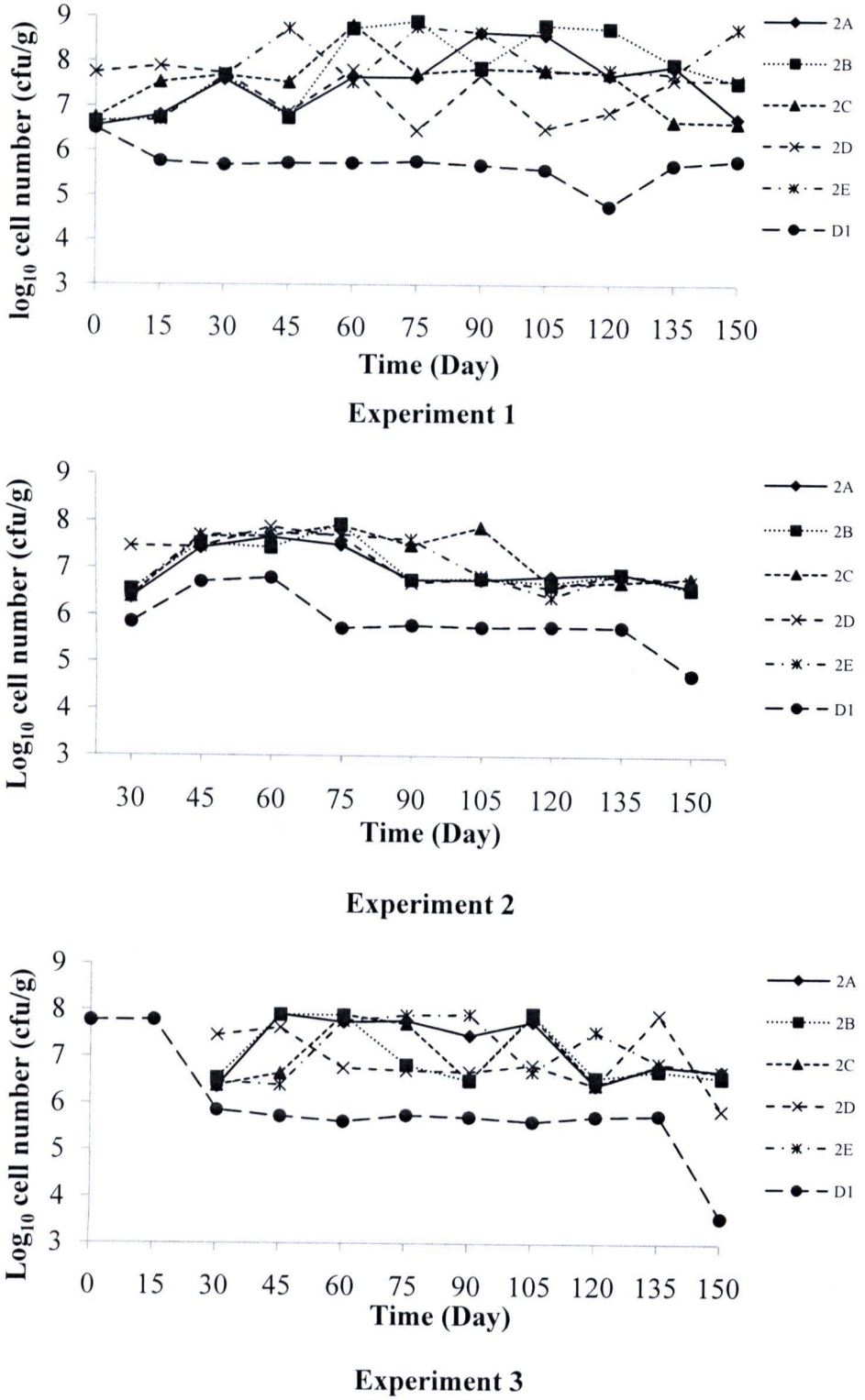
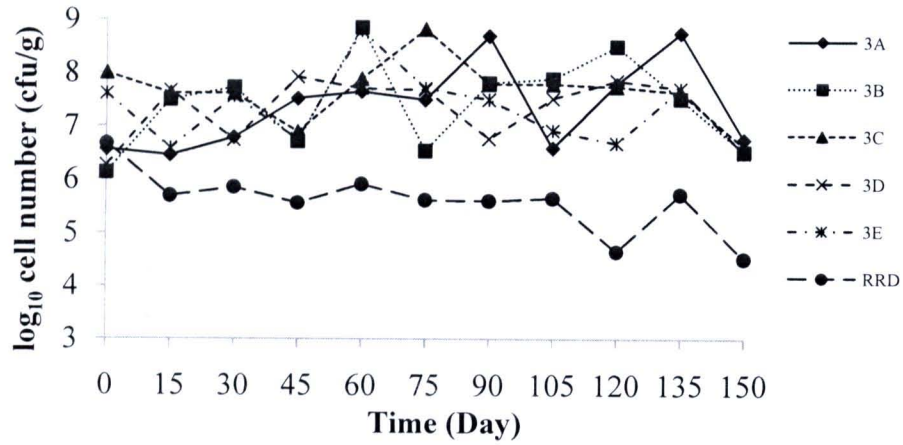
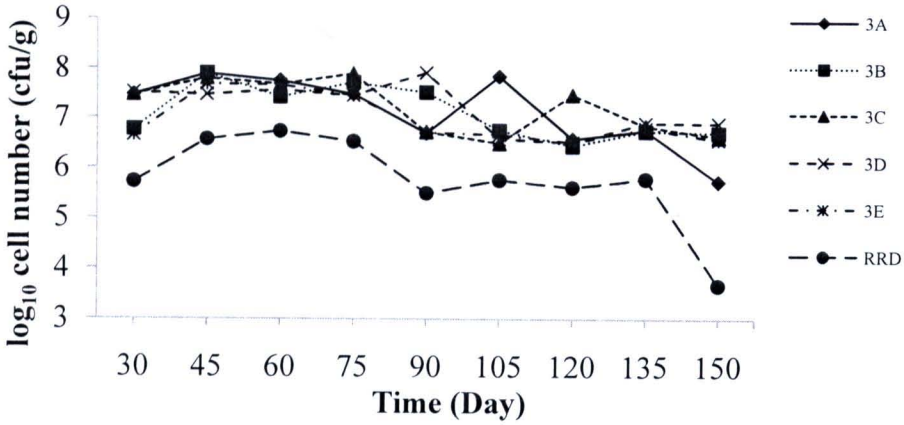


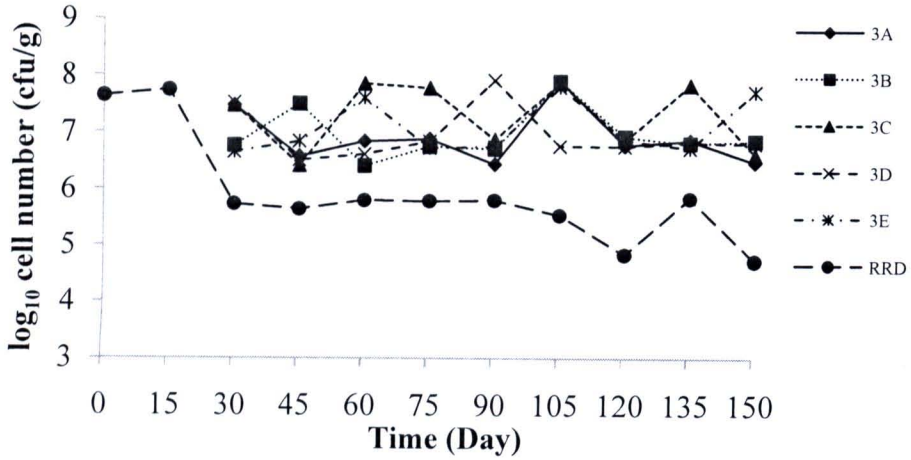
Figure 57 Viable cell count of mixed antagonists and pathogenic bacteria D1 in soil. Three experiments were applied to Pathumma plants



