INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
11–489/AKP/2012/14–3–377–382
http://www.fspublishers.org

Full Length Article

Adhesion of Wilt Causing Bacteria in *Curcuma alismatifolia* Tissue

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ABSTRACT

Curcuma alismatifolia Gagnep. or pathumma is an economic plant of Thailand, which is also called "Siam tulip". It has faced a serious problem of bacterial wilt infection for many years. Wilt causing bacteria grow well in plant xylems, obstruct the vessels and cause plant death eventually. The pathogenic bacteria isolated from infected pathumma rhizomes were identified as *Enterobacter* sp. by morphological, biochemical and molecular methods. The strain JK1 had 43% survival rate after cultivation for 1 year in soil without host. Infectivity of this strain to cause wilt disease in pathumma was evaluated. The infected pseudostems were examined under compound and scanning electron microscopes. The electron microscopic studies clearly revealed the bacterial adhesion and structural changes of plant tissues. *Enterobacter* sp. JK1 adhered to the vascular bundle walls and shrunken the plant tissue. © 2012 Friends Science Publishers

Key Words: Bacterial adhesion; Pathumma; *Enterobacter*; Bacterial wilt; Microscopy

INTRODUCTION

Curcuma alismatifolia Gagnep., also known as the Siam tulip or pathumma, is native plant of South-East Asia. An attractive flower is re-collective to a group of tulips (Bunya-atichart *et al*., 2004). Pathumma is a member of the family *Zingiberaceae*, which is found in north and northeastern regions of Thailand ranging from sea level up to 790 m above mean sea level. These ornamental plants become more popular not only in Thailand but also in foreign countries. The annual exported value of rhizomes is about 20-30 million baths (Anonymous, 2011). The important markets have been the United States of America, Japan, the Netherlands, Germany and Australia (Thongwai & Kunopakarn, 2007). The increase in demand of this plant leads to rapid genetic erosion caused by over-harvesting and land clearing (Paisooksantivatana *et al*., 2001). During cultivation, pathumma is susceptible to many diseases including bacterial wilt, which is one of the most important constraints.

The prominent wilt-causing bacterium is *Ralstonia solanacearum* (Wydra & Beri, 2006), which devastated several economic plants particularly tomato, ginger and banana resulting in crucial losses to growers. However, several reports showed that *Enterobacter* spp. could cause bacterial wilt in worldwide crops. Some strains of *E. cloacae* have been associated with rot of ginger rhizomes (Nishijima *et al*., 2004). *E. sakazakii* induced internal yellowing of papaya fruit (Keith *et al*., 2008). *E. asburiae* and *Enterobacter* spp. caused mulberry (*Morus alba*) wilt disease. *Enterobacter* sp. is Gram negative, rod-shaped and facultative anaerobic bacteria. It was previously grouped in genus *Erwinia* and has transferred to genus *Enterobacter* based on molecular and taxonomic studies (Wang *et al*., 2010).

In general, the plant pathogenic bacteria invade host cell through natural opening and interact with cell structures. The shoot system of a typical flowering plant consists of the stem and the attached leaves, buds flowers and fruits. Stems provide support to the leaves, buds and flowers. They conduct water and nutrients, and produce new cells in meristems. Internally, stems provide pathways for the movement of water and dissolved minerals from the roots to the leaves and for moving of food synthesized in leaves to roots (Rost *et al*., 2006). Whenever the ability to carry out essential functions of plant cells is interfered with pathogenic microorganisms, the activities of the cells are disrupted, altered and the plant becomes diseased. The affected plant cells and tissues determine the first disruption of plant physiological function. For some instance, the infected roots became rotten and were unable to absorb water and nutrients. The xylem vessels were interfered with translocation of water and nutrients to the top of the plant. Pathogens often cause diseases in plants by interfering the metabolism of plant cells through several substances they produced including enzymes, toxin or growth regulator. The bacterial pathogen can also use some host cell substances to support their growth. By growing and multiplying in the plant xylem and phloem vessels, the bacteria block the upward transportation of water through plant tissues (Agrios, 2005).

To cite this paper: Promsai, S., Y. Tragoolpua, A. Jatisatienr and N. Thongwai1, 2012. Adhesion of wilt causing bacteria in *Curcuma alismatifolia* tissue. *Int. J. Agric. Biol.*, 14: 377–382

Various strategies have been proposed to control wilt-causing bacteria, but it was limited in general applications or economic conditions. The holistic approaches to control wilt-causing bacteria have been used such as tolerant varieties, greenhouse cultivation, crop rotations, pesticides or antibiotics (which later accumulated in soils) or shifting cultivation (Pal & McSpadden Gardener, 2006). Nowadays, there is no single best way for crop protection against pathogens. Prior to selection of the way for eradication, understanding the mechanism of wilt disease is very important. The objective of this study was to explore the adhesion of plant pathogenic bacteria in host plant tissues in order to gain more knowledge about wilt causing bacteria in pathumma.

