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THESIS

IN VITRO CULTIVATION OF '*CANDIDATUS* LIBERIBACTER ASIATICUS', THE CAUSAL AGENT OF CITRUS HUANGLONGBING AND DETERMINATION OF ITS PRESENCE IN ALTERNATE HOST AND VECTOR IN THAILAND

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**A Thesis Submitted in Partial Fulfillment of
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Arom Jantasorn 2012: *In Vitro* Cultivation of '*Candidatus* Liberibacter asiaticus', the Causal Agent of Citrus Huanglongbing and Determination of Its Presence in Alternate Host and Vector in Thailand. Doctor of Philosophy (Tropical Agriculture), Major Field: Tropical Agriculture, Faculty of Agriculture. Thesis Advisor: Associate Professor Niphone Thaveechai, Ph.D. 100 pages.

'*Candidatus* Liberibacter asiaticus (Las).' is a phloem inhabiting bacterium that causes huanglongbing disease (HLB) and also knows as citrus greening in which presently associated with three species of α-Proteobacteria in the genus '*Candidatus* Liberibacter sp'. This research was to develop a medium to cultivate Las. A medium contains periwinkle extract and growth factor for Las bacteria to grow in the medium. Cultures obtained positive with Real time PCR at 24 hour after growth. The highest populations of '*Candidatus* Liberibacter asiaticus' bacterium culture were obtained from infected periwinkle, citrus and psyllids at 24 hours of cultivations in the developed medium. The medium composition and culture condition were determined. Fastest growth of Las obtained from cultivation at 25°C and pH 5.8, containing ATP at 2,000 ppm and glucose at 2% as an energy and a carbon sources, respectively. Scanning electron microscope, LIVE/DEAD cell, DAPI and FISH techniques were used to examination the pathogen in the culture. The morphology of '*Candidatus* Liberibacter asiaticus' in culture is a clump of cells and after treated with proteinase K the cells were separated to show individual rod shape cell. The cell cultures were confirmed by using 16s rDNA primer real time PCR. This is the first report on successful cultivation of Las with the high titer of HLB bacterium in culture. However, the Ar medium can supported growth of Las for 3 times subcultures at 48 hours as a interval. A first report on assessment of the infection frequency of HLB on psyllid vector from *M. paniculata* in Thailand was demonstrated as a potential source of inoculum. Murraya plant samples and psyllids on the Murraya plants from ten diverse geographical regions of Thailand were collected and DNA was extracted to evaluate the presence and titers of Las by real time PCR using two different methods. The data showed variation of Las levels both in Murraya and psyllids in Thailand. Different titers among individual psyllid and Murraya plant sample were observed in each province of Thailand which indicate potential insect vector and source of inoculum of HLB in Thailand.

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Student's signature	Thesis Advisor's signature

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Arom Jantasorn

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INTRODUCTION

Huanglongbing (HLB) disease is one of the most destructive diseases of citrus worldwide. Previously known as citrus greening , HLB is associated with *Candidatus* Liberibacter, phloem limited, fastidious, gram negative bacteria belonging to the alpha subdivision of *Proteobacteria* (Damteegte *et al.,* 2010; Jagoueix *et al,* 1996). The '*Candidatus* Liberibacter' genus contains three species of the expanding list, '*Candidatus* Liberibacter asiaticus' and '*Candidatus* Liberibacter americanus' which are heat tolerant species and transmitted by *Diaphorina citri,* and '*Candidatus* Liberibacter africanus', a heat sensitive species which is transmitted by *Trioza erytreae*. These species of HLB occur in Asia and Florida (Bruce *et al.,* 2005), Brazil and South Africa, respectively (Halbert *et al.,* 2004; Jagoueix *et al.,* 1994; Li *et al.,* 2006; Teixeira, 2005). '*Candidatus* Liberibacter solanacearum', a newly recognized species isolated from tomato, is genetically related to the HLB bacteria. Though it is not naturally associated with HLB in citrus plants (Lin *et al.,* 2009), but rather is associated with the emerging zebra chip disease of potato and tomatoes (Liefting *et al.,* 2008). *Candidatus* Liberibacter asiaticus has been shown to colonize plants including periwinkle and dodder and the insect vector *Diaphorina citri,*. Diagnostic tests relys on PCR and qPCR based on the Las 16s rDNA and the *rpo*JL loci and other sequences in addition to HLB symptoms (Hocquellet *et al.,* 1999; Hung *et al.,* 1999; Liao, *et al.,* 2004; Okuda *et al.,* 2005; Wang *et al.,* 2006). *Candidatus* Liberibacter asiaticus (Las), vectored by the Asian citrus psyllid (ACP, *Diaphorina citri*), is the most prevalent in the world. *M. paniculata*, an ornamental rutaceous shrub, is a preferred host for ACP. *M. paniculata* and other species in this genus are widely distributed in commercial nurseries. There is a potential risk for spreading of HLB through commercial *Murraya* spp. seedlings.

About 99% of microorganisms including all *Candidatus* Liberibacter spp. in nature are still uncultivable using general culture methods because of the complexity and lack of knowledge concerning bacteria growth *in vitro* (Joong-Jae *et al.,* 2008; Yu *et al.,* 2006). Bacterial growth requires an energy source, nutrients, and proper physiochemical conditions. It can be challenging to identify the required nutrients and conditions needed to support the growth of uncultivable bacteria in a synthetic media while avoiding the co-precipitation of the introduced chemical. Modification of growth media remains difficult since different bacteria require different nutrients in varied concentrations and forms (Alain *et al.,* 2009). Recently, success in the improvement of cultivation of uncultured bacteria has been achieved by using the following methods: use of a relatively low concentration of nutrients to increase the viability of bacterial cells, and to improve the recovery of bacteria from the natural sample (Connon *et al.,* 2009; Rappe *et al.,* 2002; Sangwan *et al.,* 2005); addition of signaling compounds as known to communicate between bacteria (Bruns *et al.,* 2002; Bruns *et al.,* 3003); use of gellan gum (Stott *et al.,* 2008; Tamaki *et al.,* 2005); combination of an unusual energy source with antibiotics to select bacteria (Konneke *et al.,* 2005); a decrease in inoculum size (Davis *et al.,* 2005); addition of an electron transporter to the media and the addition of enzymes to elevate reactive oxygen species (Stevenson *et al.,* 2004); addition of inhibitors for unneeded organisms (Leadbetter *et al.,* 2009); and finally, use of an increased incubation period to allow the development of a rare bacterial stain (Davis *et al.,* 2005; Sait *et al.,* 2002, Sangwan *et al.,* 2005; Stevenson *et al.,* 2004; Stott *et al.,* 2008).

Nutrition requirements in the medium of the '*Candidatus* Liberibacter' pathogens are not completely understood. This has hampered the isolation and cultivation of '*Candidatus* Liberibacter' species *in vitro*. Minimal nutritional media with soil extracts has been successful to culture members of soil borne *Rizobiaceae*, the closest relative to '*Candidatus* Liberibacter' species based upon 16s rDNA sequence comparisons (Jagoueix *et al*., 1994). This may provide reasonable predictions about the metabolic properties of '*Candidatus* Liberibacter' species. This information could facilitate future *in vitro* cultivation of this important bacterium.A particular method for cultivation of the '*Candidatus* Liberibacter' species has been developed, but that to date has not maintained sustained pure cultures (Davis *et al.,*

2008). However, a medium supplemented with plant extract could be useful in culturing the 'Candidatus Liberibacter' species (Sechler *et al.,* 2009). The existence of complete 'Candidatus Liberibacter asiaticus' genome sequence is a valuable tool for predicting metabolic pathways (Duan *et al.,* 2009), but the genome is not yet reliable enough to provide a complete analysis of the nutritional requirement needed to culture this bacterium. Based on the genome sequence of 'Candidatus Liberibacter asiaticus' and the presence of known and putative genes for metabolic enzymes, a requirement of nitrogen metabolism was predicted. ATP/ADP translocase, which is used to synthesize ATP and uptake energy directly from its surroundings, was present as well as phosphate, and a major component (14 genes) of the respiratory electron transport chain. Based on sequence analyses, no predictions could be made regarding a metal requirement. The presence of enzyme sequences indicates that 'Candidatus Liberibacter asiaticus' has the ability to metabolize sugar such as glucose, fructose, and xylulose. However, previously published findings (Sechler *et al.*, 2009) did not provide insight into the relative concentrations of nutrients required for optimal growth because they do not have information from 'Candidatus Liberibacter asiaticus' genome.

OBJECTIVES

1. To develop a medium to cultivate *'Candidatus* Liberibacter asiaticus' under aerobic conditions using HLB infected materials as the inoculum and to evaluate of the effect of the culture conditions on *in vitro* growth and survival of *'Ca* Liberibacter asiaticus'. The presence of *'Ca* Liberibacter asiaticus' in culture will be confirmed by using several techniques.

2. To determine the presence and titers of Las bacteria in *Murraya paniculata* and the Asian citrus psyllid (ACP) collected from *Murraya paniculata* in Thailand.
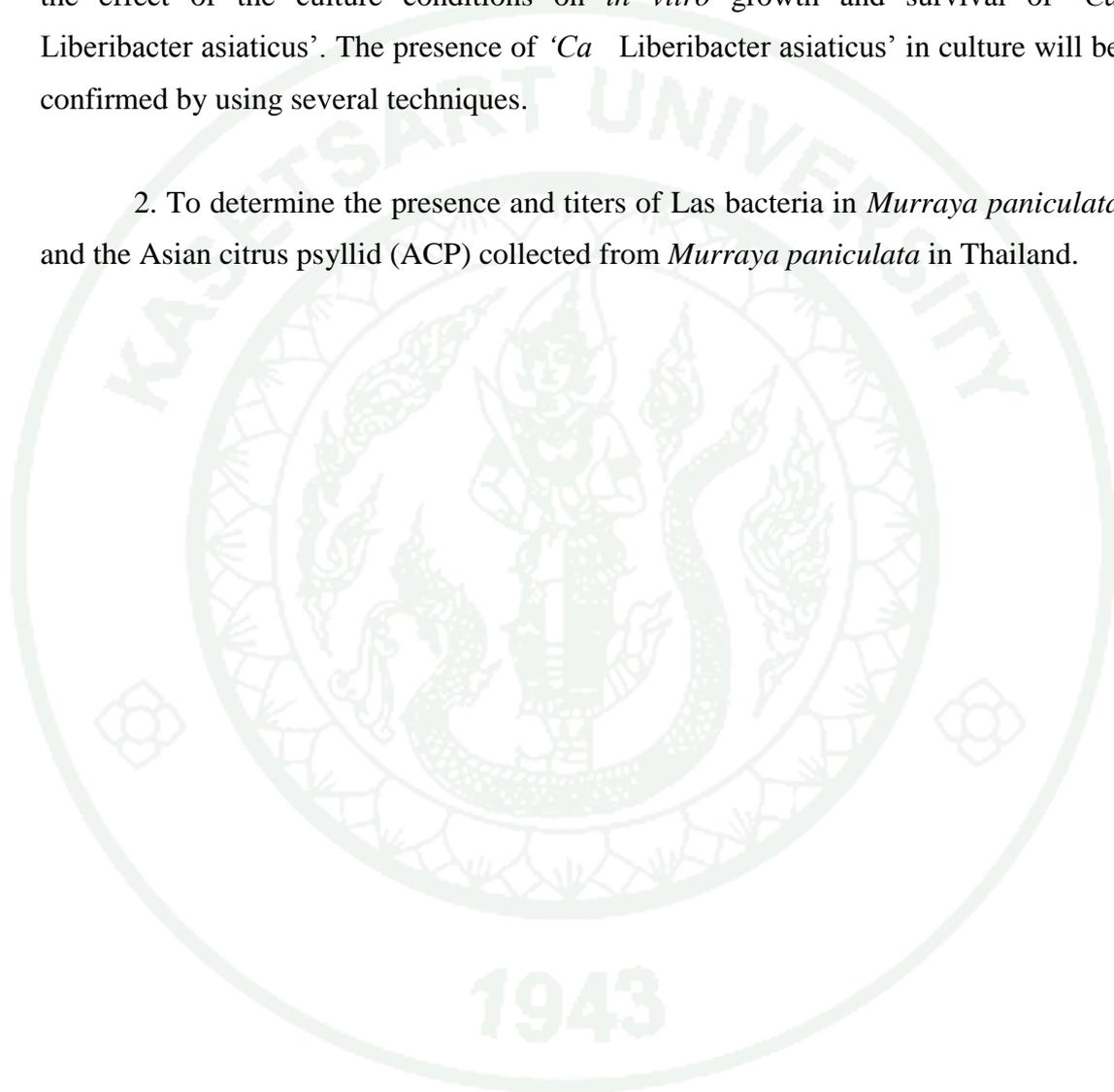
LITERATURE REVIEW

1. Causal organism

The demonstrations that greening is a graft- and insect-transmissible disease led to the conclusion that a virus was responsible for this disease (McClean and Oberholzer, 1965). Lafle'che and Bove' (1970) reported mycoplasma-type bodies in sieve tubes of sweet orange infected with HLB. The observation of cellular organisms in the phloem of HLB- infected citrus and their absence in healthy materials indicated that a prokaryotic organism was the causal agent. Similar organisms were observed in psylla (Chen *et al.,* 1973; Moll and Martin, 1973). Electronmicroscope studies suggested that the organism was a true bacterium, belonging to the Gracilicute division (Garnier and Bove', 1978). Attempts to isolate and culture the organism on artificial media and to fulfill Koch's postulates have been unsuccessful.

The causal agent of HLB is a phloem-limited bacterium that has a true cell wall, but it has not been cultured. Based on ribosomal DNA (rDNA) profiles, the bacterium was named *Candidatus* Liberibacter belonging to the alpha proteobacteria. The species, *Candidatus* L. *africanus* causing African HLB and *Candidatus* L. *asiaticus* causing Asian HLB, have been described previously.

The new species recently found in Brazil has been proposed to be *Candidatus* L. *americanus* (Teixeira, 2005). The African HLB causes symptoms only under relatively cool conditions (20-25°C) and generally has a milder effect than the Asian HLB that causes symptoms under both cool and warm (up to 35°C) conditions. The bacterium found in Florida is the Asian form.

In addition to the Liberibacter sp. that is affecting the citrus industry, a new Liberibacter species, '*Ca.* L. solanacearum', is recently associated with the emerging 'zebra chip' disease of potatoes in the U.S. and tomatoes in New Zealand (Liefting *et al.*, 2008). '*Ca.* L. solanacearum' is closely related to '*Ca*. L. asiaticus', although '*Ca.*

L. solanacearum' is not associated with citrus HLB nor has it been found in Asian citrus psyllids (Li *et al*., 2008).

2. Disease symptoms

Depending on the age of a tree and the time and stage of infection, the first symptoms of HLB usually start with the appearance of a yellow shoot. The pathogens start moving through the vascular system of the plant, specifically in the phloem sieve tube, other symptoms become evident on the tree canopy. There is a tendency for other branches to become yellow, followed by twig necrosis or dieback. A severe leaf drop that affects the density of the foliage can also be observed. Another symptom is premature fruit drop leading to enormous losses in production.

However, the most important diagnostic symptom is the presence of the blotchy mottle on leaves that crosses the veins (Gottwald *et al.,* 2007; Bové, 2006; and McClean and Schwarz, 1970). The blotchy mottle can be observed on the leaves as dark or light green patches which are not symmetrical to both sides of the mid vein. This symptom is generally more evident in the interior part of the tree canopy, and it was the only one observed in all of the studied species. With a severe infection, the dark green areas are reduced to small circular spots that contrast with a light yellow or green background. This symptom is commonly known as "green islands". It has been observed on numerous sweet orange (*C. sinensis*) plants in commercial groves and on a potted grapefruit (*C. x paradisi*) plant in a Miami nursery. It is important to know that the mottling produced by HLB can easily be confused with those produced by other diseases such as severe forms of *Citrus Tristeza Virus* or *Phytophthora* infections, or even nutrient deficiencies. On severely affected branches having acute leaf drop, some small leaves develop a symptom commonly known as "rabbit ears". This name refers to the presence of small, pointed, erect leaves having a light green or yellow color. These leaves can also be observed at the tip of new flushes. They are evident on species like sweet orange (*C. sinensis*), pummelo (*C. maxima*) and mandarin (*C. reticulata*)

Another characteristic observed on HLB infected leaves is the yellowing of the main and secondary veins (McClean and Schwarz,1970) that sometimes become enlarged, swollen, and corky (Bové, 2006.). In sour orange (*C. aurantium*) and key lime (*C. aurantiifolia*) plants, it was also possible to observe thicker and leathery leaves in advanced stages of the disease (Bové, 2006, McClean and Schwarz, 1970). Accumulation of starch grains in the parenchyma cells may explain why the leaves are leathery. One of the most interesting symptoms to look for during survey and detection activities is the presence of leaf and branch flushes with mineral deficiency-like symptoms that resemble those chlorotic patterns produced by zinc, iron, manganese, calcium, sulfur and/or boron deficiencies (Brlansky *et al*.,, 2007).

Disease detection is aided by observation of the notching caused by toxin secretion of psyllids while feeding on the leaves. This has been demonstrated the case during nursery and residential property inspections where the notching is the clue inferring that the associated mottling is due to HLB but not to a nutrient deficiency in the potted plants.

The presence of small and misshapen (lopsided) fruit is a noteworthy symptom in field surveys. However, it is not exclusive to citrus greening. Misshapen fruit can be caused by other problems such as nutrient deficiency. For this reason, it is important to analyze all symptoms on the tree. On the other hand, if small and misshapen fruit is found in a packinghouse, it is likely that the disease is present in the grove where it originated.

When diseased fruit is cut longitudinally, the axis of the fruit observed is curved (Bové, 2006). In addition, aborted seeds can be present (Gottwald *et al*., 2007; Bové, 2006; Timmer *et al*., 2002.). In pummelo (*C. maxima*) and sour orange (*C. aurantium*) fruits, aborted seeds in the affected part of the fruit and normally developed seeds in the remaining part have been simultaneously detected. Another symptom on the fruit is the orange-brown stain of the vascular columella (Bové, 2006) that has been observed on grapefruit (*C. x paradisi*) and sweet orange (*C. sinensis*) fruits. This fruit symptom should only be used as a diagnostic character when other reliable symptoms are not present.

Reduced fruit size is one of the symptoms produced by greening that is evident in groves and is responsible for losses in the fresh fruit market, but it is not the only one. The juice from fruits affected by HLB disease has been referred to as bitter and with a low content of soluble acids (Timmer *et al*., 2002), or as having a salty bitter taste (Brlansky *et al*., 2007; McClean , and Schwarz, 1970.). In relation to this, the juice tested with sour oranges (*C. aurantium*) and pomelo (*C. maxima*) proved to be insipid, coinciding with the off tasting characteristic of the fruit mentioned by Halbert (Halbert, and Manjunath, 2004). Another interesting symptom on fruit affected by citrus greening is the color inversion that tends to keep the green color in the stylar end (Bové, 2006). This characteristic was observed on pummelo (*C. maxima*) fruit in a Miami area of commercial grove. Lastly, HLB affected fruit, especially sweet orange (*C. sinenesis*), can also have a mottled appearance (Gottwald *et al*., 2007).

3. Host range

HLB causing citrus greening disease can affect almost all citrus cultivars;. All species of citrus appear to be susceptible, irrespective of the rootstock used (Aubert, 1993). Infected lemons, grapefruits and sour oranges remained non-productive, whereas Mexican limes, trifoliate oranges and some trifoliate orange hybrids showed only leaf symptoms (Chung and Brlansky, 2005). Symptoms have also been observed in *Microcitrus australasica, Swinglea glutinosa, Atalantia missionis, Clausena indica, Limonia acidisimma, Balsamocitrus dawei, Aeglopsis chevalieria, Severinia buxifolia, Murraya paniculata. Catharanthus roseus* (Periwinkle) and *Nicotiana xanthii* (Tobacco) are the only reported of non-Rutaceous hosts.

Both of the latter hosts were infected only under laboratory conditions and acted as indicator plants (Knapp *et al.,* 2004). In order to declare a species a host, the positive presence of pathogen needs to be established. Some species (*Citrus indica Tan* and *Citrus macroptera Montr*.) remained symptom-free under heavy inoculum pressure (Bhagabati, 1993). *Citrus limetta* remained symptom-free after a laboratory inoculation. Relative citrus species such as *Murraya paniculata L.* (orange Jasmine), and citrus species *C. jambhiri* (Rough lemon), *C. aurantium* (Sour orange) and *C.*

paradisi (Grapefruit) were screened against HLB in laboratory. Grape fruit was the most susceptible host, followed by the other species (Halbert and Manjunath, 2004). Only a few lemon cultivars and a few other species like *Citrus indica* and *Citrus macroptera* reportedly exhibited some tolerance or possible resistance to the bacterium. However, most of the citrus and citrus relatives are potential hosts for the citrus greening pathogen.

