The Effect of Chemical Treatment on the Suppression and Ultrastructure of '*Candidatus* Liberibacter Asiaticus' in Infected Pomelo (*Citrus maxima*)

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Abstract

Huanglongbing (HLB) is an important disease affecting citrus plants worldwide. Formerly known as citrus greening, HLB is associated with three species of *Candidatus* Liberibacter, a gram negative bacterium, phloem limited, and belonging to the α-proteobacteria. The *Ca.* L. asiaticus (Las) distributes in Florida, Asia and Thailand. The trunk injection method was used to evaluate the effectiveness on the inhibition of Las infected pomelo trees in greenhouse. The T4 treatment containing streptomycin (250 mg L^{-1}), ampicillin (2.5 g L^{-1}), penicillin G (2 g L^{-1}), and Bacicure® (2 g L^{-1}) provided the highest efficiency in reducing and suppressing the Las bacterium population with the increase mean cycle threshold (Ct) values of the qPCR from 20.8 to 36.6 which corresponding to no Las detected by conventional polymerase chain reaction (cPCR) detection. Our combination remained therapeutically effective for eight months after the last treatment. In addition, response of Las infected pomelo toward chemical treatments was elucidated using transmission electron microscope with Bacicure® and antibiotic combinations resulted in cell thickening when compared with other treatments. Moreover, the increases of starch, callose accumulation, and swelling of cell wall observed in TEM were also related to the high titer of HLB bacterium.

Keywords: citrus huanglongbing, antibiotic treatment, HLB management, ultrastructure

Introduction

Huanglongbing (HLB), commonly known as citrus greening disease, is a serious problem on citrus production worldwide since no effective controls have yet been established (Bove, 2006). All citrus industries affected by the disease suffer a decline in both production and profit (Gottwald, 2010). In Asian countries and Florida, the pathogen that causes HLB disease is *Candidatus* Liberibacter asiaticus, which is transmitted by the psyllid vector, *Diaphorina citri* Kuwayama (Hung et al., 2004; Jagoueix et al., 1994). The '*Candidatus* Liberibacterasiaticus (Las),' is a gram-negative, fastidious α-proteobacterium. The bacterium propagates within the phloem of the host plants, resulting in blotchy mottles on leaves, yellow shoots, and dieback or decline. In Thailand, HLB is a destructive disease affecting citrus trees, particularly mandarin (*Citrus reticulata*), sweet orange (*Citrus sinensis*), lime (*Citrus aurantifolia*) and pomelo (*Citrus maxima*). The citrus farming area in Thailand has reduced from 80,000 hectares in 1992 to 8,400 hectares in 2014 due to the HLB infection and the lack of effective control measures. Pomelos are great commercial value for export market, and cultivated in many areas of Thailand in which they are also HLB disease.

During the 1970s, tetracycline was directly injected into the trunk of HLB infected citrus trees in South Africa, China, Taiwan, and Indonesia. These works noted a significant reduction of HLB symptoms in treated trees. To date, there are limited control practices for preventing further spread of HLB, and all known citrus species, cultivars, or combinations of scion and rootstock are susceptible to HLB. In 2011, Zhang et al. reported that a combination of penicillin G and streptomycin was effective for controlling and suppressing the population of HLB bacterium by trunk injection procedure. This report showed that the mixture was effective in reducing the HLB bacterial population and the disease symptom. Recently, tetracycline and ampicillin with the dosage of 12,500 ppm 20 m L^{-1} were applied to Las infected citrus mandarin orchards in Chiang Mai province, Thailand. It was found that the occurrence of HLB symptoms was significantly reduced after 2 months of the last injection (Paradornuwat, 2014).

Examinations of the disease infected tissues using transmission electron microscope (TEM) revealed that the pathogenic bacterium possesses a cell wall of gram-negative type and exclusively reside within the sieve tubes of infected citrus trees (Villechanouxet.al, 1993). These properties of the bacterium were used as a basis for the detection of HLB by electron microscopy, which had been the only reliable diagnostic technique for a number of years prior to the development of detection methods based on DNA hybridization and PCR. In this study, we examined the response of different genotypes of Las-infected pomelo trees to HLB bacterium and the efficiency of chemical treatment under greenhouse condition using transmission electron microscope (TEM). These conditions allowed us to monitor the development of the disease symptom and to evaluate the effect of chemical treatment to ultra structural changes in pomelo.

Materials and Methods

Greenhouse Experiment

Las- infected pomelo (*Citrus maxima*) trees with typical symptoms of HLB were selected. The HLB infected citrus trees were tested for the presence of *Ca*.L.asiaticus bacterium using the conventional PCR (cPCR) and quantitative PCR (qPCR) with CGO3F/CGO5R and HLBaspr specific primers, respectively. The experiments were conducted in the greenhouses at Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom province, Thailand. These greenhouses contained 5 years old Las-infected pomelo trees in soil chambers. The experiments were performed for 8 months from January 2012 to August 2012. The experiment areas are located in the central part of Thailand that has a tropical wet and dry or savanna climate type with temperature between 40ºC (104ºF) and 19ºC (66ºF) (http://www.tmd.go.th/ thailand.php). The citrus trees were pruned before the experiments started.