MATERIALS AND METHODS

Bacterial strains and plant materials: The plant pathogenic bacteria were isolated from infected pathumma rhizomes. Bacteria were grown in triphenyltetrazoliumchloride (TZC) medium containing (w/v) 0.1% peptone, 0.05% glucose, 0.01% pancreatic digest of casein and 0.005% 2, 3, 5-TZC.

Curcuma alismatifolia Gagnep., cv. Chiang Mai Pink rhizomes were provided by Bua Lai Pathumma garden, Chiang Mai Province, Thailand. Plants were grown in the greenhouse at Department of Biology, Faculty of Science, Chiang Mai University. Rhizomes were planted at the beginning of May. Stems started shooting about three weeks later.

Identification of wilt causing bacteria: The selected pathogenic bacteria were identified by both conventional and molecular methods.

Conventional method: Macroscopic and microscopic morphologies were observed and described, e.g. colony growth pattern, pigment production, spore formation. Biochemical characterization were performed e.g. catalase, oxidase and urease tests; hydrolysis of gelatin, starch and esculin; utilization or assimilation of some carbohydrates (Holt *et al*., 1994).

Molecular identification: Total genomic DNA was extracted from fresh culture following Dellaporta *et al.* (1983), a nearly complete 16S rRNA gene (~1.5 kb) was amplified using the universal primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Frank *et al*., 2008). Amplification was carried out in a 50 µL reaction volume. The thermal cycle consisted of 1 min initial denaturation at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 2 min primer annealing at 58°C, 2 min extension at 72°C and a final 7 min extension at 72°C. PCR products were purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Catalog no. DF100) following the manufacturer's protocol. The purified PCR products were directly sequenced at First base company, Malaysia with primer 27F and 1492R as sequencing primers. The identities of nucleotide sequences of the 16S rRNA gene obtained were subjected to BLAST analysis with the NCBI database (http://www.ncbi.nlm.nih.gov). Nucleotide sequences were aligned using ClustalW program. Neighbor-joining method (NJ) was used in the construction of a phylogenetic tree using the program in MEGA4. The topological analysis was performed with 1000 bootstrap replicates.

Survival of wilt causing bacteria in soil without host: The bacterial strain JK1 was examined for its ability to survive in soil without host. The bacteria were cultured in TZC medium and incubated at 30°C for 24 h. The bacterial suspension containing approximately $1x10^7$-$1x10^8$cfu/mL was poured into plastic bags containing sterile soil mixture prepared from commercial soil, chaff and coir at the ratio of 3:1:1. The viable cell count was performed in order to calculate survival rate of the bacteria. One gram of soil sample was collected every 30 days after inoculation for 1 year duration. The bacterial population was assessed by the dilution plate technique (Holt *et al*., 1994). The bacterial population data were transformed to $\log^{10}$ number value.

Infectivity of wilt causing bacteria in pathumma: Plant shooting pseudostems were wounded. Concurrently, the bacterial strain JK1 was grown in TZC medium for 24 h at 30°C. A 200 mL of inoculum suspension (Optical Density at 660 nm was 0.5, which corresponded to ~1×10^8 cfu/mL) was poured in each wounded stems. Pathumma plants were inoculated with TZC broth as control. Plants were monitored for the occurrence of wilt disease. 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.

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

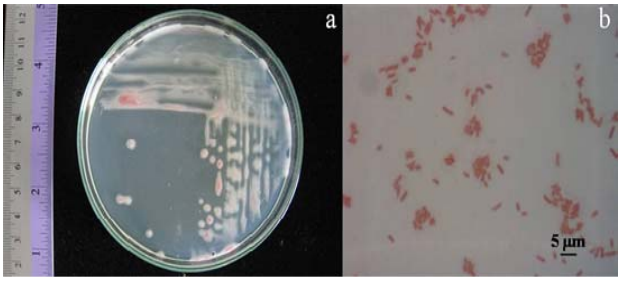
Electronmicroscopic studies: The plant stems were cross sectioned about 3-4 cm length prior to preparation for scanning electron microscopic. Briefly, a portion of stems was fixed with 2.5% glutaraldehyde at 4ºC overnight. After fixation, the cells were covered with 1% osmium tetraoxide (OsO_4) in 0.1 M phosphate buffer, incubated at 4ºC for 2 h, washed three times in phosphate buffer. The stems were subsequently dehydrated in ethanol, critical point-dried, mounted on scanning electron micrograph stubs, sputter-coated with gold and observed by a scanning electron microscope.