4. Cultivation of the HLB pathogen

The causal agent of citrus HLB is restricted in phloem sieve tube of host plant that has not been grown in culture. Previous studies on fastidious prokaryotic plant pathogen in culture have met with limited success. The phloem restricted spiroplasmas were first isolated in the early 1970s, but none of the phloem limited phytoplasmas have been isolated in culture. All of fastidious xylem limited gram negative bacteria such as *Xylella fastinosa* have been isolated. The bacterium grew on a medium modified from the PD2 medium developed for isolation of *Xylella fastinosa*, the causal agent of Pierce's disease of grapevines. The medium had been modified to support better growth of another fastidious bacterium that had been isolated originally on the PD2. Davis *et al.*(2008) studied on development of the medium to grow *Candidatus* Liberibacter asiaticus in culture. Growth of the babaco bacterium was evaluated on different media formulation that also would support growth of *Candidatus* Liberibacter asiaticus. Examination of the cultures indicated that *Candidatus* Liberibacter asiaticus was present in co-culture with other bacteria. Most traditional media for culturing bacteria favor organisms that grow in isolation, in high density, and in high concentrations of nutrient (Keller and Zengler, 2004). Some recent techniques to culture fastidious organisms that do not grow in these conditions have been developed to simulate nutrient poor bacterial environments with minimum media (Janssen *et al.*, 2002). Minimum media enhanced with soil extracts have been used successfully to culture members of soilborne Rhizobiaceae, the closest relative to *Candidatus* Liberibacter asiaticus (Jagoueix *et al.*, 1994). Therefore, a medium supplemented with an environmental extract may be useful for culturing *Candidatus* Liberibacter asiaticus. In a study on the development of a new medium (Sechler *et al.*, 2009), Liber A was designed and used to successfully cultivate all three *Candidatus*

Liberibacter spp. isolated from samples around the world. Two cultures of the HLB pathogen including *Candidatus* Liberibacter americanus and *Candidatus* Liberibacter asiaticus on Liber A medium were found to be pathogenic on citrus.

5. Detection

Field diagnosis of HLB is difficult because of the non-specific nature of foliar symptoms. Since it is easy to confuse HLB leaf symptoms with nutrient deficiencies and other disease or stress related factors, positive confirmation with fruit symptoms is often required in the field. Prior to the more recently positive identification of the causal agent with molecular techniques, the only method for confirmation was inoculation of biological indicators such as sweet orange, Orlando tangelo (Schwarz, 1968) or Ponkan mandarin (Matsumoto *et al.,* 1968).

There are various traditional methods for detecting Liberibacter species. The electronic microscopy (EM) was the only reliable detection method for the bacterium since the first EM observation of a "mycoplasma-like organism" in phloem tissues of HLB-infected citrus in 1970 (Lafleche & Bove' 1970) until the first DNA probe developed specifically to detect the bacterium (Villechanoux *et al.,* 1992). However, the EM observation was time-consuming and unable to distinguish species of Liberibacters. A method based on the identification of a fluorescent gentiosyl-glucoside from infected fruits and bark was developed in 1968 (Schwarz 1968) for confirmation of the disease. However, this method soon proved non-specific since stressed trees contained the same marker (Schwarz, 1970). Monoclonal antibodies were raised for ELISA tests first in 1987 (Garnier *et al*., 1987), but they were too isolate-specific to be used for detection of different isolates of the bacterium associated with the disease. Two DNA probes were developed specific to "*Ca.* L. *asiaticus"* and *"Ca.* L. *africanus"* (Villechanoux *et al*., 1992), but the detection sensitivity of the dot-blot hybridization using these probes was similar to that of EM (Jagoueix *et al*., 1996).

Recently, an iodine reaction (IR) kit was developed (Onuki *et al*., 2002) based on the elevated starch accumulation in HLB-diseased citrus leaves (Schneider, 1968).

However, the IR method is only a little better than observation of visual symptoms of the disease. Field HLB diagnosis based on symptoms is usually difficult because none of the symptoms is specific (Bové, 2006). The biological indexing by plant indicators such as sweet orange (Schwarz, 1968) and Ponkan mandarin (Matsumoto *et al*., 1968) was another confirmatory test of HLB. However, the transmission percentages varied from 54.7 to 88.0% for "*Ca*. L. *asiaticus*" and 10.0 to 45.2% for "*Ca*. L. *americanus*" even by the very efficient "seedling inoculation method" for citrus diseases (Li *et al*., 1996) with a large budstick up to 4 cm in length from HLB symptomatic twigs (Lopes *et al*., 2009).

The polymerase chain reaction (PCR), first described in the mid-1980s has since become a powerful technique for the selective amplification of DNA or RNA sequences. In the detection of HLB, it is necessary to identify the causal agent of the disease unambiguously, rapidly and at a level of infection that is not visually apparent. Ribosomal genes are particularly appropriate targets for PCR-directed identification, as the genes occur in high copy numbers, are highly conserved and are flanked by spacer regions that contain comparatively variable sequences. DNA sequence data on ribosomal genes can be obtained by PCR with broad-range primers (universal primers) that anneal to the highly conserved ribosomal gene sequences and amplify across regions that contain nucleotide variation. Planet *et al*. (1995) developed a PCR technique whereby a fragment of the *rplKAJL-rpoBC* operon (b operon) (Jagoueix et al., 1994) of the Asian Liberibacter strain from Poona (India) and the African Liberibacter was amplified. This section of the Liberibacter genome represents a conserved region of the 16S rDNA of the Liberibacter spp. Three primers have been developed and are currently commercially used during PCR detection of HLB (Jagoueix 1996; Hocquellet *et al*., 1999). Currently the PCR technique is ISO 17025 accredited and is being used for commercial detection of HLB in suspected plant materials by Plant Pathology Laboratories, University of Pretoria, South Africa.

Conventional PCR. The first sets of 16S rDNA-based primers (OI1/OI2c and OA1/OI2c) were designed specific for conventional PCR to detect 'Ca. L. asiaticus' and 'Ca. L. africanus', yielding the same size of 1160 bp of 16S rDNA fragments (Jagoueix *et al*., 1996). A time-consuming enzyme digestion of the 1160 bp PCR

products with *XbaI* is needed to distinguish the two Liberibacter species. In China, one specific primer set was developed for detection of '*Ca*. L. asiaticus' (Tian *et al*., 1996). In 1999, the conventional PCR primer set A2/J5 was developed based on the β- operon ribosomal protein gene (Hocquellet *et al*., 1999). This set of primers allows identification of the two Liberibacter species directly by the PCR ampicon size of 703 bp for "*Ca*. L. asiaticus" and 669 bp for '*Ca*. L. africanus'. Also in 1999, another primer set was developed specific to '*Ca*. L. asiaticus' based on DNA fragments directly obtained from HLB-infected citrus in Taiwan (Hung *et al*., 1999). This set of primers produces an amplicon of 226 bp only with strains of '*Ca*. L. asiaticus' not with strains of '*Ca*. L. africanus'. Since none of the primer sets above could detect the new Liberbacter species '*Ca*. L. americanus' , a new set of 16S rDNA-based primers (GB1/GB3) was developed, producing an amplicon of 1027 bp (Teixiera *et al*., 2005). In Thailand, Jantasorn *et al*. (2006) developed the specific primer GB and GE to detect *Candidatus* Liberibacter asiaticus specifically targeting the *rpl*J gene of HLB pathogen.

The universal 16S rDNA-based primer set fD1/rD1 (Weisburg *et al*., 1991) was used in the first round of the nested PCR assays to improve the detection sensitivity of single conventional PCR assays with the 16S rDNA-based primer sets OI1/OI2c for '*Ca*. L. asiaticus' (Deng *et al*., 2007) and GB1/GB3 for '*Ca*. L. americanus' (Teixiera *et al*., 2005). The primer set was also nested with another 16S rDNA-based primer set CGO3F/CGO5R for detection of '*Ca*. L. asiaticus' in *Murraya paniculata* (Zhou *et al*., 2007). The nested conventional PCR was still at least 10 to 100 fold less sensitive than real-time PCR (Teixiera *et al*., 2008). The loop-mediated isothermal amplification (LAMP) method was developed for detection of Liberibacters in under-equipped laboratories without a thermal cycler (Okuda *et al*., 2005). In 2008, LAMP was developed into a cycleave isothermal and chimeric primer-initiated amplification of nucleic acids with probe technology (Cycleave ICAN) (Urasaki *et al*., 2008). Cycleave ICAN method is a little more sensitive than conventional PCR, and can be used for detection of the Liberibacters in screening laboratories without a thermocycler.

Real-time PCR. The first TaqMan probe of real-time qPCR for Liberibacter detection was designed in 2004 (Liao *et al*., 2004) based on the 16S rDNA fragment amplified by the conventional PCR primer set OI1/OI2c (Jagoueix *et al*., 1996) from a HLB-infected citrus plant in Fujian, China. However, its amplicon was 441 bp which was out of the amplicon size range (50-250 bp) for real-time PCR (Wang and Seed, 2006). This too long amplicon leads to decreased PCR efficiency and detection sensitivity with a low detection limit of 1,000 copies of templates of cloned plasmid DNA per reaction.

In 2005, species-specific TaqMan probe-primer sets, HLBaspr, HLBafpr and HLBampr were developed for detection and identification of the three known species of Liberibacter in complex PCR with the positive internal control TaqMan probe-primer set COXfpr targeting the host plant cytochrome oxidase gene (Li *et al*., 2006a). The low detection limits of the three HLB probe-primer sets are down to 1 to 10 copies of Liberibacter's 16S rDNA per reaction and their PCR efficiency is up to 99.90%. In addition, the low detection limit and the PCR efficiency of the positive internal control probe-primer set COXfpr are almost the same as those of the HLB probe-primer sets, which allows accurate estimation of the ratio of the Liberibacter DNA to the host plant DNA in total DNA extracts obtained from infected plants. The HLBaspr set has been successfully applied in detection, identification and quantification of '*Ca*. L. asiaticus' in host plants of citrus (Tatineni *et al.,* 2008) and in vector psyllids (Manjunath *et al*., 2007).

Based on a β-operon protein gene of a DNA isolate obtained from HLB-infected citrus in Quangxi, China in 2006, one TaqMan probe-primer set CQULA04f/r/p10 was developed specifically for detection and quantification of '*Ca*. L. asiaticus' (Wang *et al*., 2006). This primer pair was also employed in the SYBR Green real-time PCR (Wang *et al*., 2006). In 2008, another β-operon-based primer set was developed in Brazil for SYBR Green real-time PCR to study the distribution and quantification of '*Ca*. Liberibacter americanus' in citrus plants (Teixeira *et al*., 2008). The PCR efficiency and sensitivity of the two β-operon-based qPCR primer and/or probe sets were similar to those of the 16S rDNA-based TaqMan probe-primer sets HLBaspr and HLBampr (Li *et al*., 2007; 2006). However, the former two sets

produced real-time PCR results of Ct values about 2 cycles higher than the later two sets. This is due to that there are three operons of 16S rRNA per genome of the bacterium while only one copy of β-operon per genome. So, the detection sensitivity of the 16S rDNA-based real-time PCR is a little higher than that of the β-operon-based one in assay performance for real plant and insect samples. In addition, the real-time PCR assays are at least 10 to 100 fold more sensitive than the conventional PCR methods for detection of Liberibacter species associated with HLB (Teixeira *et al*., 2008; Li *et al*., 2006 and Wang *et al*., 2006;).

A very sensitive and stable positive internal control TaqMan probe-primer set WGfpr was successfully developed in 2007 for real-time qPCR multiplexed with HLBaspr,HLBafpr or HLBampr for detection, identification and quantification of the three unknown species of Liberibacter in vector psyllids (Li *et al*., 2008b). The quality of DNA extraction from Liberibacter-suspected citrus psyllids can be evaluated by the positive internal control. '*Ca*. L. asiaticus' was readily detected and quantified in a single infected nymph or adult psyllid even if it was in a sample composed with other 100 Liberibacter-free nymphs or adults. The discovery of the elevated ratios of '*Ca*. L. asiaticus' DNA to the Asian citrus psyllid DNA by the multiplex real-time PCR has been successfully used in the Liberibacter genome sequencing project (Duan *et al*., 2009).

Multiple genetic loci for detection are to date the most popular and the most confidential method for detection of Liberibacter species associated with HLB. The conserved 16S rRNA can be used as a housekeeping gene for detection of Libertibacter species if primers and/or probes are designed on its species signature (domain-specific) region (Li *et al*., 2006a; Teixieira *et al*., 2005 and Jagoueix *et al*., 1996;). These species-specific primers and/or probes can detect all isolates of each of the three species of Liberibacter associated with HLB and do not react with the new Liberibacter species, '*Ca*. L. solanacearum' associated with zebra chip disease of potato (Li *et al*., 2009), neither other bacteria causing false positive results. However, 16S rDNA-based but domain-beyond primers and/or primers could yield false positive detection results.

6. Transmission

The HLB bacteria can be transmitted to citrus plants by the insect vector , the Asian citrus psyllid (*Diaphorina citri*) and African citrus psyllid (*Trioza erytreae*), dodder (*Cuscuta sp*.), or grafting. Psyllid transmission is the naturally process that occurs in the field. Adult psyllid is small in a range of 3 to 4 mm, or 0.12 to 0.16 inch with mottled brown wing. The adult may survive for several months depending on temperature (Halbert and Manjunath 2004). The Asian citrus psyllid can transmit HLB from the fourth nymphal instar through the adult stage with a latent period as short as 1 day or as long as 25 days (Xu *et al*., 1988). Although no experimentally proven, it is thought that the bacterium multiplies in the psyllid.

The Asian citrus psyllid completes its life cycle on citrus and its close relatives. All life stages (egg, nymphs, and adult) can be found on the new growth or shoot tips. As this insect feeds, it injects a salivary toxin that causes the developing shoots to be malformed, twisted, curled, or laterally notched. In severe cases, the shoot tip will die. In addition, infested leaves may be covered with white, waxy deposits from the psyllids and sooty mold that grow on the large amounts of honeydew excreted by the psyllids (Halbert and Manjunath, 2004). In both Brazil and Florida, the Asian citrus psyllid was found before symptoms of HLB were observed, and this event could certainly occur in California.

HLB pathogen was first transmitted experimentally by grafting (Chen, 1943), thereby establishing the causal agent as a pathogen. Natural spread was demonstrated by exposing healthy seedlings in an infected citrus orchard (Schwarz, 1964), and the vector in Africa was identified shortly thereafter as the citrus psylla, *T. erytreae* (McClean and Oberholzer, 1965). The vector of the disease in Asia was then identified as another species of psylla, *D. citri* (Capoor et al., 1967).

T. erytreae exists in Africa from the Red Sea coast through East and Central Africa to South Africa, as well as in Cameroon. It is also found in Yemen, Madagascar, Mauritius, and, before bio-eradication, Reunion. More recently it has also been described in Madeira Island (Jagouiex *et al*., 1996). It is sensitive to

excessive heat, and thrives in cooler, higher areas 500 m and more above sea level. *D. citri*, on the other hand, is found in hotter, lower lying areas throughout south and south-east Asia, as far west as eastern Iran and Saudi Arabia, in Reunion, Mauritius, St Helena, Guadeloupe, Brazil, Florida (Knapp *et al*., 1998) and, most recently, Venezuela (Cermeli et al., 2000), Texas (French *et al*., 2001), and Mexico (D.Thomas, pers.comm. 2002). Samples have also been collected in Argentina, Bahamas, Cuba, Dominican Republic and Puerto Rico. The heat preferences of the two species correspond to that of the two forms of HLB, although it has been shown experimentally that both species can transmit both forms (Massonie *et al*., 1976). Recently *D. citri* was reported from northern Irian Jaya (West Papua) province in Indonesia near Papua New Guinea (PNG) (Davis *et al*., 2000). During a survey of northern Australia, PNG and adjacent regions by the Australian Quarantine and Inspection Service (AQIS), it was found that the eradication campaign near Sorong failed and that HLB established more than 1000 km to the east (Davis *et al*., 2000). This raised concerns of movement of planting materials or ornamental hosts of *D. citri* that can result in further spread of the disease. Thus far PNG and north Queensland remain psylla and HLB-free.

Under experimental conditions, the pathogens of HLB can be transmitted by some species of dodder (Ke *et al*., 1988; Garnier and Bove, 1983 and Raychaudhuri *et al*., 1974,).

7. Control and Management

HLB disease is spread by insect vectors and the pathogen is limited in the phloem. Control of the HLB vectors and the pathogen of citrus greening disease are involved all aspects of an integrated pest and disease management program. The following is a review of the HLB vectors and HLB disease management. This review focuses on chemical, biological and cultural controls which were found to reduce the vector population and HLB disease severity. HLB free planting materials should be used in order to reduce disease severity and incidence in the area to establish a citrus orchard.

Applying insecticides to control the vector

When the insect and the pathogen of HLB are present, conceivably, the easiest HLB control strategy is chemical control with the use of pesticides. The use of pesticides will reduce the population of HLB psyllid vectors (Graça and Korsten, 2004; Aubert, 1990). Spraying pesticides is expensive; so doing it would depend on the economy of individual producers. Gottwald *et al*. (2007) found that commercial citrus production has access to the machinery or commercial grove care companies to accomplish chemical sprays for psyllid control. However, the necessity of additional sprays is both costly depending upon the economics of individual producers which can be marginally feasible. Although there is much information on efficacy of various insecticides and programs for control of psyllid vectors, the effect of this practice on HLB increase and spread remains largely anecdotal and undocumented. An even greater challenge is the large population of HLB positive residential citrus trees where regulated chemical control is not an option.

In a study conducted in China, 10-13 sprays per year were required during flush periods to rehabilitate citrus production in a HLB infected area (Roistacher, 1996). *D. citri* was controlled using systemic insecticides like dimethoate and monocrotophos (Graca, 1991). Methomyl or melathion sprays on citrus trees at intervals of 10 - 12 days from March to May were not effective against citrus greening. In some cases, 44% dimethoate EC, 50% melathion EC and 40.64% carbofuran FP showed economically good control of the psyllid (Chen, 1998). Two patent applicators are available in South Africa that calibrates the dose based on the diameter of the tree (Buitendag and Broembsen, 1993). Supriyanto and Whittle (1991) and Shivankar *et al*. (2000) also had success with trunk applications. The best time for trunk application is just prior to spring flush (Rao *et al*., 2000). Synchronized chemical control is very important for farmers in a citrus production area affected by HLB. Also the use of sticky cards is recommended for monitoring *D. citri* in order to time control action (Aubert, 1990; Aubert, 1988). In Asia, a range of insecticides, mostly organophosphates and pyrethroids, are used in very intensive spray programs to kill nymphs and eggs of the HLB vector on flush growth. In Southeast Asia, control has not been achieved with 35-52 applications of synthetic pesticides a year (Aubert,

1990). These sprays often comprise four or more active ingredients (some of which are highly toxic and banned). Fruit quality is not improved by heavy use of pesticides; heavy pesticide use destroys natural enemies and results in serious outbreaks of minor pests such as citrus red mite, *Panonychus citri*. In South Africa, applying systemic insecticides aimed specifically at psyllids to tree trunks has been the most effective (Davis, 2005).

Using antibiotics to control the HLB pathogen

In Florida, two chemical agent, penicillin G sodium and 2,2-dibromo-3-nitrilopropionamide (DBNPA), were found to be effective at eliminating or suppressing the '*Ca* Liberibacter asiaticus' bacterium in this periwinkle refeneration system. When treated with penicillin G sodium at 50 μg/ml, all plants regenerated from '*Ca* Liberibacter asiaticus' infected cutting were '*Ca* Liberibacter asiaticus' negative as determined by both nested polymerase chain reaction and quantitative real time PCR (Zhang *et al.,* 2010)

Antibiotics were also used to control the greening disease pathogen. Different types of antibiotics were injected into infected citrus trees, such that the injection of antibiotics temporarily relieved the crop plant of symptoms (Shelton and Badenes, 2006; Tonhasca and Byrn, 1994; Buitendag and Broembsen, 1993; Shamsudin *et al*., 1990 and Su and Chang, 1976;). Injecting antibiotics has been recommended as part of an integrated management program in India (Nariani, 1981).