Antibiotic Treatment Using Trunk Injection Method

A randomized complete block design (RCBD) with four replications was used. Three branches of the same tree were selected as treatment units for each replication. The Las infected pomelos were injected with 30 mL of each chemical (Table 1) using an Avoject syringe injector (a catheter-tripped 60-mL syringe; Aongatete Coolstores Ltd., NZ). The tapered tip was fitted into a 19/64-in (7.5-mm) diameter hole, approximately 3 cm deep, drilled into the tree. Applications of chemicals were conducted monthly for four months (January 2012 to April 2012). Then, there was no chemical treatment in the next 4 months (May 2012 to August 2012). After the last treatment of each chemical and every month afterward, citrus leaves were collected for DNA extraction and the bacterial population was evaluated by qPCR with HLBaspr specific primers (Li et al., 2006).

DNA Extraction

Total DNA was extracted from leaf midribs using the modification of PVP method as described previously (Zhou et al., 2011). Briefly, 0.2 g of fresh tissues of leaf midribs were frozen in liquid nitrogen, and quickly ground to a fine powder with a mortar and pestle. Citrus powder was placed in a 2mL screw-cap tube (USA Scientific, Ocala, FL) containing 800 µL extraction buffer (100 mM Tris-Base, 50 mM EDTA, 500 mM NaCl, 2.5% PVP), and mixedusing FastPrep®-24 Homogenizer (MP Biomedicals, LLC, OH, USA). Ground samples were treated with 80 µL of 20% SDS and incubated at 65ºC for 15 min. Subsequently, the samples were treated with 400 µL of 5 M potassium acetate. The samples were immediately placed on ice for at least 15 min, and then centrifuged at 14,000 rpm for 15 min. The upper phase was transferred to a new 1.5 mL tube and the DNA was precipitated using 600 µL or 2/3 volume of cold isopropanol. The DNA pellets were washed twice with 1 mL of 75%

Table 1 List of chemicals used in trunk injection experiments.

Treatment	Chemicals
T1	Tetracycline (2 g L^{-1})
T2	Tetracycline (2 g L^{-1}), Bacbicure® (2 g L^{-1})
T3	Streptomycin (250 mg L^{-1}), ampicillin (2.5 g L^{-1}), penicillin G (2 g L^{-1})
T4	Streptomycin (250 mg L^{-1}), ampicillin (2.5 g L^{-1}), penicillin G (2 g L^{-1}), Bacbicure® (2 g L^{-1})
T5	Bacbicure® (2 g L^{-1})
T6	Water (Control)

ethanol and dried using a speed vacuum. The pellets were then dissolved in 50-100 µL of distilled water and kept at -20 °C.

Detection of '*Ca.*L.asaiticus' Using Conventional PCR (cPCR)

The presence of Las bacterium was confirmed using the CGO3F (5' RGGGAAAGATTTTATTG GAG3')/CGO5R (5'GAAAATAYCATCTCTGAT ATCGT3') primers (Zhou et al., 2007) targeting 798 bp of 16S rRNA. The PCR reaction was performed in a 20 µL reaction mixture containing 10 µL of 2x buffer D (Epicentre Biotechnology, Madison, WI, USA), 250 nmol of forward and reverse primers, 1.25 U of *Taq*-DNA polymerase (New England BioLads Inc., Ipswich, MA, USA) and 1-2 µL of template DNA. The PCR cycles were initiated with 95 ºC for 3 min, followed by 40 cycles of 94ºC for 30 seconds, 52ºC for 30 seconds, 72ºC for 1 minute, and the final extension at 72ºC for 10 minutes. The amplified PCR products were separated by electrophoresis in 1% agarose gels (1x TAE buffer) containing ethidium bromide (0.5µg mL^{-1}) and photographed under an UV illuminator.

Detection of '*Ca.*L.asaiticus' Using Quantitative PCR (qPCR)

The quantitative real time PCR was performed with specific primers and probes (Li et al., 2006) HLBasf, (5'-TCGAGCGCGTATGCGAATACG-3'), HLBasr, (5'-AGACGGGTGAGTAACGCG-3') and HLBasp, (5'-GCGTTATCCCGTAGAAAAAGGT AG-3') using LightCycler® 480 system (Roche Applied Science, Mannheim, Germany). The 15 µL TaqMan PCR reaction mixture contained 7.5 µL of TaqManPCR master mix (Roche Applied Science), 250 nmol of each primer, 150 nmol of probe, and 100 ng of DNA template. The qPCR amplification setting included 95ºC denaturation for 5 minutes, followed by 40 cycles of 94ºC for 3 seconds and 60ºC for 30 seconds.

Statistical Analysis

The data were analyzed using Analysis of Variance (ANOVA) and signification difference between treatments compared by Duncan's multiple range test (DMRT) method with statistical analysis system (SAS Version 9,SAS Institute Inc., USA) was used to separate the treatment means at $P \leq 0.05$.

