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Full Length Research Paper

Ultrastructures of *Candidatus* Liberibacter asiaticus and its damage in huanglongbing (HLB) infected citrus

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***Candidatus* Liberibacter asiaticus is not cultured in media and there is insufficient information on the movement of the pathogen in citrus plants. Samples were collected from infected citrus plants grown in Ulu Pakar, Terengganu, Malaysia and they show typical symptoms of the huanglongbing (HLB) disease. Polymerase chain reaction (PCR) using specific primer pairs of OI1 and OI2c was conducted to assess the presence and to amplify the *Candidatus* Liberibacter asiaticus in infected plants. The samples were then examined under transmission electron microscope for the determination and identification of *Candidatus* Liberibacter asiaticus. The spherical and rod shaped particles of this agent were found in phloem cells. The length of the bacteria ranged from 594.57 to 1368.16 nm (mean 930.09 nm) and its width ranged from 201.68 to 811.15 nm (mean 410.61nm). Cell wall membranes were irregular in shape and were of different thickness. Damage was caused by *Candidatus* Liberibacter asiaticus penetrating through the cell wall and their movement between cells. This study was conducted to confirm the presence of *Candidatus* Liberibacter asiaticus pathogen in citrus plant using transmission electron microscopy (TEM) and to identify the cell wall modifications of the phloem.**

Key words: Citrus greening disease, huanglongbing, transmission electron microscope.

INTRODUCTION

Citrus include oranges, grapefruit, tangerines, mandarins, lemon and lime, and is grown in more than 100 countries in tropical, subtropical and Mediterranean climates. It is one of the important fruit crop grown in the world. Major setbacks to citrus production involve inefficient crop management and susceptibility to pests and diseases. Huanglongbing (HLB) disease, also known as citrus greening disease, is one of the most destructive diseases of citrus. It infects citrus in more than 40 countries all over the world. Citrus grown in Malaysia have been infected since 1990 and large areas of citrus orchards had to be eradicated. The pathogen belongs to the genus Candidatus Liberibacter. It is a phloem limited and fastidious bacteria. HLB pathogen is transmitted by citrus psyllid *Diaphorina citri* in Asia and America, and *Trioza erytreae* in Africa (Carmo et al., 2005; Susan and Keremane, 2004).

HLB has been threatening citrus industry in the world. America used to be free from HLB, but symptoms of the disease were recognized and the agent was detected by polymerase chain reaction (PCR) in March 2004 in the State of São Paulo, Brasil, and in August 2005 in Florida, USA, two of the largest citrus growing regions in the

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Abbreviations: HLB, Huanglongbing; **TEM,** transmission electron microscopy; **PCR,** polymerase chain reaction; **CTAB,** cetyl trimethyl ammonium bromide; **SC,** sodium cacodylate.

world (Carmo et al., 2005). HLB has destroyed an estimated 60 million trees in Africa and Asia (Timmer et al., 2003). Typical symptoms of HLB disease on citrus leaves of infected trees include reduced size, yellowing, blotchy mottle or variegated type of chlorosis with small upright leaves, followed by leaf drop and twig dieback at later stages. Some of these symptoms (midrib yellowing) are similar to the symptoms caused by nutritional deficiencies (Bové, 1974).

HLB is an uncultured phloem- limited bacterium that was first characterized in 1994 on the basis of the 16S rDNA sequence analysis and shown to be a new genus in the α-Proteobacteria (Jagoueix., et al., 1994). PCR method is specific and at least sensitive for the detection of different Liberibacter species (Hocquellet, et al., 1999).

Detection of *Candidatus* Liberibacter spp. is based mainly on conventional PCR (Jagoueix et al., 1996) and quantitative real-time PCR (Diva do Carmo, 2005) with species-specific primers developed based on 16S rDNA and β-operon fragments. *Candidatus* Liberibacter asiaticus can also be detected with an electron microscope (Garnier and Bove, 1983). Its particles have elongated sinuous rod like structures, 0.15 - 0.25 µm in diameter. They can be observed by electron microscopy in the sieve tubes of infected trees (Moll and Martin, 1973). The aim of this study was to detect and identify *Candidatus* Liberibacter asiaticus using PCR and transmission electron microscopy (TEM) and to find the cell wall modification of the phloem.