Leaf symptoms of HLB were reduced when tetracycline hydrochloride was used and complete control of HLB was obtained by using penicillin and carbendazim (Cheema et al., 1986). Tetracycline can also be used to treat budwood by being deeply absorbed in soluations of tetracycline hydrochloride at 1,000 μg/ml for 2 hours or 500 μg/ml for 3 hours (Zhao, 1981).

The best results of the tetracycline treatment were observed when it was used through injection to control the HLB, and the spring season is the best time for injection (Graça and Korsten, 2004; Aubert and Bove, 1980). Trunk injections of

tetracycline hydrochloride have also been successful in Taiwan, China, Reunion and the Philippines (Graca, 1991). In South Africa, tetracycline used up to 20 g/adult tree with high capacity compressors at 10 kg/cm^2 on citrus trees which were infected by greening disease (Buitendag, 1993). Trees were also treated by relight tetracycline and the symptoms of HLB were reduced.

Using mineral oils to reduce disease severity

Horticultural mineral oils (HMOs) have been developed as alternative treatments to control some vectors and reduce the disease caused by these vectors. Using mineral oils have many advantages. It is less damaging to the environment and less disruptive to the biocontrol of other pests. In citrus, using mineral oil is effective in the control of the citrus psyllid which is a vector of the HLB. Mineral oils are also effective in controlling other pests such as citrus red mite, aphids, scale insects and fungal diseases such as greasy spot, algae, thus leaving trees and fruits clean. It can also reduce the severity of virus which is transmitted by aphids. HMOs and machine oils were as effective as omethoate and diflubenzuron (Rae *et al*., 1997). The oils suppressed egg laying (oviposition) by adult female psyllids. It was found that the use of HMOs as a control measure had no phytotoxicity. The foliage and fruit were clean and free of pests as compared to the foliage on trees that were treated by synthetic chemicals. The use of mineral oils also has some disadvantages. For example, the heavy mineral oils may injure the tree and phytotoxicity may occur. However, using mineral oils do not allow female leaf miner and psyllid to lay eggs on oil deposit because the oil moves into the spiracles of the insects. Mineral oils are usually used at the concentrations of 1 to 2%. In Sarawak, as high as 5% of mineral oils was used which did not induce phytotoxicity to the trees (Andrew and Holford, 2007).

Biological control of HLB vectors

There are different approaches to controlling vectors of HLB such as, cultural or biological control measures. In biological control, the parasites *Tamarixia radiate* and *Tamarixia dryi* were found effective against HLB vectors. Nymphs of both psyllid species are parasitized by hymenopterous ectoparasites *T. dryi* Waterston and

T. radiatus Waterston, which has been used to accomplish biological control of vector populations (Chiu *et al.,* 1988; Aubert and Quilici, 1984; Etienne and Aubert, 1980; Catling, 1969). The parasites significantly reduced the psyllid populations and damage of HLB (Chiu *et al.,* 1988). *Diaphorencyrtus aligarhensis* has also been found in parasitoid *D. citri* (Tang, 1989). Field observations in Florida where *T. radiatus* was introduced indicate that the effect of biological control can range broadly from 4 to 70% reduction in psyllid populations (da Graça *et al*., 2007). In Mauritius, biological control of *T. erytreae* was much more effective than biological control of *D. citri*. There are several reasons for this. First, the initial population of *T. erytreae* was much lower than that of *D. citri*. *T. erytreae* reproduces principally on citrus, which is regularly treated with pesticides, whereas *D. citri* utilizes *M. paniculata*, which is unsprayed and is ubiquitous as an ornamental throughout the island. The climate on much of the island also is more suitable to *D. citri* than to *T. erytreae*. Second, the parasite of *T. erytreae* (*Tamarixia dryi* Waterston) has an alternate host in the common psyllid *T. litseae*, whereas *T. radiata*, the parasite of *D. citri*, has no alternate host (Halbert and Manjunath, 2004). In general, biological control of the pathogen has not been well studied and both Asian and African types of greening disease are able to infect the citrus plants (Garnier and Bove, 1996).

Eliminating the source of inoculums

The effectiveness of the removal of diseased trees is directly related to the latency of infection. The first occurrence of visual symptoms can be dramatic in some trees yet subtle in others. It is generally recommended that diseased trees should be removed but multiple asymptomatic and potentially infected trees or trees with subclinical symptoms must be recognized because it may probably exist in the vicinity. It is unknown how much these early stage infected trees, including asymptomatic trees and and those with limited symptoms, contribute to inoculum dispersal. Depending on the diligence and speed with which the individual grove manager removes trees after discovery, these early stage infections may contribute to more or less inocula to an epidemic. Removal of the diseased trees may be more effective if we take this subclinical portion of the population of infected trees into account and develop a threshold of tolerance that we will accept. Entire blocks of

commercial citrus should be removed when the acceptable level of disease severity is surpassed since the potential infection capability exist (Graça *et al*., 2007).

There are three main aspects to managing citrus greening disease: propagation of clean nursery stock, psyllid control and removal of potential inoculum sources (Wu *et al*., 2000; Su *et al*., 1986). In South Africa, control measures were done by removing the infected plants and applying chemical control measures to keep psyllid populations at a minimum (Roux *et al*., 2006; Baniqued, 1998). HLB inoculum reduction on individual branches affected by HLB will not allow the tree to produce good fruit and the diseased branches usually do not recover. As the bacterium spreads slowly through infected trees, removing part of the tree showing symptoms can be useful depending on the age of the tree and the infection level. Young trees which are less than 4 years and those not bearing fruits but showing symptoms should be eradicated and replaced, whereas trees with fruit should only be pruned. Trees infected up to 50-70% should be eradicated. Tree owners are encouraged to remove these trees from the site if possible. The following recommendations for African greening disease were also made in 1993; however, tree destruction or pruning must be done when psyllid populations are at a minimum, otherwise the disturbance will increase tree to tree spread of both psyllids and disease (Buitendag and Broembsen, 1993).

In many Pacific Islands, it may be possible to reduce inocula when psyllid populations are naturally low, simply by timing the work carefully. This would avoid the environmental impact and costs of killing psyllids prior to working on trees. If a distinct wet season occurs, psyllid numbers will be at their lowest towards the end of the season, making this the ideal time to get rid of HLB infected branches and trees. Replacement trees must be known as HLB free and should be obtained either from disease indexed nursery stock, or from disease free areas.

Using resistant citrus rootstocks to reduce the HLB severity

Using HLB free seedlings is the most effective way to reduce the severity of greening disease. After planting these disease free seedlings, proper maintenance and

monitoring should be followed in order to keep them healthy. Good orchard management plays a very important role in controlling the pathogens. The emphasis is on improving the orchard environment. The layout and design should ensure good ventilation and exposure to sunlight, good soil management includes efficient fertilization program and grassy cover in orchards where rainfall is heavy. While these practices may not keep the trees free of greening and virus diseases indefinitely, they will prolong their useful lifespan and keep the trees productive for some years even after they have been infected.

Citrus plant is normally propagated through grafting. It is done by taking a scion from a disease free citrus plant and placing it into the disease free rootstock. Selection of healthy plant source materials is very important so as not to spread HLB disease. Pathogen free rootstocks are obtained through a shoot tip grafting (STG) system. The main thing to do is to keep the orchard free from any pests, especially HLB vectors. However, infected trees inside an orchard must be immediately eradicated, including its alternative host plants. Rootstock selection in citrus is chosen primarily on criteria including cold tolerance and disease resistance (particularly resistance to virus disease) in the growing area (Ferguson, 2003). In some cases, the rootstock can affect the occurrence of the HLB disease symptoms (Kapur et al., 1982). In South Africa, the percentage of greening in Valencia oranges was higher on trifoliate orange rootstock than on empress Mandarin or Troyer *citrange*. The trifoliate rootstock caused an extension of the growth flesh period thereby extending the feeding time for the HLB (Vuuren and Moll, 1985).

In contrast, no differences were found in a Chinese study on the effects of 13 rootstocks on symptoms in Ponkan mandarin (Lin, 1963). Unfortunately, no readily available source of resistant citrus has yet been identified either for conventional breeding or transgenic improvement systems. It is anticipated that the efforts in pathogen and host sequencing and bioinformatics will point to resistant genes or pathogen vulnerabilities thus paving the way for future incorporation of disease resistance mechanisms. At the present time, the citrus industry is composed of numerous commercial cultivars and species, a vast majority of which are moderately to highly susceptible to HLB. Thus when sources of resistance are identified, this

resistance will have to be incorporated, perhaps using transgenic technology, into a large number of cultivars and species to meet with current marketing demands.

Other than this, there are 23 rough lemon strains, 8 of which namely 'Australian', 'Florida', 'Helseth', 'Milam', 'Miri', 'South Africa-I', 'South Africa-II' and 'Volkamar' lemon remained free from HLB infection (Kapur *et al.,*1982). In 'Jambiri', 'Jullundri Khatti' and local rough lemon, 100% infection was obtained. The infection in other strains varied from 20 to 80%. The tolerant rough lemon strains were re-evaluated by re-inoculation and re-indexing. 5 strains, namely 'Milam', 'Miri', 'South Africa-I', 'South Africa-II' and 'Volkamar', showed no infection. Of the remaining 3 strains, Australian rough lemon showed visual symptoms of greening on 'Pineapple' sweet orange indicator. The other 2, i.e. 'Florida' and 'Helseth', although not showing any visual symptoms in the indicator bud shoots, were found positive for greening marker substance, thus showing their susceptibility to greening. As to the 10 other rootstocks, none was found free from infection. In addition, 100 % infection was observed in Chinese box orange. In others, the infection varied from 60 to 80% (Kapur *et al*., 1982).

Reducing HLB severity by inter cropping

In Vietnam, the severity of HLB was controlled by inter planting citrus with guava which apparently inhibits the psyllid vector. It was reported that this inhibition could be in two ways. One is that the vector directly is attracted to the guava and killed when it feeds presumably by some toxin; the other way is that the vector is indirectly repelled or confused by some released volatile substances. However, the exact effect is unknown but preliminary data are encouraging (Beattie *et al*., 2006).

The effect of interplanting crops or mixed crops to deter insect pests and diseases via a "push-pull" strategy is not new, but rarely used in western agriculture (Isman, 2006; Tonhasca and Byrn, 1994). The potential of this strategy and its integration with more traditional management tools such as chemical vector control and rouging are being examined in commercial citrus plantings in Florida but are not recommended at this time due to insufficient research data (Graça and Korsten, 2004).

Hirohisa *et al*. (2008) conducted a study on different models of infected HLB disease of new citrus orchards in Tan Phu Thanh village in Japan. The models were:

A) Supplemental mixed planting,

B) Intercropping Cam Sanh and mango,

C) Quit Duong solitary planting with Murraya traps of psylla,

D) Intercropping of Quit Duong and mango.

Trees with HLB symptoms were 76.3% in orchard A, 19.7% in orchard B, 5.1% in orchard C and 3.3% in orchard D. The study showed that the situations were different among models of orchard. In orchard B, the ratio of infected plants in bed set intercropping showed decrease tendency from near to far side from Chanh Num spreader bed, while a non-intercropping bed shows no influence of the distance. In orchard C, infected trees are mainly located near a Chanh Giay bed and efficacy of Murraya traps was not clear. The results suggest that the Chanh Num and Chanh Giay were dangerous spreader and distance from them is an important factor for early HLB transmission into new orchards (Hirohisa *et al*., 2008).

MATERIALS AND METHODS

1. Sample collection

Infected periwinkle was collected from a greenhouse at the USDA-ARS, Fort Pierce, Florida, USA. Infected citrus leaves showing symptomatic blotchy mottle were collected from Picos Farm at the USDA-ARS. All samples were used for preparing '*Candidatus* Liberibacter asiaticus' inocula.

2. Preparation of Periwinkle leaf and flower extract

Periwinkle leaves and flowers were collected from healthy periwinkle in the greenhouse and washed by tap water, and dried. The leaf and flower tissues were squeezed with no addition of buffer using Diedmont Machine & Tool (Dayton Electric MFG CO.,Chicago, IL). The resultant materials were transferred to a 50 -mL Falcon tube and centrifuged at 5,000 rpm for 20 minutes. The supernatant was transferred to a fresh 50-mL Falcon tube and sonicated on ice by using a Branson Sonifier 450 (Terra Universal, Danbury, CT) at 5 µm amplitude. After sonication, the samples were pooled into 50-mL centrifuge tubes and spun at 14,000 rpm for 30 minutes using a Sorval W/a SLA-1500 rotor. The supernatant was then decanted into a fresh Falcon tubes, filter sterilized using 0.2 µm filters (Millipore) and store at 4ºC prior to use.

3. Bacterial isolations and culture media.

The midveins of leaves were cut out by using a razor blade and surface sterilized in 95% ethyl alcohol for 5 minutes. The samples were then rinsed in sterile water for 5 minutes and placed into a 10% bleach solution for 10 minutes. The midveins were washed in sterile water two times for 5 minutes each and placed in between two layers of sterile paper towel set in Petri dishes.

To prepare the Las inoculum, the midveins were cut into small pieces by using a sterile razor blade in a Petri dish. The pieces of midveins were placed into a 50-mL Falcon tube and NT buffer (0.85%NaCl, 1.5% arabinose) was added. The tube was incubated at room temperature for 30 minutes and centrifuged at 8,000 rpm for 1 minute. The supernatant was collected for Las inocula, and 100 uL was added to the media as described below.

Psyllids were fed in a cage with HLB infected trees. After feeding for a varied period of time, psyllids were collected and steriled in 75% ethyl alcohol for 5 minutes. They were rinsed in sterile water for 5 minutes and placed into a 5% bleach solution for 10 minutes. The psyllids were washed in sterile water two times for 5 minutes each and placed in between two layers of sterile paper towel in Petri dishes to dry. The psyllids were smashed by using mini pestle in 2-mL eppendorf tubes containing 200 ul NT buffer. The tube was incubated at room temperature for 30 minutes and centrifuged at 8,000 rpm for 1 minute. The supernatant was collected for Las inocula, and 100 uL was added to the media as described above.

The Ar medium consisted of 6 g of K_2HPO_4, 2g of KH_2PO_4, 2.55 g of $NaNO_3$ per liter as a source of electron acceptors for denitrification under anaerobic conditions, 3 g of NaCl per liter, and the pH was adjusted to 5.8 before autoclave. After autoclaving, the medium was cooled to room temperature at which the following was added to the medium: ATP at a concentration of 2,000 ppm, 10% periwinkle leaf or flower extract, 2.5% sodium hydroxide, and 2% glucose.

The cultures were incubated in an shaker incubator with 2,000 rpm at 25℃ for 24 hours. , One milliliter of the culture was removed from each flask. Three samples were collected for each temperature and pH treatment. The flasks were returned to the shaker after each sampling. This study was performed twice. A separate experiment was conducted for the Las bacterial growth at 25℃ and at a pH 5.8. Cultures were sampled 1 mL every 24 hours for 14 consective days.

4. DNA extraction from HLB cultures

The cell suspension was centrifuged at 15,000 rpm for 15 minutes, and the supernatant was discarded. The cell pellets were resuspended in 100 uL of lysozyme buffer (10 mM Tris-HCl pH 8.0, 20% sucrose), and 5 uL of lysozyme (50 mg/mL) was added to the cell suspensions, and incubated at 37°C for 1 hour. After incubation, 2 uL of Protenase K (50 mg/mL) was added and incubated at 65°C for 30 minutes. The sample was then treated twice with a freeze and thaw using liquid nitrogen and a 65°C water bath. The sample was transferred to a 2-mL tube with screw cap containing 5 glass beads. The tubes were placed in a Fast Prep-24 and homogenized for 3 minutes. Four hundred microliters of extraction buffer (0.1M NaCl, 0.2 M sucrose, 50 mM EDTA, 50 mM Tris-HCl pH 8.0, 1.25% SDS) were added and the mixture was transferred into a fresh 1.5-mL micro-centrifuge tube. The sample was mixed by inverting 20-30 times and incubated in a water bath at 65°C for 20 minutes. After incubation, 186 µl of solution III (5 M potassium acetate, 3 M glacial acetic acid) was added followed by 200 µl dichloromethane (CH_2Cl_2) and vortexed vigorously for 20 seconds and placed on ice for 10 minutes. The samples were centrifuged at 12,000 rpm for 10 minutes. The supernatant was transferred into a 1.5-mL tube which contained 500 µl of isopropyl alcohol. Tubes were inverted 5-10 times and centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant was carefully decanted and the pelleted DNA was washed once with 70% ethanol. The DNA pellet was dried by speed vacuum. 20-50 µl of distilled water was added to the tube of dried DNA and 2 µl of DNA sample were used for PCR analysis.

5. DNA Extraction from plants

The methods for DNA extraction followed Alexander *et al*., (2007). DNA extraction was done from midrib of infected plants and healthy plants excised with razor blades. A portion of 0.5 g of midribs was cut into small pieces with a razor blade, frozen with liquid nitrogen, and then ground into powder with a mortar and pestle. Six hundred microlitter extraction buffer (0.1M NaCl, 0.2M sucrose, 50mM EDTA, 50mM Tris-HCl pH 8.0, 1.25% SDS) was added and the mixture was transferred into a 1.5 ml micro-centrifuge tube, then was mixed by inverting the tube

20-30 times. Samples were incubated in a water bath at 65°C for 15 min. After incubation, 226 µl solution III (5M Potassium acetate, 3M Glacial acetic acid) and 200 µl of dichloromethane (CH_2Cl_2) were added, vortexed vigorously for 20 sec. and placed on ice for 10 min before the tube was centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into a fresh tube containing 600 µl of isopropyl alcohol for precipitation of DNA. To pellet DNA, tubes were inverted gently 5-10 times and centrifuged at 14,000 rpm for 5 min. The supernatant was carefully decanted and the pellet DNA was washed once with 70% ethanol. The DNA pellet was air-dried and 20-50 µl of distilled water was added. Two microliters of DNA sample were used for PCR analyses.

6. Conventional PCR amplification

All the primers used for conventional PCR in this study are list in Table 1. DNA amplification was performed in a final volume of 20 µl containing 10 µl of 2X buffer D (Epicentre Biotechnologies, Madison, WI, USA), 250 nmol of each forward/reverse primer, 1.25 units of *Taq* DNA polymerase (New England BioLabs Inc., Ipswich, MA, USA), and 1 to 2 µl of genomic template DNA from '*Candidatus* Liberibacter asiaticus' which was extracted from nidribs of orange jasmine, psyllid vectors and '*Candidatus* Liberibacter asiaticus' cultures. Conditions at which PCR was performes were: denaturation at 95°C for 3 min, followed by 35 cycles of amplification with denaturation at 94°C for 45 sec, annealing at melting temperature of each primer pairs for 45 sec (as shown in Table 1), and DNA extension at 72°C for 1 minutes with a final extension at 72°C for 10 minutes using a thermocycler (Perkin-Elmer 9600/Applied Biosystem, Bedford, MA). The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer, the gel was stainedwith ethidium bromide and amplified DNA bands were viewed under UV-transilluminator.

7. Real time PCR quantification of '*Candidatus* Liberibacter asiaticus' genome in HLB culture

Primers and TaqMan probes labeled with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) were used for detection of '*Candidatus* Liberibacter

asiaticus' (Li *et al*., 2006). The 5' terminal nucleotide of the probe was labeled with hexachlorofluorescein (HEX) reporter dye (Sigma, St Louis, MO) and the 3' nucleotide with Black Hole Quencher (BHQ-1(Sigma, St Louis, MO). The primers were designed such that the annealing temperature of the primer is close to that of the primer set used for detection of '*Candidatus* Liberibacter asiaticus'. DNA samples were tested by real time PCR for the presence of '*Candidatus* Liberibacter asiaticus'. Real time PCR was performed using primers HLBasf/r and probe HLBp based on the 16s rRNA gene (Li *et al*., 2006), and primer LJ900f/r targeting on a gene in the prophage region (Kent *et al*., 2011) (Table 2). Quantitative real-time PCR was carried out with primers and probes for '*Candidatus* Liberibacter asiaticus' as described in Li *et al*. (2006). Real time PCR amplifications were performed with Mastercycler Realplex Real time PCR system (Eppendorf Inc., Hauppaugeny, NY, USA) using TaqMan Universal PCR Mastermix (Applied Biosystems, Beverly, MA, USA) in a volume of 15 ul reaction. The standard amplification protocol was 95°C for 15 min followed by 40 cycles at 94°C for 15 s and 58°C for 60 s. All reactions were performed in triplicate with positive, healthy, and water control, and the mean value of the threshold cycle (Ct) was presented with standard deviation. For direct quantification of total DNA, each sample was measured using a Nano-Drop spectrophotometer (Thermo Scientific Inc., Tunnell Blvd., Maiami OK, USA).