Anatomical Study of Infected Cells by Transmission Electron Microscope (TEM)

The Las infected and healthy pomelo samples were collected from the greenhouse at 16 weeks and 32 weeks after treatment. The healthy sample was obtained from disease-free nursery of the Department of Agriculture, Ministry of Agriculture and Co-operatives, Thailand. Each leaf was rinsed with sterile water. Citrus midribs were separated from the whole leaf and cut into 1 to 4 mm. For sample preparation, a fixation procedure was used in which they were fixed with 2% freshly prepared paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 under vacuum for 1 hour. The small midribs were then washed 3 times with phosphate buffer, post-fixed in 1% osmium tetroxide for 1 hour, dehydrated for 30 minutes in each of an ethanol series (30%, 50%, 70%, 80%, 90%, and 100%, respectively). The samples were then infiltrated and embedded in Spurr's resin. The 120 nm thick of the resin was cut using Leica EM UC7 (Leica Microsystems (SEA) Pte Ltd, Singapore) and placed on formvar-coated grids and stained in 1% aqueous uranyl acetate (Liefting et al., 2009). Sections were viewed on a transmission electron microscope (JEOL model JEM-1220, JEOL Ltd, Tokyo, Japan).

Results

Effect of Chemical Compounds on HLB Population in Infected Pomelo

The HLB bacterium titer in pomelo trees was evaluated using qPCR with the HLBapsr primers(Li et al., 2006), and cPCR, with CGO3f/CGO5r primers (Zhouetal., 2007). The expected size of PCR product from CGO primers was 750 bp. The results indicated a high titer of bacteria in the pomelo trees prior to the treatment (week 0) with the Ct mean values ranging from 20.8 to 24.1 (Table 2). Treatments of antibiotics and combinations of antibiotics and Bacbicure® were listed in Table 1. The most drastic decrease in bacterial titer was observed in T1, T2, T3, and T4 after the last injection (12^{th} week) with the increase of the Ct mean value ranging from 30.2 to 36.6 (Table 2), corresponding to cPCR results as no Las populations (Figure 1). The chemical treatments in T3 and T4 effectively reduced HLB populations as shown in Table 2 in which the mean Ct value of T3 was increased from 24.1 to 33.5 and T4 was the most effective in reducing HLB population by increasing the mean Ct value from 20.8 to 36.6 at the 16^{th} week of the experiment. The Las infected pomelo leaves in T3 and T4 displayed no HLB symptoms and HLB population of 16 weeks after treatments (Figure 1). The pomelo trees in T4 were the most effective recovered from the HLB disease after treatments with a combination of streptomycin (250 mg L^{-1}), ampicillin (2.5 g L^{-1}), penicillin G (2 g L^{-1}), and Bacbicure® (2 g L^{-1}) as shown by leave morphology in Figure 1. In addition, results from qPCR (Table2) and cPCR (Figure1) confirmed that the Las bacterial populations in T4 became smallest at the 16^{th} week of the experiment. However, the Las bacterial populations in T3 and T4 were gradually increased after the last chemical treatment at the 12^{th} week (Table 2), in which the Ct mean values of T3 and T4 were not significantly different ($P \leq 0.05$) at the 28^{th} week and the 32^{nd} week of the experiment. The results from T4 at the 32^{th} week indicated that the Las bacterial population gradually increased since the last treatment. In addition, the Las infected pomelo leave of T4 slightly showed botchy mottling symptom after 32 weeks. Although the effect of T1 and T2 was weaker than those of T3 and T4, T1 and T2 were also able to significantly reduce the

Table 2 Quantitative real-time PCR (qPCR) data of '*Ca*. L. asiaticus' from pomelo tree leaves. The experiments were conducted in the greenhouse at Kasetsart University Kamphaengsaen campus, Nakhon Pathom province, Thailand for 32 weeks after the first treatment.

Injection time[1/] (week)	Mean Ct value (± SD)[2/]					
	T1[3/]	T2[3/]	T3[3/]	T4[3/]	T5[3/]	T6[3/]
1st injection (0)	22.1 ± 3.4 ab	20.8 ± 1.4 a	24.1 ± 4.7 b	20.8 ± 1.1 a	20.9 ± 1.1 a	20.8 ± 3.3 ab
2nd injection (4)	25.0 ± 1.9 b	27.2 ± 3.3 bc	29.7± 2.8 cd	31.2 ± 4.4 d	22.5 ± 0.5 a	20.8 ± 0.9 a
3rd injection (8)	26.1 ± 1.7 b	28.7 ± 4.9 c	31.5 ± 2.4 d	33.5 ± 1.9 d	22.7 ± 1.2 a	23.4 ± 1.5 a
4th injection (12)	29.3 ± 0.9 b	31.6 ± 3.9 c	32.9 ± 2.1 c	35.6 ± 1.4 d	23.3 ± 1.4 a	23.8 ± 1.9 a
No injection (16)	31.5 ± 1.1 c	30.2 ± 2.8 c	33.5 ± 1.3 d	36.6 ± 1.1 e	24.2 ± 1.4 b	22.6 ± 1.5 a
No injection (20)	31.2 ± 1.4 b	31.0 ± 2.8 b	32.4 ± 1.8 b	35.5 ± 0.5 c	23.3 ± 2.1 a	22.1 ± 1.7 a
No injection (24)	30.1 ± 1.2 c	30.9 ± 1.3 c	31.6 ± 1.7 c	34.1 ± 1.3 d	25.4 ± 2.9 b	19.9 ± 1.9 a
No injection (28)	28.5 ± 1.5 c	29.1 ± 1.5 c	33.0 ± 1.3 d	32.5 ± 1.3 d	24.2± 2.7 b	21.1 ± 2.5 a
No injection (32)	27.1 ± 1.6 c	27.9 ± 1.5 c	30.9 ± 0.9 d	31.6 ± 1.2 d	24.6 ± 1.6 b	21.3 ± 1.2 a

[1/] The chemical injections were performed 4 times in monthly interval at 0-12 weeks and there was no injection at the 16^{th} week onward.

