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Ultrastructure and Cell Wall Thickness Modification and Its Detection After Chemical Treatments in Huanglongbing Infected Citrus Plants

Hajivand Shokrollah¹, Thohirah Lee Abdullah² and Kamaruzaman Sijam³

¹Department of Plant and Seed Improvement,
Agriculture and Natural Resources Research Center of Qazvin, Qazvin,

²Departments of Crop Science, Faculty of Agriculture,
University Putra Malaysia, Serdang,

³Department of Plant Protection, Faculty of Agriculture,
University Putra Malaysia, Serdang,

¹Iran

^{2,3}Malaysia

1. Introduction

Citrus is the leading fruit crop grown in the world. Many factors are known to limit the production and processing of citrus. Unfortunately many diseases such as Huanglongbing (HLB) have been threatening citrus industry in the world. The disorder of citrus described as “greening” disease has caused great damage in the citrus industry and is known as Huanglongbing (HLB) disease (Bove, 2006). It was detected in most citriculture areas of Asia, Africa, USA and most *Citrus* species are susceptible to this disease.

The HLB disease pathogens are fastidious phloem-limited bacteria in the genus *Candidatus Liberibacter*. The isolate from South Africa has been named *Candidatus Liberibacter africanus*, and the isolate from Asia has been named *Candidatus Liberibacter asiaticus* (Jagoueix et al., 1994). Symptoms of HLB were observed in sweet orange trees near the city of Araraquara in São Paulo State. This was the first reported case of HLB from the American Continent (Coletta-Filho et al., 2005; Teixeira et al., 2008). A new type of HLB (*Candidatus Liberibacter*) species is genetically characterized, and the bacterium is designated as “*Candidatus Liberibacter psyllauros*”. This bacterium infects the psyllid *Bactericerca cockerelli* and its solanaceous host plants potato and tomato, potentially resulting in “psyllid yellowing” (Hansen et al., 2008).

Virus and bacteria such as HLB pathogen can be detected using transmission electron microscope (TEM). This method has been used to detect the HLB pathogen since 1993. The shape and length of bacteria can be determined by TEM but the disadvantage of TEM methods is that it required special equipment (Bove and Garnier, 2002). Different protocols have been developed to detect the bacteria disease in plant by Roistacher (1991). A “mycoplasma-like organism” was observed in citrus phloem tissue infected with citrus

greening disease using a transmission electron microscope (TEM) (Bove and Garnier, 2002). Electron microscopy measurements on thin sections showed that the filamentous forms of the liberibacteria have a diameter of 0.2–0.3 micrometer (μm). Variations in diameter occurred between organisms and sometimes within a single organism. Round forms were larger (0.5 μm) with a less dense cytoplasm and often showed plasmolysis. The *Candidatus Liberibacter* spp., envelope was a membranous wall characteristic of Gram-negative bacteria, but the peptidoglycan layer was hardly visible (Garnier et al., 1984). There is no evidence of flagella or pili. *Candidatus* L. asiaticus and *Candidatus* L. africanus cannot be distinguished on the basis of morphology. As far as it is known, none of the HLB strains have been obtained in a media culture (Bove and Garnier, 2002).

However, the HLB organisms were about 2000 nanometer (nm) long and 100–200 nm in diameter. Similar bodies were observed in both vectors of the citrus greening disease, *Ttioza erytreae* (Moll and Martin, 1973) and *Diaphorina citri* Kuwayama (Chen et al., 1973). The greening organism was compared with citrus stubborn organism (spiroplasma) by Saglio et al., (1971), the study showed that the outer membrane of the greening organism was much thicker (25 nm) than that of the spiroplasma (10 nm). HLB bacterium was found to be Gram-negative bacteria type (Garnier et al., 1984).

Molecular detection methods have been difficult to develop since the greening organism hasn't been cultured (Halbert and Manjunath, 2004). Recently, molecular methods such as polymerase chain reaction (PCR) have become available to detect HLB disease and provided the credible detection of HLB disease. *Candidatus Liberibacter* spp., in infected plants and psyllid vector has been detected using DNA technology. Halbert and Manjunath (2004) reported that Jagoueix et al. (1996) used universal primers for general amplification of prokaryotic, 16S ribosomal DNA (rDNA).

Based on sequence information, primers have been developed to amplify 1,160 base pair (bp) region of ribosomal DNA for detection of greening by PCR. Ribosomal DNA primers have been used widely for detection of both forms of HLB. These primers have do not amplify 16S ribosomal sequences of other citrus pathogens.

Detection of citrus HLB pathogens from asymptomatic tissue is inconsistent by any known method. Similarly, the molecular assays sometimes are complicated to run, and results are not always reliable. Clearly, more accurate, timely, and robust detection methodologies are needed. The outputs of the study may improve fruit quantity and quality, and reduce the risk of HLB in Malaysian citrus industry and citrus industries around the world. Therefore, the present research was carried out to detect *Candidatus Liberibacter asiaticus* (Ca. L. a) pathogen causing HLB disease in infected *Citrus reticulata*, and to determine the cell wall thickness modification in infected citrus after chemical treatments using TEM technique.

2. Materials and methods

2.1 Research area description

The research was conducted in Kuala Berang, Terengganu, Malaysia, between Jan 2008 and October 2009. The state of Terengganu overlooks the South China Sea on the east coast of Malaysia. It has a strong tropical monsoon climate, with a relatively uniform temperature ranging between 21 degree centigrade ($^{\circ}\text{C}$) and 32 $^{\circ}\text{C}$. Between January and April, the weather is dry and warm, with consistent high humidity in the lowlands ranging between 82 and 86 percent annually. Terengganu's average rainfall ranges from 2,032 millimetre

(mm) to 2,540 mm per year, with the most rain falling between November and January. The orchard of *Citrus reticulata* cv. Limau madu was cultivated in 2000 and it has been unproductive since that time because of HLB (Fig.1).