Table 1 List of primers used in this study for conventional PCR

Primer	Sequence (5'-3')	Tm (°C)	Ref.
LJ524f	AGCCATTCCTACTGCCAAAG	56	this study
LJ427r	CTCCACGGCTTTAGACATTG	56	this study
LJ383f	AGAGACAGTAAGGAGGCAATC	56	this study
LJ384r	GCGAGATGATAACCGACGAG	56	this study
LJ667f	GTTGACGCTGTTGATGACGATG	60	this study
LJ668r	TCCGCCGTATCCATAAAGAACTG	60	this study
LJ846f	AACCGAAGTACCTATGCAAG	54	this study
LJ848r	TCAAATCTATTACTTCACTCATCC	54	this study
LJ161f	TCCCCATAATTTTCTAACATC	52	this study
LJ162r	AACTGCTCCCAAGAACTGC	52	this study
LJ119f	GCTCTCTGTATCTTGCCAAC	54	this study
LJ120r	AATACACCCAAAAATCCTCTC	54	this study
LJ1124f	TGGGATCTTCTATTGGAGC	52	this study
LJ518r	ATGCCCCAATCAAAACAAAG	52	this study
LJ532f	CCCTATAACAGAAAATCCTAAC	56	this study
LJ533r	TTGCGTTGGATTTAGTTGCTG	56	this study

Table 2 List of primers and probes used in this study for real time PCR

Primer	Sequence (5'-3')	Loci in Las genome	Ref.
LJ900f	GCCGTTTTAACACAAAAGATGAATATC	prophage gene	Zhou *et al*., 2011
LJ900r	ATAAATCAATTTGTTCTAGTTTACGAC	prophage gene	Zhou et al., 2011
Lasf	CTTACCAGCCCTTGACATGTATAGGA	16S rRNA	-
Lasr	TCCCTATAAAGTACCCAACATCTAGG	16S rRNA	-
HLBasf	TCGAGCGCGTATGCGAATACG	16S rRNA	Li *et at*., 2006
HLBr	GCGTTATCCCGTAGAAAAAGGTAG	16S rRNA	Li *et at*., 2006
HLBp	*AGACGGGTGAGTAACGCG**	16S rRNA	Li *et at*., 2006

*6-FAM™ at 5' –end; **Iowa Black FQ at 3' –end

8. Construction and screening of 16S rDNA of 'Candidatus Liberibacter asiaticus' clone libraries

The 16S rDNA genes were amplified from extracted DNA using oligonucleotide primers (27F 5'AGAGTTTGATCMTGGCTCAG3' and 1492r 5'TACCTTGTTACGACTT3'). The voluame of 20 μL reaction containing 10 μL of 2X buffer D (Epicentre Biotechnologies, Madison, WI, USA), 250 nmol of each forward/reverse primer, 1.25 units of *Taq* DNA polymerase (New England BioLabs Inc., Ipswich, MA, USA), and 1 to 2 μL of genomic template DNA from 'Candidatus Liberibacter asiaticus' cultures. Thermocycling conditions included 3 minutes of denaturation at 95°C, 45s of primer annealing at 54°C, and 45s of primer extension at 72°C. This cycle was repeated 35 times. PCR products were digested by *Ban*I restriction enzyme and cloned by using the TOPO-TA cloning kit with the pCR 2.1 vector (Invitrogen, Grand island, NY, USA) according to the manufacturer's protocol.

Clone libraries were screened by blue/white colonies and plasmid DNA was extracted from *E. coli* cultures of recombinant clones grown for 24 hours in 96-well microtiter plates containing 1 mL of Luria-Bertani (LB) media per well using the Nucleospin® Plasmid Quick Pure kit (Macherey- Nagel Inc., Bethlehem, Bedford, MA , PA). The LB medium was prepared by adding 5 g of yeast extract, 10 g of tryptone, and 10 g of NaCl into 1 liter of deionized water. DNA sequencing was performed in the U.S. Horticulture Research Laboratory Core Genomics Facility using BigDye Terminator version 3.1 and the 3730x1 DNA analyzer (Applied Biosystems, Bedford, MA). The 16S rDNA sequences were classified by partial nucleotide sequencing and BLAST analyses (version 2.0; National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/ BLAST/]).

9. Sample preparation for scanning electron microscopy

The Las samples in liquid media were centrifuged at 10,000 rpm for 30 minutes. The supernatant was discarded, and the pellets were fixed in a solution of 2.5% glutaraldehyde in 75 mM Phosphate buffer at a pH7.4 for 1 hour. The resultant

samples were rinsed three times for 15 minutes in 50% of 75 mM phosphate buffer. After rinsing , samples were dehydrated in ethanol at progressing concentrations of 30%, 50%, 70%, 80%, 85%, 90%, 95%, and three times with 100%, three times with HDMS solution each for 15 minutes. The samples were dried at 55ºC in hot air-oven for 10-16 hours, coated with gold and visualized using a scanning electron microscope (Hitachi S-4800, Tokyo, Japan).

10. Sample preparation for fluorescence *in situ* hybridization

Suspensions of pellets were placed on the slide and air dried, and the samples were fixed to the slide using 4% paraformaldehyde (PFA) overnight. The slide was rinsed twice with 1X phosphate buffer saline and dried. One hundred uL of 0.5mg/mL lysozyme solution was added to the dried cell smear on the slide. The slide was incubated at room temperature for 30 minutes, and washed twice with 1X PBS buffer. The cell smear was treated with 100 uL of 0.1 ug/mL protenase K and incubated at room temperature for 10 minutes. The slide was washed three times with 1X PBS buffer and dried. Eighty microliters of hybridization buffer were added to the cell smear. The hybridization buffer consisted of 0.9 M NaCl, 20 mM Tris-HCl (pH 7.5), 40% formamide, 0.1% (wt/vol) sodium dodecyl sulfate, and 1 ng of TEXAS labeled probe per microlitter. The smears were covered with plastic cover slips, and the slides were incubated in a buffer-saturated hybridization chamber at 46ºC for 2.5 hours. After hybridization, the cover slips were eased off using warm (56ºC) washing buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.5) at 56ºC. The slides were washed in 50 mL of washing buffer at a temperature of 56ºC,   then transferred to room temperature and buffered for 5 minutes into the dark. Slides were washed in distilled water and dried. Once the slide was dry, a cover slip was mounted on the slide using anti-fade reagent (Invitrogen Inc., Grand island, NY, USA). The slide was placed on a slide warmer overnight. The other method was determined with 0.5 µg (wt/vol) of 4', 6-diamidino-2-phenylindole (DAPI) as a DNA stain under illumination with a UV excitation filter block.

The slides were examined using an Olympus epifluorescence microscope (Olympus AX70 provis, Vancouver, BC, Cannada) equipped with Texas Red dual-band filter set (Chroma Technology Corp., Brattleboro, Vt.). 'Candidatus Liberibacter asiaticus' cultures were identified as multiple clusters of bright fluorescent in multiple fields of view. Images were obtained with a color charge couple device camera connected to a computer system.

11. Sample preparation and viability of Las using LIVE/DEAD® BacLight™ kit

Las inocula were prepared as described above on a shaker at 25°C for 24 hours. Cell suspensions were centrifuged at 10,000 rpm for 30 minutes. The supernatant was discarded and the pellet was resuspended in 100 uL of 0.85% NaCl or appropriate buffer. One hundred microliters of this suspension were added to 1.5- mL microcentrifuge tube containing either 800 uL of 0.85% NaCl for bacterial live cells or 800 uL of 70% isopropyl alcohol to kill the bacteria. Both samples were incubated at room temperature for 1 hour and mixed every 15 minutes. After incubation, the samples were centrifuged at 12,000 rpm for 10-15 minutes. The supernatant was discarded and the pellet was resuspended in 100 uL of 0.85% NaCl. The samples were then centrifuged at 12,000 rpm for 10 minutes. Finally, the pellet was resuspended in 50 uL of 0.85% NaCl.

Direct counts of viable and total bacteria were also obtained with BacLight viability kit (Molecular Probes Grand island, NY, USA). Samples were incubated in the dark room at room temperature for 5 to 10 minutes. Two BacLight stains, SYTO9 and propidium iodine, dissolved in DMSO, were mixed together (1:1). An aliquot of 50 uL of samples were mixed with 0.3 uL of a mixture of SYTO 9 and propidium iodine nucleic stains from a LIVE/DEAD® BacLight™ kit, which were then vortexed and incubated in the dark for 15 minutes at room temperature. A 5 uL aliquot was spotted on a slide and an 18 mm square cover slip was placed on top. The population of total bacteria was determined by counting green and red micro-organisms under the excitation and emission maxima of 480 and 500 nm for these dyes for SYTO9, and 490 and 635 nm for propidium iodine, respectively. All counts by viability were

determined in two independent experiments in which the live cells of bacteria with intact plasma membrane were fluoresce green, and the dead or injured cells with compromised membrane were fluoresce red. In accordance with the manufacturer's instructions, all green cells were considered viable and red cells were considered dead. Images were recorded with a digital camera (Olympus, Vancouver, BC, Cannada)

12. Inoculation of host plants with 'Candidatus Liberibacter asiaticus'

Healthy periwinkle and citrus plants were grown from seeds in an insect-proof greenhouse at ambient temperature. Las cells from 20 mL of Las cultures were resuspended in 10 mL 1X PBS buffer after washing twice in 1X PBS buffer, and inoculated periwinkle or citrus plants by root soaking or leaf injection. For root soaking method, young periwinkle and citrus seedlings with 3 to 4 leaves were pulled out and the soil was washed away from the roots. The roots of each plant were soaked in a small vial cut from the bottom of 15 mL Falcon tube which contained 1 mL of Las cell suspension overnight. The inoculated seedlings were re-planted in pot soil and maintained in the insect-proof greenhouse. For Las inoculation by injection, 100 uL of cell suspensions were injected into leaf midribs on healthy periwinkle and citrus seedlings and maintained in the same greenhouse. Starting when after inoculation leaves from periwinkle and citrus seedlings were collected once every few months for DNA extraction until symptoms developed.

13. Assessment of 'Candidatus Liberibacter asiaticus' in Murraya paniculata and Diaphorina citri

13.1 Collection Murraya paniculata Plants

Characterization on symptom expression of Huanglongbing naturally infected citrus and relative plants were determined. The Murraya samples from diverse geographical regions were collected in Thailand. The collection sites of Murraya plants were selected in provinces of the Northern Region, the Central Plain and the Eastern Region. All of the murraya plants were established from cutting or

macrottings. Symptomatology of Huanglongbing on Murraya appeared as the same symptoms reported elsewhere including vein yellowing and the leaf symptom similar to zinc deficiencies and symptomless .

13.2 Collection of psyllids

Adults of *D. citri* were collected from both visually healthy and symptomatic plants belonging to *Murraya paniculata*, and related genera from diverse geographical regions in Thailand, such as commercial groves, retail, resident sites and discount garden centers in different provinces of Thailand. Adult psyllids were collected using an aspirator. The insects were catalogued and stored in 75% ethanol in 2 ml tube for further analysis of Lasand non-Las infections. The psyllids were assigned unique sample number and stored -20°C until processed.

RESULTS

1. Culturing and media conditions

We developed a medium for '*Candidatus* Liberibacter asiaticus' multiplication as well as isolation of the inoculum from plant midvein. We determined that '*Ca.* Liberibacter asiaticus' grew on glucose and ATP as a carbon source and biological energy of the cell, respectively. The HLB bacteria can growth on broth media 6 hour after inoculation. The positive of HLB in culture was detected by RT-PCR by using HLBas/r and HLBp TaqMan (Li *et al*., 2006). There are three inoculum sources from HLB-affected plants. Figure 1. represented the growth of Las from periwinkle, citrus and psyllid culture on broth media at 0, 6, 12, 24, 48, 72, and 168 hours. The higher populations of Las were about 24-48 hours after inoculation.

Confirmation '*Ca.* Liberibacter asiaticus' grew in culture by using LJ00fr (SYBR Green1) targeting hyv_I gene, LasLong (SYBR Green) targeting 16S rDNA, and HLBaspr protocol were performed on samples from Las bacterial culture isolated from different HLB-affected materials. '*Ca*. Liberibacter asiaticus' was detected by all three methods in six samples. The titers of Las in these six samples from four HLB-infected materials were highly variable using three different primer and probes targeting different loci. All the resulted were shown in Table 3.

The colonies of '*Candidatus* Liberibacter asiaticus' on Ar agar media were irregularly shaped and very small in size when observed after 2 to 4 days at 25°C. The observation was confirmed by the analysis of cell under the microscope (Figure.2) and the clumping of Las bacterium in Ar liquid medium were observed under the microscopic at 100X magnification (Figure 3). The colonies that were increased in size and viability were lost after 2 to 3 weeks. On solid media, single colonies were clumps of '*Ca.* Liberibacter asiaticus' cells and visible after 4 days under scanning electron microscopy (Figure 12B). In liquid culture of '*Ca*. Liberibacter asiaticus' can be subcultured three times to the new media (Figure. 4). However, the bacterial titer remained unchange for another four times, and declined thereafter. To further examine the identity of the observed 16S rDNA libraries of '*Ca.* Liberibacter

asiaticus' in the media, the 16S rDNA gene was amplified, cloned and sequenced. A BLAST search revealed 88% identity with a partial sequence of the 16S rDNA gene of '*Ca.* Liberibacter asiaticus' Genbank accession CP001677 and 4%, 6% and 2% belongs to other uncultured bacteria *Novospinggobium*, Stenotrophomonas and others respectively. Thus, '*Ca.*Liberibacter asiaticus' appeared to be more aboundent, which is clear indication of growing in the medium.

Use of periwinkle extracts at a final concentration 10% enhanced the growth of the Las bacterium based on RT-PCR results. The results show that periwinkle leaf extract prepared from expanded whole leaves supported growth better than periwinkle flower extract prepare from flowers (Figure 5). However, the citrus extract prepared from young fully expanded whole leaves and citrus juice prepared from young fruit did not enhance the growth of HLB in Ar media.
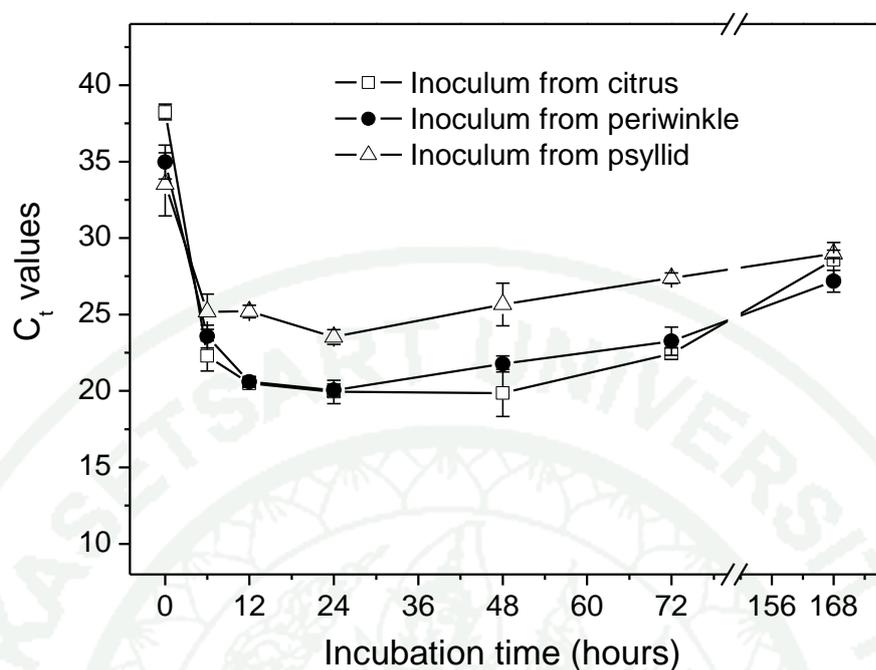
Figure 1 Growth of '*Candidatus* Liberibacter asiaticus' isolated from HLB- affected
periwinkle, citrus and psyllids vector (*Diaphorina citri*) in Ar broth
medium over a period of 14 days detecting by real time PCR.
Vertical bars represented standard errors of the mean from two
experiments. (No amplification was detected from control)

Table 3 Quantitative measurment of '*Candidatus* Liberibacter asiaticus' from the culture samples using qPCR targeting on 16S rRNA gene with TaqMan and SYBR, hyv_I- and hyv_{II}- gene with SYBR