Experiment 1

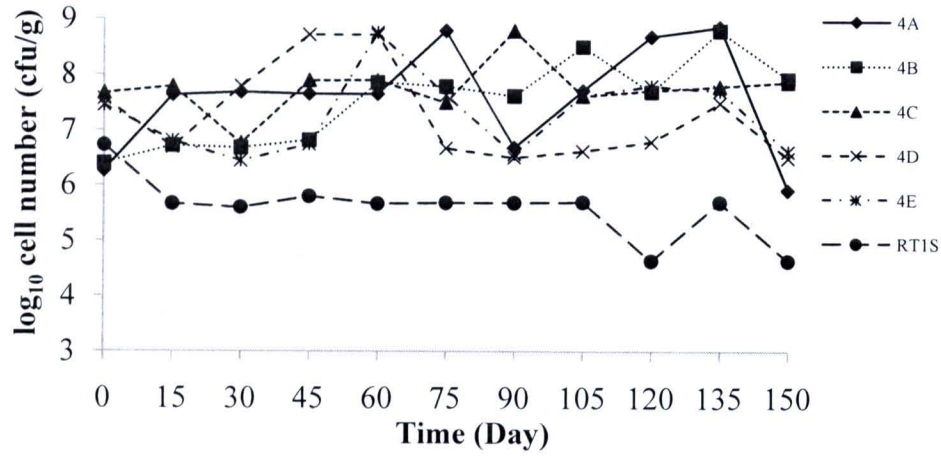


Experiment 2

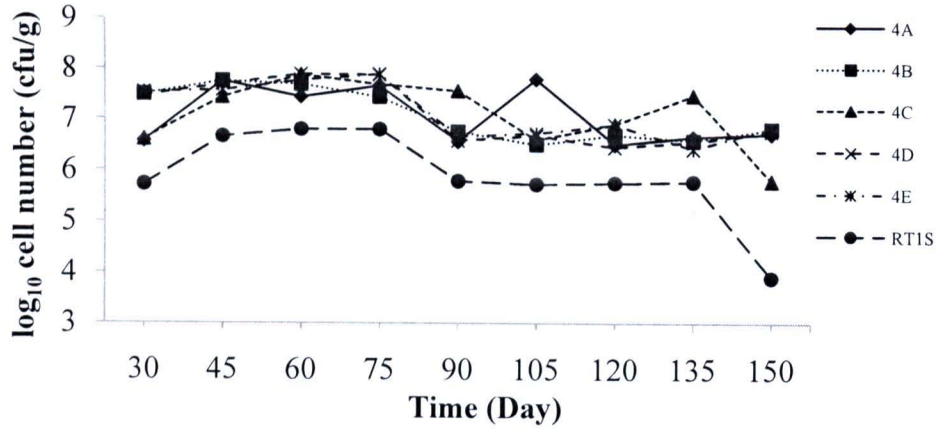


Experiment 3

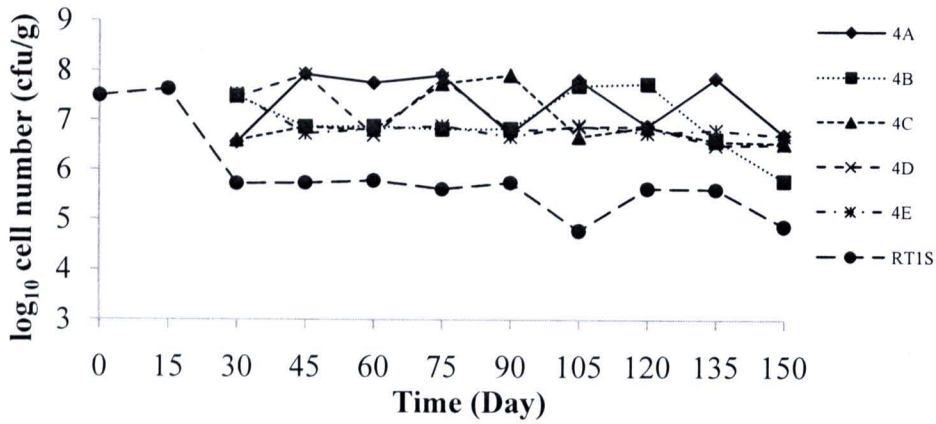
Figure 58 Viable cell count of mixed antagonists and pathogenic bacteria RRD in soil. Three experiments were applied to Pathumma plants



Experiment 1



Experiment 2



Experiment 3

Figure 59 Viable cell count of mixed antagonists and pathogenic bacteria RT1S in soil. Three experiments were applied to Pathumma plants

9.2 Greenhouse study in 2009

The application of the antagonistic bacteria and wood vinegar for control wilt-pathogens were repeatedly evaluated. The treatment of all experiments was not shown the wilt incidence except only the treatment 1D in experiment 1 while the disease incidences of the control plants were 33-66% (Table 36; Figure 60, 61).

After antagonists and pathogenic bacteria were co-applied to Pathumma rhizomes and soil before cultivation, the final cell concentration of antagonistic bacteria were declined by 12.5% on average while pathogenic bacteria PT1J, PT2X, RRD and RT1S were declined by 30, 15, 31 and 16%, respectively (62-65).

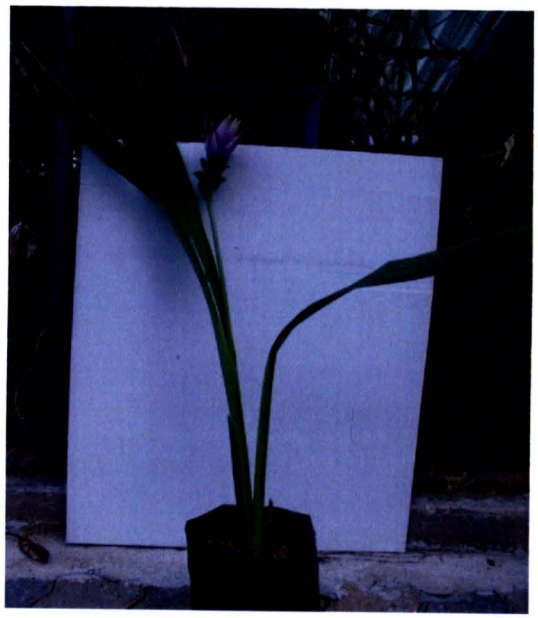
In experiment 2, when the antagonists and pathogenic bacteria were co-inoculated in Pathumma pseudostems, the quantities of antagonists were declined by 11% on average while pathogenic bacteria PT1J, PT2X, RRD and RT1S were declined by 24, 38, 40 and 35%, respectively.

The treatment of antagonists in shooting plants after the inoculation of pathogens in rhizomes resulted in bacterial reduction by 12% on average for the antagonists while pathogenic bacteria PT1J, PT2X, RRD and RT1S were declined by 37, 24, 40 and 26%, respectively.

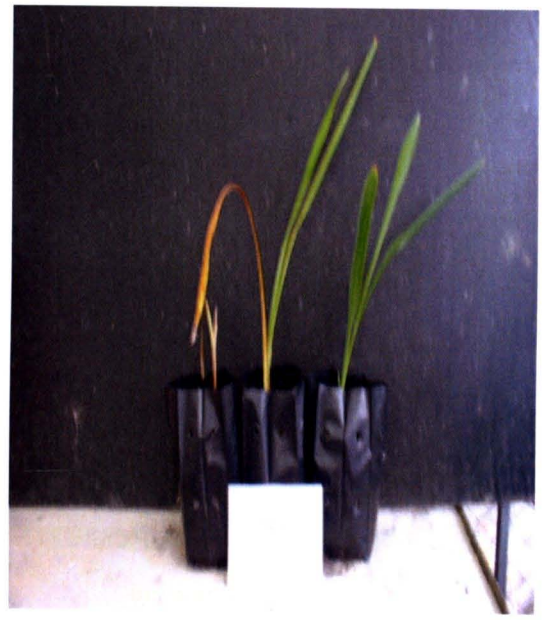
The use of wood vinegar for wilt disease control demonstrated that the amounts pathogenic bacteria PT1J, PT2X, RRD and RT1S were reduced by 25, 23, 38 and 36%, respectively. It is interesting that this by-product chemical could be effective in term of wilt-bacterial reduction, however, it caused the plant and soil dry. Thereby, the proper concentration should be investigated before use.

Table 36 The disease incidence (%) of plants after the application of antagonistic bacteria in Pathumma pot conducted in 2009

Treatment	Disease incidence (%)		
	Experiment 1	Experiment 2	Experiment 3
1A	0	0	33
1B	0	0	0
1C	0	0	0
1D	33	0	0
1E	0	0	0
1W	0	0	0
2A	0	0	0
2B	0	0	0
2C	0	0	0
2D	0	0	0
2E	0	0	0
2W	0	0	0
3A	0	0	0
3B	0	0	0
3C	0	0	0
3D	0	0	0
3E	0	0	0
3W	0	0	0
4A	0	0	0
4B	0	0	0
4C	0	0	0
4D	0	0	0
4E	0	0	0
4W	0	0	0
Control pathogen	66	33	66



(A)



(B)

(C)

(D)

Figure 60 Examples of (A) uninoculated and (B) disease plant control. (C, D) The plant treated with mixed antagonistic bacteria showing no wilt symtomps

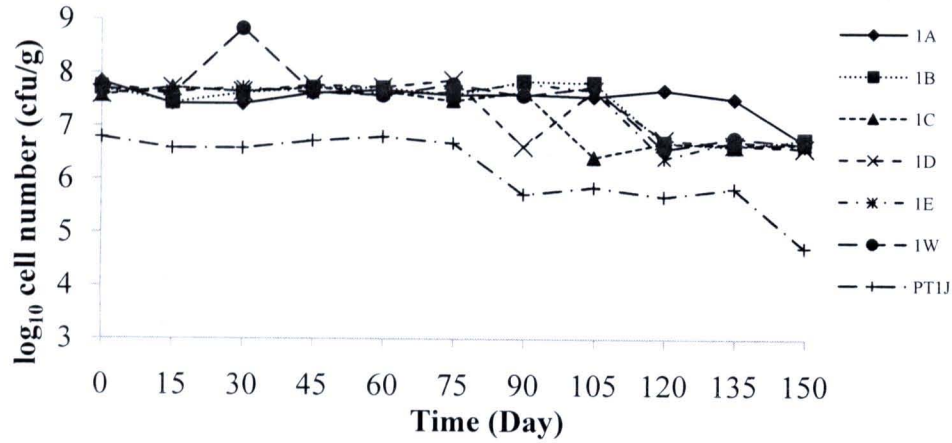
(A)