RESULTS AND DISCUSSION

Identification of wilt causing bacteria: The bacterial

isolate JK1 was selected for further study due to its ability to inhibit growth of pathumma plants. On TZC media, after 24 h of incubation at 30°C, the bacterial colony was pink color surrounded by white slime layer. The strain was Gram negative, short-rod and catalase positive (Fig. 1). The morphological and biochemical identification revealed that it was *Enterobacter* (Table I). The strain identification was confirmed by 16S rRNA gene determination. Phylogenetic analysis based on 16s rRNA gene sequencing showed that the isolate JK1 was *Enterobacter asburiae* (Fig. 2). Presumably, bacteria isolated from infected pathumma rhizome collected from fields was *Ralstonia solanacearum*, however, this research demonstrated that *Enterobacter* could be a causative agent of wilt disease in pathumma as well as *Ralstonia*. The scientific documentation of *Enterobacter* infection is limited; however, some species were reported to cause the major diseases in many crops. Onions were infected by *E. cloacae* that cause discolored and flaccid in bulbs. Similarly, 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

Fig. 1: The isolate JK1 on TZC agar (a) and Gram stain of the isolate JK1, 100x objective lens (b). Magnification: (b) x 1000



(*Hylocereus* spp.) in Malaysia. The symptom of the infected stems 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). Already described wilt disease caused by *R. solanacearum* was characterized by flaccid wilted leaves without discoloration and defoliation.

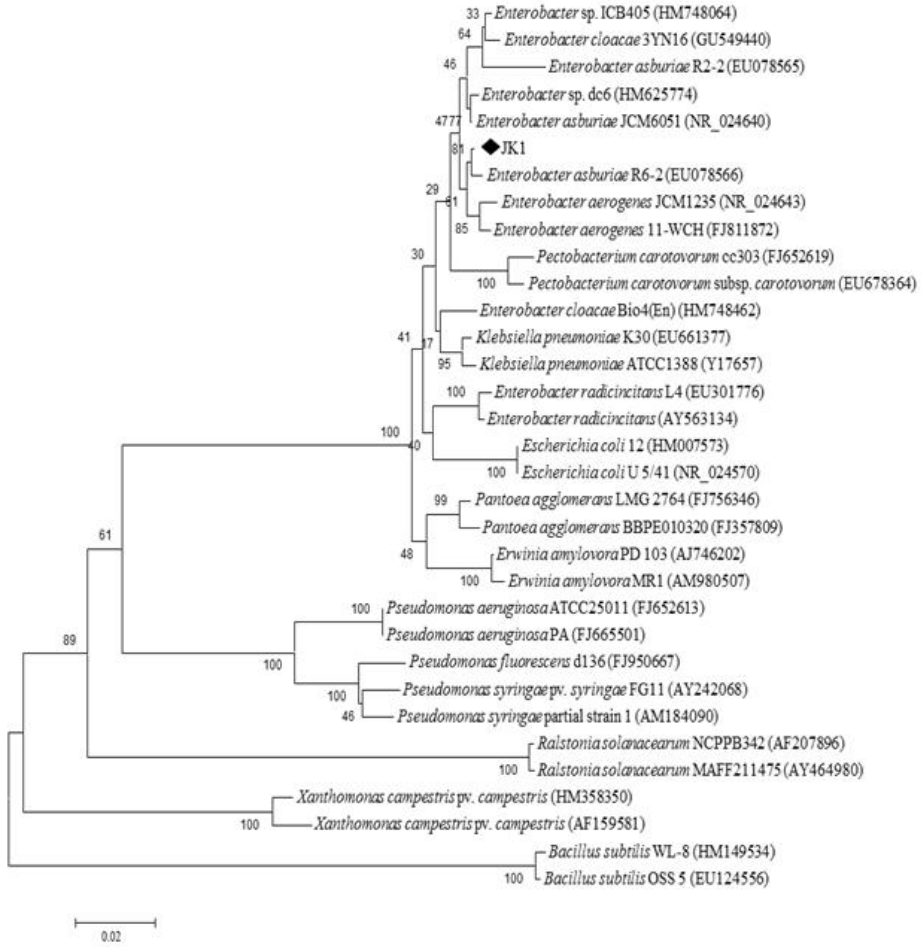
Infectivity of wilt causing bacteria in pathumma: After 7-10 days of bacterial inoculation, wilt disease showed early leave discoloration and defoliation. The infected plants grew slowly compared to the control plants. The bacterial strain might enter the vascular system via opening wounds,