[2/] Ct mean values are from the qPCR detection of *Ca*. L. asiaticus 16S rDNA gene using HLBaspr primers. The qPCR data are presented as the mean values from an independent assay followed by the standard deviation (SD).

[3/] Different letters in the same row indicate significant differences in Ct mean values, whereas the same letter indicates non-significant differences at $P \leq 0.05$

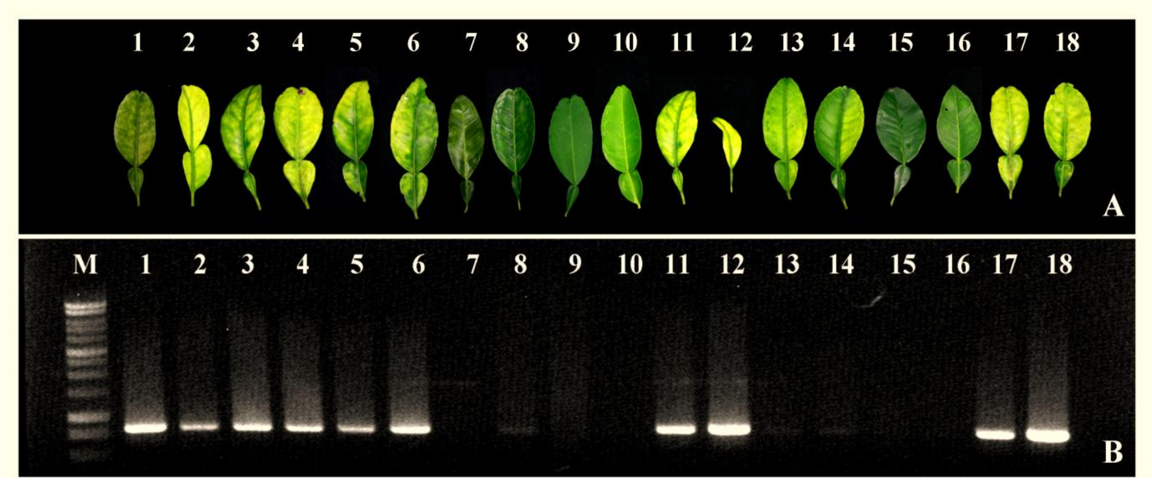

Figure1 The comparison of leave symptoms and cPCR amplification of Las infected pomelo (*Citrus maxima*) using CGO3f/CGO5r primers at 0 (pretreatment), 16, and 32 weeks. Panel A represents the infected pomelo leaves. Panel B represents the results from PCR amplification. Lanes 1 to 6: DNA from T1 to T6 of Las infected samples at 0 week (pretreatment), Lane 7 to 12: DNA from T1 to T6 of Las infected samples at16 weeks of treatment. Lane 13 to 18: DNA from T1 to T6 of Las infected samples at 32 weeks of treatment. The 1-kb DNA ladder (Promega) is shown in the far left lane (M).

Las bacterial population ($P < 0.05$) comparing to T5 and T6 (water control). The decrease of Las-bacterial population in T1 was indicated by the lower bacterial titer with the Ct value of 31.5 at the 16th week, which was similar to the Las-bacterial populations in T2 corresponding to the low bacterial titer with the Ct value of 31.6 at the12th week. However, the Las bacterial titer quickly increased after 12 weeks when compared with T4 (Table 2, Figure 2). The HLB disease symptoms of Las infected pomelo leave in T1 and T2 were observed at the 32th week of the experiments. In contrast, the Las bacterial population from the Bacbicure® (T5) and water control (T6) treatments resulted in the Ct mean value between 19.9 and 25.4 throughout the experiments (Table 2, Figure 1). Interestingly, the Las bacterial population in T5 was significantly different from T6 ($P \leq 0.05$) at the16th, 24th, 28th, and 32nd weeks (Table 2). From these results, it could be concluded that Bacbicure® suppressed the Las bacterial population and enhanced the effectiveness of antibiotic mixtures.