A total of 72 trees of *Citrus reticulata* were selected for this experiment. A randomized complete block design (RCBD) with three replications was used. Three trees were selected as a treatment unit for each replication for each treatment. Gibberellic acid (GA₃), antibiotics, foliar fertilizer and a combination of those chemicals were tested (Table 1). Aqueous solutions of the treatments were prepared and sprayed on whole trees. Moreover, 2 grams per litre (g/L) of antibiotic was injected into the stem at 15 to 25 centimetre (cm) above the soil (Fig.2). The treatments were applied before flowering and during fruit set formation (Fig.3). The following parameters were evaluated to measure the severity of the HLB disease and the effects of the treatment on plant rehabilitation. HLB detection using conventional PCR, ultrastructure of HLB and cell wall thickness modification using transmission electron microscopy (TEM) were carried out.



Fig. 1. Overview of HLB-infected *Citrus reticulata* in Hulu Paka, Terengganu, Malaysia.

Treatments	
T1	*Antibiotic (Oxi-tetracycline) 2 g/L (as injection)
T2	Gibberellic acid (GA ₃) 15 mg/L (as foliar application)
T3	Foliar fertilizer (Vita-Grow) 5 ml / L (as foliar application)
T4	Antibiotic (2 g/L) + GA ₃ (15 mg/L)
T5	Antibiotic (2 g/L) + foliar fertilizer (20 ml / 4 L)
T6	GA ₃ (15 mg/L) + foliar fertilizer (20 ml / 4 L)
T7	Antibiotic (2 g/L)+ GA ₃ (15 mg/L)+ foliar fertilizer (20 ml / 4 L)
T8	Control (non treated)

*Antibiotic was injected in the trunk of the tree 15 cm above the soil surface.
A total of 72 trees of *Citrus reticulata* on a RCBD with 3 replications were used.

Table 1. List of chemical treatments to reduce the HLB disease severity and improve fruit quantity and quality.

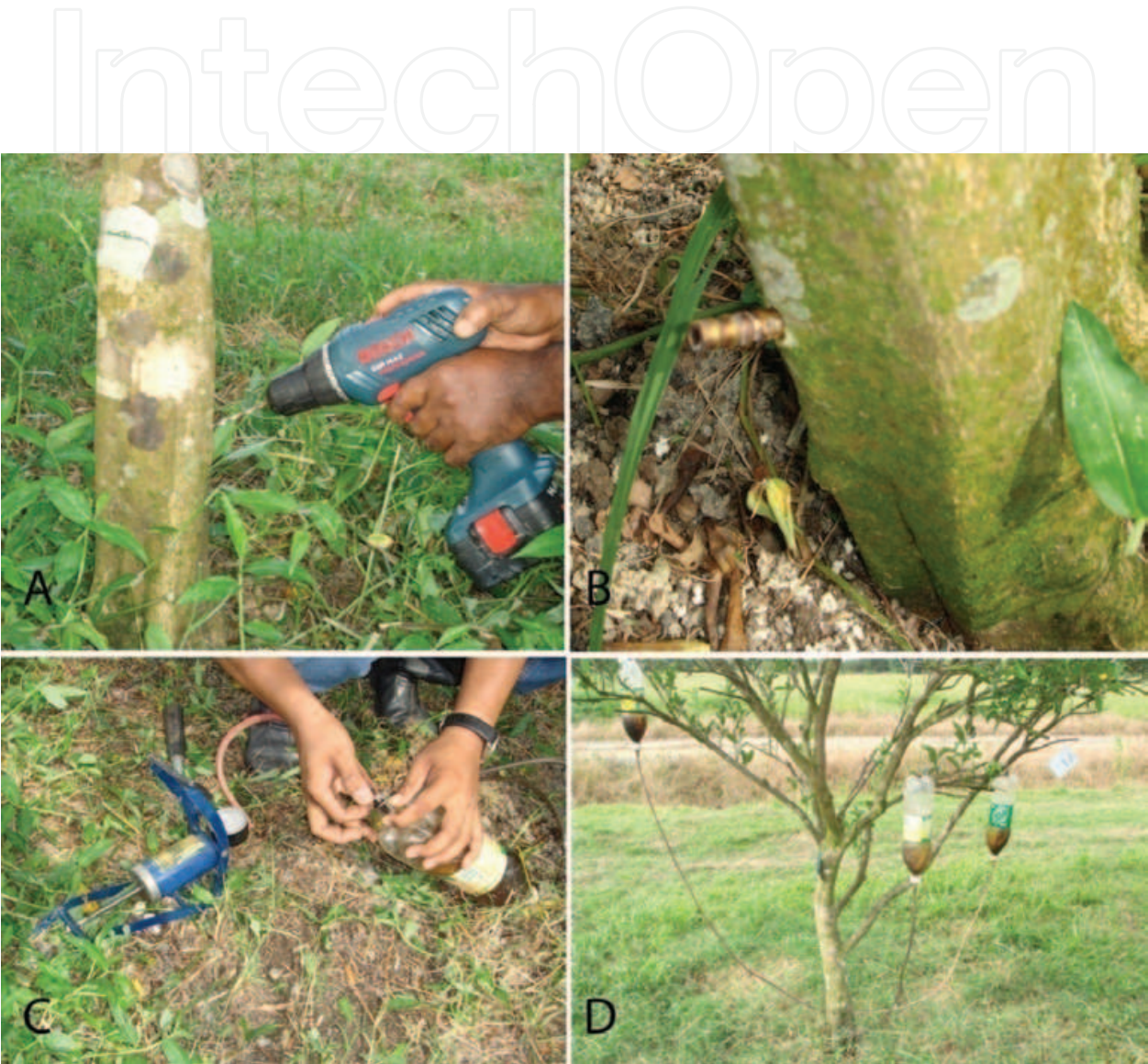


Fig. 2. Injection of antibiotics; (A) making a hole on the trunk of the tree, (B) placing the tube, (C) preparing the antibiotic container, (D) an overall view of the whole injection set up.



Fig. 3. Chemical treatments (GA_3 and foliar fertilizer) application before flowering (A) and after flowering (B) using tractor mounted sprayer.