MATERIALS AND METHODS

Sample collection and HLB detection using conventional PCR

Samples were collected in an area of Kuala Berang, Terengganu. The state of Terengganu overlooks the South China Sea on the east side of Malaysia. It has a strong tropical monsoon climate, with relatively uniform temperature within the range of 21 to 32°C. The weather from January to April, is dry and warm, with humidity in the lowlands being consistently reaching 82 – 86% throughout the year. Terengganu's average rainfall is 2286 per year with most rain falling between November and January. Samples were collected in an orchard of *Citrus reticulate* cv. Limau Madu which was cultivated in 2000. Citrus leaves with typical symptoms of HLB, notably blotchy mottling and midrib yellowing were collected. Typical symptom of HLB is blotchy mottling, and midrib yellowing. DNA was extracted from the midribs of leaves by modifying the method of Hung et al. (2000). The leaves of the citrus were soaked with liquid nitrogen and pounded using mortar and pestle for DNA extraction. DNA was extracted from HLB-infected tissue using cetyl trimethyl ammonium bromide (CTAB). The pellets were washed with 70% ethanol, dried and resuspended in 100 µl TE buffer. Then, PCR was performed using 25 µl of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM of dATP, dTTP, dCTP and dGTP, 50 ng forward primer, 50 ng reverse primer, 0.75 units of Taq DNA polymerase and 200 ng genomic DNA. The thermal cycle condition was: 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 pair, composed of the forward primer of OI1 (5'-GCG CGT ATG CAA TAC GAG CGG CA-3') and reverse primer of OI2c (5'-GCC TCG CGA CTT CGC AAC CCA T-3'), was used to amplify the 16S ribosomal DNA fragment. Amplification of DNA was determined by electrophoresis on 1.2% agarose gel for about 30 - 45 min; ethidium bromide was used for staining and visualized under UV light.

HLB detection, *Candidatus* Liberibacter asiaticus size measurement and cell modification of HLB infected citrus using transmission electron microscopy

Terminal shoots of C. *reticulata* trees with typical symptoms (blotchy mottled and midrib yellowing) were collected and washed. Midrib were taken from the leaves and chopped into 2 - 5 mm pieces, similar to the TEM techniques described by Aubert (1990). The samples were fixed in 5% glutaraldehyde buffered in 0.1 M phosphate buffered saline (PBS), pH 7.4. The samples were dried in an oven under vacuum at 60°C for two days. After that, the samples were washed with 0.1 M sodium cacodylate (SC) buffer three times in changes of 30 min, respectively, at 30 min intervals. Subsequently, samples were post-fixed with 1% osmium tetroxide for one day at 4°C. The samples were then washed again three times with SC buffer. After dehydration process with a series of ethanol concentrations (35, 50, 75, 95 and 100%) for 1 h, at 1 h intervals, the samples were infiltrated and embedded in Epon 812. After polymerization, ultra thin sections (60 - 90 nm) was carried and cut using diamond knife and ultra microtome. Golden sections were double stained with uranyl acetate and lead citrate for 15 and 30 min, respectively. Sections were stained first in uranyl acetate for 15 min and then in lead citrate for 30 min before examination under transmission electron microscope (magnification, 30-100Kv) for the detection and identification of *Candidatus* Liberibacter asiaticus. The sizes of of 9 *Candidatus* Liberibacter asiaticus isolates were measured.