(B)

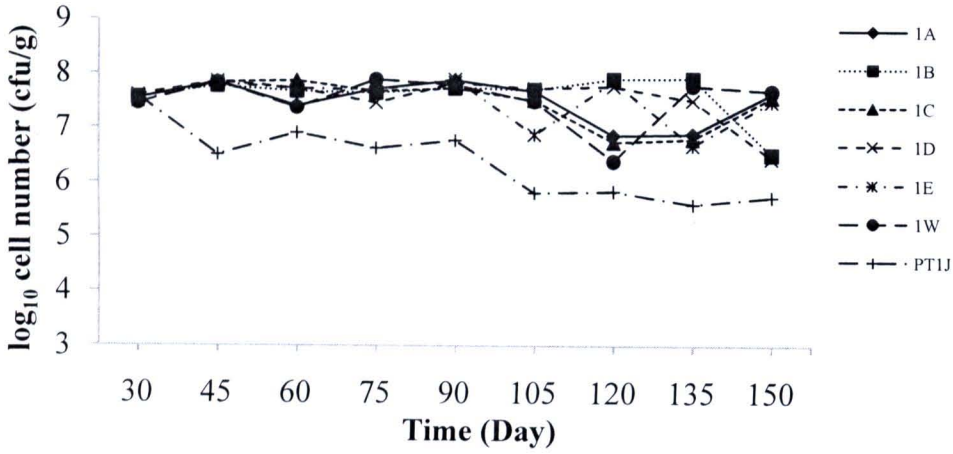
(C)

(D)

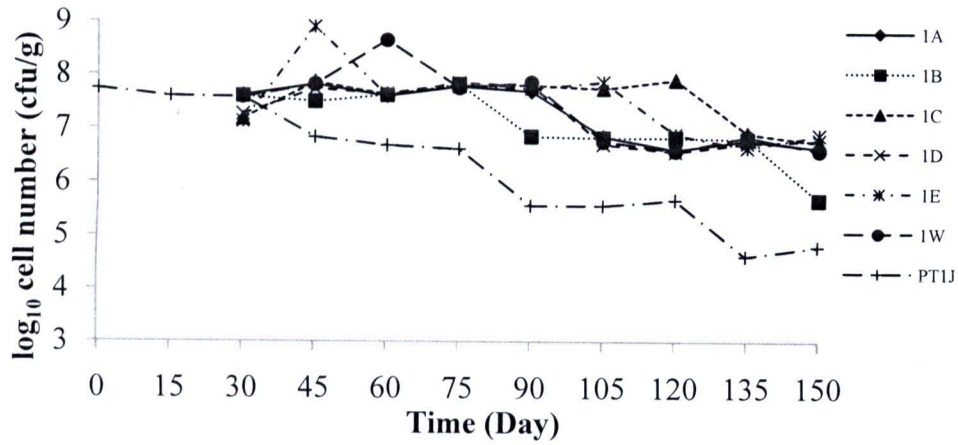
Figure 61 Examples of the plant treated with mixed antagonistic bacteria showing no wilt symptoms



Experiment 1

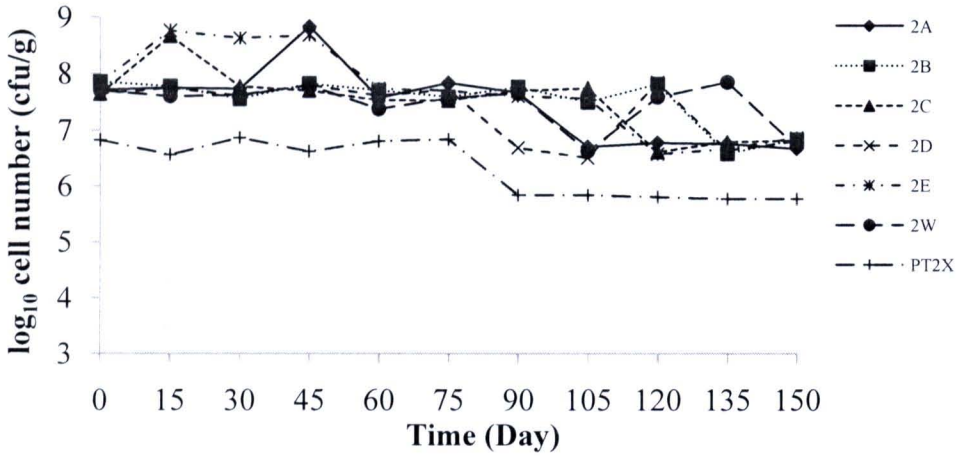


Experiment 2

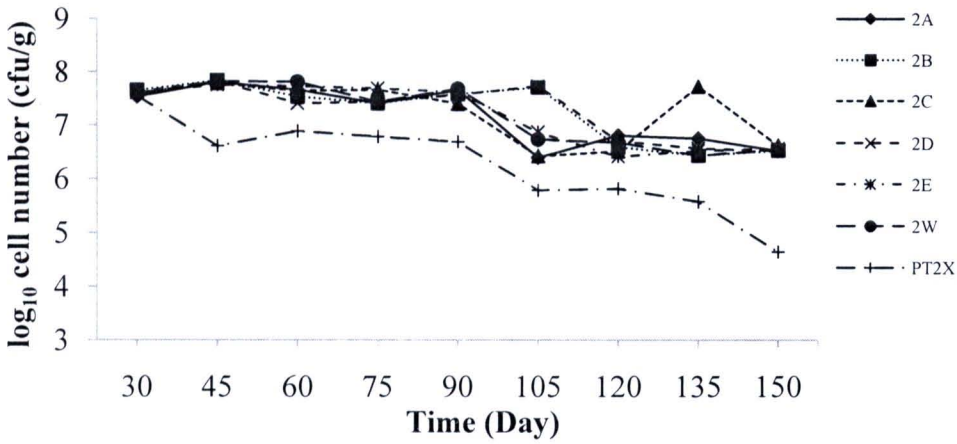


Experiment 3

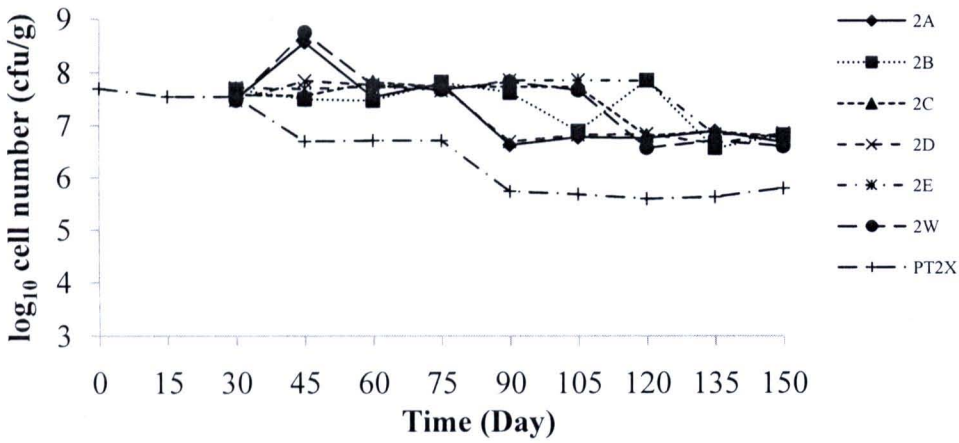
Figure 62 Viable cell count of mixed antagonists and pathogenic bacteria PT1J in soil. Three experiments were applied to Pathumma plants



