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Huanglongbing (HLB) Incidence on 2-3 Years Old Tangerine Trees (*Citrus reticulata*) Grown from Disease-free Nursery Stock

Akarapisan, A.[1,2*], Kuenpech, W.[1] and Srimai, K.[1]

[1]Division of Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand; [2]Center of Excellence on Agricultural Biotechnology(AG-BIO/PERDO-CHE), Bangkok 10900, Thailand.

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Abstract *Huanglongbing* (HLB) previously referred to as greening disease is a bacterial disease of citrus trees, and a major problem worldwide. HLB affects citrus trees by blocking the phloem or the vascular system of the tree, limiting its ability to uptake nutrients. Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psyllidae) is the vector, transmitting *Candidatus* Liberibacter asiaticus (Las). In this study in 2012-2014, occurrence of HLB disease after infection was monitored in an experimental planting of 589 tangerine trees (*Citrus reticulata*) grown from disease-free seedlings in a nursery in Chiang Mai, Thailand. An early symptom of HLB on tangerine is the yellowing of leaves on an individual limb or in one sector of a tree's canopy. Field trees can be identified with suspected HLB infection by their foliar but verification requires DNA detection methods. Las can be detected in DNA extracted from infected plants and psyllids by polymerase chain reaction (PCR). An efficient method for DNA extraction from infected plants and psyllids was studied. Polymerase chain reaction (PCR) was performed by using Las606/LSS primer to detect symptomatic leaves. Specific primers, forward primer Las606 (5'- GGA GAG GTG AGT GGA ATT CCG A-3') and reverseprimer LSS (5'- ACC CAA CAT CTA GGT AAA AAC C -3') were used for amplification of the16S rDNA of *Candidatus* Liberibacter asiaticus (Las), producing specific bands of 500 bp. The study area showed an increasing percentage of disease incidence, from 0.84% in 2012 to 4.41% in 2013 and 11.54% in 2014. It appears that there is a high incidence of HLB-infected trees at the edges of the plantation. One of potential HLB pathways is infected Asian citrus psyllids from natural movement. The Asian citrus psyllid vector of HLB has a wide host range, can achieve high populations at citrus vegetative flush, can be spread over long distances, and its control demands both continuous inspection and regular insecticide applications.

Keywords: *Huanglongbing* (HLB), *Candidatus* Liberibacter asiaticus (Las), the Asian citrus psyllid, tangerine trees (*Citrus reticulata*)

[*] **Corresponding author:** Akarapisan, A.; **Email:** angsana.a@cmu.ac.th

Introduction

Huanglongbing (HLB), previously called citrus greening disease is caused by a phloem-limited bacterium, '*Candidatus* Liberibacter asiaticus' (Las) also vectored by the Asian citrus psyllid *Diaphorina citri* (Susan *et al.*, 2012). *D. citri* is known range of distribution covers tropical and subtropical Asia, including India, Burma, Thailand, Nepal, Hong Kong, Ryukyn Islands, the Philippines, Malaysia, Indonesia, Ceylon, Pakistan, Afghanistan, Reunion, and Mauritius (James H, 2005). The disease spreads when a bacteria-carrying psyllid flies to a healthy plant and injects bacteria as it feeds on it (Grafton-Cardwell *et al.,* 2013). HLB can spread rapidly in an abandoned citrus grove. These abandoned groves may serve as vector reservoirs, as well as a source of the presumed causal agent for huanglongbing in Florida, *Candidatus* Liberibacter asiaticus (Las). Affected trees at an advance stage of disease show dieback of twig and gradually decline (Akarapisan *et al.,* 2008). In China the disease was reported to kill young tree within 1-2 year (Da Graca, 1991). In 1994 with the sequencing of 16S rDNA, HLB was shown to be a new genus in the α-Proteobacteria subdivision (Jagoueix *et al*., 1994). A polymerase chain reaction (PCR) detection method, based on the amplification of 16S rDNA, was developed in 1996 (Jagoueix *et al*., 1996). A new PCR detection method based on the amplification of ribosomal protein genes, which allows for direct identification of both species by the size of the amplified DNA, was developed in 1999. This PCR method is specific and sensitive for the detection of both the two different Liberibacter species (Hocquellet *et al*., 1999). The purpose of this survey, in 2012-2014, was to discover whether Las-positive psyllids and Las-positive tangerine trees could be found on nursery stock in Chiang Mai, Thailand.

Materials and methods

Sources of leaves sample and psyllids

Tangerine leaf samples were selected based on visual HLB symptoms. A early symptom of HLB on tangerine trees is the yellowing of leaves on an individual limb or in one sector of a tree's canopy. Each leaf samples contained 20-40 leaves and were cut and placed inside transparent plastic bags, labeled properly and kept in a portable cool box. Adults of the Asian citrus psyllid *Diaphorina citri*, from both visually healthy and symptomatic plants, were collected for this study. Five *D. citri* from each sample were stored in a microfuge tube at -20 °C until used for disease assessment.