Sources of inoculums	Culture codes	Type of periwinkle extract	Real time PCR Ct value by:		
			16S rRNA gene-based HLBasfpr	16S-rRNA Las Long	hyv_I -and hyv_{II}- based LJ900fr
HLB infected periwinkle	P$^+$-PWE	Leaf extract	20.60	18.62	14.83
	P$^+$- FW	Flower extract	21.91	19.57	15.05
HLB infected citrus	C$^+$-PWE	Leaf extract	22.30	19.24	16.09
	C$^+$-FW	Flower extract	22.45	20.52	15.08
HLB infected psyllids	Psy-FW	Flower extract	24.08	22.63	18.41
HLB infected Mealybug	ML-FW	Flower extract	28.25	27.76	19.78
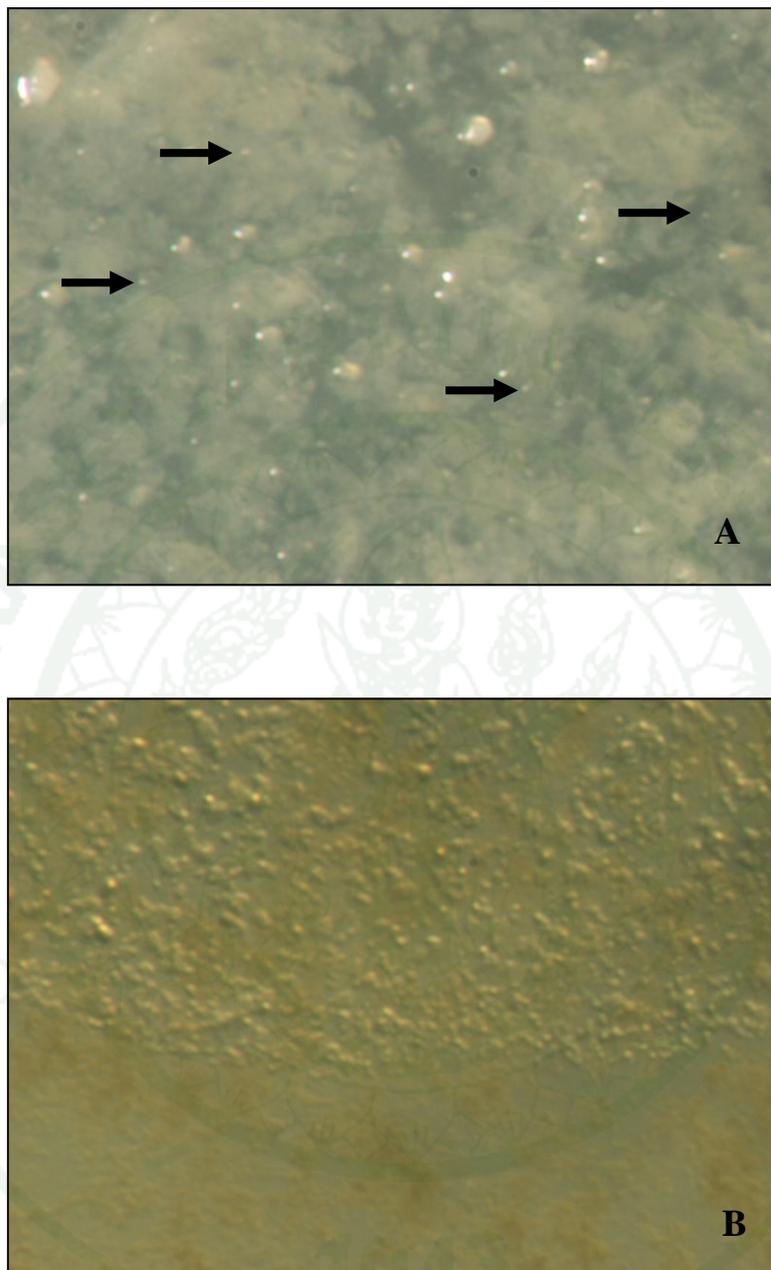
Figure 2 Single colony of '*Candidatus* Liberibacter asiaticus' on solid Ar medium
incubated at 25ºC. Steriomicrographs of Las colony with top amd bottom
light sources; A, top view angle and B, Incline view angle at 4 days after
incubation (100X magnification)
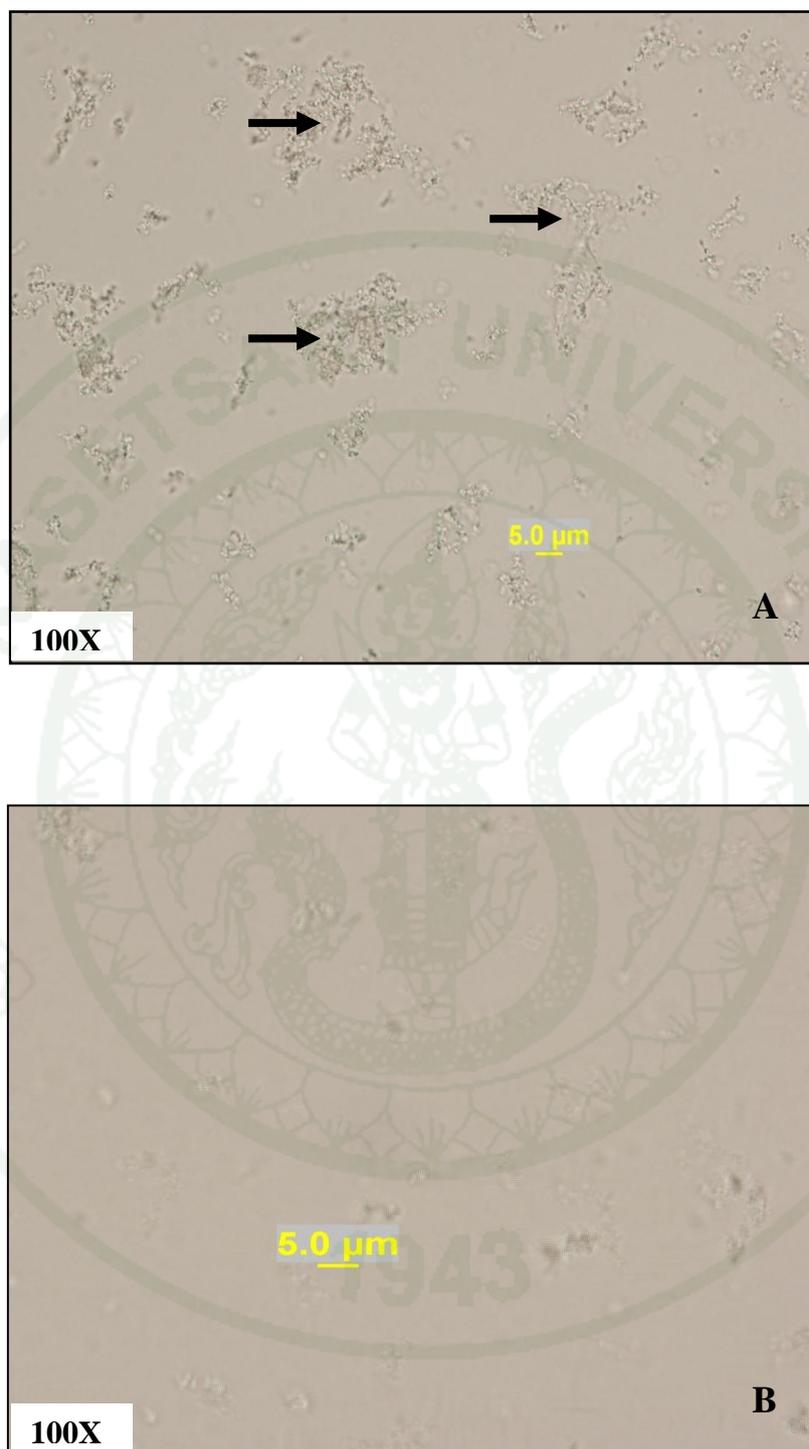
Figure 3 Clumping cells of '*Candidatus* Liberibacter asiaticus' (Las) in Ar broth medium incubated at 25ºC.A), Micrograph of Las at 4 days after incubation (100X magnification); and B), No Las in control culture
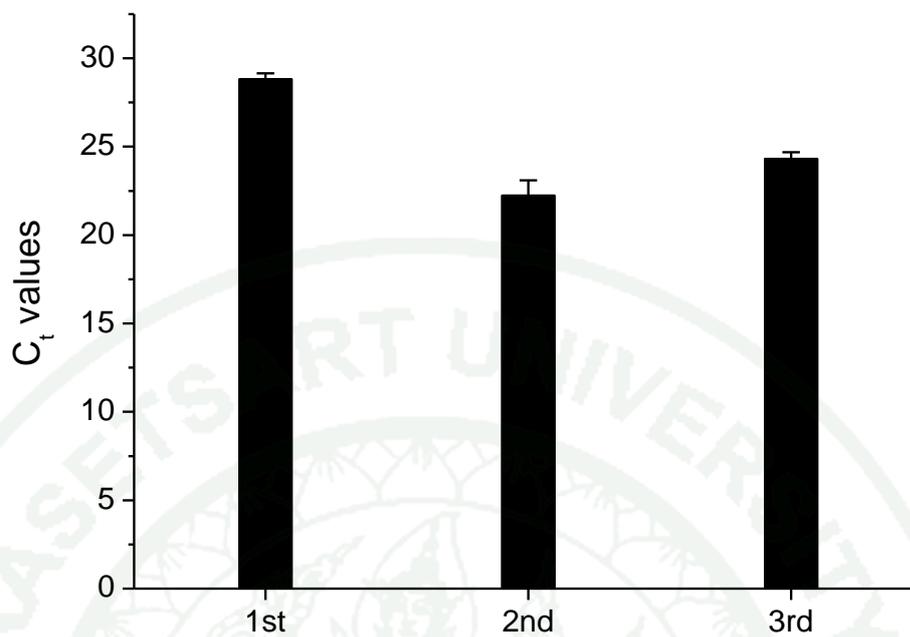
Figure 4 Growth of '*Candidatus* Liberibacter asiaticus' in 48 hours sub-cultures interval with new Ar broth medium. The Ct values were determined using Las-specific16S rDNA primer and probe (HLBasfpr**).**
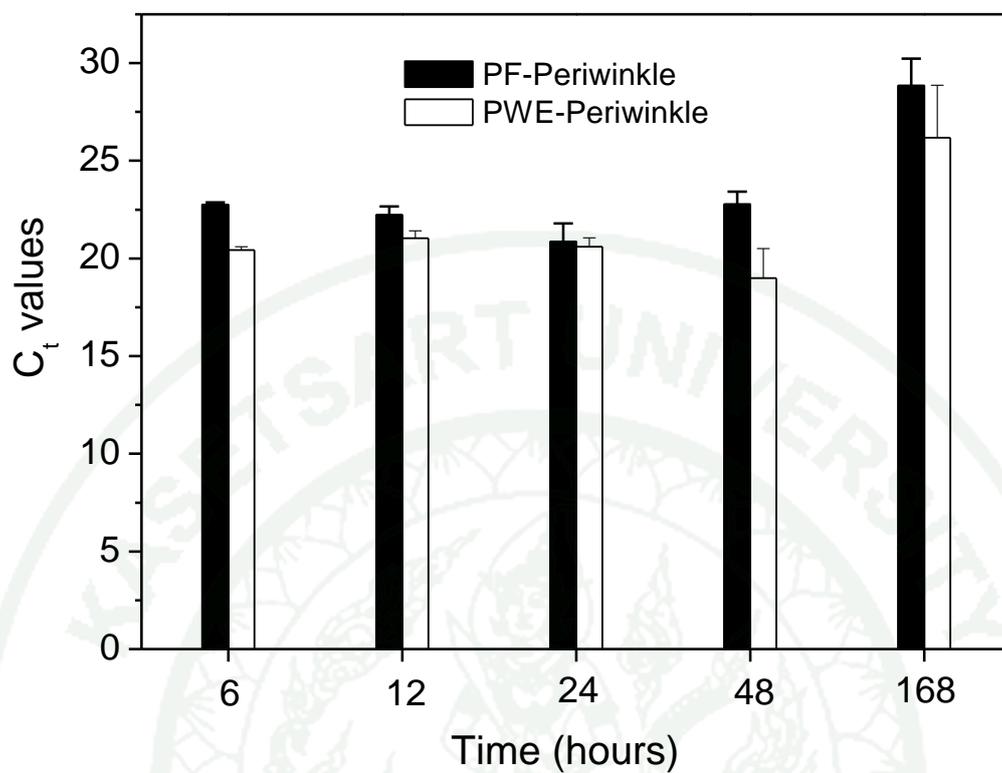
Figure 5 Effect of periwinkle leaf extract and periwinkle flower extract as
subplements on the growth of '*Candidatus* Liberibacter asiaticus'in Ar
broth medium.

2. Conventional PCR detection

Based on the genome sequence of 'Candidatus Liberibacter asiaticus' CP001677, we designed eight set of specific primers to confirm the presence and growth of 'Candidatus Liberibacter asiaticus' in the culture. First, LJ524f/LJ427r and LJ 383f /LJ384r located in SC1 and SC2 prophage region were used to amplified 583 bp, and 1,602 bp amplicons respectively (Figure 6 A and B),. This primer can be confirmed presence or absence of prophage gene region in 'Candidatus Liberibacter asiaticus' in medium.

In order to conform 'Candidatus Liberibacter asiaticus' can be grown in synthetic Ar medium PCR reactions of the genomic DNA from culture and healthy plant extracts were performed using another six Las-specific primer pairs, namely, LJ667/668, LJ846/848, LJ161/162, LJ532/533, LJ119/120, and LJ1124/518. After PCR amplification, all six primer pairs yield bands with expected size from 'Candidatus Liberibacter asiaticus' culture but not healthy control (Figure 6, 7 and 8). confirming.

A total of 65 PCR confirmation reactions (covering approximately 8,210 bp of 'Candidatus Liberibacter asiaticus' genome) were run against Las bacteria cultures inoculated from HLB-affected citrus, periwinkle, psyllids, and mealy bug inoculm, respectively (Figure 6, 7 and 8), to determine if these culute contain 'Ca. Liberibacter asiaticus' sequences.
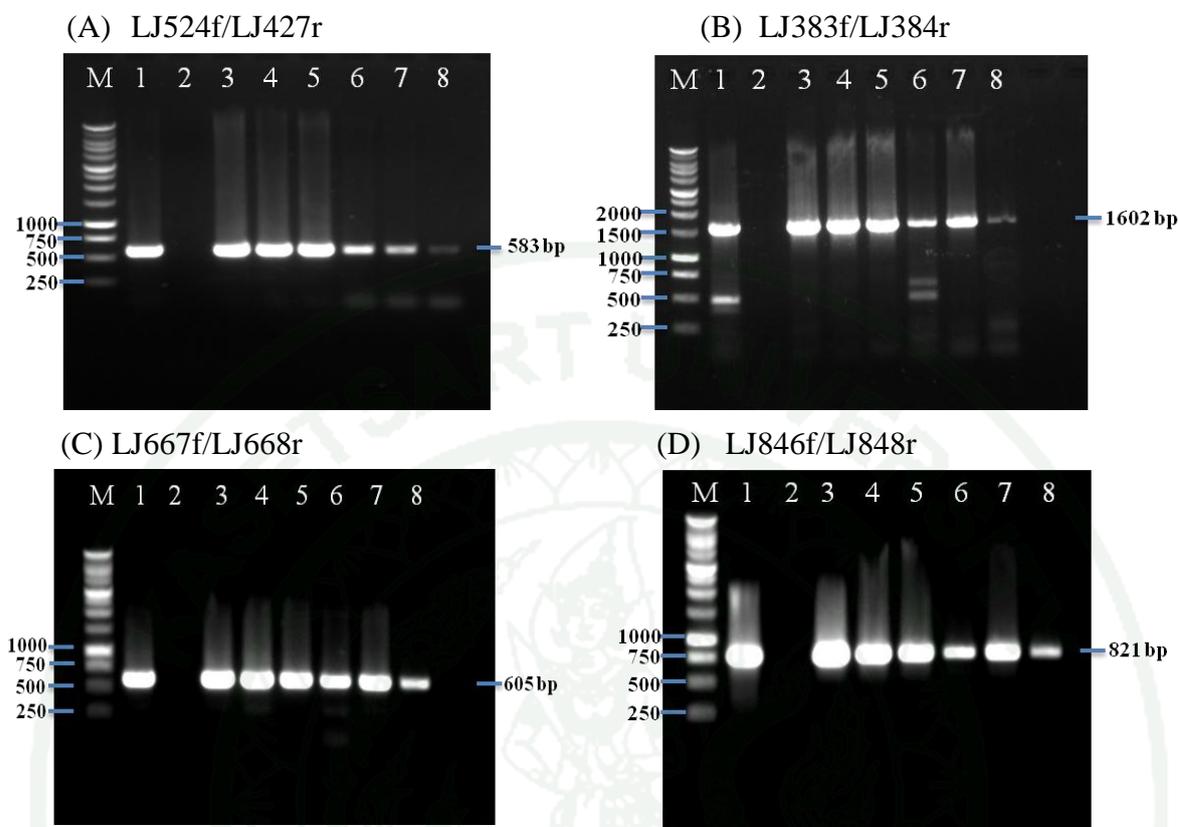
Figure 6 PCR detection of 'Candidatus Liberibacter asiaticus' isolated from
culture growing in Ar medium using primer sets (A),LJ 524f/LJ427r,
(B), LJ383f/LJ384r, (C), LJ667f/LJ668r and (D), LJ846f/LJ848r. **Lane M**:
1kb DNA Ladder (Promega); **lane 1**, DNA from 'Candidatus Liberibacter
asiaticus' (Las) positive infected citrus; **lane 2**, water; **lane 3**, DNA from
Las culture isolated from infected periwinkle and grown in Ar medium
containing periwinkle leaf extract (PL); **lane 4**, DNA from Las culture
isolated from infected periwinkle and grown in Ar medium containing
periwinkle flower extract (PF); **lane 5**, DNA from Las culture isolated from
infected citrus and grown in Ar medium containing PL; **lane 6**, DNA from
Las culture isolated from infected citrus and grown in medium containing
PF; **lane 7**, DNA from Las culture isolated from infected psyllids; **lane 8**,
DNA from Lasculture isolated from infected mealybug.

Figure 7 PCR detection of '*Candidatus* Liberibacter asiaticus' isolated from
culture medium using primer sets (A), LJ161f/LJ162r, (B), LJ532f/LJ533r,
and (C), LJ119f/LJ120r. **Lane M**: 1kb DNA Ladder (Promega); **lane 1**,
DNA from '*Candidatus* Liberibacter asiaticus'(Las) positive infected citrus;
lane 2, water; **lane 3**, DNA from Las culture isolated from infected
periwinkle and grown in Ar medium containing periwinkle leaf extract
(PL); **lane 4**, DNA from Las culture isolated from infected periwinkle and
grown in Ar medium containing periwinkle flower extract (PF); **lane 5**,
DNA from Las culture isolated from infected citrus and grwn in Ar medium
containing PL; **lane 6**, DNA from Las culture isolated from infected citrus
and grwun in Ar medium containing PF; **lane 7**, DNA from Las culture
isolated from infected psyllids; **lane 8**, DNA from Las culture isolated from
infected mealybug;

Figure 8 PCR detection of '*Candidatus* Liberibacter asiaticus' grown in
culture Ar medium using primer sets LJ1124f/LJ518r.

Lane M: 1kb DNA Ladder (Promega);

Lane 1, DNA from '*Candidatus* Liberibacter asiaticus'(Las) healthy
periwinkle from greenhouse;

Lane 2, DNA from Las culture isolated from infected periwinkle and grown
in Ar medium containing periwinkle leaf extract (PL);

Lane 3, DNA from Las culture isolated from infected periwinkle and grown
in medium containing periwinkle flower extract (PF);

Lane 4, DNA from Las culture isolated from infected citrus and grown in Ar
medium containing PL;

Lane 5, DNA from Las culture isolated from infected citrus and grown in
medium containing PF;

Lane 6, DNA from Las culture isolated from infected psyllids;

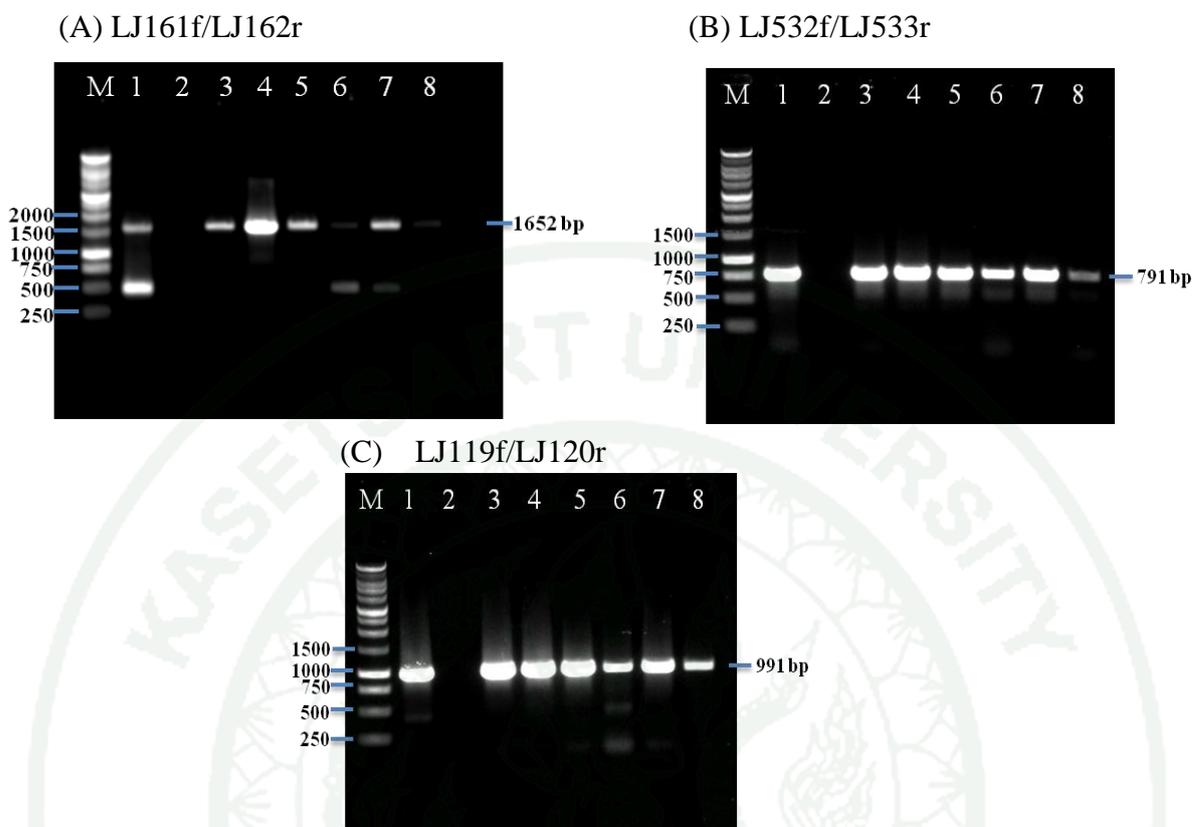**Lane 7**, DNA from Las culture isolated from infected mealybug;
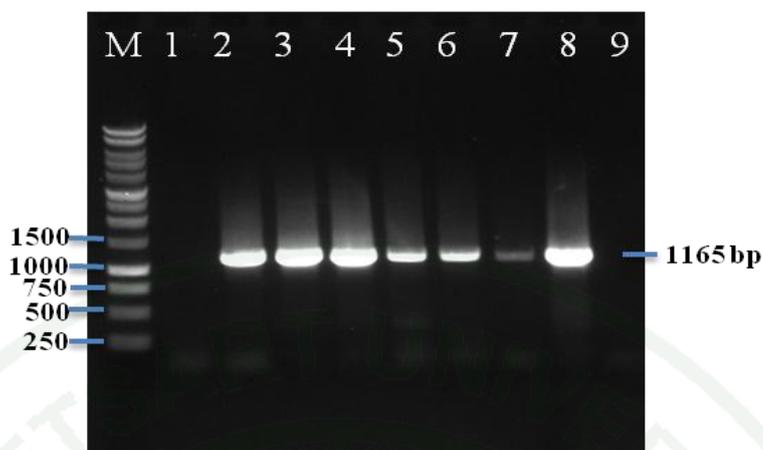
Lane 8, DNA from Las positive citrus from greenhouse;

Lane 9, negative control (water)

3. Effect of temperature and pH

Broth pH considerably affects the Las populations and behavior of the bacterial community in the media. The optimum starting pH and temperature have been investigated in previous studies. The effect of pH and temperature are of primary importance to the bacterial growth. Prelimilary studies on Ar medium showed that the media without pH adjustment was not suitable for the bacterial growth (data not shown.). The effect of pH in media culture was investigated by varying it between pH 3.8 to pH 7.8 with different temperature. As shown in Table 4 (A, B and C) The bacterial populations reached to the highest level at a pH of 5.8 and temperature of 25°C. The bacterial average C_t was 25.91 at a pH 5.8, but decrease slightly to 26.47 and no detection at the pH of 6.8 and 7.8, respectively. The maximum bacterial population was around 48 hours. It was interesting to observe lower bacterial titers at 35°C regardless of pH of the culture. In alkaline pH, '*Candidatus* Liberibacter asiaticus' bacterial titers were decreased more dramatically at pH higher than 7.8. However, the bacterium could grow at a pH 4.8-5.8 in 30°C and 35°C but the titers were lower than those at pH 5.8 at 25°C.