2.2 Transmission Electron Microscopy (TEM) examination

A TEM technique described by Childers and Achor (1991) was used as TEM examination. New terminal shoots of the infected citrus trees were collected and washed. Midribs were removed from the leaves and chopped into small pieces, which were fixed in 4% glutaraldehyde for 12 to 24 hours at 4°C and washed using a 0.1molar cacodylate buffer at three stages, each for 30 minutes (min). The specimens were first rinsed in the buffer followed by post-fixation in 1% osmium tetroxide for 2 hours at 4°C. It was washed for a second time and dehydrated through a series of acetone from 35%, 50%, 75%, 95% and 100%, each for 30 min. Specimens were later infiltrated with acetone and resin mixtures at 1/1, 1/3 and 100 resin (two stapes) for 24, 12 and 2 hours, respectively, and embedded into beam capsules and filled with resin. It was polymerized at 60°C for 72 hours.

Thick sectioning (1micromolar) was done using a glass knife and an ultra-microtome (Leica UCT, Australia) to cut ultra-thin (60-80 nm) sections. The samples were placed onto a glass slide and stained with toluidine blue. Subsequently, these were dried on a hot plate and the stain was washed. Ultra-thin sections of the samples were cut with a diamond knife on an LKB-Huxley ultra-microtome (Cambridge, UK) and stained with 2% uranyl acetate (10 min). This was followed by filtered alcohol (50%) and washed by double distilled water prior to examination using a transmission electron microscope (TEM) at 60 kilovolts (kV).

For each treatment, six cells were randomly chosen to measure the thickness of cell walls and the middle lamella. The thickness of cell walls and middle lamella were measured using three points for each cell and the average thickness was used for comparison.

2.3 Polymerase Chain Reaction (PCR) test

The leaves of new citrus flushes were collected from the plots every two months after treatment. DNA was extracted from the midribs of the leaves using the method described by Hung et al. (2000). This was followed by grinding the tissue in a mortar and pestle using liquid nitrogen until there were no longer any large pieces of tissue remaining. DNA was extracted from the HLB-infected tissue using the hexadecyltrimethyl ammonium bromide (CTAB) method. The pellets were washed with 70% ethanol, dried and diluted with 100 micro litres (µl) of Tris-EDTA (TA) buffer (pH 8.0). The DNA quality was checked by electrophoresis on 1% agarose gel.

Conventional PCR was performed using 25µl of reaction mixture containing 20 milimolar (mM) Tris-HCl (pH 8.0), 50 mM KCl, 4 mM MgCl₂, 0.2 mM of dATP, dTTP, dCTP and dGTP (the 5'-triphosphates of deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine respectively), 50 ng forward primer, 50 ng reverse primer, 0.75 units of Taq DNA polymerase and 200 ng genomic DNA. The thermal cycle conditions were: one cycle at 95°C for 2 min, 35 cycles at 95°C for 40 sec, 60°C for 1 min and 72°C for 1 min followed by a 72°C extension for 10 min. Specific primer pairs, composed of the forward primer of OI1 (5'-GCG CGT ATG CAA TAC GAG CGG CA-3') {The four bases of DNA are adenine (abbreviated A), cytosine (C), guanine (G) and thymine (T)} and reverse primer of OI2c (5'-GCC TCG CGA CTT CGC AAC CCA T-3'), were used to amplify the 16S ribosomal DNA fragment. Amplification of DNA was determined by electrophoresis on 1.2% agarose gel for approximately 30-45 min and visualized by ethidium bromide staining. PCR products were purified directly on spin columns (Qiagen PCR purification kit).

2.4 Statistical analysis

A quick method was adopted to analyse the PCR product using a 1% agarose type III (Sigma brand). The gel was run in a TBE buffer (Tris base, boric acid and 2 mM EDTA, pH 8.0). The resolved DNA bands were detected by staining the gels with 0.5µg/mL of ethidium bromide, followed by destaining with distilled water. The electrophoresis was turned on for 30-40 min using a high voltage (100V) and finally photographed under UV illumination (SYNGENE; GENE Genius Bio Imaging System). A one kilo base pair (1Kbp) DNA ladder was used as a convenient marker for size estimates of the products.

Data of cell thickness modification were collected and analyzed by using analysis of variance (ANOVA), and significant differences between treatments were compared by the Duncan's multiple range tests (DMRT) method with a statistical analysis system (SAS Version 9 -TS M0) to separate the treatment means at the 0.05% level of probability. The percentage data were converted to their arcsine-square root equivalents in order to normalize data sets prior to analysis.

3. Results

3.1 TEM detection of HLB, ultrastructure of the pathogen and cell wall thickness modification after treatments using TEM examination

Results have shown abundant bacteria cells damaging the cell wall in sieve tube cell at 30-70 kV magnification (Fig. 4 and 5) in infected samples. TEM detection of HLB has shown that the number of bacteria in sieve tubes is higher in leaves with strong mottle. According to these results, the organisms are seen surrounded by a cell wall. Electron micrographs of sieve tube elements of infected *Citrus reticulata* leaf at high magnification (30-100 kV) were observed (Fig.4). In figures 4 and 5 there were two shapes of *Candidatus liberibacter asiaticus* that were present; spherical and rod shape. Electron micrographs of sieve tube elements of the infected *Citrus reticulata* leaf at high magnification (30-100 kV) were observed (Fig.4 -Fig.6). The HLB bacteria organisms were found to be surrounded by a cell wall (Fig.5 and Fig.6; T8). Electron micrographs of the sieve tube of *Citrus reticulata* leaf showing the fusion confluent of middle lamella and cell wall structure are shown in (Fig.6; T8). The cell wall and middle lamella were damaged by the *Candidatus Liberibacter asiaticus*, which penetrated through the cells (Fig. 6; T8).

The obvious change in the total cell wall thickness, relating to the change of the cell wall and middle lamella thickness, were observed using TEM study (Fig.6; T1-T7) after treatments. The comparison of means of the total thickness of cell wall, cell wall thickness and middle lamella thickness are presented in table 2 at the 0.05% probability level.