Experiment 1

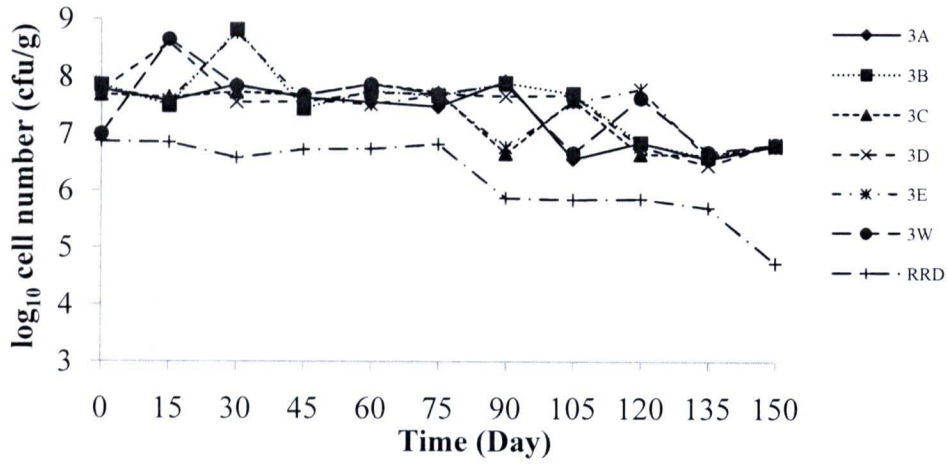


Experiment 2

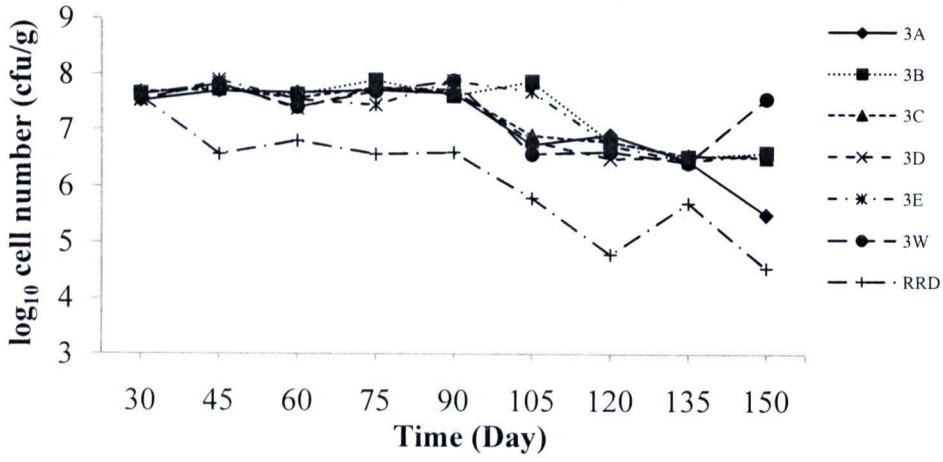


Experiment 3

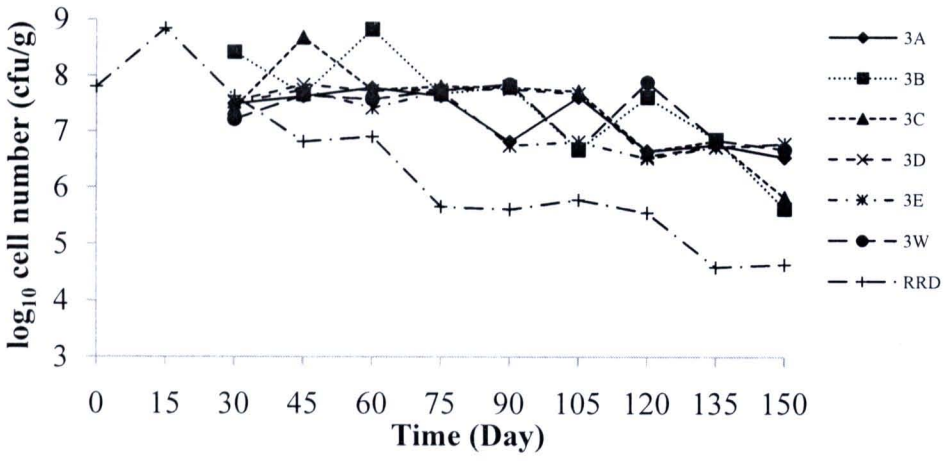
Figure 63 Viable cell count of mixed antagonists and pathogenic bacteria PT2X in soil. Three experiments were applied to Pathumma plants



Experiment 1



Experiment 2



Experiment 3

Figure 64 Viable cell count of mixed antagonists and pathogenic bacteria RRD in soil. Three experiments were applied to Pathumma plants

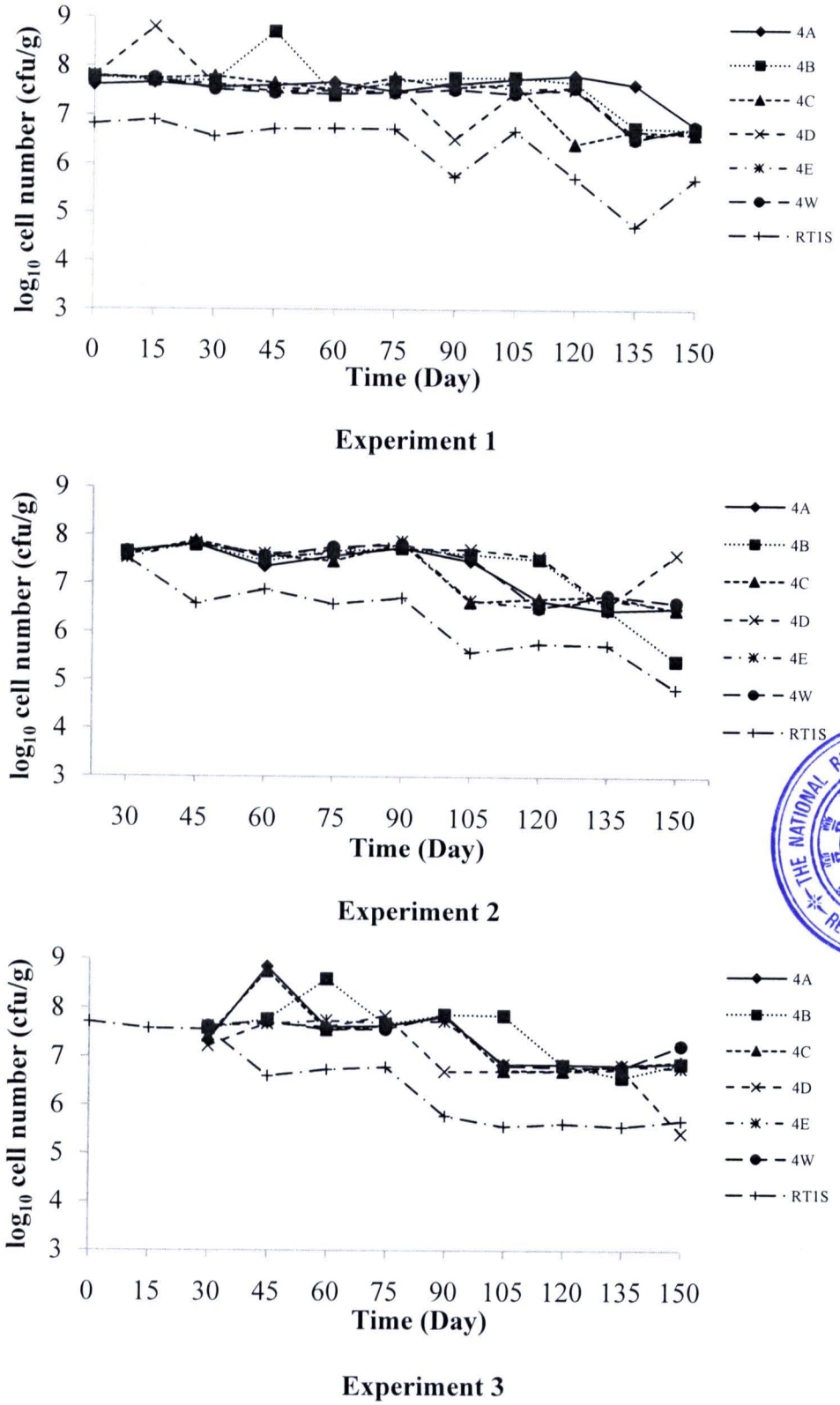


Figure 65 Viable cell count of mixed antagonists and pathogenic bacteria RT1S in soil. Three experiments were applied to Pathumma plants

9.3 Greenhouse study in 2010

When the mixed cultures of the antagonistic bacteria were introduced into Pathumma rhizomes and soil in pots upon cultivation as well as the pathogens, the plants showed slight wilt symptoms (Figure 66, 67). The disease incidences of antagonists treatment particularly in experiment 3 were increased compared with the previous study in 2008 and 2009 (Table 37). The amounts of antagonistic bacteria increased by 3.4% on average while the pathogenic bacteria PT1J, PT2X, RRD, RT1S and R227 declined by 24, 23, 37, 38 and 37%, respectively, after 5 months of examination (68-72).

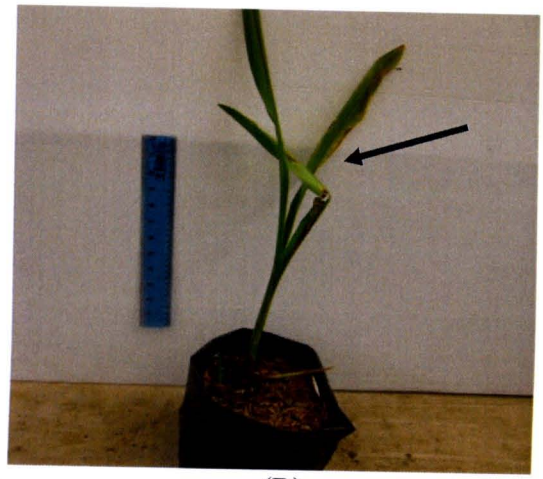
Meanwhile, when antagonistic bacteria were co-applied with the pathogens on shoots of Pathumma in pots, the amounts of antagonists declined by 9% on average while pathogenic bacteria PT1J, PT2X, RRD, RT1S and R227 declined by 24, 23, 30, 39 and 28%, respectively.