4. Effect of ATP

Figure 9 depicts the in vitro growth for '*Candidatus* Liberibacter asiaticus' isolated from periwinkle plant at 25°C with different concentration of ATP that were added into the media. The Las bacteria grew more quickly at ATP final concentration of 2,000 ppm during the exponential phase. At this concentration, bacterial titers were 22.34 and 23.36 at Ct value of after 2 and 4 days, while the ATP concentration of 500 ppm and 1,000 ppm were 23.75 and 23.93 at 6 days, respectively. Likewise, the ATP concentration 4,000 ppm that bacteria grew but yielded lower titers than the results above. Each concentration in these studies reached approximately the different maximum density and declined thereafter. No growth was detected after incubations for 2 weeks at 25°C.

Table 4 Effect of temperature and pH on growth of '*Candidatus* Liberibacter asiaticus'. A) at 25°C and pH 3.8 to 7.8; B) at 30°C and pH 3.8 to 7.8, and C) at 35°C and pH 3.8 to 7.8

A) At 25°C

pH of medium	Average Ct value at 25°C by using HLBas/r and HLBp TaqMan					
	0 day	1 day	2 days	3 days	1 week	2 weeks
3.8	nd	nd	nd	27.3	27.9	29.6
4.8	nd	nd	29.94	27.85	27.23	29.00
5.8	nd	25.87	25.91	26.13	26.81	29.06
6.8	nd	nd	26.47	26.61	28.15	29.25
7.8	nd	nd	nd	nd	nd	nd

[nd]/ non amplification detected. All control treatments were not amplified.

B) At 30°C

pH of medium	Average Ct value at 30°C by using HLBas/r and HLBp TaqMan					
	0 day	1 day	2 days	3 days	1 week	2 weeks
3.8	nd	nd	29.76	29.81	29.95	33.89
4.8	nd	nd	26.48	27.07	27.38	30.75
5.8	nd	nd	26.67	26.82	27.1	29.06
6.8	nd	nd	26.95	28.02	28.19	30.29
7.8	nd	nd	nd	nd	nd	nd

[nd]/ non amplification detected. All control treatments were not amplified.

C) At 35°C

pH of medium	Average Ct value at 35°C by using HLBas/r and HLBp TaqMan					
	0 day	1 day	2 days	3 days	1 week	2 weeks
3.8	nd	nd	27.70	28.58	30.60	35.80
4.8	nd	nd	27.16	27.67	27.87	30.31
5.8	nd	26.38	26.42	26.67	27.35	29.50
6.8	nd	nd	27.33	nd	nd	nd
7.8	nd	nd	nd	nd	nd	nd

[nd]/ non amplification detected. All control treatments were not amplified.
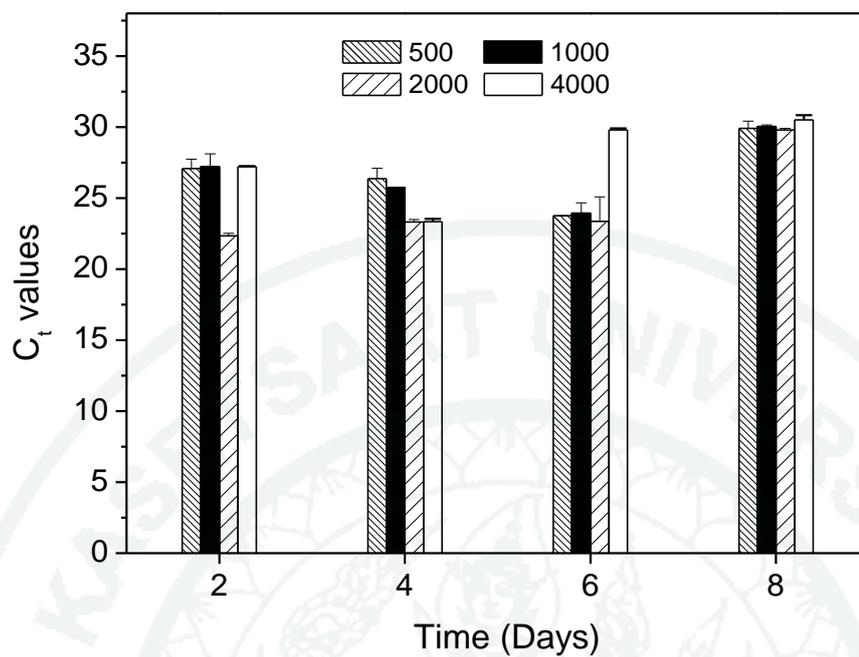
Figure 9 The effect of ATP concentrations at 500 to 4000 ppm to support
'*Candidatus* Liberibacter asiaticus' growth in the medium by real time PCR

5. Effect of carbon source

Carbohydrates are important carbon and energy source for cultures of 'Candidatus Liberibacter asiaticus'. Effects of various carbon sources on bacterial production have been reported. In order to identify a suitable carbon source for 'Candidatus Liberibacter asiaticus' cultures, different concentrations of carbohydrates such as arabinose and glucose were studied in the media containing K_2HPO_4, KH_2PO_4, $NaNO_3$ and NaCl. Other carbon sources including fructose, sorbitol, and sucrose were also tested in preliminary experiments and found that they did not help the growth of 'Candidatus Liberibacter asiaticus' culture (data not shown). The results for glucose and arabinose are shown in Figure 10 A and B. It appears that 2% of glucose was a better carbon source than 2 % of arabinose for Las culture. When 2% of glucose was used as a carbon source, the Las population reached the highest. . With 2% of arabinose as a carbon source, the population after 6 days incubaction was higher than 1% and 3%, but was lower compared to 2% of glucose. The other concentrations i.e. 1% and 3% of glucose or arabinose were not the best for cell growth. With increased concentration of glucose to 2%, the populations of Las bacteria increased from 24 to 72 hours and decreased thereafter. On the contrast, the mixture of glucose and arabinose makes it possible to increase Las populations very slightly (data not shown). Since this increase is not really significant compared to the use of glucose and arabinose alone, the subsequent experiments were carried out with 2% of glucose.

Figure 10 Influence of glucose and arabinose in Ar medium on growth of
‘*Candidatus* Liberibacter asiaticus’(Las). (A) glucose ;(B) arabinose. The
rate of Las growth in the medium detected by using real time PCR. Values
are the mean of four replication with a bar of standard deviation

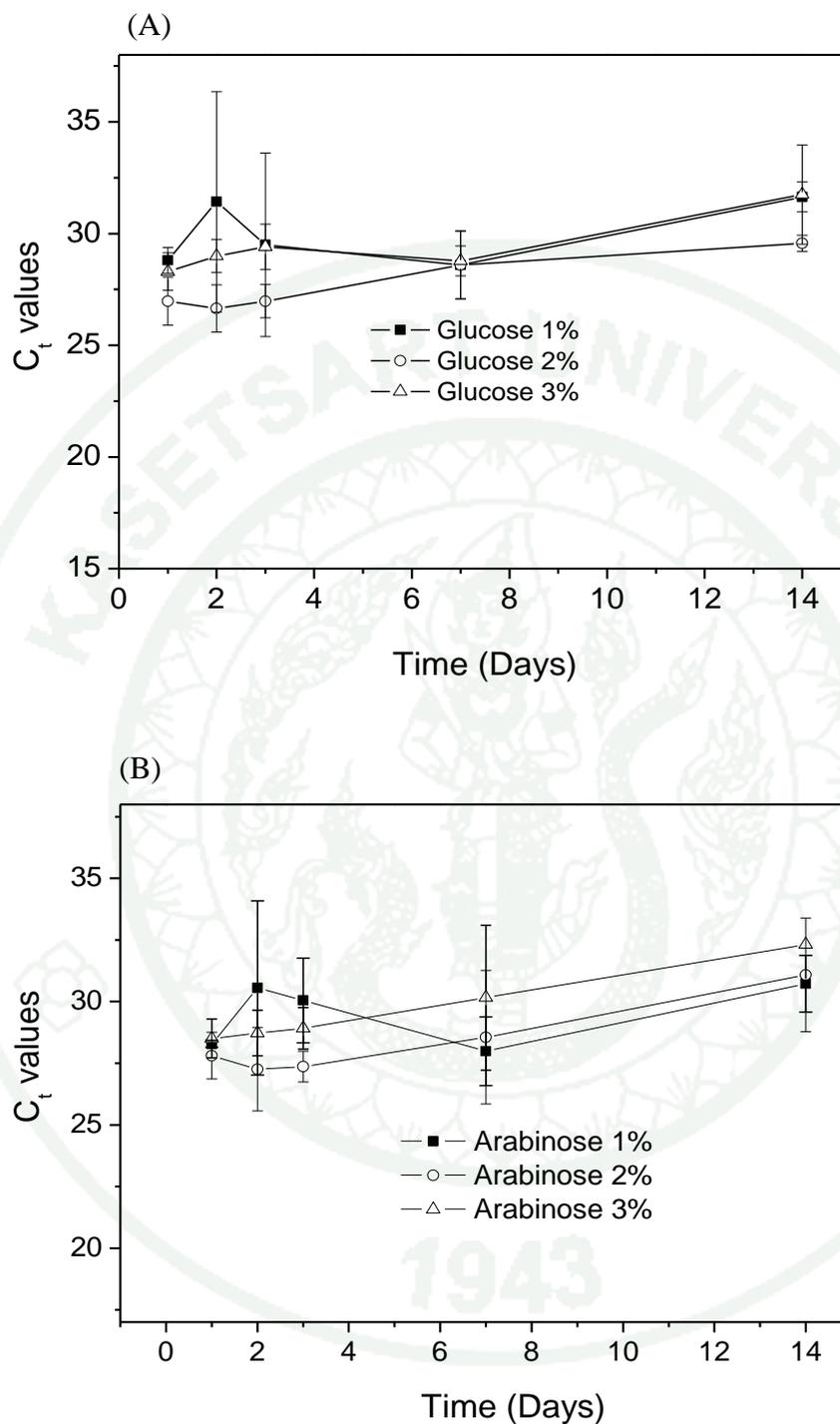**6. 16S rRNA of '*Candidatus* Liberibacter asiaticus' clone libraries**

A total of 197 sequences from '*Candidatus* Liberibacter asiaticus' cultures grown from HLB infected periwinkle were subjected to sequence analysis with subsequent online homology searched using two databases, Genbank which implements the BLAST algorithm and the RDP database which implements with the SIMILARITY_RANK program (Table 5). Although there are no exact 16S rDNA similarity limits for defining specific texa such as genus and species, in general, species definition requires sequence similarities greater than 98%. Most sequences from the 147 colonies in our libraries can be identified from periwinkle chloroplast DNA because of the medium containing periwinkle leaf and flower extracts, 44 colonies belong to '*Candidatus* Liberibacter asiaticus', and 6 colonies belong to uncultured bacteria. Sequences of about 88% of the bacterial clones have a similar level with 16S rRNA database sequence of '*Candidatus* Liberibacter asiaticus' in the range of 100% and those of 12% of the bacterial clones have a similarity level with 16S rRNA of uncultured bacteria, respectively. The similarity level value for a majority of our bacterial sequences of 16S rRNA libraries from '*Candidatus* Liberibacter asiaticus' culture was similar to 16S rRNA of '*Candidatus* Liberibacter asiaticus'. This indicated that the sequences from our library are from *Candidatus* Liberibacter asiaticus because in general bacteria cannot grow in Ar medium.

7. Viability of '*Candidatus* Liberibacter asiaticus' in culture

This experiment was undertaken to investigate the viability of bacterial cells of Las in culture. The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit was used to estimate viable or dead cells of Las bacteria. Figure 11 A and B shows that the total of viable cells of '*Candidatus* Liberibacter asiaticus' in the culture at the beginning of the study (4 days after inoculation). About 80% of clumping bacteria were proved to be viable by fluorescent staining.

Table 5 Characterization of 16S rRNA libraries from '*Candidatus*: Liberibacter asiaticus' culture

Host	16S rRNA clone resulted		
	Number of colony	**Percentage of total colony**	**Percentage of 16S-rRNA libraries of bacterial clone can grow in culture (50 colony)**
Periwinkle chloroplast	147	74.62	-
'*Candidatus* Liberibacter asiaticus'	44	22.34	88
Uncultured bacteria-*Novosphingobium*	2	1.02	4
Uncultured bacteria	3	1.52	6
Uncultured bacteria-*Stenotrophomonas*	1	0.5	2
Total	**197**	**100**	**100**

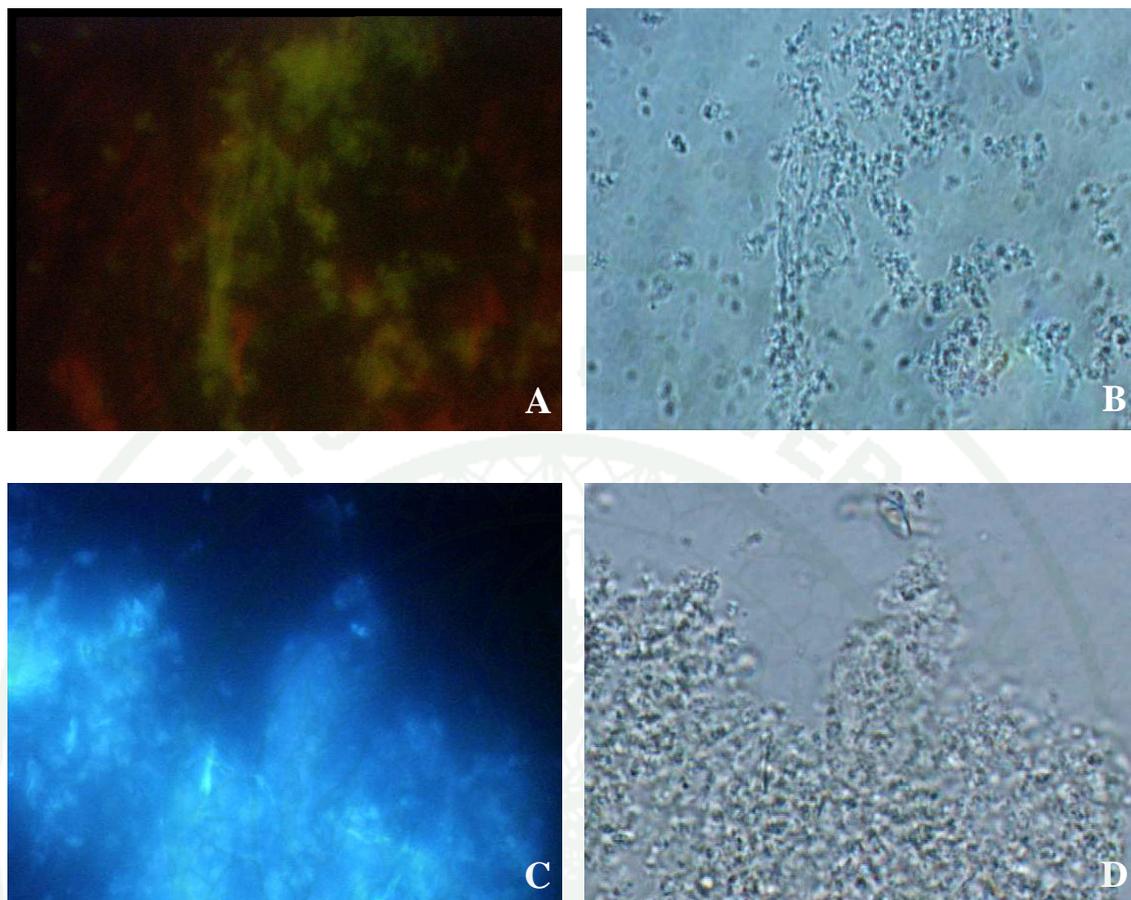**Figure 11** Viability and DAPI of '*Candidatus* Liberibacter asiaticus'bacteria
in culture after growth in Ar broth medium for 3 days. (A) epifluorescence
micrograph of Live/DEAD cell of '*Candidatus* Liberibacter asiaticus'
using *Bac*Light kit, (B) light microscopy of clumping cell of Las bacteria,
(C) epifluorescence micrograph using DAPI technique, and (D) light
microscopy of the Las cells

8. Electron microscopy

All strains of 'Candidatus Liberibacter asiaticus' isolated from citrus and periwinkle formed aggregates under the experiment conditions. Growth of strains in 50-mL flasks often led to clumping of cells, evident by light microscopy observations. Las cultures were usually turbid and contained a clumping of cells abundant in suspensions in the medium. However, light microscope analysis showed that all strains tested formed clumps and microcolonies to some degree (Figure 3). The scanning electron microscope results showed the bacterial cells grown in liquid medium were clumps of cells; the typical clumping was irregularly shaped in both samples (Figure 12A). The morphology of bacteria formed thick clumping of cells, and the clumps of cells were composed of cells tightly attached to each other, immersed in an abundant exopolymeric matrix, organized mostly in tandem and formed a varied size of irregularly intertwined clumps, where individual cells were often indistinguishable. After treatment with protenase K (10 ug/mL), the bacteria were separated from clumps to individual cells. The cells are pleomorphic, but more cells were typical as rod shaped. Elongation and globular shape were not often observed for these cultures (Figure 12 C and D).

9. Fluorescence in situ hybridization (FISH)

FISH was performed on a selection of relevant strains from bacterial cultures. All hybridizations were performed under a standard condition that is at 46°C with formamide. Under these conditions, a 16S rDNA probe was hybridized with 'Candidatus Liberibacter asiaticus'. The Las bacteria emitted hybridization signal with the 16S rDNA probe when visualized with the appropriate filter in epifluorescence microscopy. Figure 13 shows the epifluorescence micrograph of 'Candidatus Liberibacter asiaticus' cells grown in culture. The Las culture was hybridized with TEXAS red labelled probe. We frequently observed fluorescent aggregates on clumping of 'Candidatus Liberibacter asiaticus' cells in the culture samples, similar to one previously observed in scanning electron microscopy. These probes were able to be used successfully to detect Las microorganisms in culture.

Figure 11 C and D shows the clumps cell strain stained by DAPI. Results from these two techniques confirmed that Las bacteria clump in liquid media.