Evaluation of Phloem Morphology After the Chemical Treatment at the 16th Week by TEM

PCR analyses from leave midribs were performed using HLBaspr and CGO3f/CGO5r primers targeting the 16sRNA of Las bacteria to confirm the presence of '*Ca*.L. asiaticus' in infected leaves and the absence of Las bacteria in healthy leaves. The new flushes of Las infected pomelo leaves in T1, T2, T3, T4, T5, and T6 (water control) at the 16th week were collected (Figure 1), and the phloem structures were examined and compared by TEM. The anatomical analysis of leaf petioles was performed at the 16th week. The cross-section of healthy pomelo leave was shown in Figure 2A. The phloem cell wall and cambium layer of infected leaves appeared to be thinker than those of healthy leaves (Figure 2B). Accumulation of starch was present in phloem parenchyma cells of the infected leaves but was absent in the healthy leaves (Figure 2A and 2B). The most striking structural change noted in the Las infected leaves was a significant swelling of the middle lamella between cell walls surrounding sieves element (Figure 2B). In addition, a cross-section of pomelo leaves from the 16th week after chemical treatments was shown in Figure 3. Transmission electron micrographs of T4 showed normal cells with no starch accumulation and no callose deposition in sieve elements as shown in Figure 3D. Further observation of the T3 leaves showed that most of them had a relatively small amount of callose lining the sieve element pore, which is significantly different from T4. From TEM, the cell wall in T4 treatment appeared to be thinker than the one in T3. In addition, the phloem areas of T1 and T2 contained more extensive deposition of amorphous callose (Figure 3A and

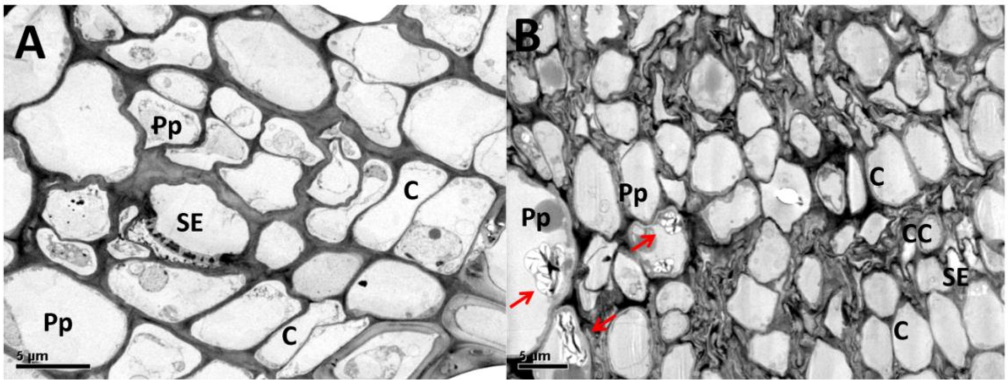

Figure 2 Anatomical analysis of midrib phloem tissues of the healthy pomelo leave (A), and the Las infected leave (B) using transmission electron microscope (TEM).The cross section area shows the xylem (X), cambium layer (C), sieve element (SE), companion cell (CC) and phloem parenchyma (Pp). The arrows indicate starch accumulation on sieve element in phloem tissue.