When the pathogens were applied to cultivated Pathumma, while the antagonistic bacteria were introduced to the shooting plants, the amounts of mixed antagonists declined by 9% on average while pathogenic bacteria PT1J, PT2X, RRD, RT1S and R227 declined by 24, 12, 24, 36 and 26%, respectively. Moreover, wood vinegar had also an effective to inhibit growth of wilt-bacterial strains in Pathumma pots. The pathogenic bacteria were declined by 30-40%.

Table 37 The disease incidence (%) of plants after the application of antagonistic bacteria in Pathumma pot

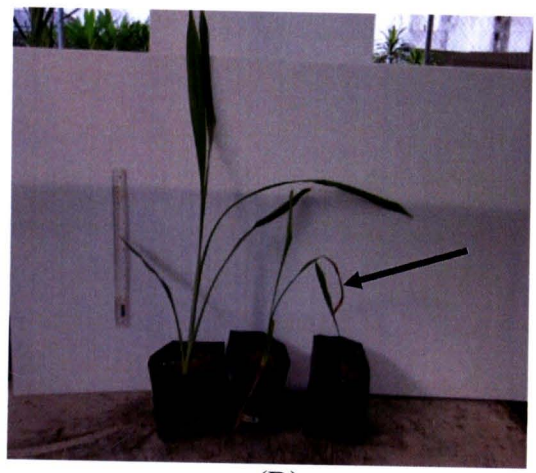
Treatment	Disease incidence (%)		
	Experiment 1	Experiment 2	Experiment 3
1A	0	0	100
1B	0	0	100
1C	0	0	66
1D	33	0	33
1E	0	0	66
1W	0	0	66
2A	0	0	66
2B	0	0	66
2C	33	0	66
2D	100	0	100
2E	33	0	33
2W	0	0	0
3A	0	0	33
3B	0	0	66
3C	0	0	66
3D	0	0	0
3E	0	0	33
3W	0	0	0
4A	0	0	0
4B	0	0	0
4C	0	0	0
4D	0	0	0
4E	0	0	0
4W	0	0	33
5A	0	0	33
5B	0	0	33
5C	0	0	33
5D	0	0	0
5E	0	0	33
5W	0	0	0
Control pathogen	66	66	66

(A)



(B)

(C)



(D)

(E)

(F)

Figure 66 Examples of (A) uninoculated and (B) disease plant control. (C, D) The plant treated with mixed antagonistic bacteria showing wilt symtomps. (E,F) The plant treated with mixed antagonistic bacteria showing no wilt symtomps

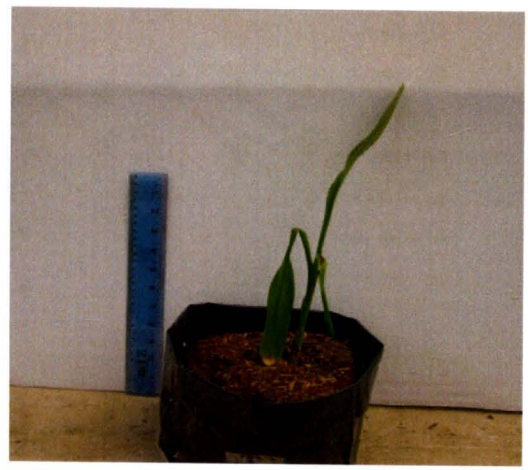
(A)

(B)

(C)

(D)

(E)



(F)

Figure 67 Examples of (A-E) plant treated with mixed antagonistic bacteria compared with (F) wilt disease plant control

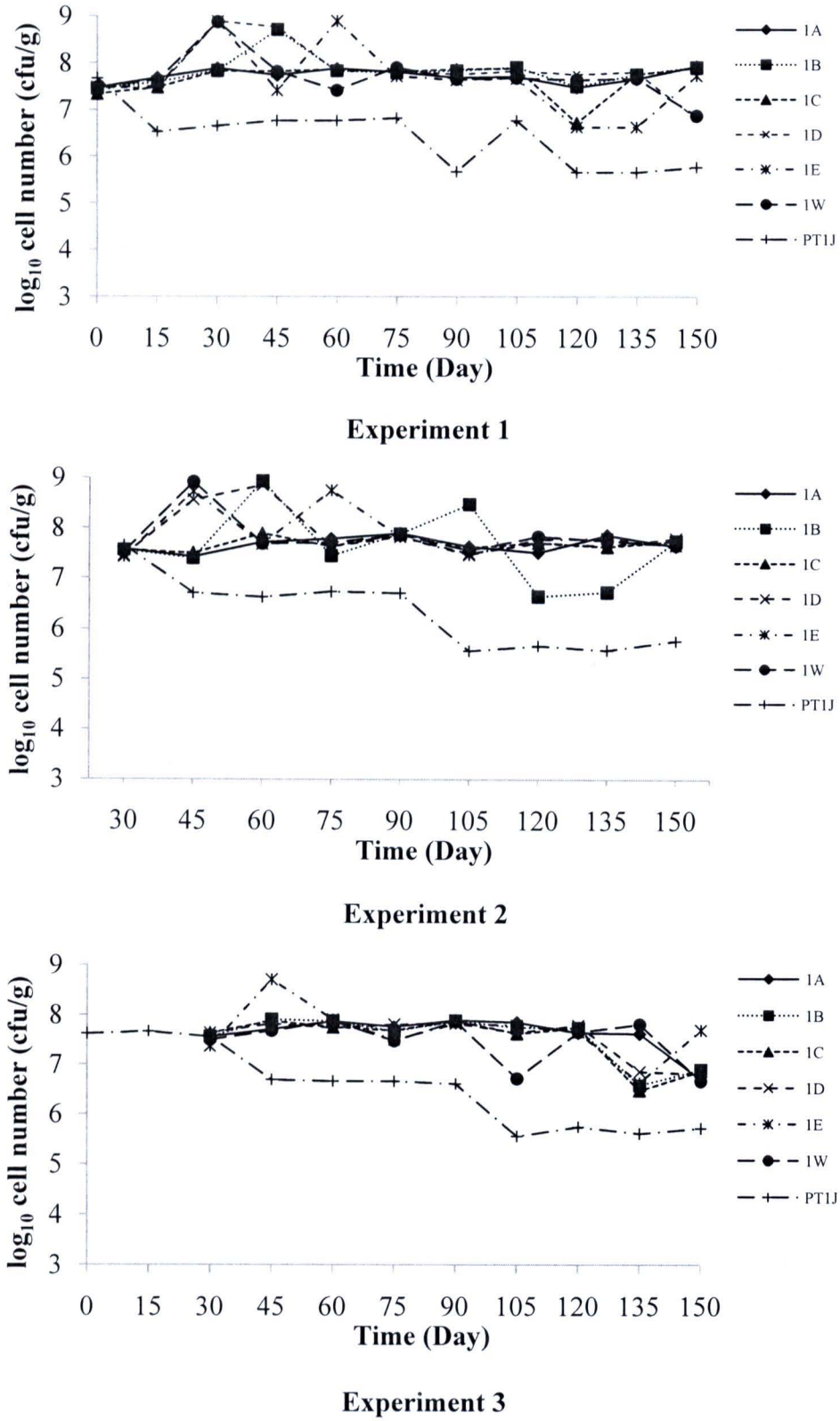
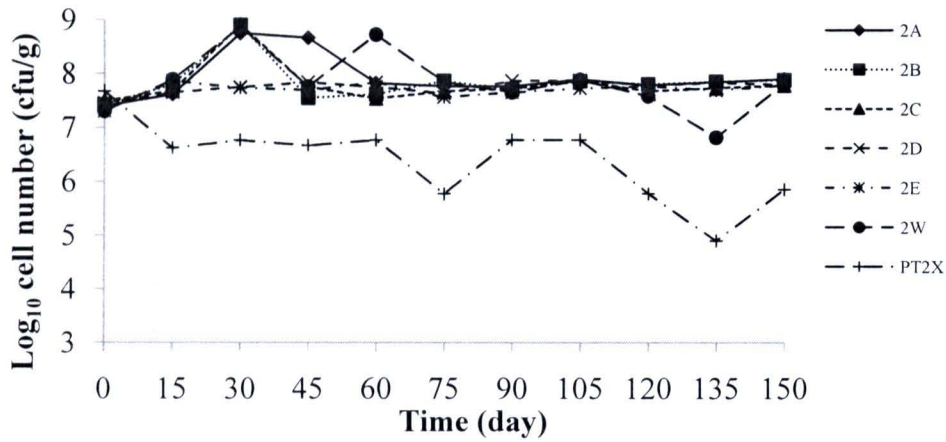
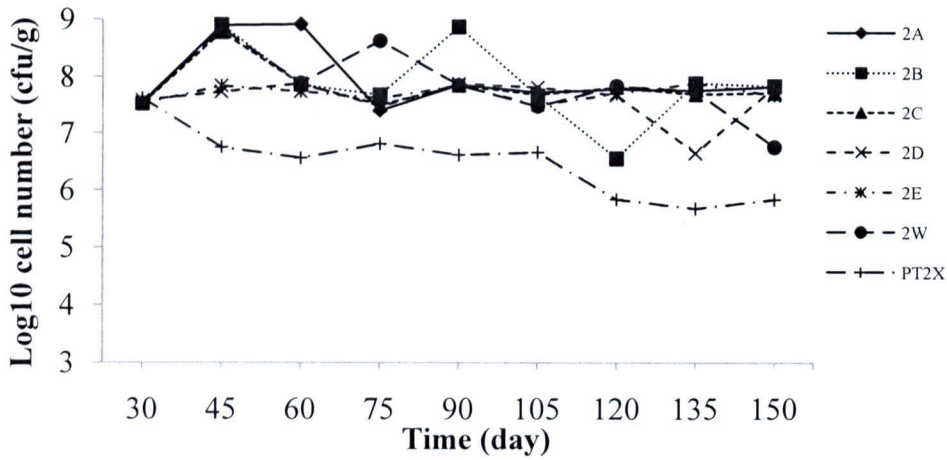