Total cell wall thickness was measured and was found to be statistically significant for certain treatments (Table 2). T4 (942.72 nm), T7 (811.62 nm) and T5 (766.07 nm) had the highest total cell wall thickness compared to the control treatment (T8; 214.07 nm) while a low rate of total cell wall thickness was measured for T3 (43.31 nm), T2 (469.78 nm) and T1 (564.96 nm). There was no significant difference for T1, T2 and T3, which were used as direct treatments in this study.

However, a significant difference in the cell wall thickness was observed among the treatments compared to the control (Table 2). Thick cell walls were observed for T4 (580.82nm) followed by T7 (476.39 nm), while thin cell walls were recorded for T8 (as control; 86.33 nm) followed by T3 (209.83 nm), T2 (212.64 nm) and T1 (305.53 nm).

Significant differences between the treatments were also observed concerning the thickness of the middle lamella (Table 2). A thick middle lamella was recorded for T7 (403.15 nm) and T4 (361.90 nm), while a thin middle lamella was observed for T8 (126.98 nm), T3 (223.48 nm), T1 (254.72 nm), T2 (256.20 nm), T6 (278.7 nm) and T5 (289.68nm). These were found to be statistically similar (Table 2).

Treatment*	Cell wall	Middle lamella	Total thickness
T1	305.53 cd	254.72 b	564.96 dc
T2	212.64 de	256.20 b	469.78 d
T3	209.83 de	223.48 b	433.31 d
T4	580.82 a	361.90 a	942.72 a
T5	425.69 bc	289.68 b	766.07 b
T6	378.65 bc	278.75 b	671.66 bc
T7	476.39 ab	403.15 a	811.62 ab
T8	86.33 e	126.98 c	214.07 e

* Means within a column with different letters are significantly different and means followed by the same letters are not significantly different according to DMRT test at $P \leq 0.05$ levels.
T1: Antibiotic. T2: GA₃. T3: foliar fertilizer. T4: Antibiotic + GA₃. T5: Antibiotic + foliar fertilizer. T6: GA₃ + foliar fertilizer. T7: Antibiotic + GA₃ + foliar fertilizer. T8: Control.

Table 2. Mean Comparison of cell wall, middle lamella and total thickness of cells after treatments using Duncan multiple range test.

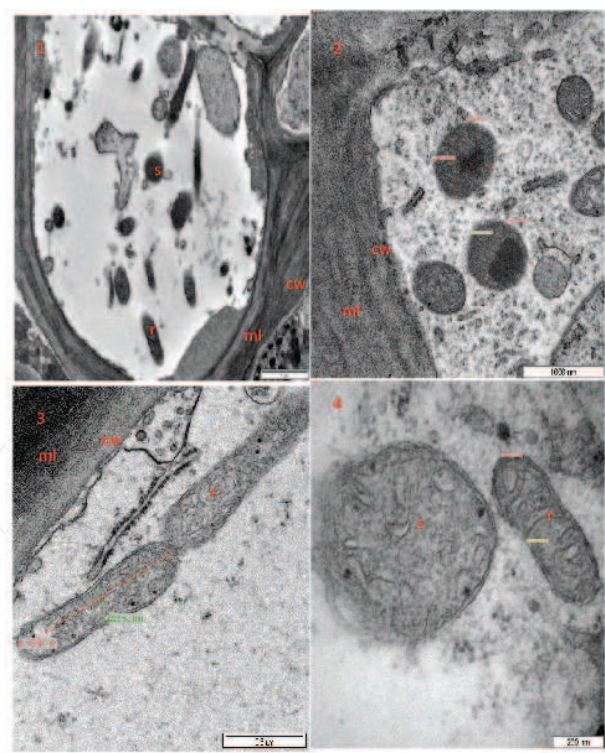


Fig. 4. Electron micrographs of the vascular system from the infected midrib of *C. reticulata* showing abundant bacteria cells damaging the cell wall (cw) and middle lamella (ml) in sieve tube cell (Fig.1-2) at magnification, 30 kV; and cross section of sieve tube cells containing spherical (s) and rod (r) shape bacteria (Fig.3-4) of HLB disease at high magnification (100 kV).