RESULTS AND DISCUSSION

Identification of HLB using conventional polymerase chain reaction

Weak band after amplification of DNA fragment of *Candidatus* Liberibacter asiaticus was isolated from blotchy mottling symptoms on the gel (Figure 1, lane 3). Amplification products from samples with midrib yellowing symptoms yielded detectable DNA bands (Figure 1, lanes 5 - 7). Our results confirmed the previously reported study regarding the difficulty in detection of *Candidatus* Liberibacter asiaticus by PCR. In Brazil, most PCR reactions gave negative results, even though leaves with strong symptoms of blotchy mottle were used (Texeira et al., 2005).

HLB infection was confirmed by PCR results in the plants showing typical symptoms of this disease in this study. PCR with specific primers OI1/OI2c and A2/J5 were used for detection of *Candidatus* Liberibacter asiaticus in Thailand and it resulted in obtaining of the bands of expected sizes 1160 and 703 bp, respectively (Aubert, 1990). The bacteria was also detected using PCR with OI1 and OI2c primers in citrus grown in Peninsular Malaysia (Ahmad et al., 2008) and in 15 citrus species in Malaysia (Hajivand et al., 2009). Results obtained by Deng et al. (2007) showed that nested PCR was more

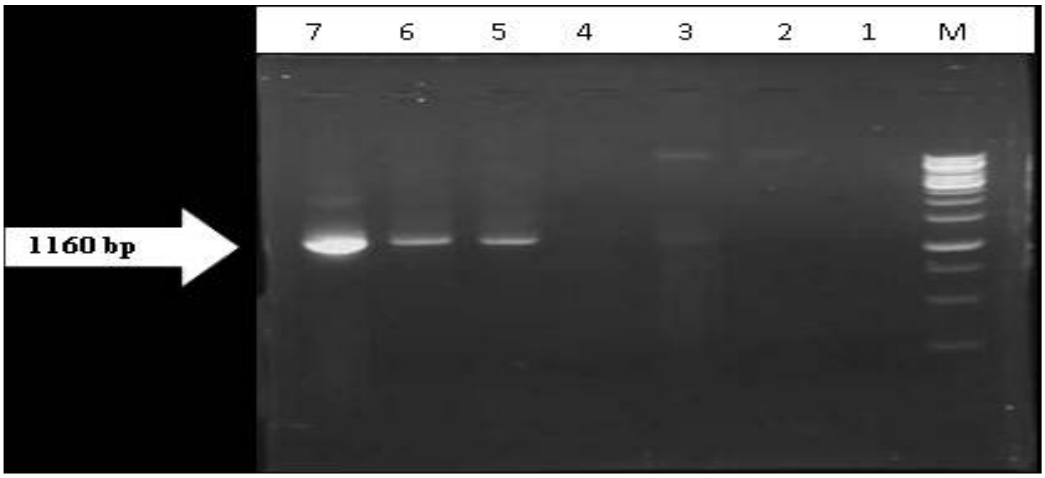

Figure 1. 16s rDNA fragments with molecular weight of 1160 bp were successfully amplified from the infected samples. M: Marker (100 bp invitogen); line 1: water; line 2: negative sample; line 3: blotchy mottling symptoms; line 4: negative sample; line 5 - 7: midrib yellowing symptoms.