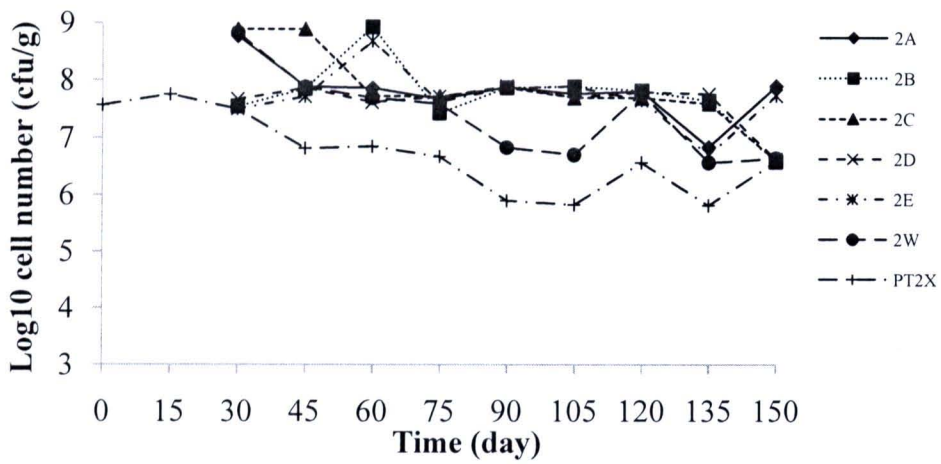
Figure 68 Viable cell count of mixed antagonists and PT1J in soil. Three experiments were applied to Pathumma plants



Experiment 1



Experiment 2



Experiment 3

Figure 69 Viable cell count of mixed antagonists and PT2X in soil. Three experiments were applied to Pathumma plants

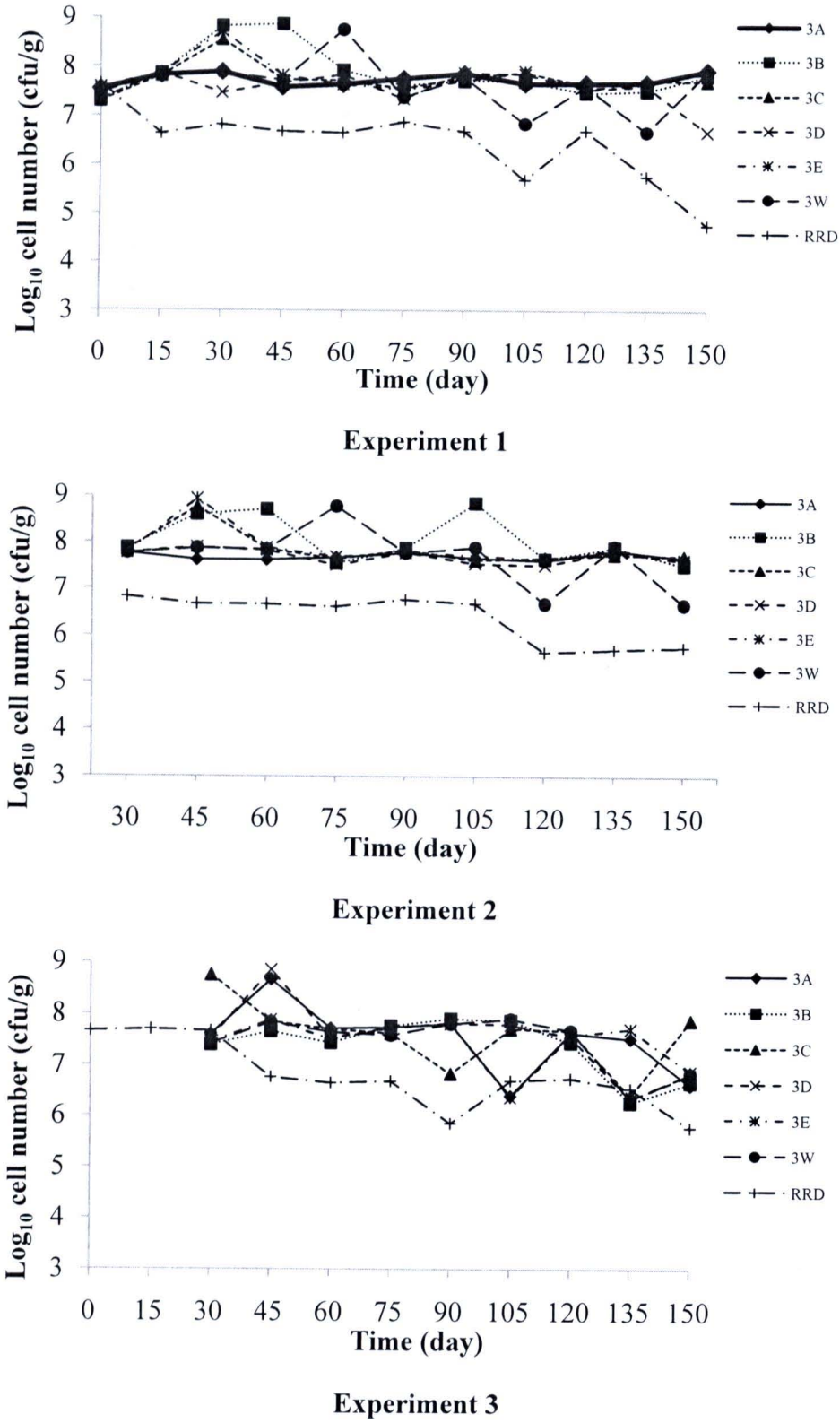


Figure 70 Viable cell count of mixed antagonists and RRD in soil. Three experiments were applied to Pathumma plants