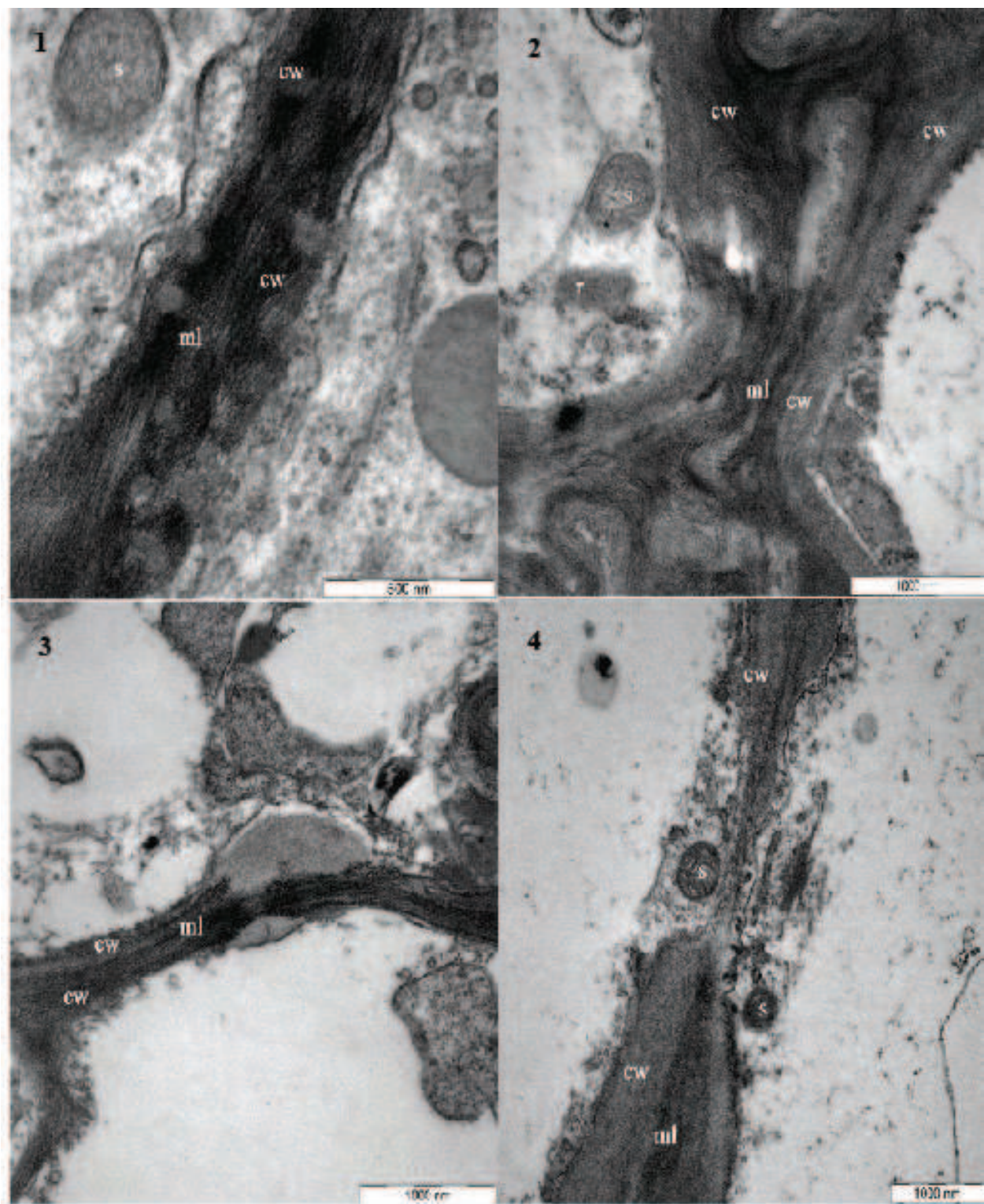


Fig. 5. Electron micrographs of the sieve tube of *Citrus reticulata* leaf; section (1 and 2) showing the fusion confluent of middle lamella (ml) and cell wall (cw) structure (1-mag. at 70 kV and 2-mag., at 30 kV magnification). Cross section of sieve tube (3 and 4) showing damaged cell wall (cw) and middle lamella (ml) caused by *Candidatus Liberibacter asiaticus* penetrating through the cells (3-mag. at 30 kV and 4-mag. at 20 kV).

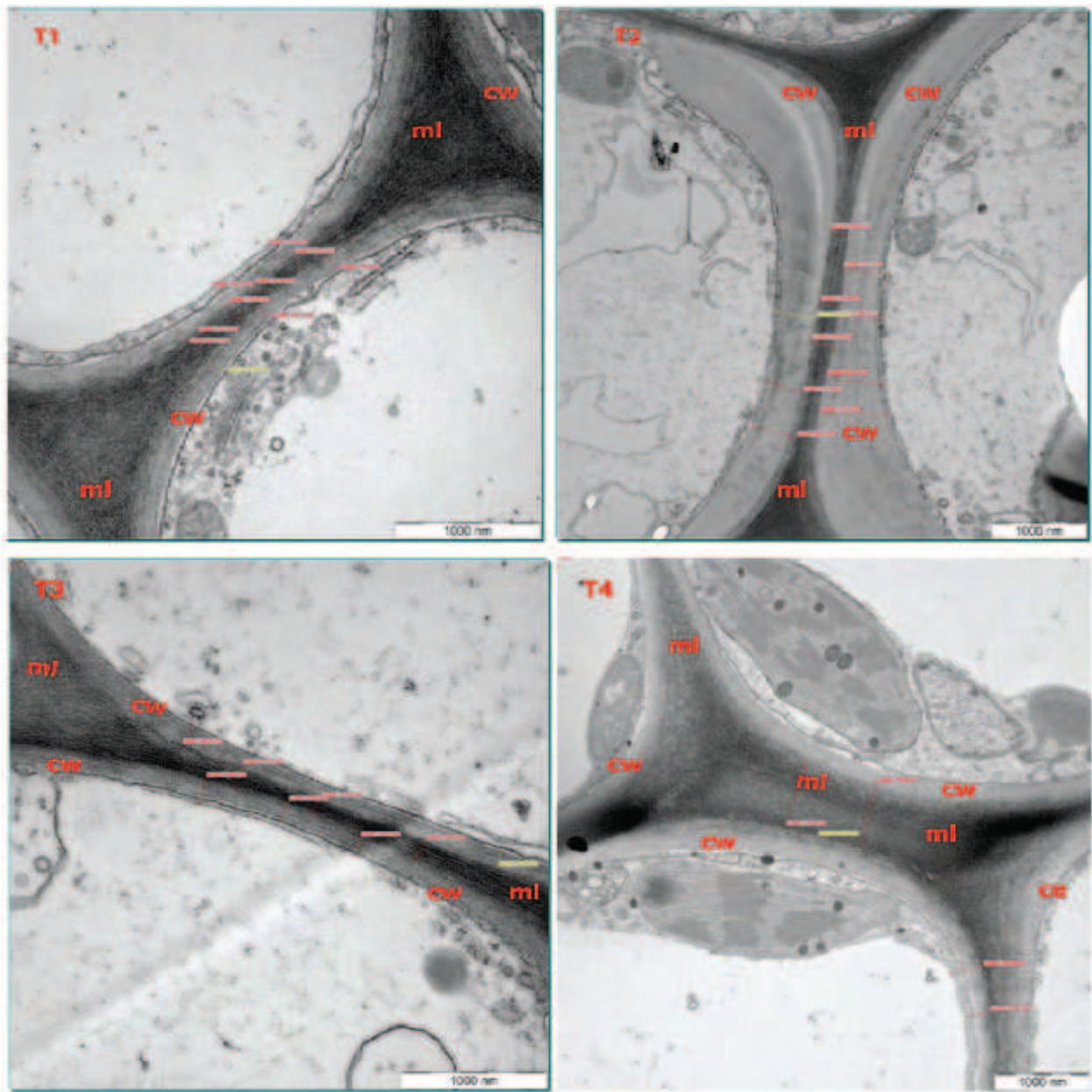


Fig. 6. Electron micrographs of the sieve tube of *Citrus reticulata* leaf: section showing the middle lamella and cell wall structure and cell wall thickness (mag at 70 kV magnifications). T1: Antibiotic. T2: GA3. T3: foliar fertilizer. T4: Antibiotic+GA3. T5: Antibiotic + foliar fertilizer. T6: GA3 + foliar fertilizer. T7: Antibiotic + GA3 + foliar fertilizer. T8: Control.