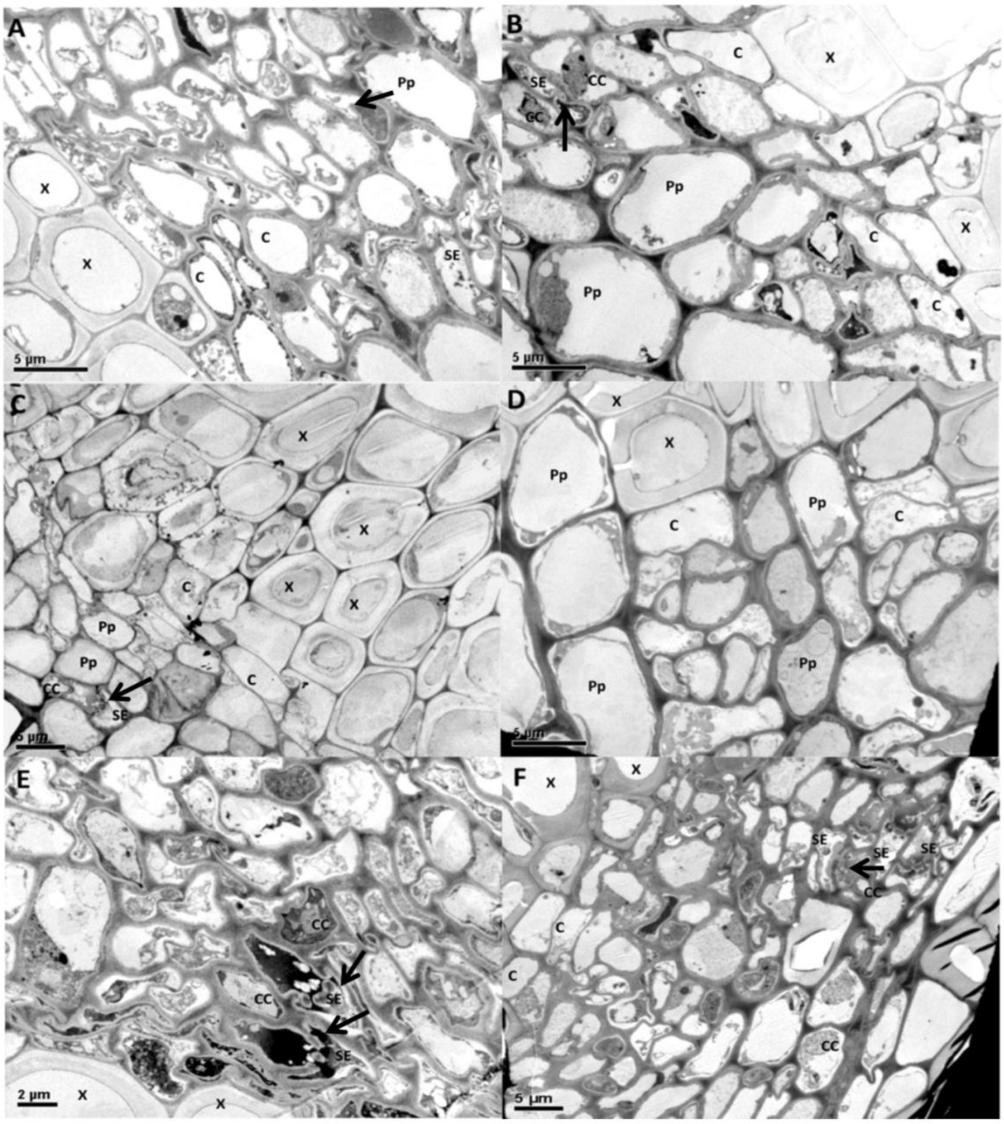

Figure 3 Transmission electron micrographs of phloem tissue of leaf petioles extended from xylem to phloem fibers illustrating from various treatment A. T1 treatment; B. T2 treatment; C. T3 treatment; D. T4 treatment; E. T5 treatment; and F. T6 treatment at 16 weeks after treatment. The cross section shows the xylem (X), cambium layer (C), sieve element (SE), companion cell (CC) and phloem parenchyma (Pp). The arrows indicate callose deposition on sieve element in phloem tissue.

3B). The sieve element morphology of tissues in T1 and T2 were similar to those of T3, T4, and the control, which contained dark, round deposition of starch as shown in Figure 3A and 3B. However, the sieve element and companion cells confined within areas with swollen middle lamella appeared to show signs of compression. In addition, the Bacbicure® treatment may have an effect on the cell wall and the middle lamella thickness in which the cell wall thickness was significantly increased with T2, T4, and T5 treatments compared to the T1, and T3, respectively. The sieve element in T5 and the T6 (control) also contained dark material, round deposition of starch (Figure 3E and 3F), which made it possible to be easily recognized in the absence of an obvious sieve plate as HLB infected cells. In addition, the qPCR results confirmed that the Las bacterial titer of T4 at the16th week was decreased as evidenced by the increase of the Ct mean value to36.6 (Table2) and no PCR product of Las was detected by cPCR (Figure 1B).

Evaluation of Phloem Morphology after Chemical Treatment at the 32nd Week by TEM

The phloem tissues were collected from mature flushes at the 32ndweek after chemical treatment. The new flush demonstrated significant anatomical changes in the phloem structure when compared with samples from healthy tree. In order to investigate whether '*Ca*. L. asiaticus' itself could block sieve pore, midribs from the HLB infected citrus leaves were collected for TEM analysis. Most sieve elements and companion cells and, occasionally, cambium cells were collapsed as shown in Figure 4. The phloem tissue of pomelo leaves demonstrated a completely collapsed sieve element with an accumulation of starch. The sieve element was also plugged with the callose-like and p-protein-like materials (Figures 4 and 5). The results from T6 demonstrated the distinct symptoms of HLB disease on the younger flushes (Figure1). In the 32nd week sample, the phloem tissue of T1 and T6 revealed a large number of bacterial-like

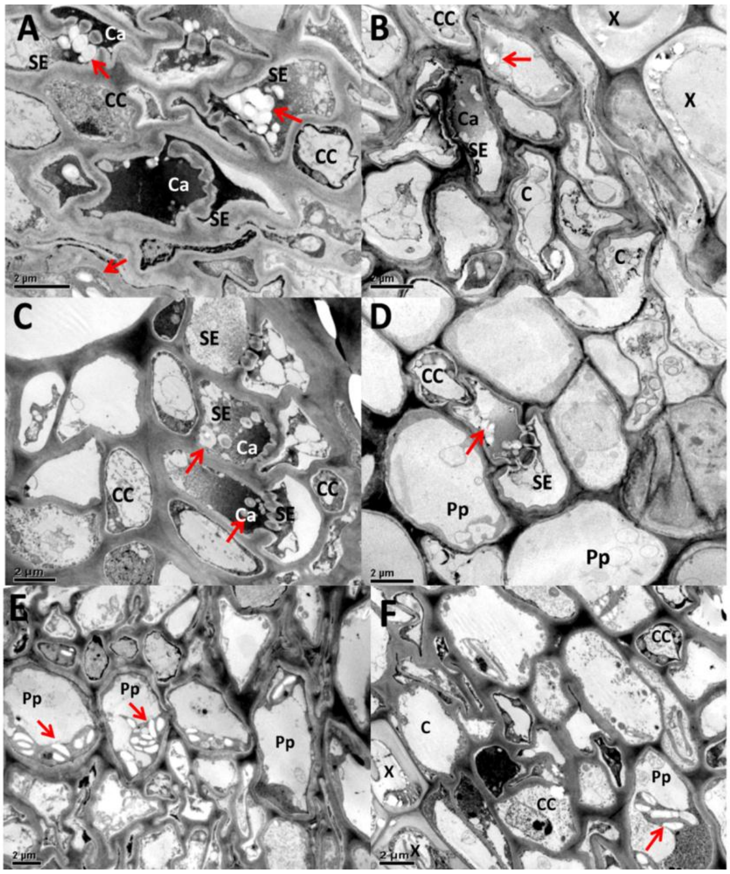

Figure 4 Transmission electron micrographs reveal sieve element area and starch accumulation of A). T1 treatment; B). T2 treatment; C). T3 treatment; D). T4 treatment; E). T5 treatment and F). T6 treatment of Las-infected pomelo leave at the 32th week after chemical treatment. The red arrows indicate starch accumulation on sieve element in phloem tissue. X, xylem; SE, sieve element; C, cambium layer, CC, companion cells; Pp, phloem parenchyma; Ca, callose.