DNA extraction from citrus tissues and psyllid bodies

DNA was extracted from leaf midribs and veins by CTAB (cetyltrimethylammonium bromide) method as previously described by Ruangwong and Akarapisan (2006).

DNA was extracted from adult psyllid (*D. citri*) bodies by CTAB method (modified from Ruangwong and Akarapisan, 2006). The psyllid bodies in microfuge tube were ground finely with 125 µl of cold grinding buffer and additional 125 µl of cold grinding buffer. The extract was centrifuged at 10,000 g for 5 minutes at 4°C and the supernatant collected. After centrifugation at 14,000 g for 25 minutes, the pellets were resuspended in 250 µl of CTAB buffer and incubated at 60°C for 30 minutes. Then, 250µl of chloroform/isoamyl alcohol (24:1) was added to the mixture and centrifuged at 7,000 g for 5 minutes. The aqueous supernatant phase was collected and combined with an equal volume of isopropanol, followed by centrifugation at 14,000 g for 15 minutes. The pellets were washed with 200 µl of 70% ethanol, dried, and resuspended in 20 µl of sterile water.

Primers and thermal cycles

All samples were assayed by PCR with the specific primers; forward primer Las606 (5'- GGA GAG GTG AGT GGA ATT CCG A-3') and reverse primer LSS (5'- ACC CAA CAT CTA GGT AAA AAC C -3') were used for amplification of the16S rDNA of *Candidatus* Liberibacter asiaticus (Las). The PCR reaction was carried out in 25 µl of reaction comprised of 1 µM of each primer, 0.2 mM of each four dNTPs, 10X PCR buffer, 2.5 mM MgCl2, 0.5 units *Taq* DNA polymerase (Invitrogen) and 1 µl DNA template. The PCR thermal cycle conditions were: one cycle at 95°C for 8 minutes; 40 cycles at 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 1 minutes; followed by 72°C extension for 5 minutes (Peltier-Based Thermal cycler A100).

Analysis of PCR products by electrophoresis

The PCR products were analyzed by gel electrophoresis using a 1% agarose in TE buffer (Tris base, boric acid and 0.5M EDTA [pH 8.0]) and stained with ethidium bromide. Gel was visualized and analyzed by the GEL documentation (SYNGENE; GENE Genious Bio Imaging System).

HLB distribution and incidence

In 2012-14, tangerine trees (*Citrus reticulata*) grown from disease-free in nursery in Chiang Mai, Thailand were observed for HLB disease

symptoms. Infected trees were continually monitored. Observation data on the quantity of HLB infection in the tangerine tree and psyllid dispread were collected for six times. The quantity percentage comparisons were confirmed by the PCR analysis. The data were used for dispersions models of HLB infection in tangerine tree and orchard management.

Results and Discussion

Sources of leaves sample and psyllids

The study conducted a *huanglongbing* (HLB) disease and the vector survey in tangerine (*Citrus reticulata*) orchard of 589 tangerine tree grown from disease-free nursery stock in Chiang Mai, Thailand in 2012-14 (Fig. 1.). A part of orchard showed early symptom of HLB on tangerine, yellowing of leaves on an individual limb or in one sector of a tree's canopy. For each target tangerine grove, thirty of leaves sample were collected including and HLB-infected *Candidatus* Liberibacter asiaticus, which displayed as two different symptoms - yellow shoot symptom (Fig. 2.B) and chlorosis with green vein symptom (Fig. 2. C). Two of healthy leaves sample were collected from trees with no overt HLB symptom. In the same way, the early symptoms of HLB is leaf yellowing that usually starts from one branch or one part of the tree, new leaves which are similar to zinc deficiency symptom (Jagoueix *et al.*, 1997). Six samples of adult Asian citrus psyllid (*Diaphorina citri*) as they fed on a host tree that showed nearly symptoms of HLB (Fig. 2.A) were collected at three times (June and July 2013 and February 2014). Xu *et al.* (1988) showed that adults as well as nymphs acquired and transmitted the causal bacterium. The adult psyllid remains in the plant canopy feeding on mature leaves or migrate to new areas where new flush is available (Brlansky and Rogers, 2007).

Figure 1. The tangerine (*Citrus reticulata*) orchard which seedlings from disease-free nursery stock in Chiang Mai, Thailand was surveyed and sampled in 2012-14.