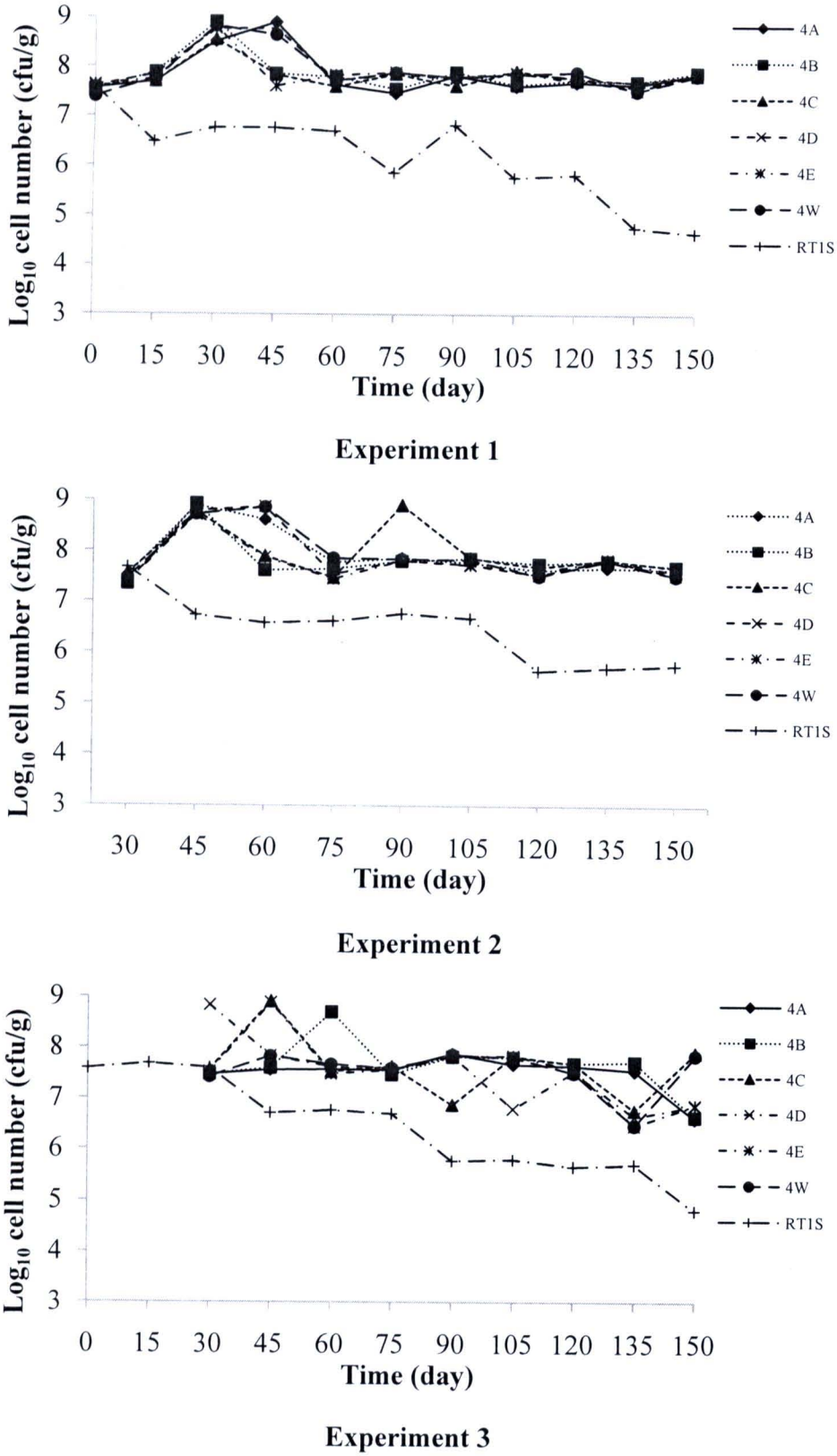


Figure 71 Viable cell count of mixed antagonists and RT1S in soil. Three experiments were applied to Pathumma plants

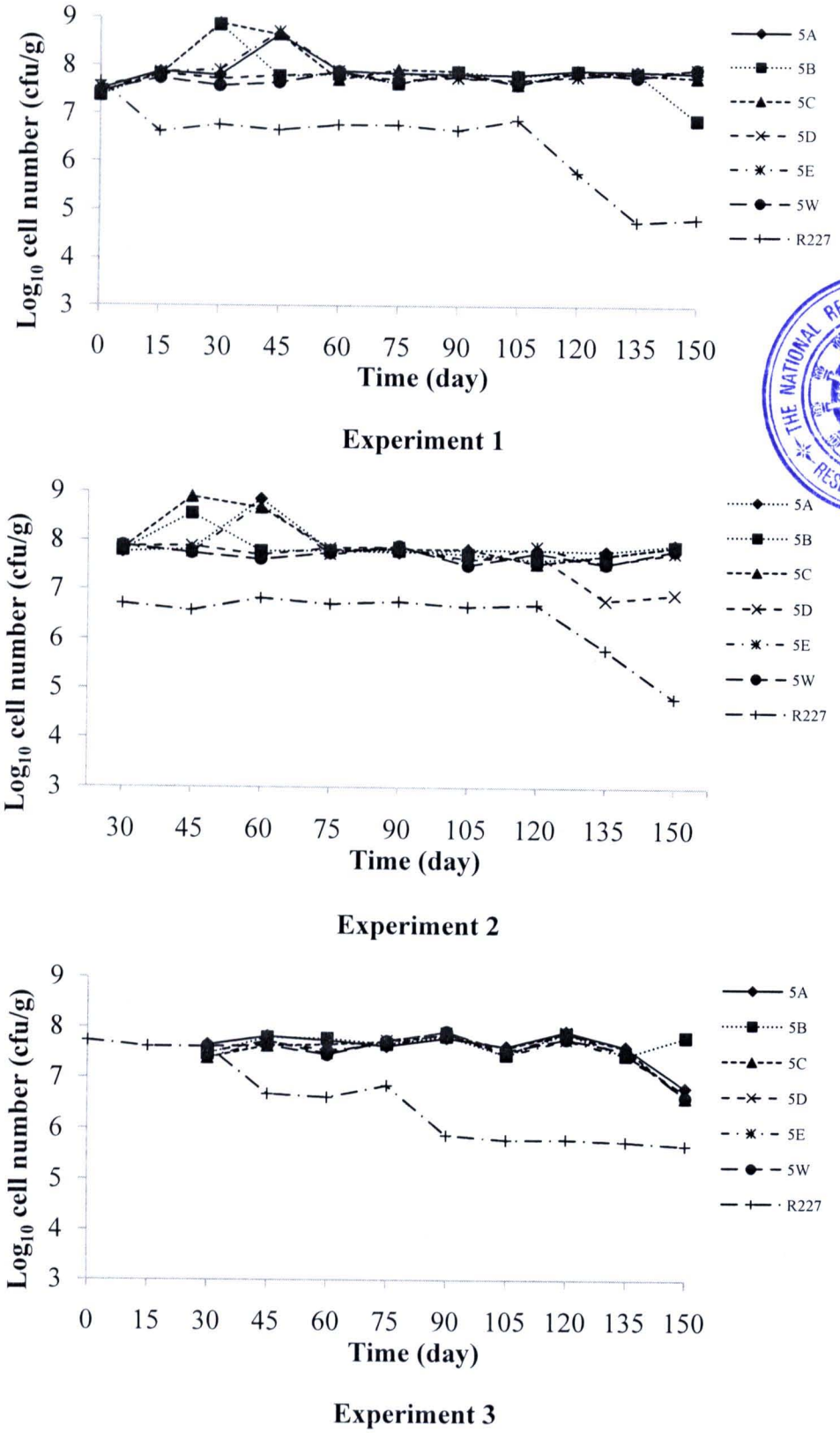


Figure 72 Viable cell count of mixed antagonists and R227 in soil. Three experiments were applied to Pathumma plants

According to the results of wilt causing bacterial growth inhibition in greenhouse assay, it was indicated that the antagonistic bacteria isolated from various soils were effective in controlling bacterial wilt caused by *Enterobacter* spp. There were several reports demonstrated that *Bacillus* and *Pseudomonas* could be biocontrol agents against plant pathogenic bacteria. Maleki *et al.* (2010) proposed that *Ps. fluorescens* CV6 isolated from cucumber rhizosphere could inhibit the growth of *Phytophthora drechsleri*. Abdullah *et al.* (2008) revealed that *B. amyloliquefaciens* inhibited the growth and production of mycelia and sclerotia. There was approximately 33-60% reduction in the number of sclerotia. In seedlings bioassay, this strain also protected over 80% of tomato, squash and eggplant seedlings inoculated with *S. sclerotinum*. Guo *et al.* (2004) demonstrated that *R. solanacearum*, the causal agent of bacterial wilt of tomato could be restricted by fluorescent *Pseudomonas* J3 and *Bacillus* BB11. The disease was reduced up to 90% in the field experiments. Schisler *et al.* (2002) stated that the antagonist *B. subtilis* AS 43.3 reduced disease severity and incidence by as much as 96 and 78%, respectively.

Many factors should be considered for the use of antagonistic bacteria as biocontrol agents. The reduction of inoculum densities of the pathogen in soils helped in reducing the disease incidence of soil borne disease (Das *et al.*, 2008) but some experiments demonstrated that the antagonists could not protect the plants from bacterial infection. In this study, the application of antagonists before or together with plant cultivation was the suitable time for disease prevention. The main problem of biological control was its low consistency and reliability under field condition due to a high variability in efficacy. The agar assay, although easier to perform, was limited by the absence of a plant that can greatly affect the ability of an amended

bacterium to survive, colonize and repress pathogens. The *in vivo* assay was more representative of condition to which the amended bacteria will be exposed once field trials are initiated. The inoculum was one of the realized factors for biocontrol assessment. The coinoculant had been proposed to use as favorable inoculums due to the synergistic effect to enhance the biocontrol methods. This conclusion was supported by the fact that a combination of strains as a biocontrol agent, utilizing multiple antimicrobial traits might prove to be advantageous by ensuring that at least one of the bio-control mechanisms will function under the unpredictable ecological conditions faced by the released PGPR strains (Radja Commare *et al.*, 2002). In addition, the antagonists should not be risk for the environment. This study showed that *Ps. aeruginosa* could be beneficial to the plant but it is an opportunistic pathogen in human and animal (Chin-A-Wong *et al.*, 1998). Hence, it was suggested that this strain should be replaced by other organisms or used as antimicrobial substance producer.

The further studies should be evaluated the biocontrol efficacy of antagonistic bacteria in the field particularly with a previous history of the disease. The application of biological control agents in the field is inherently more variable and challenging than in the greenhouse.

10. Designation of mixed culture products

10.1 Liquid formulation products

To select the most effective carrier, several medium were tested for the maximum growth of antagonistic bacteria. The preliminary results revealed that molasses and soybean whey were the most efficient medium for bacterial growth

(Table 38). After secondary screening, it was found that molasses was the best liquid formulation product. This medium was further evaluated for shelf-life of antagonistic bacteria for 90 days. The cell number of *B. subtilis* SP15, *Ps. mosselii* SP38, *Ps. mosselii* SP46 and *Ps. aeruginosa* SP58 were increased by 1.4%, 1.2%, 15% and 1.7%, respectively (Figure 73).