cells with polymorphic shape in sieve element areas (Figure 5). These samples were confirmed as HLB positive by qPCR (Table 2) and cPCR (Figure 1B) analyses. Structural analysis with higher magnification showed a double membrane layer of cells (Figure 5B and 5D), a structure that indicated the differentiation between fastidious bacteria and phytoplasma. These bacteria were present in different forms such as rod- and spherical-shape. In addition, anatomical structure of infected areas of leaf petioles in T1, T2, T5, and T6 showed swelling cell walls when compared with T3, T4, and the healthy plant. The T4 and T3 treatments demonstrated significant anatomical changes in phloem tissue compared to other samples as shown in Figure 4. From the micrographs of T4 in Figure 4D, it was apparent that the minimal amount of callose deposition in sieve pores was observed. Moreover, a small amount of starch was accumulated. However, the starch accumulation was also present in phloem parenchyma cells in T3 leaves, but not in T4 and healthy leaves. Interestingly, the examination of phloem tissue in the 32nd week from leaf petioles in T4 revealed a similar cell structure to those from the healthy plants (Figure 4D and Figure 2A). However, sieve pore of samples from T1 and T2 treatments was plugged with the callose-like and parenchyma cells and collapsed due to HLB infection (Figure 4A and 4B).The black areas were significantly expanded in size, and many cells contained large starch grains (Figure4E, and 4F) and enriched cytoplasm compared with cell in T4 tissue (Figure 4D). As far as the pattern of starch distribution was concerned, petiole of HLB infected trees in the 32ndweek contained large amount of starch in parenchyma cells both within the sieve element and surrounding in parenchyma tissue (Figure 4 and 5). Further examination of tissue samples collected from T5 demonstrated a progressive increase in severity of ultra-structural aberration of the infected phloem; accumulation of starch gains, plugged sieve pore and swelling of middle lamella when compared with another treatment and appeared to be correlated with the disease development (Figure 4E). In addition, the phloem areas of T5 indicated the structural changes similar to T6 (water control) as shown in Figure 4F. Interestingly, all of those changes occurred in a period of 32 weeks and were apparently correlated with the progression of the disease symptoms and PCR detections of Las bacteria populations.

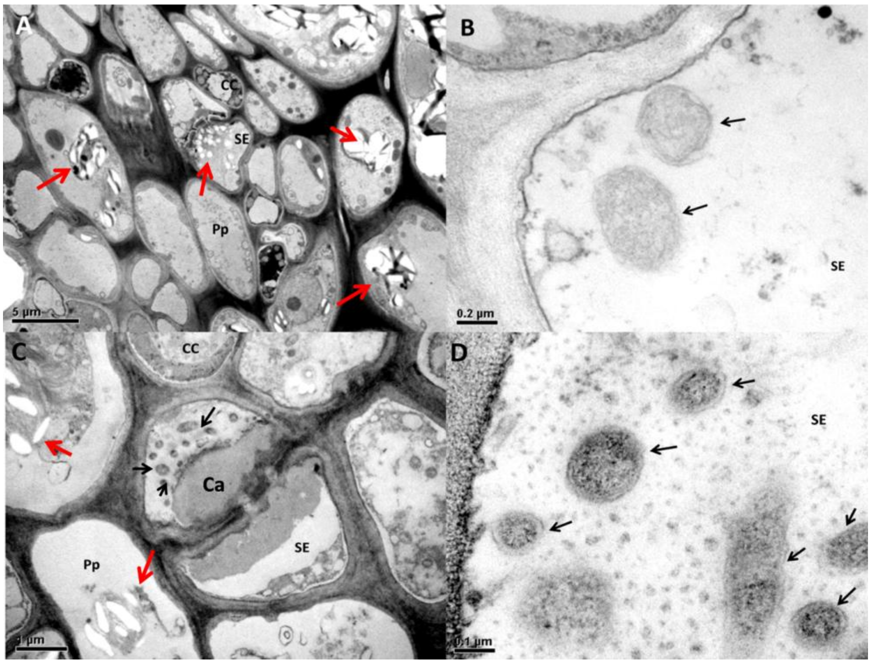

Figure 5 Transmission electron micrographs of sieve element from petioles of infected pomelo trees at the 32nd week after treatment show large numbers of '*Candidatus* Liberibacter asiaticus' bacterium. Panel A and C represent T1 and T6 treatment. Higher magnification of micrographs (B and D) shows double membranes (black arrow) surrounding the bacterial cells. The red arrows indicate starch accumulation on sieve element in phloem tissue X, xylem; SE, sieve element; C, cambium layer, CC, companion cells; Pp, phloem parenchyma; Ca, callose.