Table I: Morphological and biochemical identification of the isolate JK1

Test	JK1	*Enterobacter asburiae*	*Enterobacter agglomerans*	*Enterobacter aerogenes*	*Enterobacter cloacae*	*Enterobacter (Erwinia) dissolvens*
Gram stain	-	-	-	-	-	-
Catalase production	+	+	+	+	+	+
Oxidase production	-	-	-	-	-	-
Esculin hydrolysis	+	+	d	+	d	+
Indole production	-	-	[-]	-	-	-
Methyl red	-	+	d	-	-	-
Voges-Proskauer	+	+	d	+	+	+
Citrate (Simmons)	+	+	d	+	+	+
H_2S production	-	-	-	-	-	-
Urea hydrolysis	+	d	[-]	-	d	+
Phenylalanine deaminase (24h)	-	-	[-]	-	-	-
Lysine decarboxylase	+	-	-	+	-	-
Motility	-	-	[+]	+	+	+
Gelatin hydrolysis, 22°C	+	-	-	-	-	-
D-Glucose, acid production	+	+	+	+	+	+
D-Glucose, gas production	+	+	[-]	+	+	+
Acid production when fermented						
L-Arabinose	+	+	+	+	+	+
Cellobiose	+	+	d	+	+	+
Dulcitol	-	-	[-]	-	[-]	-
Glycerol	-	[-]	d	+	d	-
myo-Inositol	-	-	[-]	+	[-]	d
Lactose	+	[+]	d	+	+	d
Maltose	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
Melibiose	+	-	d	+	+	+
Raffinose	+	d	d	+	+	+
L-Rhamnose	+	-	[+]	+	+	+
D-Sorbitol	+	+	d	+	+	+
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)

Fig. 2: Neighbor-joining tree based on 16S rRNA gene sequences showing the position of the isolate JK1. The sequence of *Bacillus subtilis* WL-8 and OSS 5 were used as an out group. 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



rapidly multiply, adhere and block xylem vessels.

Survival of wilt causing bacteria in soil without host: To evaluate the persistence of wilt-bacterial strains in natural soil, *Enterobacter*sp. JK1 was inoculated into soil without pathumma plants. The number of pathogenic cells was declined by 56% after 1 year of inoculation (Fig. 3). A 43% survival rate of the strain JK1 indicated that this wilt bacterial isolate could survive more than 1 year in soil. 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 & Allen, 2000). Breukers *et al*. (2006) reported that *R. solanacearum* had a survival period in potato planting soil up to 2 years. It is interesting that the survival of pathogenic bacteria is 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.

Study of bacterial adhesion in plant tissue under compound light and scanning electron microscopes: The light micrographs showed the possible association of wilt causing bacteria in cell parenchyma in xylems and vascular bundles (Fig. 4). It could not reveal the adhesion of bacteria apparently due to the contrast of plant cell and bacteria. In addition, the changes of plant cell structures between control and infected plants were not different.

To deeply understand the adhesion of the bacteria to the plant cell, morphological study was performed using electron microscopic technique. Scanning electron microscopy showed that some parts of infected stem were shrunken (Fig. 5-e) compared with the healthy stem. This study indicated that the infected plant had structural changes. However, little is known about the early events in

Fig. 3: Average populations of *Enterococcus* sp. JK1 in soil

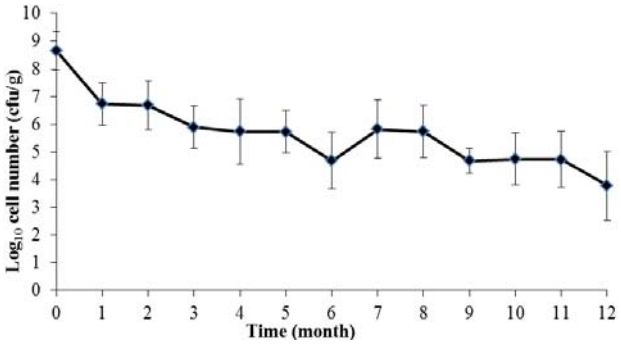
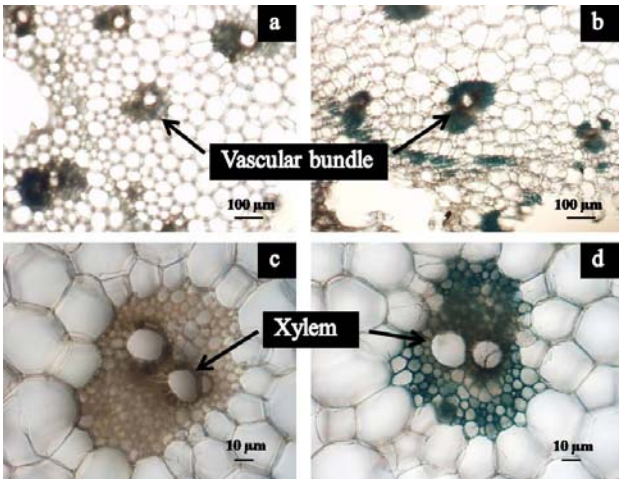