10. Inoculation of host plants with '*Candidatus* Liberibacter asiaticus' culture

Two-month old periwinkle and citrus seedlings were removed from soil and placed into tubes with Las cell cultures of periwinkle and citrus strains resulted in symptoms throughout the whole plant. The inoculated periwinkle and citrus plants began to wilt and the surrounding leaves became yellowing after 1 to 2 months. By 3 months, the entire leaf showed mottling and yellowish (Figure 14 and 15). These symptoms were similar to HLB symptoms; however, test results by real time PCR were negative. After 4 months, twenty of inoculated plants were detected positive by real time PCR. Some of them were positive with 16s rRNA primers. The average C_t value was about 34 to 36 with 16s rRNA primers. Seedlings inoculated with water served as a control (data not shown). Currently, the inoculation of periwinkle and citrus seedlings with '*Candidatus* Liberibacter asiaticus' from cell cultures in Ar.
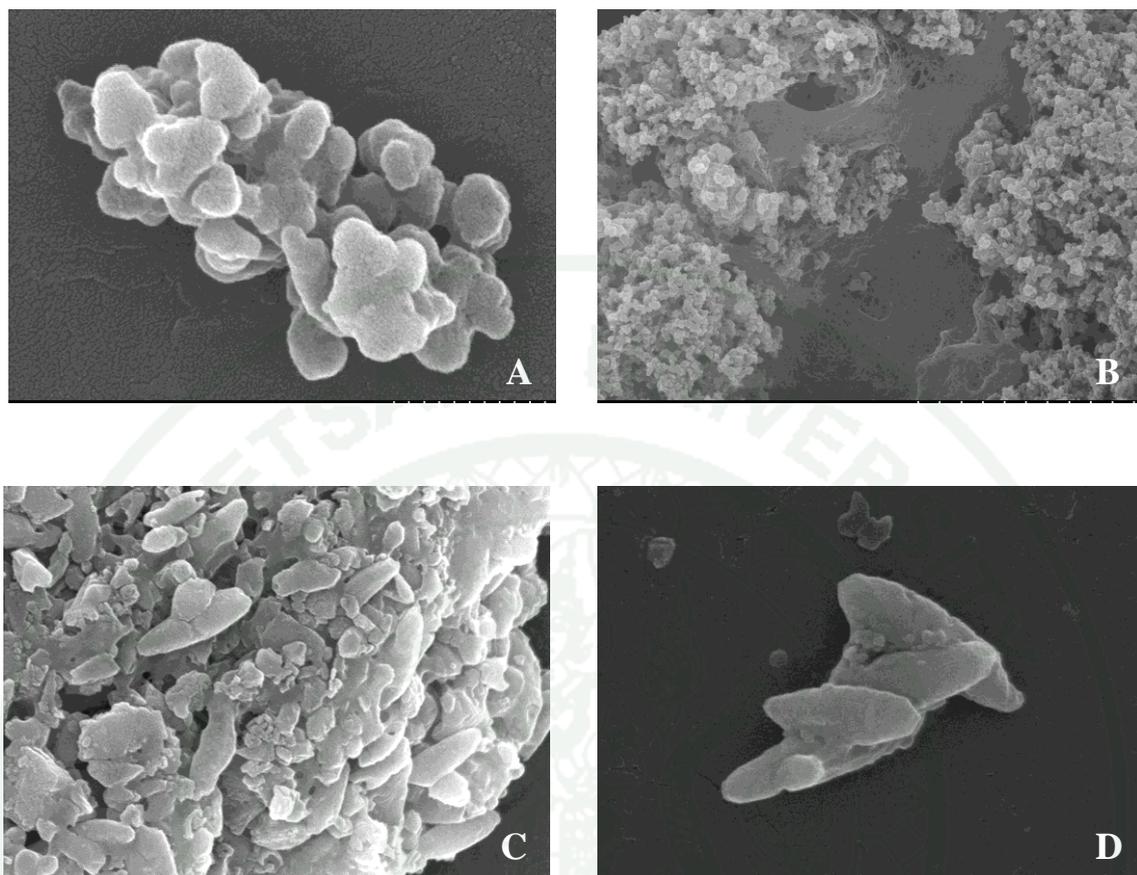
Figure 12 Scanning electron micrograph of '*Candidatus* Liberibacter asiaticus' in
Culture Ar medium. **A**. clumping cells in liquid medium, **B.** clumping
cells of '*Candidatus* Liberibacter asiaticus' in solid medium, and **C and D.**
'*Candidatus* Liberibacter asiaticus' in liquid media after treated with
protenase K
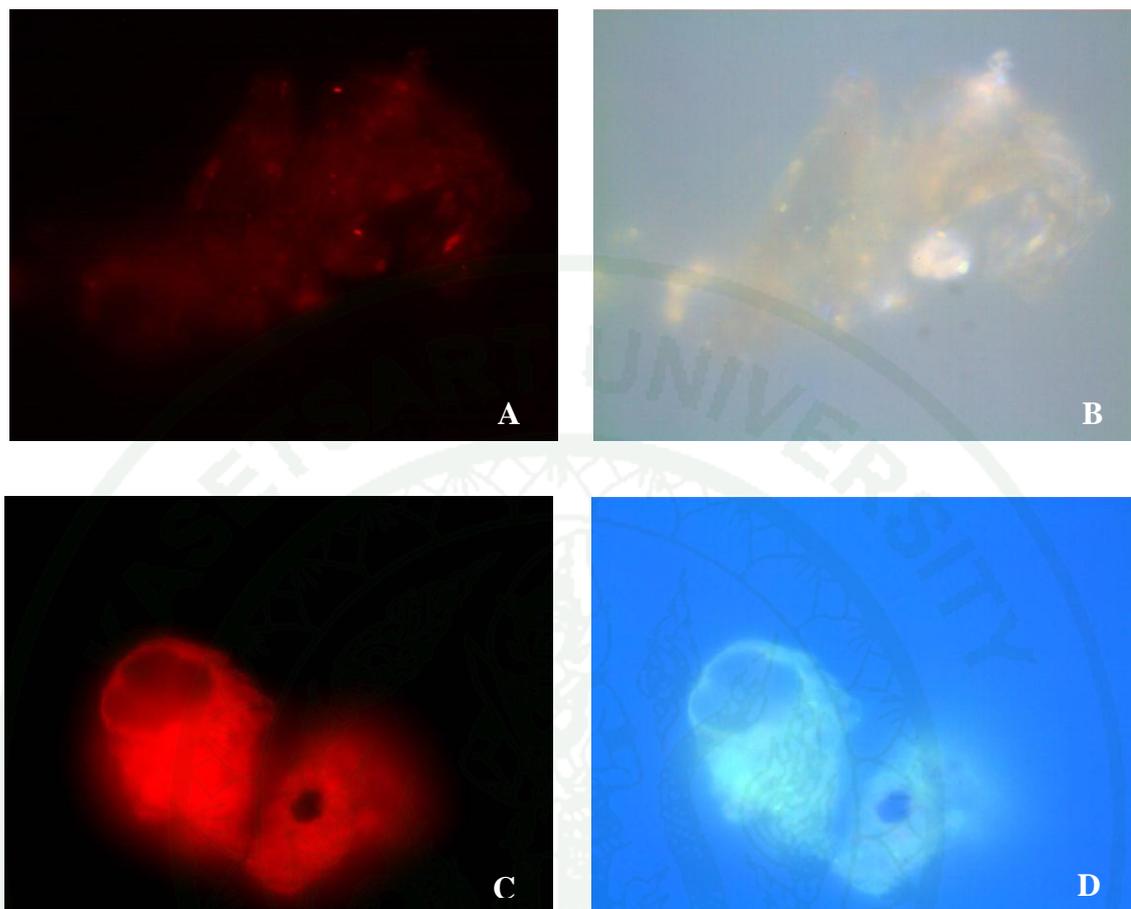
Figure 13 In situ hybridization micrographs of '*Candidatus* Liberibacter asiaticus'
cells in cultures. **A** and **C**. '*Candidatus* Liberibacter asiaticus' culture cells
detected under TEXUS red fluorescence filter, and **B** and **D**. detected
under light microscopy
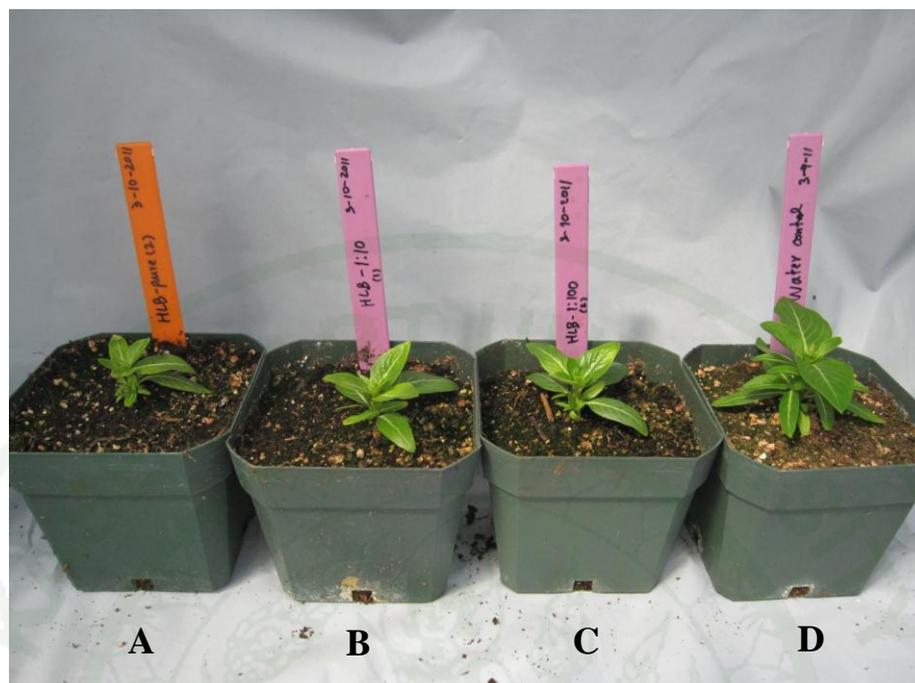
Figure 14 Pathogenicity test of '*Candidatus* Liberibacter asiaticus' (Las) from Ar
medium culture. yellowing and wilt growth of periwinkle at two months
after inoculation with '*Candidatus* Liberibacter asiaticus' from Ar edium
culture. A, the cell suspension of Las culture no dilution. B, the cell
suspension of Las culture at 1:10 dilution. C, the cell suspension of Las
culture culture at1:100 dilution. D, water inoculated control
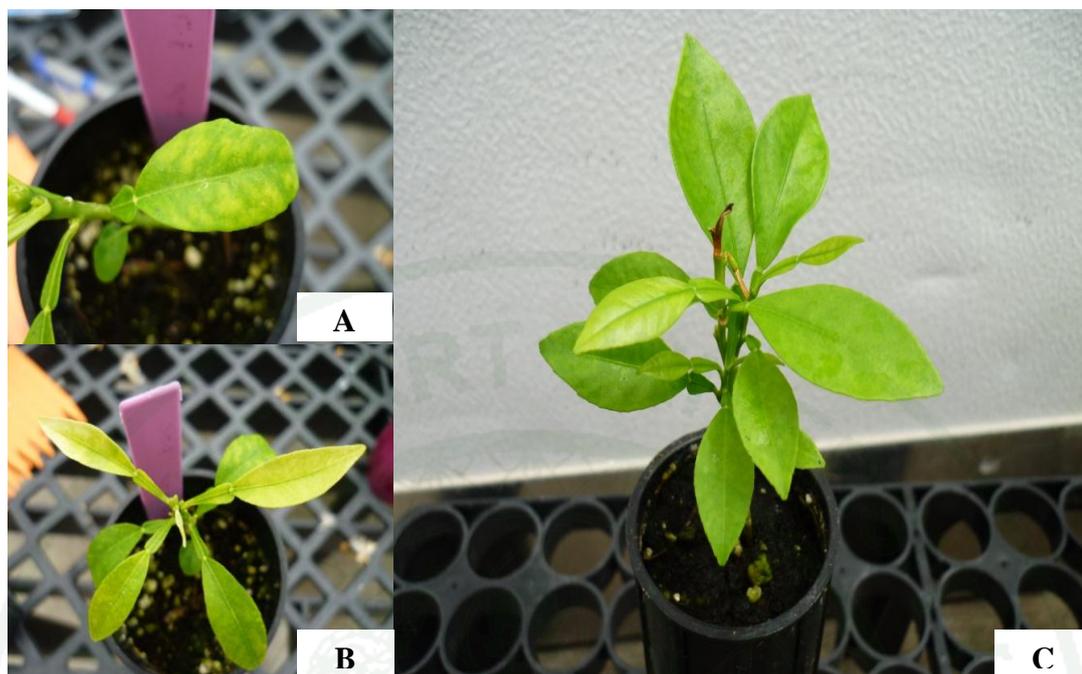
Figure 15 Pathogenicity test of '*Candidatus* Liberibacter asiaticus' (Las) from Ar medium on citrus seedling.A, mottling symptoms on inoculated citrus seedling after three month. Distal portion of leaf was removed for a real-time polymerase chain reaction assay. B, Yellowing and narrow leaf symptoms on inoculated citrus seedling. C, Healthy from control inoculated with water

11. Assessment of 'Candidatus Liberibacter asiaticus' in *Murraya paniculata* and *Diaphorina citri*

Prevalence of Las in psyllid samples

Over the course of the experiment, 232 psyllids samples collected from Murraya plants at different locations in Thailand were tested to determine the presence of Las and to evaluate the Las population levels. The number collected for each site and date was varied. Only 39 (16.8%) psyllids from 10 provinces were positive for Las by the LJ900 method. The Ct values for the psyllids where Las was detected using the LJ900 primers ranging from 17.57-29.87 (Table 2). The psyllids from which we isolated the highest number of copies of hyv_I/hyv_{II} also had a detectable number of copies of the 16S rRNA gene of Las. Fourty-two (18.1%) psyllids were positive for Las by using HLBaspr primers. The Ct values were highly variable with Ct values ranged from 16.12 to 36.74 using primers and probe targeting on 16S rRNA gene (Table 6).

Prevalence of Las in *Murraya paniculata* samples

The Murraya samples covering all fourteen provinces in Thailand collected from the month of January, March, July, and August 2010 were retained in the analysis. Of these 44 samples, six tested positive for the hyv_I/hyv_{II} target DNA sequence. The positive samples represented in Pathum Thani, Nakhon Pathom, Chanthaburi, Rayong and Trat provinces. We were able to amplify 16S rRNA of Las from 20 of the 44 samples that some samples tested positive for hyv_I/hyv_{II} (Table 7).

Table 6 Detection of '*Candidatus* Liberibacter asiaticus' from psyllids (*Diaphorina citri)* collected from different provinces of Thailand by two primer set of real time PCR

Location (Province)	Totals	LJ900f/r[a]		HLBaspr[b]	
		No. of positive sample	Range of Ct values (Ct ≤30)	No. of positive sample	Range of Ct values (Ct ≤36.9)
Bangkok	27	2	28.69-28.98	6	33.47-36.66
Lop Buri	23	1	29.85	3	33.95-35.06
Nakhon Pathom	22	2	19.39-27.93	1	16.12
Pathum Thani	24	6	27.16-29.65	3	35.90-36.74
Samut Sakhon	20	3	25.45-29.31	1	24.79
Saraburi	24	2	28.61-29.40	4	29.33-36.63
Chanthaburi	27	4	17.57-29.87	6	21.85-36.44
Chon Buri	20	4	24.36-29.93	2	33.02-33.43
Nakhon Nayok	22	4	25.65-29.44	2	33.93-34.83
Kamphaeng Phet	23	11	24.40-29.76	14	29.83-36.65
Total	**232**	**39 (16.8%)**		**42 (18.1%)**	

[a]Primers LJ900f/r target the hyv_I/hyv_{II} gene of Las prophages

[b]Primer HLBaspr target 16S rRNA of Las

Table 7 Detection of '*Candidatus* Liberibacter asiaticus' from *Murraya paniculata* collected from different provinces of Thailand by real time PCR

Location (Province)	Totals	LJ900f/r		HLBaspr	
		No. of positive sample	Range of Ct values (Ct ≤30)	No. of positive sample	Range of Ct values (Ct ≤36.9)
Bangkok	5	0	-	0	-
Lop Buri	1	0	-	1	27.89
Nakhon Pathom	5	1	29.11	4	33.27-34.11
Pathum Thani	6	1	26.88	4	32.44-34.60
Samut Sakhon	1	0	-	0	-
Samut Songkhram	1	0	-	0	-
Saraburi	2	0	-	1	28.20
Chanthaburi	9	2	25.69-29.45	3	32.09-35.33
Chon Buri	2	0	-	0	-
Nakhon Nayok	2	0	-	1	30.91
Rayong	4	1	29.17	3	27.36-36.45
Trat	4	1	29.25	3	32.23-36.43
Kamphaeng Phet	2	0	-	0	-
Total	**44**	**6 (13.6%)**		**20 (45.5%)**	

DISCUSSION

HLB-associated bacterium '*Candidatus* Liberibacter asiaticus' has not been continuously cultured *in vitro*. The inability to grow the bacteria has made it difficult to study the disease and develop a successful control method. To our knowledge, this is the first report in which a high titer of '*Candidatus* Liberibacter asiaticus' from citrus, periwinkle and psyllids was detected in a culture using real time PCR detection of the 16s rDNA. In this work, a culture medium, named Ar medium, was designed and optimized to culture '*Candidatus* Liberibacter asiatcus' isolated from citrus, periwinkle plants and psyllids. We determined that '*Candidatus* Liberibacter asiaticus' grew on the Ar medium and the addition of periwinkle extract and ATP was considered essential for '*Candidatus* Liberibacter asiaticus' growth.

Using a modified medium (referred as Ar medium) which was incorporated with both monobasic and dibasic potassium phosphate, periwinkle extract and ATP, '*Candidatus* Liberibacter asiatcus' was proved to grow in liquid culture as soon as 6-24 hours. By using specific primers specifically HLBas/r and HLBp derived from '*Candidatus* Liberibacter asiatcus' genome, TaqMan real time-PCR was performed to identify the Ct value of the bacteria culture, thus confirming the presence of the bacteria in the culture (Figure 1) (Li *et al.,* 2006). The bacterial average Ct values were 19.94, 20.05, and 23.53 at 24 hours from HLB infected citrus, periwinkle, and psyllids, respectively (Figure 1). The Ct values of the culture reduced after 24 hours post-inoculation. The Ct value at time zero was higher than 33.00. The change in Ct values over time demonstrated the growth of the bacteria in liquid media. Although periwinkle extracts could be made from fresh periwinkle flowers or leaves, autoclaving destroyed all activity. There appeared to be something degraded such as a nutrient and protein that was inactived by autoclaving.

The formulation of the Ar medium was determined in part by genomic sequence information. The predicted metabolic pathways of '*Candidatus* Liberibacter asiatcus' were determined with the use of the whole sequenced genome (Duan *et al.,* 2009). '*Candidatus* Liberibacter asiaticus' retained pathways for glycolysis, ATP/ADP translocase, and nonoxidative pentose phosphate biosynthesis but lacked

key enzymes involved in oxidative phosphorylation and most of the components of the tricarboxylic acid (TCA). Vahling et at. (2010) also reported the presence of ATP translocase and NttA gene in '*Candidatus* Liberibacter asiaticus' which expressed in *E. coli* the protein functioned as a transfer system for ATP. Through the use of an ATP/ADP translocase, '*Candidatus* Liberibacter asiaticus' can behave as energy parasites and scavenge ATP from their host (Duan *et al.,* 2009). Based on the this information, varying concentrations of ATP were added to the culture media and it was determined that '*Candidatus* Liberibacter asiaticus' bacteria grew more quickly in the presence of ATP at a final concentration of 2,000 ppm.

Based on the presence of predicted glycolysis pathways, various carbohydrates were supplemented to the media as a potential carbon and energy source for '*Candidatus* Liberibacter asiatcus'. '*Candidatus* Liberibacter asiaticus' is able to use glucose and arabinose as carbon and energy sources (Figure 9). Comparison of the glucose and arabinose experiments revealed glucose as a carbon source resulted in a high cell population of '*Candidatus* Liberibacter asiaticus'. It may also be possible that *Candidatus* Liberibacter asiatcus grows on other carbon sources such as TCA cycle intermediates. *Xylella fastidiosa*, a xylem-limited bacterium, can grow *in vitro* on media supplemented with all TCA cycle intermediates with the exception of isocitrate, but could be recovered from plants that had citrate (Lemos *et al.,* 2003). In addition, glucose has been shown to sustain *X. fastidiosa* growth if it is used as the sole carbon source (Lemos *et al.,* 2003). Because carbohydrates are diluted in xylem sap, their impact on the bacterium's metabolism in comparison with organic acids must be addressed.

The energy production of '*Candidatus* Liberibacter asiaticus' is solely dependent on substrate-level phosphorelation through glycolysis pathways. Glycolysis is the major pathway for the catabolism of monosaccharide. Based on the enzymes predicted by the '*Candidatus* Liberibacter asiaticus' genome sequencing data, the bacterium has the ability to metabolize glucose, fructose and xylose. This is further shown by the ability to culture the bacteria in media containing glucose but fructose and xylose not effective to culture HLB. However, how these sugars are transported into the cell remains unclear. In uncultured Termite Group 1 bacteria,

phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) is important for fructose and other sugars transferred to the cell (Schwoppe *et al.,* 2002). Although the HPr (Ser) kinase gene *hpr*K is a pseudogene of bacteria, regulation of carbohydrate assimilation does not work via this PTS (Hongoh *et al*., 2008). Interestingly, in '*Candidatus* Liberibacter asiaticus' genome, the only phosphotransferase system (PTS) protein identified within the genome was a SgaT homolog of gene in the PTS. Considering the multiple ABC transporter proteins in the '*Candidatus* Liberibacter asiatcus' genome, it is highly possible that these proteins may be involved in sugar transport.