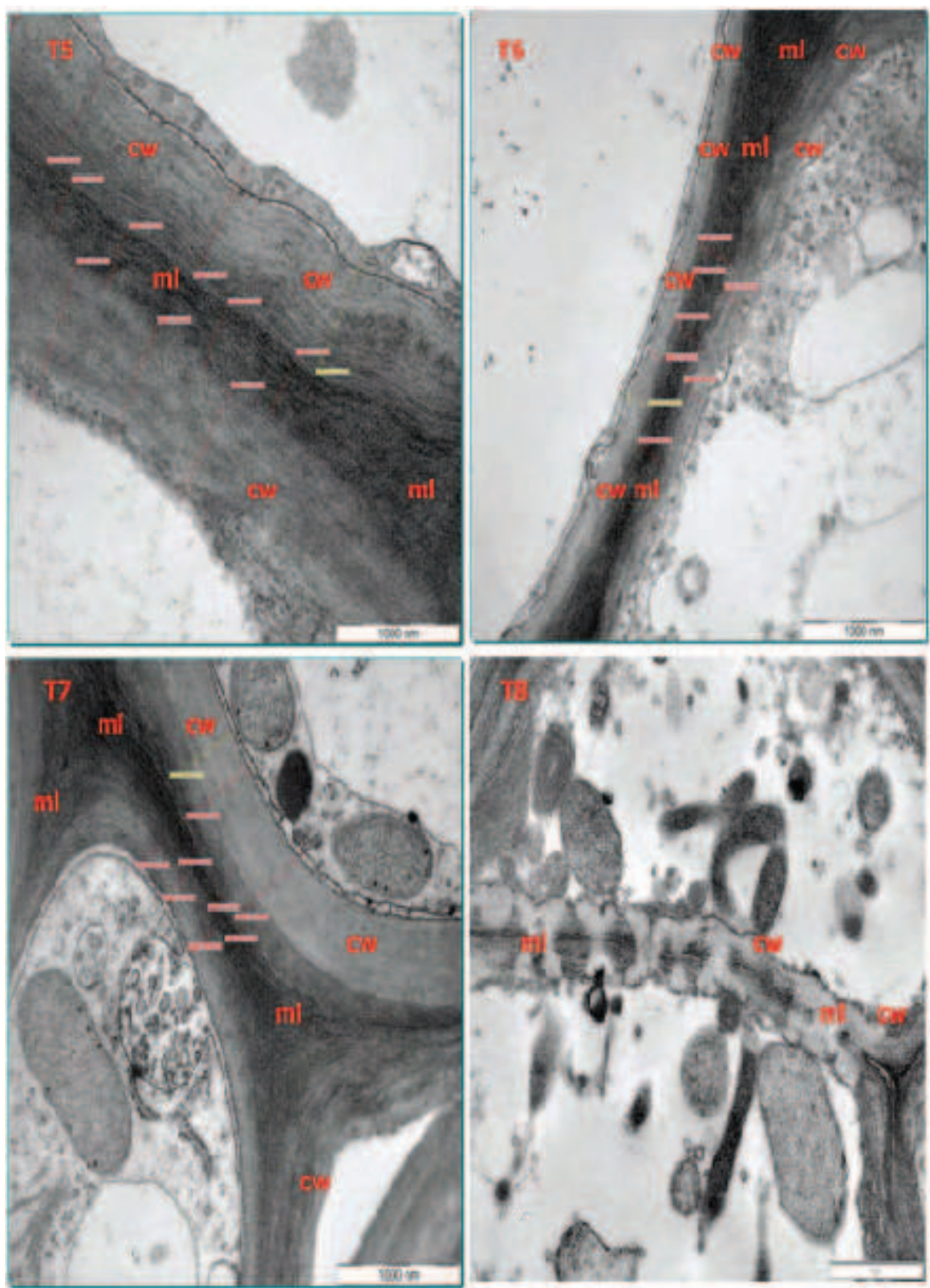


Fig. 6. (Continue): Electron micrographs of the sieve tube of *Citrus reticulata* leaf: section showing the middle lamella and cell wall structure and cell wall thickness (mag at 70 kV magnifications). T5: Antibiotic + foliar fertilizer. T6: GA3 + foliar fertilizer. T7: Antibiotic + GA3 + foliar fertilizer. T8: Control.

3.2 Detection of HLB bacteria using conventional PCR

For PCR detection of the *Candidatus liberibacter asiaticus*, blotchy mottled and midrib yellowing leaves were used. One pair primers: f-OI1 and r-OI2c for amplification of 16S rDNA was used. Line 4 of Fig.7 shows strong band of midrib yellowing symptoms while line 5 shows weak band of blotchy mottling symptoms after conventional PCR amplification. It is true to state, as it is sometimes written, that it is difficult to detect liberibacters. Therefore HLB infection was confirmed by PCR amplification in the typical symptoms. A polymerase chain reaction (PCR) with specific primers OI1/OI2c and A2/J5 were used for detection and produced specific bands of 1160 bp and 703 bp, respectively in Thailand. These were amplified from diseased leaves whereas no product from healthy citrus plants could be obtained.