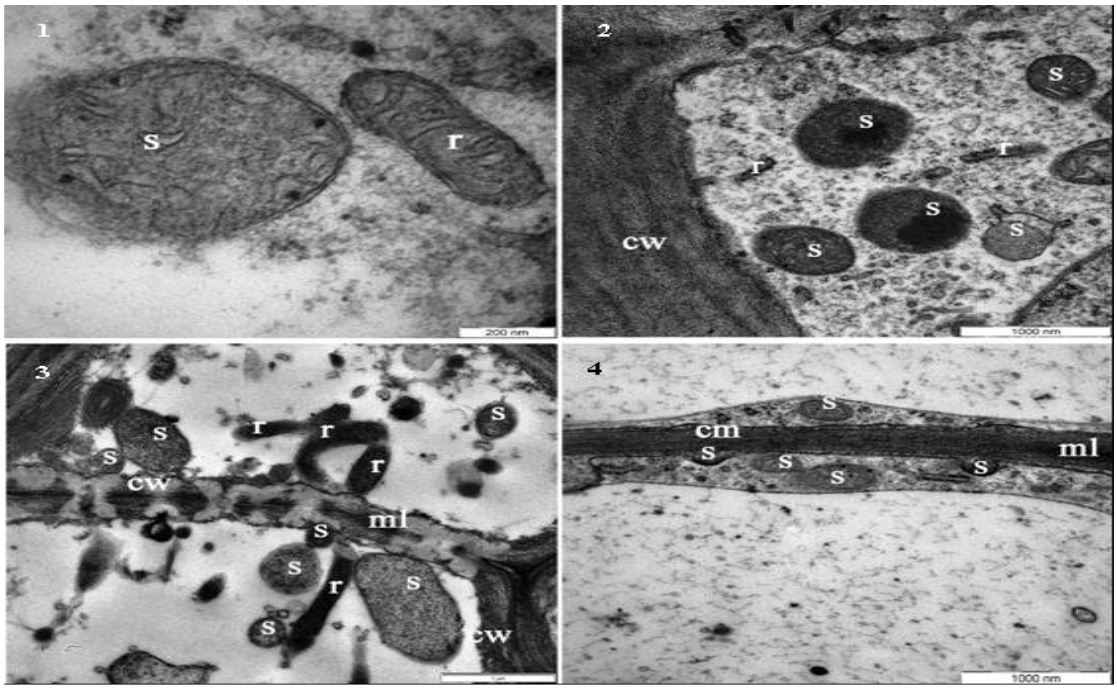

Figure 2. Electron micrographs of the vascular system (1) of *C. reticulata* showing sieve tube cells containing spherical (s) and rod shape bacteria (r) from the infected midrib with blotchy mottling symptoms of HLB disease at high magnification (100 kV). Cross section of sieve tube (2 – 4) showed abundant bacteria cells damaging the cell wall (cw) and middle lamella (ml) in sieve tube cell. 2: magnification, 30 kV; 3 and 4: magnification, 70 kV.

sensitive. Non-nested PCR using primers OI1/OI2c did not yield any detectable DNA bands from both symptomatic and asymptomatic leaves (Deng et al., 2007).

Ultrastructures of *Candidatus* Liberibacter asiaticus

Sieve tube elements of infected *C. reticulate* leaves were observed using electron microscopy. Particles of *Candidatus* liberibacter asiaticus of two different shapes (spherical and rod) were observed under microscope at high magnification (30-100 kV) (Figure 2). A long rod-shaped gram negative organism from African greening-infected citrus leaf midribs was isolated in 1984 (Garnier et al., 1984). The ultrastructure of this organism was described as similar to that of the organism observed in

Table 1. Size of *Candidatus* Liberibacter asiaticus isolates.

Number of bacteria	Length (nm)	Width (nm)
1	879.15	403.7
2	782.55	267.12
3	1155.08	316.09
4	1134.2	225.50
5	803.34	796.84
6	723.67	811.15
7	594.57	201.68
8	1368.16	315.33
9	930.09	358.10
Mean	930.09	410.61