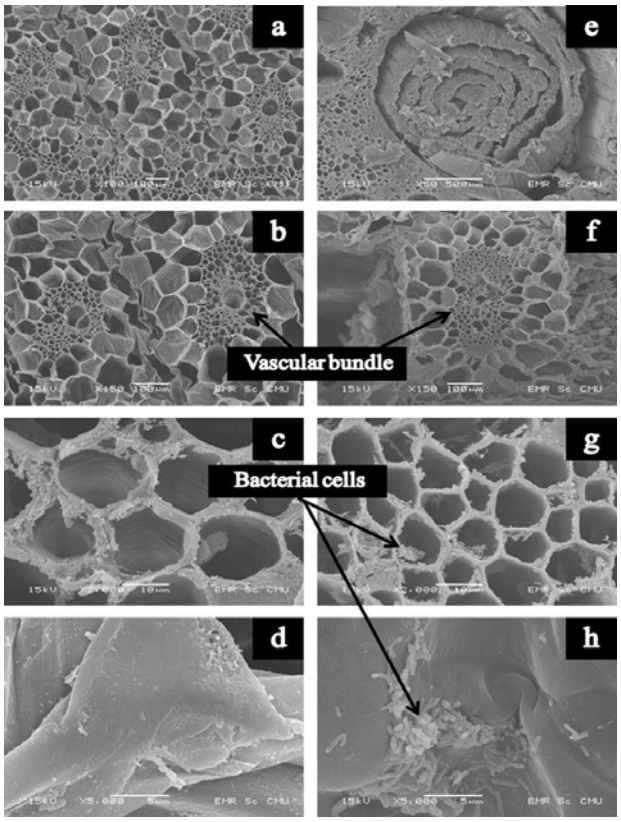
Fig. 4: Light micrographs of cell parenchyma and vascular bundles of control plant (a,c) and infected plant (b,d). Magnifications: (a,b) x 40; (c,d) x 100



the development of the symptoms and which factors contribute to the systemic colonization of host plants by the bacteria. It may be caused by the host plant defense mechanisms or some compounds from bacteria. 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 (Fig. 5g, h), but how they adhere and multiply in vascular bundles had not yet understood.

There are no reports about the adhesion and colonization of *Enterobacter* in *Curcuma* plants. The previous studies demonstrated colonization or morphological changes solely due to *R. solanacearum* (Wydra & 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 & Beri, 2006). Grimault *et al*. (1995) also found that root invasion was not

Fig. 5: Scanning electron micrograph of pseudostem tissue: Control plant (a-d), Infected plant (e,f) and Infected plant with bacterial adhesion(g,h). Magnifications: (a) x 100; (b,f) x 150; (c, g) x 2000; (d, h) x 5000; (e) x 50



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 cause 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.

CONCLUSION

This is the first report of using scanning electron microscope for investigation of bacterial-plant interaction in

pathumma. This study revealed the destruction of plant cells caused by pathogenic bacteria. Additionally, the survival rate in soil without host of *Enterobacter* sp. JK1, a causative agent of wilt disease in pathumma, would be useful for further monitoring the effects and mechanisms of the bacteria on pathumma in order to control and minimize the bacterial wilt disease by green chemistry in the future.

Acknowledgement: This research was supported by grant from the office of the Higher Education Commission, Ministry of Education, Thailand under the program of Strategic Scholarship for Frontier Research Network for the Ph.D. Program Thai Doctoral Degree. We sincerely thank Dr. Sumalee Pruksakorn for her valuable comment on molecular study. We thank the Department of Biology, Faculty of Science, Chiang Mai University and the Graduate School of Chiang Mai University for all support.

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(Received 20 August 2011; Accepted 05 December 2011)