The results presented in Table 4 showed that the average Ct value of '*Candidatus* Liberibacter asiaticus' bacteria was lower grown at the incubation temperature 25°C. These results differ from the work by Sechler et al., (2009) who concluded that higher recovery of '*Candidatus* Liberibacter asiaticus' bacteria at the incubation temperature of 28°C, which was similar to the temperature of the environment from which the bacteria were obtained (28°C-30°C). Interestingly, in this study, an increase in bacterial populations of '*Candidatus* Liberibacter asiaticus' was observed when the temperature was 25°C. The incubation temperature was crucial maybe because the temperature change likely resulted in a longer generation of the bacteria. Additionally, this work has shown that Las bacterial populations decreased at a high pH values particularly above pH 7.8. '*Candidatus* Liberibacter asiaticus' bacterial densities were higher at pH 5.8 than at pH 3.8, 4.8, 6.8 and 7.8 for all temperatures investigated. For *Xylella fastidiosa* CVC strains, the impact of pH on biofilm formation could be related to a quorum sensing effect, as gene regulation by bacterial density is predicted by the analysis of the genome of *Xylella fastidiosa* (Scarpari *et al.,* 2003). It is possible that at a certain pH, the concentration of cells reaches a threshold allowing transcription of genes involved in aggregation and maintenance of bacteria. In fact, the growth of '*Candidatus* Liberibacter asiaticus' decreased with an increase of pH in the medium when it was higher than 5.8. It is known that the pH in the medium influences the expression of proteins in *Escherichia coli* (Hickey *et al.,* 1990), and 28 genes were found to be acid inducible in *Escherichia coli* (Tuckey *et al.,* 2002).

The growth of '*Candidatus* Liberibacter asiaticus' decreased concomitantly with the increase of the culture medium pH and temperature. In reversible way, '*Candidatus* Liberibacter asiaticus' cells presumably are killed by phages in culture media which was proved positive by using LJ900f/r primers (data not shown). This is additional evidence of viability under growth conditions, even with decreased growth rate during the short time.

Several microscopic methods were used to further confirm the presence of '*Candidatus* Liberibacter asiaticus' in liquid media. We showed viable cells of '*Candidatus* Liberbacter asiaticus', using the LIVE/DEAD *Bac*Light stain with a combination of SYTO9 and propidium iodide (PI) (Boulos *et al.,* 1999). The physiological state of the bacteria may affect both the number of binding sites for stains and permeability of the membrane. As shown in Figure 10 A and B, culture conditions are suitable for '*Candidatus* Liberibacter asiaticus' in our study. The value of high viable cell ratio for '*Candidatus* Liberibacter asiaticus' did not decrease until three days after incubation. To produce good results using *Bac*Light kit, nucleic acids present in the culture of '*Candidatus* Liberibacter asiaticus' must be sufficient to be stained by PI and SYTO9. Additionally, the DAPI technique for observing the characteristic light blue fluorescence in culture of '*Candidatus* Liberibacter asiaticus' was found reliable for cell growth in the medium. DAPI stained DNA in '*Candidatus* Liberibacter asiaticus' fluorescences as discrete foci in medium. However, DNA with high AT content enhances the binding of the AT-specific fluorochome DAPI (Schweizer *et al.,* 1978). On the other hand, the mitochondrial DNA fluorescence is quickly quenched when exposed to UV light, whereas the '*Candidatus* Liberibacter asiaticus' fluorescence remains stable for at least several minutes under UV exposure.

Fluorescence in situ hybridization (FISH) was also used to detect, identify, and confirm the presence of '*Candidatus* Liberibacter asiaticus' in culture. To our knowledge, no molecular probe exists for quantitatively visualizing the '*Candidatus* Liberibacter asiaticus' pathogen. We have shown that the '*Candidatus* Liberibacter asiaticus' in situ hybridization assay with Las specific probes that target 16S rDNA of '*Candidatus* Liberibacter asiatcus' can be used for direct identification of '*Candidatus* Liberibacter asiaticus' in culture medium. The results were performed with smears

made from liquid culture, and determined of results is conducted to microscopy, such that the FISH assay procedure simply adds the high specificity of 16S rDNA specific probes to standard microbiological procedure (Oliveira) to provide the definitive identification of '*Candidatus* Liberibacter asiaticus' in an amount of time not possible by conventional methods. The '*Candidatus* Liberibacter asiatcus' FISH assay procedure resembles previously published FISH methods for studying localization of '*Candidatus* Liberibacter asiaticus' pathogen in psyllids (Ammar *et al.,* 2011). The assay used several FISH protocols and three nucleic acid probes to investigate the distribution of '*Candidatus* Liberibacter asiaticus' in psyllid organs by confocal laser scanning microscopy. They demonstrated that '*Candidatus* Liberibacter asiaticus' was present in several organs and tissue of Asian citrus psyllid. In this case, many microorganisms were only detectable with DAPI but not with the FISH probe, suggesting that bacteria with rRNA content below the FISH detection level were presumably dead. However, part of the '*Candidatus* Liberibacter asiaticus' cells showed bright FISH signal, which is consistent with a high ribosomal content. This argues for a recently biological activity of the microorganism. FISH using the 16S rDNA probe might facilitate early detection of '*Candidatus* Liberibacter asiaticus' cells from culturing in histologic specimens and is a promising tool for rapid and accurate diagnosis of HLB pathogens. Furthermore, the use of FISH in this study yet again confirms the presence of Las in the newly designed liquid media.

Lastly, scanning electron microscope (SEM) in this study demonstrated that '*Candidatus* Liberibacter asiaticus' was indeed present in the culture and was a clumping bacterium. These data correlate with previous *Bac*Light kit stain, DAPI and FISH images of the bacteria grown in the culture, showing large aggregates and clumping of cells. Recent electron microscopy studies of '*Candidatus* Liberibacter asiaticus' in infected citrus tissue (Kim *et al.,* 2009) and dodder (*Cuscuta indecora*) (Hartung *et al.,* 2010) served to highlight that microscopic analyses either have not been done or the results have not been included in published studies on seed transmission of '*Candidatus* Liberibacter asiaticus'. Electron microscopy has been used several times to photograph bacteria in the phloem cells of citrus and periwinkle plants with symptoms of HLB. In such case, two or more distinct morphologies i.e. an elongated form and a round formwere observed among bacteria in single tissue

sections, (Garnier *et al.,* 1983 and Garnier *et al.,* 1976). Because Koch's postulates have not been completed for '*Candidatus* Liberibacter'spp. and phytoplasmas associated with symptoms indistinguishable from those of HLB (Chen *et al.,* 2009 , Teixeira *et al.,* 2008), it is important to determine if the bacteria with different morphologies present in tissue sections are produced by a single population of cells. Thus, '*Candidatus* Liberibacter'spp. is apparently pleomorphic. The culture of '*Candidatus* Liberibacter asiaticus' is pleomorphic and irregularly shaped comprised of clumping cells. Final confirmation that the pleomorphic and clumping cells are in fact those of '*Candidatus* Liberibacter' spp. will require antibodies specific for '*Candidatus* Liberibacter' spp. to label the bacteria in culture and phloem cells.

Results from this study showed that inoculation methods are not quite effective for inoculation of citrus and periwinkle with '*Candidatus* Liberibacter asiaticus' cultures. However, the results could have been due to inoculum concentrations rather than the inoculation method. The time needed for the development of systematic symptoms was much shorter for seedlings than the old trees. Symptom development and expression in a greenhouse with temperature control may be more variable than symptom expression in a natural environment. The inoculated seedlings showed wilt symptom after inoculation at 24 hours and a generalized symptom was the same as HLB symptom at 3 months, with symptoms progressing to distinct leave yellowing and mottling symptom by 3-4 months after inoculation. Instead, studies on inoculating plants including this one relied upon real time PCR to indirectly detect presence of bacteria by detection of bacterial DNA. In this study, using real time PCR we established the protocol for inoculation of periwinkle and citrus and resulted in low titer of '*Candidatus* Liberibacter asiaticus' bacteria in inoculated plants. The range of Ct value was 34 to 36 in inoculated periwinkle plants after 2 month inoculation. This remains the possibility that HLB may be a syndrome with some population of '*Candidatus* Liberibacter asiaticus' and not entirely responsible for the '*Candidatus* Liberibacter asiaticus' bacteria culture. In addition, the trees used in this work were too young. We cannot complete Koch's postulates by inoculating periwinkle and citrus trees because the plants have to be sensitive with environment conditions or the inoculation method been not effective.

In summary, the results described above will be useful for research on 'Candidatus Liberibacter asiaticus' culture and for improving the chance to cultivate the HLB pathogen. They may be useful in future after other functional genome studies have been completed of this bacterium. In this work, bacteria was repeatedly cultured in the newly designed liquid Ar media which consists of 6 g of K_2HPO_4, 2 g of KH_2PO_4, 2.55 g of $NaNO_3$, and 3 g of NaCl per liter. Based on optimization studies, the addition of ATP at a concentration of 2,000 ppm, 10% periwinkle leaf or flower extracts, 2% glucose of glucose stock (50% wt/vol), and 2.5% sodium hydroxide for a final pH of 5.8 in the liquid media and incubation at 25 °C provided the best growing conditions for 'Candidatus Liberibacter asiaticus'.

Assessment of 'Candidatus Liberibacter asiaticus' in Murraya paniculata and Diaphorina citri

The presence of Las infection in *Murraya paniculata* samples using a detection method with the LJ900fr primers targeting on hyv_I/hyv_{II} prophage region was extremely low. Seventeen plant samples that amplified using the 16S rRNA primers had a high Ct value, similar to what we have observed in running these primers against other species of bacteria (unpublished). The only sample in this experiment that would be considered positive with low Ct value by 16S rRNA detection method was psyllid where amplification was detected using the HLBaspr primers. There are conflicting reports about whether transovarial transmission of Las occurs in *D. citri* (Pelz-Stelinski *et al*., 2010 and Xu *et al*., 1988). If transovarial transmission occurs, it is possible that this infection originated from an infected female that migrated to *M. paniculata* from citrus.

M. paniculata infections were detected when using primers targeting the bacterial 16S rRNA gene than with using primers targeting the hyv_I/hyv_{II} tandem repeat sequence. The discrepancies between the two primer sets could occur for several reasons. The most likely is that more infections were detected using the 16S rRNA primers because of higher sensitivity of that method and 16S rRNA regions are the best characterized regions, highly conserved among the species of 'Candidatus Liberibacter spp. The *M. paniculata* plants were positive with high Ct value by 16S

rRNA primer. This suggests that '*Ca*. Liberibacter asiaticus' either was decreased multiplying in plant or that *M. paniculata* is not a good alternate host for Las which may contain some inhibitors.

The rate of Las infection of *D. citri* that developed on *M. paniculata* is low relative to that of *D. citri* that develop on citrus, and also lower than infection rates published for the potato-tomato psyllid, *Bactericera cockerelli* and another Liberibacter species, '*Candidatus* Liberibacter solanacearum' (Liefting *et al.,* 2008). Using conventional and quantitative PCR, the infection level of *D. citri* collected from infected citrus in the field is 45-82% (Ammar *et al*., 2011; Hung *et al*., 2004; Subandiya *et al*., 2000). The infection rate of   *Bactericera cockerelli* with '*Ca*. Liberibacter solanacearum' was 19-20% when the psyllids were collected directly from their host plants (Wen *et al*., 2010). The low rate of infection and level of bacterial titer in both plant and a psyllid sample raises the possibility that *M. paniculata* may have a unique resistance trait for Las. Some citrus cultivars do not display severe symptoms when they contact Las but they have a bacterial load similar to diseased citrus (Folimonova *et al*., 2009) and several orders of magnitude higher than we found in *M. paniculata*. The fact that *M. paniculata* has both a low level of infection and a low titer of bacteria in infected plants despite psyllid movement to and from citrus and high levels of HLB incidence in the local citrus population indicates that a trait of the plant may be restricting reproduction or movement of the bacteria.

The results of this work also suggest that '*Ca*. Liberibacter asiaticus' interacts with *M. paniculata* as an opportunistic pathogen. The failure to find any symptoms could be specifically associated with liberibacter infection on the natural infected trees.

CONCLUSIONS

1. The results described above will be useful for research on 'Candidatus Liberibacter asiaticus' improve to the cultivation of the HLB pathogen. This result also lead to future research in the functional genome studies of this bacterium. Based on this work, bacteria was repeatedly cultured in the newly designed Ar liquid media which consists of 6 g of K_2HPO_4, 2 g of KH_2PO_4, 2.55 g of $NaNO_3$, and 3 g of NaCl per liter. Based on optimization studies, the addition of ATP at a concentration of 2,000 ppm, 10% periwinkle leaf or flower extract, 2% glucose of glucose stock (50% wt/vol), and 2.5% sodium hydroxide for a final pH of 5.8 in the liquid media and incubation at 25 °C provided the best growing conditions for 'Candidatus Liberibacter asiatcus'

2. The Las bacterial populations were able to increase by 15,000 times, reaching approximately 10^7 cells /ml within 24 hours. However, the bacterial titer remained staggering for another 24 to 48 hours, and declined thereafter. The bacterial cells tended to clump together, and grew poorly after several transfers.

3. The real time primers namely, LJ00fr (SYBR Green1) targeting hyv_I gene, LasLong (SYBR Green) targeting 16S rDNA, and HLBaspr (standard 16S rDNA TaqMan® based Las detection) protocol with a standardized were performed on samples from Las bacteria cultures isolated from different HLB-infected materials.

4. In this study, a PCR approach was taken to confirm the presence of 'Ca L. asiaticus', to determine whether the organism was present in culture. PCR performed with primers designed to each the 'Ca L. asiaticus' sequences confirm that eight location sites of primers were present in 'Ca L. asiaticus' culture but not healthy and negative controls.

5. 16S rDNA libraries of 'Candidatus Liberibacter asiaticus' in the media was constructed, and the 16S rDNA gene was amplified, cloned and sequenced. A BLAST search revealed an 88% identity with a partial sequence of the 16S rDNA gene of 'Candidatus Liberibacter asiaticus' in Genbank (Accession CP001677) and a 4%, 6%

and 2% identity with the gene of uncultured bacteria *Novospinggobium*, uncultured bacteria, and uncultured bacteria *Stenotrophomonas* respectively. Thus, 'Candidatus Liberibacter asiaticus' appeared to be growing more in the medium.

6. The scanning electron microscope results showed the 'Candidatus Liberibacter asiaticus' bacterial cells grown on liquid medium were clumps; the typical clumping was irregularly shaped in both samples from solid and liquid medium. The clumps were composed of cells tightly attached to each other, immersed in an abundant exopolymeric matrix, organized mostly in tandem and formed a varied size of irregularly intertwined clumps. After treatment with protenase K (10ug/mL), the bacteria were separated to individual cells. The morphology of cells was pleomorphic; the typical cell was rod shaped.

7. Fluorescence in situ hybridization (FISH) was able to be used successfully to detect Las microorganisms in culture and DAPI technique confirmed the resulted that Las bacteria were clumps in liquid media.

8. *Bac*Light staining has several advantages; it is reliable, rapid and easy to use and could also be used as a tool for measuring viable 'Candidatus Liberibacter asiaticus' bacteria in culture. The major limitation in applying these direct measurement techniques is the requirement of equipment and quantified personnel , which cannot be met for all facilities.

9. *'Candidatus* Liberibacter asiaticus' can be cultivated in the Ar medium with limited growth. This is first report of real growth of Las bacteria *in vitro*, which will shed light on future improvement for Las cultivation, and provides the opportunity to initiate functional genomics study of this intriguing bacterium.

Assessment of 'Candidatus Liberibacter asiaticus' in *Murraya paniculata* and *Diaphorina citri*

1. Symptomatic *M. paniculata* plants were tested and proved to be positive for Las bacterium, indicating *M. paniculata* plants are the host of the Las bacterium in Thailand though the Las titers were lower than those from HLB-affected citrus plants.

2. Most ACP collected from *M. paniculata* show relatively low titers of '*Ca* L. asiaticus' except a few, which may be migrated from nearby HLB-affected citrus plants.

3. It is the first report that the prophage gene was undetectable in the Las isolates from Murraya host.

4. As a preferred host of ACP, *M. paniculata* may play an important role on epidemiology of HLB disease in Thailand.

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APPENDIX

Appendix A

Ar medium

<u>Chemical preparation before culture:</u>

1. Las basic media (1L):

    K_2HPO_4 6g

    KH_2PO_4 2g

    $NaNO_3$ 2.55g

    NaCl   2.5g

Adjust pH to 5.8, add H_2O to 1 liter then autoclave;

2. 50% glucose for media: 50g of glucose add to 100mL of H_2O, dissolved in $65^{o}C$ water bath and filter sterilize, final con. 2%;

3. 100,000ppmATP for media: 1g of ATP in 10mL of H_2O, dissolved and filter sterilize, final con. 2000ppm; (1ppm=1mg/L)

4. 30% H_2O_2 for media: filter sterilize, final con. 2.5%

5. Periwinkle extract for media: prepare fresh (no more than 2d), grinding without any buffer, transfer to 50mL tube, 5000 rpm for 20min, transfer supernatant to 2mL tube, centrifuge at maxim speed 20min for 3 times, transfer supernatant and filter sterilize. Final concentration 10%;

6. Solution for initial inoculums: 1.5g of Arabinose and 0.85g of NaCl to 100mL water, filter sterilize

7. Lysozyme buffer: 10mM Tris-HCl, pH8.0, 20% sucrose;

8. Lysome: 100mg/mL;

9. Proteinase K: 50mg/mL;

10. DNA extraction buffer: same as psyllid extraction buffer with 1% Sarcrosyl

11. 70% Ethanol

12. 20% Bleach

13. 70% EtOH

Preparation of autoclaved implement before culture:

1. Autoclaved H_2O

2. Paper towel

3. Beaker

4. Mortar and pestle

Surface sterilize of plant material

1. Wash leaves with H2O;

2. Add 70% EtOH to leaves and shake for 5 min;

3. Wash two times with water;

4. Add 20% bleach to leaves and shake for 10 min;

5. Wash two times with water, dry the leaves;

6. Cut midribs from leave sample, move to hood;

7. Surface sterilize with 70% EtOH for 5min, and wash two times with dH_2O

8. Add 20% Bleach for another 10min, and wash two times with autoclaved water;

9. Dry midribs with autoclaved paper towel.

Prepare for periwinkle or flower extracts used in the culture medium

1. Squeeze the leave or flower tissue without any additional buffer using Diedmont Machine & Tool from Dayton Electric MFG CO. (Chicago, IL 60648 USA);

2. Transfer the juice to 50mL Falcon tube and centrifuge at 5000rpm for 20min;

3. Transfer the supernatant to the new 50mL tube, Sonicate 5 x 1 min at 5 μm amplitude, while keeping the sample on ice In between sonication steps;

4. Aliquot the plant extracts to 2mL tube and spin down at maximum speed for 15min;

5. Sonicate again as above on ice;

6. Aliquot the plant extracts to 2mL tube and spin down at maximum speed for 15min, repeat it to three times;

7. Filter sterilized the supernatant and keep at 4^oC before use.

Prepare for initial inoculums and culture

1. Cut midribs with flame sterilized razor blade in petri dish;

2. Grinding midribs to fine powder with mortar and pestle without any buffer;

3. Transfer grinded midrib tissue to 50 mL of Falcon tube, add appropriate NT solution;

4. Incubate for about 30-60min at room temperature;

5. Transfer supernatant to 2mL tube, remove the most of plant debris by centrifuge at 8000rpm for 1.5min, and transfer supernatant to a new tube, use 100uL of initial inoculums for DNA extraction.

6. Add 100uL of initial inoculum to culture media containing 2% glucose, 200ppm ATP, 2.5% H_2O_2 and 10% periwinkle extract in Las basic media

7. Collect 1mL of culture every 24h, spin down cell at 15000rpm for 15min, use pellet for DNA extraction.

DNA extraction

1. Add 100uL of lysozyme buffer to cell pellet, add 2uL of 100mg/mL lysome to the cell suspension, and incubate at $37^{o}C$ for 1h;

2. Add 2uL of 50mg/mL Proteinase K to cell suspension and incubate at $65^{o}C$ for 30min;

3. Freeze and thaw for two times using liquid nitrogen and $65^{o}C$ water bath;

4. Transfer all cell suspension solution to a 2mL column tube with 5 glass bead, grinding in Mini bead beater for 3min;

5. Add 400uL of psyllid extraction buffer, vortex and incubate at $65^{o}C$ water bath for 30min;

6. Add 500uL of phenol: chloroform, mix by vertex and centrifuge at 12000rpm, $4^{o}C$ for 10 min;

7. Carefully transfer 400uL supernatant to a new tube containing 2X volume of cold 95% EtOH, mix by invert tube 8-10 times and precipitate DNA at 12000 rpm, $4^{o}C$ for 10 min;

8. Wash pellet one time with Cold 70% EtOH, and vacuum dry pellet for 5min;

9. Dissolve DNA with 30uL DNAse/RNAse free water.

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