Both soybean whey and molasses is a waste product from the industry. It is known to be a good fermentation medium, rich in protein, fat, carbohydrates and fiber (Lee *et al.*, 2006). However, the major problem of these carriers is that it is easy to contaminate with *Bacillus* and fungi due to the high value of water activity.

Table 38 The growth of antagonistic bacteria in various media

Medium	Antagonist			
	SP15	**SP38**	**SP46**	**SP58**
2% (w/v) potato starch	++	+++	+	+
2% (w/v) potato starch + 2% (w/v) sucrose	++	+++	+	+
5% (w/v) potato starch + 2% (w/v) sucrose	+++	+++	++	++
Soybean whey	+++	++	++	++
50% (v/v) Soybean whey	+++	++	++	++
2% (v/v) molasses	++	++	++	++
5% (v/v) molasses	+++	+++	+++	+++
2% (v/v) molasses + 2% (w/v) soybean flour	++	++	++	++

Symbols: + low growth turbidity

++ moderate growth turbidity

+++ high growth turbidity

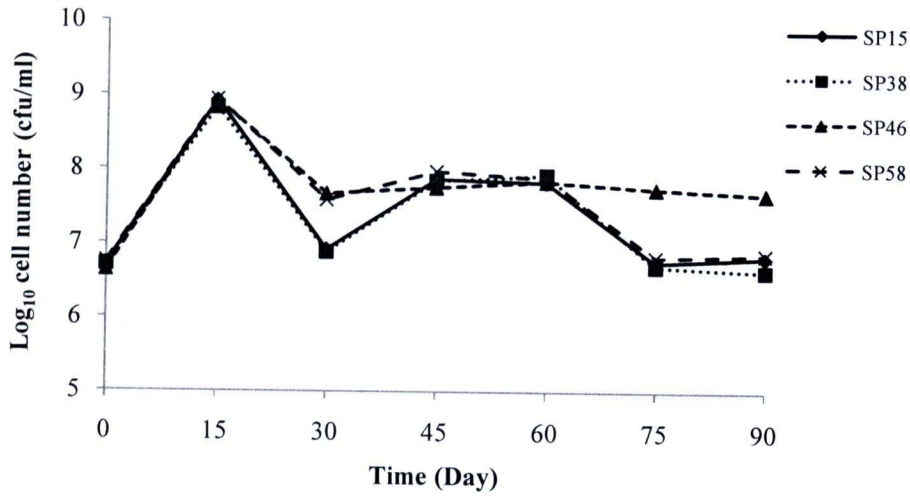


Figure 73 Viable cell count of antagonistic bacteria in 5% (v/v) molasses after incubation for 90 days at room temperature

10.2 Dry formulation products

To evaluate the optimal carrier materials for antagonistic bacteria, 3 materials including soil, coir and chaff were examined for the survival in these carriers for 60 days. The soil was the most eligible carrier material for all antagonistic bacteria. However, it was interesting that the population changes of all bacteria in coir were slightly different from the soil. All bacteria were still survived after incubation for 2 months (figure 74-76). The coir could be replaced for the soil since it was agricultural waste and the cost was lower than soil.

The dry formulation is one of solid state fermentation (SSF) that had low value of water activity (A_w). This formulation is considered that its advantage is over liquid formulation in term of the stability of potency storage and transportation costs. The dry formulation is lower weight and not required low temperature for long term storage (Bryant, 1994).

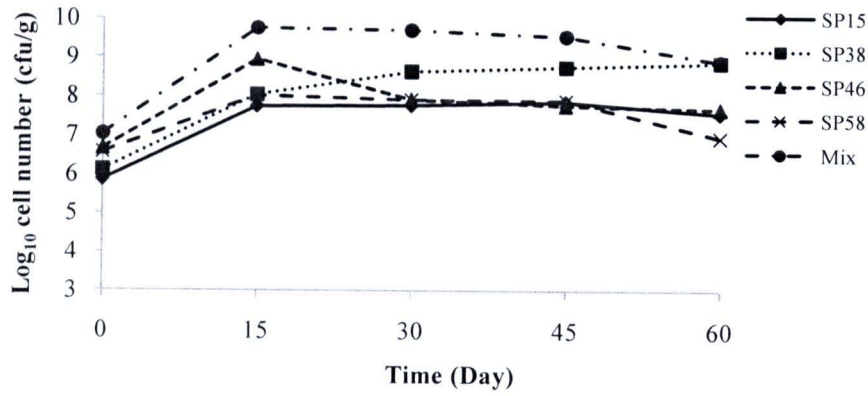


Figure 74 Viable cell count of antagonistic bacteria in soil after incubation for 60 days at room temperature

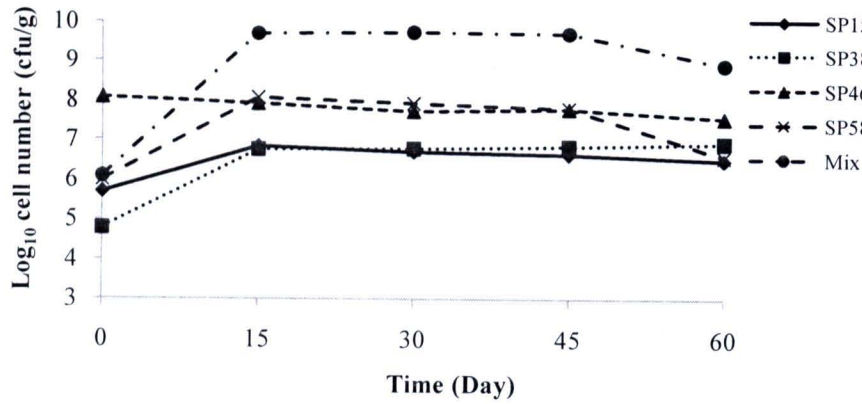


Figure 75 Viable cell count of antagonistic bacteria in coir after incubation for 60 days at room temperature

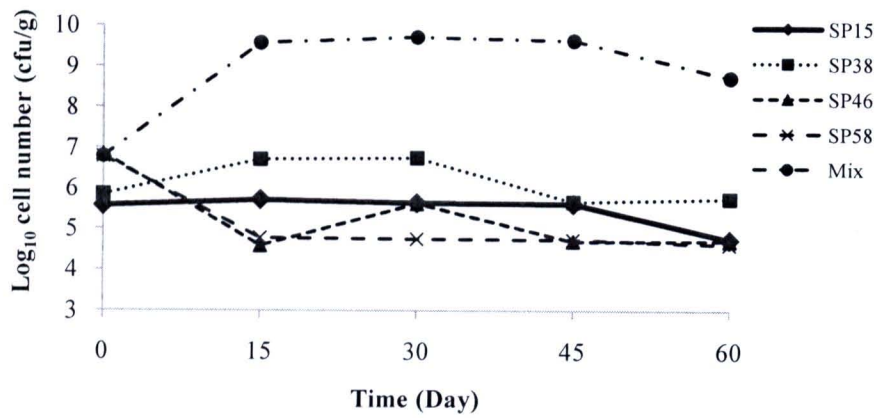


Figure 76 Viable cell count of antagonistic bacteria in chaff after incubation for 60 days at room temperature

Bacillus species have been reported to be effective in the biocontrol of multiple plant diseases owing to their production of several broad-spectrum antibiotics and their longer shelf life as a result of their ability to form endospores (Lee *et al.*, 2006). Other researches have reported the formulation of *Bacillus* and *Pseudomonas*. Lee *et al.* (2006) revealed that the wettable powder formulation N1E, based on corn starch and olive oil were evaluated for disease control activity of *B. licheniformis*. It was found that the disease control value of N1E on tomato plants was 90.5% against *Botrytis cinerea*. Radja Commare *et al.* (2002) reported that talc-based formulation of *Ps. fluorescens* PF1 and FP7 were tested against sheath blight and leaffolder in rice. In the field trials the maximum of 62.1% sheath blight and 47.7% reduction in leaffolder incidence was noticed in the mixture treatment containing chitin and its efficacy was equivalent to standard fungicide and insecticide treatment. Rabindran and Vidhyasekaran (1996) presented that they had developed a peat formulation in which *Ps. fluorescens* PfALR2 survive well for at least 2 months. A combined application of peat-based formulation to seed, root soil and foliage was the most effective method for control of the disease caused by *Rhizoctonia solani* in the field.

Formulation plays a significant role in determining the final efficacy of a BCA-based product, as do the processed of discovery, production and stabilization of the biomass. Although many microorganisms have exhibited promising biological control activity on a small scale, it remains a major challenge to successfully formulate biological control organisms. Successful scale-up production of the organisms and effective formulation is essential for the stable and economical development of pesticides (Schisler *et al.*, 2004). To develop a successful

formulation protocol for biological control agents, selection of an appropriate carrier material is important. The carrier should contribute to the attachment and longevity of the biological control activity in the field (Lee *et al.*, 2006). In addition to the above considerations, the need for low cost, lack of phytotoxicity and low visible crop residue on saleable commodities must be considered (Bryant, 1994).