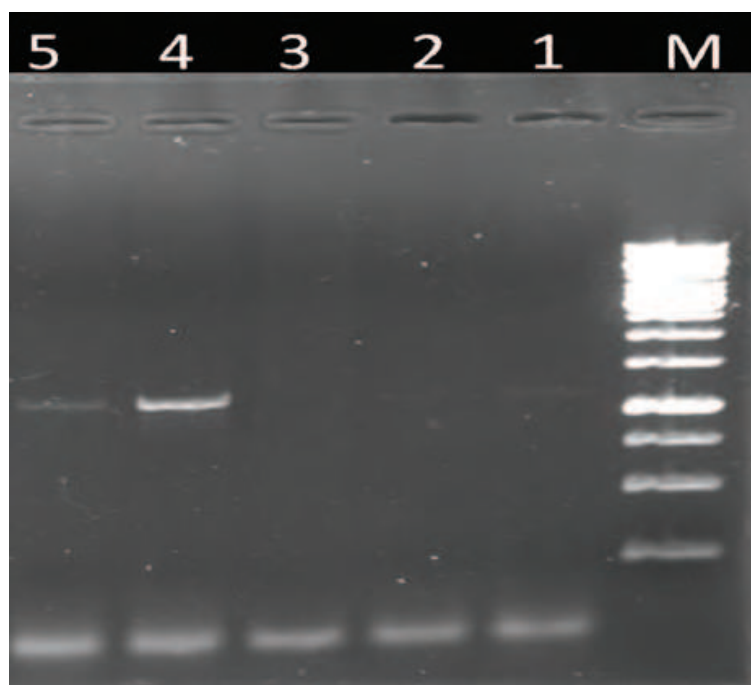


Fig. 7. 16S rDNA fragments with molecular weight of 1160 bp were successfully amplified from the infected samples; (M): Marker (100 bp invitrogen); (line 1): Water; (line 2 &3): Negative sample; (line 4): Midrib yellowing; (line 5): Blotchy mottling symptoms; (line 5-7).

A conventional PCR of 16S rDNA fragment with 1160 bp was successfully amplified and the results are presented in Figs.8 and 9 after treatments. Samples of *Citrus reticulata* leaves were collected and transferred to the pathology Lab to detect the *Candidatus Liberibacter asiaticus* (*Ca. L. a*). Conventional PCR was not able to detect *Ca. L. a* bacteria after 35 cycles of PCR with primer pair OI1 and OI2s under T1 (antibiotic) and T7 (antibiotic + GA3 + foliar fertilizer) (Fig.8). HLB was detected on T2 (GA3), T3 (foliar fertilizer), T4 (antibiotic + GA3), T5 (antibiotic + foliar fertilizer), T6 (GA3 + foliar fertilizer) and T8 (no treatment = as control) (Fig.8). Second conventional PCR with the same primer pair (OI1 and OI2s) was also conducted to detect HLB bacteria on the leaves of *Citrus reticulata* under different treatments. However, products of about 1160bp in size were obtained from the leaves of *Citrus reticulata* under all treatments after the second conventional PCR with primer pair OI1 and OI2s. These included T1, T2, T3, T4, T5, T6, T7 and T8 (Fig.9).

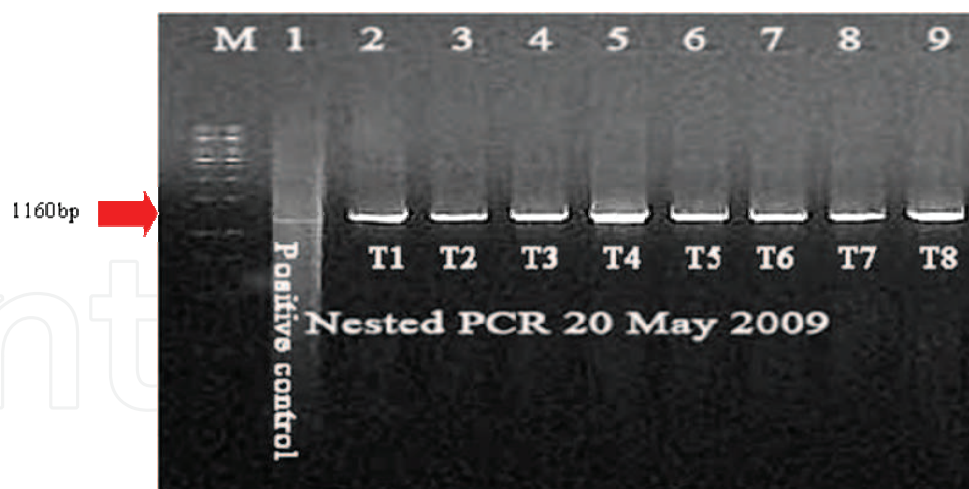


Fig. 8. Conventional PCR of 16s rDNA fragment with molecular weight of 1160 bp were successfully amplified from the leaves of *Citrus reticulata*. T1: antibiotic, T2: GA3, T3: foliar fertilizer, T4: antibiotic +GA3, T5: antibiotic +foliar fertilizer T6: GA3+ foliar fertilizer T7: antibiotic + GA3 + foliar fertilizer T8: control.