Discussion

Several citrus-producing countries are suffering from HLB disease. The disease is difficult to manage, and maintaining production of citrus in areas where HLB is widespread has been proven to be challenging and expensive. HLB has not been eradicated from many regions (Gottwald, 2010). The chemical controls have been successfully utilized for the reduction of Las infection (Su and Chang, 1976; Zhang et al., 2011). Our study demonstrated the relationship between symptom expression and the chemical treatment using transmission electron microscope and PCR analys is. A combination of streptomycin (250 mg L^{-1}), ampicillin (2.5 mg L^{-1}), penicillin G (2 g L^{-1}), and Bacbicure® (2 g L^{-1}) was the most effective for the suppression of HLB and disease symptom recovery. In addition, a mixture of antibiotic had higher antimicrobial activity against the Las bacteria than did the individual component alone or another tested antibiotic (tetracycline) or resistant-induced agent (Bacbicure®) as shown in Table 2. It is worth mentioning that Bacbicure®, also known as merpazole, is a type of induced systemic resistant (ISR) chemical used in suppression of *Xanthomonas* sp., the causal agent of bacterial plant diseases (Lertsuchatavanich et al., 2006).

Ampicillin and penicilln G are the bactericidal antibiotics, which are active against gram-positive and gram-negative aerobic and anaerobic bacteria by inhibiting bacterial cell-wall biosynthesis (Davies et al., 2007). In addition, penicillin G is taken up rapidly by plants, and is relatively nonphytotoxic as reported in an earlier study. Streptomycin is an amino glycoside antibiotic. It was first registered as a pesticide in 1955 for use in controlling bacterial pathogen of certain agricultural and nonagricultural crops (Brian, 1957). The mode of action of streptomycin is to inhibit the ribosome function by binding to the bacterial 30S subunit causing the misreading of mRNA (Luzzatto et al., 1968). In order to decrease the antibiotic resistant in bacteria, we combined tree antibiotics in a cocktail application, which had an apparent synergetic or additive effect and permitted a significant reduction of streptomycin dosage.

Our combination enhanced antibiotic activity against the HLB bacteria and remained at a therapeutically effective level for 8 months as compared with the application of tetracycline alone or other cocktail of antibiotics without Bacbicure®. The Bacbicure® and antibiotics may have an effect on the cell wall causing middle lamella thickening, which was observed from electron micrograph (Figure 3B and 3D). Development of effective control measures for the HLB disease requires a clear understanding of interactions between the vector, the pathogen, and the citrus tree. The transmission electron microscope (TEM) is a reliable detection method for the HLB bacterium. Based on TEM observation, both T3 and T4 represented normal phloem cells in the 16th week samples. The T4 treatment demonstrated a thick cell wall and no swelling of middle lamella as shown in Figure 3D. However, both treatments showed plugging of sieve pores with the callose-like material, necreosis of sieve element and companion cells, and excessive starch accumulation in phloem parenchyma cells in the 32nd week after the termination of chemical treatment. An increasing level of microscopic aberrations was observed as the symptoms progressed from mild yellowing to sever chlorosis, mottling, thickening, and occasionally vein corking of leaves. Most of the anatomical changes are typical for later stage of HLB infection including plugging of sieve pores with the callose. In addition, excessive starch accumulation in the phloem parenchyma cells has been described earlier for samples from infected field trees (Folimonova and Achor, 2010). Moreover, the increase of swelling of cell wall and middle lamella was also related to the high titer of HLB bacterium. In our work, we found that tetracycline has an effect on phloem cell morphology and phytotoxicity of plant cell as shown in Figure 3A and 3B. In addition to phytotoxicity associated with tetracycline, another reported problem was a rapid reinfection of trees following the antibiotic treatment (Van Vuuren, 1977). In 2013, Sruamsiri and coworkers monitored the presence of tetracycline in different parts of *Citrus reticulata* after trunk injection (Sruamsiri et al., 2013). The dose for trunk injection was 12,500 ppm (40 mL). It was found that only 120 ppb of tetracycline was detected in a fruit after 60 days of injection, and the amount of the antibiotic decreased dramatically after 90 days. However, antibiotics should be carefully utilized in agriculture to prevent environmental and food contamination.

To the best of our knowledge, this is the first report on the effect of chemical treatment in Las infected pomelo tree using TEM, qPCR and cPCR analyses. There are also other potential applications of the most effective treatment (T4) for rescuing the valuable citrus plant material that have been infected by HLB bacteria and ensuring that such plants are then HLB free and suitable for bud woods or rootstock and transfer to HLB-free areas. It is worth mentioning that we observed the highest number of bacteria in presymptomatic younger flushes, which likely represented a good source of the bacterium available for psyllid transmission of the HLB disease. In addition, the farmer should be advised to use our antibiotic combination monthly for four months and should be applied continuously. The establishment of integrated HLB disease management with an antibiotic application program may provide a way to immediate protection of pomelo trees and, thereby, minimize the likelihood of a pathogen reservoir and increase plant productivity.

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