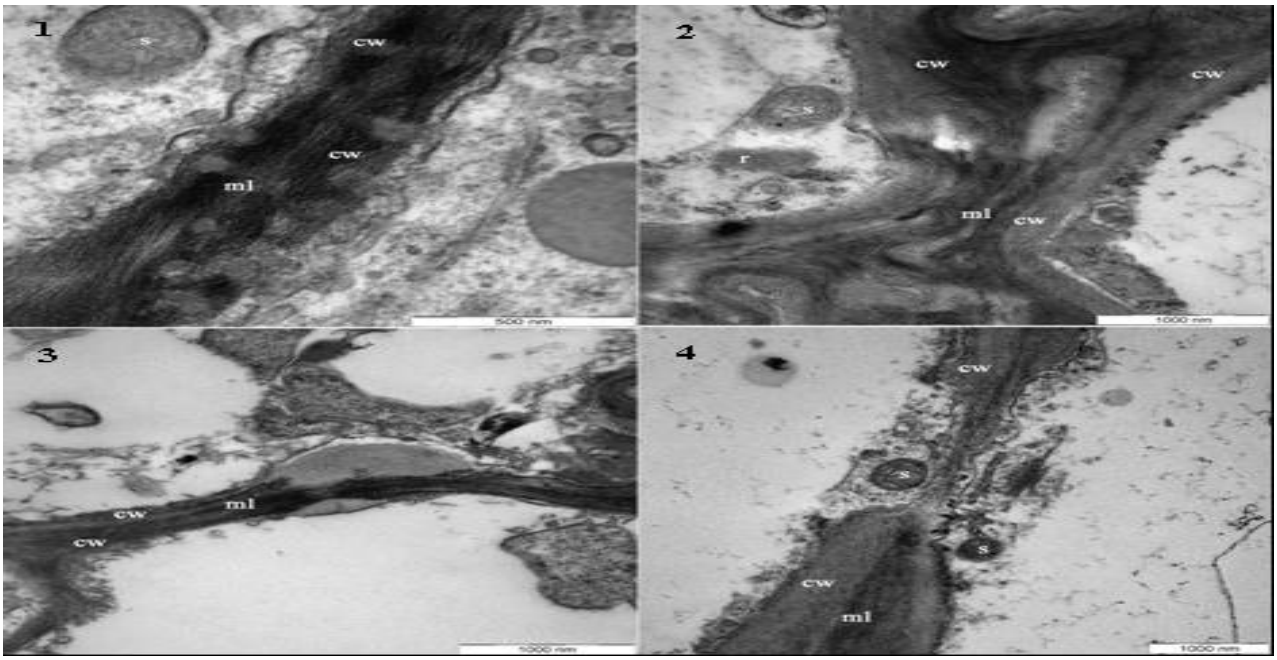

Figure 3. Electron micrographs of the sieve tube of *C. reticulata* leaf. Section 1 and 2 showing the fusion confluent of middle lamella (ml) and cell wall (cw) structure; 1- magnification at 70 kV and 2- magnification at 30 kV. Cross section of sieve tube (3 and 4) showing damaged cell wall (cw) and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

greening-infected citrus, periwinkle and insect vectors (Ariovich and Garnett, 1984). On a solid medium, it formed small round colonies with predominantly long rod-shaped cells near the edges, but rounder cells in the oldest parts. In this experiment, length and width of the HLB isolated from Terengganu were also measured (Table 1). The length of the bacteria ranged from 594.57 to 1368.16 nm (mean 930.09 nm) and its width ranged from 201.68 to 811.15 nm (mean 410.61nm). Bové et al. (2006) using a transmission electron microscope, observed a "mycoplasma-like organism" in citrus phloem tissue infected with citrus greening disease. The organisms were about 2000 nm long and 100 - 200 nm in diameter. Similar bodies were soon observed in both vectors of the citrus greening disease, *Trioza erytreae* (Moll and Martin, 1973) and *Diaphorina citri* (Bove, 2006). Abundant bacteria cells damaging the plant cell wall in sieve tube cell were observed at 30 - 70kV magnification (Figure 2). TEM detection of HLB in Asia and African has shown that the number of bacteria in sieve tubes is higher in leaves with strong mottle than in those with mild mottle (Bove, 2006). The microscopy observation showed that the bacteria particles were localized (Figure 3).

Conclusion

Microscopy observation of the sieve tube cells in the

leaves showed that *Candidatus* Liberibactera asiaticus strain infecting *C. reticulata* from Terengganu were spherical and rod shaped. Damage was caused by this pathogen to the cell wall and cell membranes. Cell wall and cell membranes were irregular in shape and of different thickness. Damages of plant cells were caused by *Candidatus* Liberibacter asiaticus as a result of penetration of the bacteria through the cell wall and its movement between the cells.

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