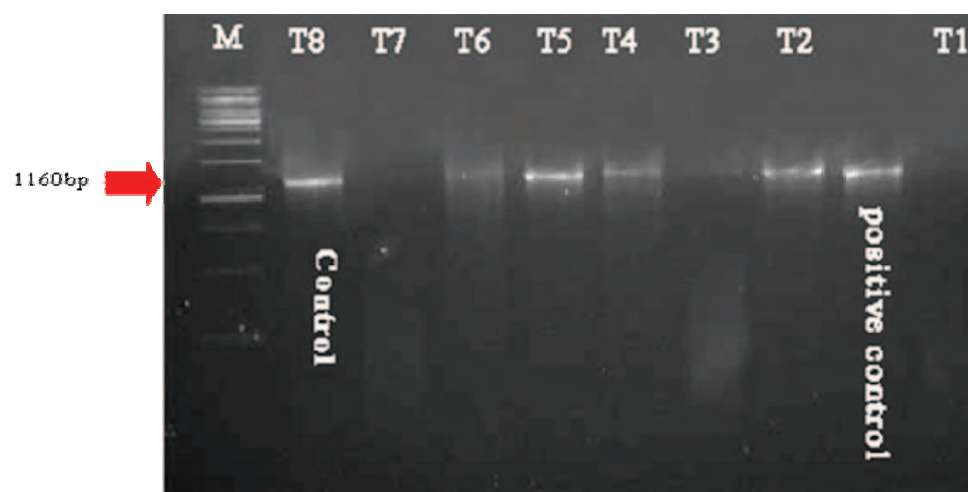


Fig. 9. Second conventional PCR of 16s rDNA fragment with molecular weight of 1160 bp were successfully amplified from the leaves of *Citrus reticulata*. T1: antibiotic, T2: GA3, T3: foliar fertilizer, T4: antibiotic +GA3 T5: antibiotic +foliar fertilizer T6: GA3+ foliar fertilizer T7: antibiotic + GA3 + foliar fertilizer T8: control.

4. Discussion

The chemical treatments had an effect on the cell wall and middle lamella thickness. The cell wall thickness was significantly increased with the T4 and T7 treatments compared to the control (T8). However, Agrios (1997) reported that after pathogen penetration the plants usually respond by forming one or more types of structure namely, cytoplasmic defence reaction, cell wall defence structure and histological defence structure which are successful in defending the plant from the next invasion.

Middle lamella and, finally, the total thickness of cell wall were significantly increased for T4 and T7 compared to the control. For cell wall defence structures, three main models have

been detected in the plant. First, the plant produces amorphous, fibrillar material on the outer layer of the cell wall of parenchyma cells in contact with incompatible bacteria, which subsequently, surrounds and traps the bacteria and controls their multiplication. Second, the cell walls thicken by producing cellulosic material that infuses with a phenolic compound, which increases the cell wall resistance to pathogen penetration. Finally, callose papillae are deposited on the inner side of the cell walls within minutes after injury by microorganisms (Agrios, 1997; Lindenthal, 2005).

When the pathogen penetrates the host, the pathogens may produce a mechanical signal by the physical pressure on the plant cell. It was found that the stimulation of a mechanical signal induces the generation and accumulation of phytoalexin in cucumber and significantly enhances the resistance (Zhao et al., 2005). Some changes were observed in the structural and permeability of cell membranes. These include the release of molecules that are important in signal transduction within and around the cell after an attack by pathogens; the release and accumulation of reactive oxygen species; the activation of phenol oxidases; and the oxidation of phenolics (Agrios, 1997).

Ahmad et al. (2008) detected HLB in peninsular Malaysia using OI1 and OI2c primer. In general, HLB-infected citrus plants were stunted with twig dieback and showed open growth (Bove, 2006; Gottwald et al., 2007; Graca, 1991). Among the growth regulators, considerable work has been done with respect to the effect of pre-harvest applications of GA3 on citrus quantity and quality (Ladaniya, 2004), however, there is little or no information about the effects of GA₃ on HLB-infected fruits.

To our knowledge, this is the first report on the effects of chemical treatments in citrus HLB-infected by TEM study. Cell wall and middle lamella thickness were also increased with the application of a combination of T4 and T7. This study has shown that in non-treated samples irregularity of the cell wall increased, and the middle lamella and cell wall became disorganized compared to the treated samples. The TEM study of the HLB-infected sieve tube of *Citrus reticulata* leaf also showed the fusion confluent of the cell wall and middle lamella structure. A cross-section of the sieve tube showed the damaged cell wall and middle lamella caused by *Candidatus Liberibacter asiaticus* penetrating through the cells. The TEM investigation of the vascular system of HLB-infected *Citrus reticulata* showed spherical and rod shaped bacteria from the midrib with blotchy mottling symptoms of the disease. Finally it can be concluded that, the findings of this research are important and useful for detection the HLB and control programmes elsewhere. Choosing a strategy to control the HLB disease is imperative. Control of HLB is difficult if inoculum sources are widespread. Different chemicals were used to increase the life span of citrus mandarin in a HLB-infected orchard.

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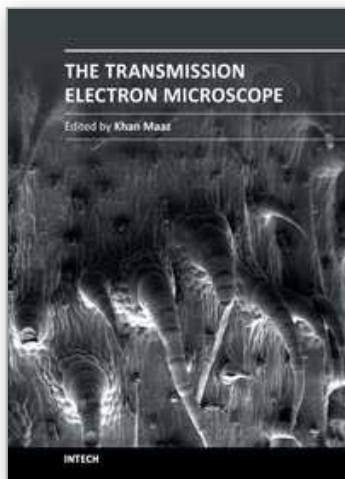
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The Transmission Electron Microscope

Edited by Dr. Khan Maaz

ISBN 978-953-51-0450-6

Hard cover, 392 pages

Publisher InTech

Published online 04, April, 2012

Published in print edition April, 2012

The book "The Transmission Electron Microscope" contains a collection of research articles submitted by engineers and scientists to present an overview of different aspects of TEM from the basic mechanisms and diagnosis to the latest advancements in the field. The book presents descriptions of electron microscopy, models for improved sample sizing and handling, new methods of image projection, and experimental methodologies for nanomaterials studies. The selection of chapters focuses on transmission electron microscopy used in material characterization, with special emphasis on both the theoretical and experimental aspect of modern electron microscopy techniques. I believe that a broad range of readers, such as students, scientists and engineers will benefit from this book.

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Hajivand Shokrollah, Thohirah Lee Abdullah and Kamaruzaman Sijam (2012). Ultrastructure and Cell Wall Thickness Modification and Its Detection After Chemical Treatments in Huanglongbing Infected Citrus Plants, The Transmission Electron Microscope, Dr. Khan Maaz (Ed.), ISBN: 978-953-51-0450-6, InTech, Available from: <http://www.intechopen.com/books/the-transmission-electron-microscope/ultrastructures-and-cell-wall-thickness-modification-after-chemical-treatments-in-huanglongbing-infe>

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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