Figure 2. Asian citrus psyllid (*Diaphorina citri*); waxy exudate from nymphs (circle) and adult Asian citrus psyllid feeding on host (A). Different symptoms of *huanglongbing* (HLB) disease observed on tangerine tree (*Citrus reticulata*); yellow shoot symptom (B) and chlorosis with green vein symptom (C).

DNA extraction and Analysis of PCR products from citrus tissues and psyllid bodies

The DNA from all of leaf and psyllid samples was extracted by CTAB (cetyltrimethylammonium bromide) method and Polymerase chain reaction (PCR) analyses using Las606/LSS primer to detect symptomatic leaves. The amplified 16S rDNA of *Candidatus* Liberibacter asiaticus (Las) produced specific bands of 500 bp (Fig. 3.). Sampling was conducted at three different times, with 5 samples taken in July 2013, 5 samples in October 2013, and 22 samples in January 2014. Twenty of the HLB-symptomatic leaf samples showed amplified DNA after electrophoresis on 1% agarose gel. A specific band of about 500 bp was obtained with extracts from HLB-infected *Candidatus* Liberibacter asiaticus including 4 samples in 2013, and 16 samples in 2014.

The samples of adult Asian citrus psyllid (*Diaphorina citri*) were collected in February 2014 from HLB-infected tree that showed the amplified DNA. Figure 3 shows examples of DNA extracted from an HLB-infected leaf (lane 1), adult Asian citrus psyllid (lane 2 and lane 3) and HLB positive control (lane 5). No amplification was observed in DNA extracted from healthy leaves sample (lane 4). Hocquellet *et al.* (1999) report, the amplification of the 16S ribosomal operon was developed and proved to be efficient for the detection *Candidatus* Liberobacter asiaticum. In 2004, PCR-based assay was developed for monitoring Las in vector psyllids bodies (Hung *et al.*, 2004).

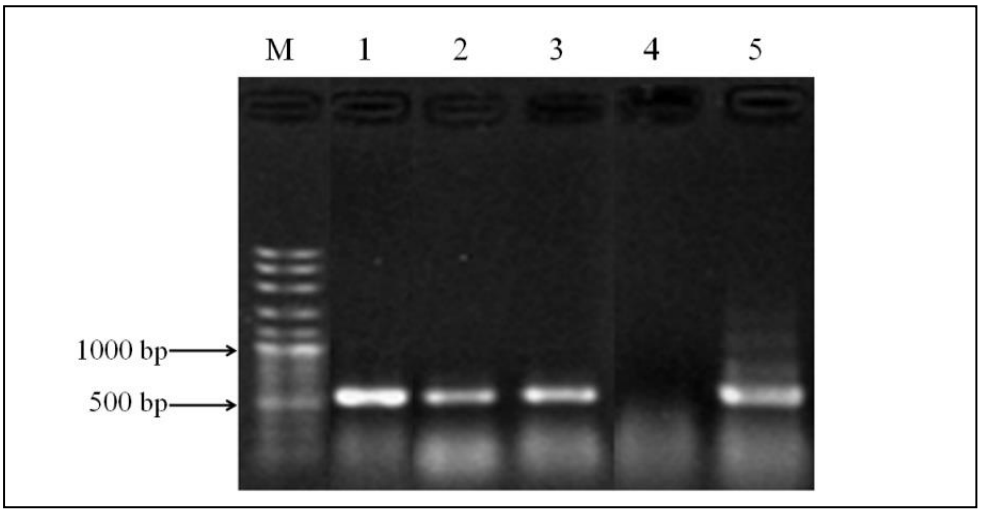

Figure 3. Agarose gel electrophoresis of the amplified. 16s rDNA from the tangerine tree (*Citrus reticulata*) and adult Asian citrus psyllid was amplified with the primer LAS606/LSS in a 1% agarose gel. M, molecular marker, 100 bp VC (Invitrogen); Lane 1 = *huanglongbing* (HLB) symptomatic plants; Lane 2 = adult Asian citrus psyllid feed on HLB-infected tangerine in February 2014; Lane 3 = adult Asian citrus psyllid feed on HLB-infected tangerine in February 2014; Lane 4 = healthy tangerine (negative control); Lane 5 = HLB-infected tangerine (positive control).

HLB distribution and incidence

Occurrence of HLB disease after infection was monitored in six times - September 2012, March 2013, July 2013, October 2013, January 2014 and March 2014 - in an experimental planting of 589 tangerine trees (*Citrus reticulata*) grown from disease-free nursery stock in Chiang Mai, Thailand. Two methods, visual rating and PCR analyses, were used to compare HLB among the tree. The study area showed an increase the percentage of disease incidence ranging from 0.84% in 2012 to 4.41% in 2013 and 11.54% in 2014. It appears that there is a high incidence of HLB-

infected trees at the edges of the plantation. In the dispersions model of HLB infection (Fig. 5), which shows the state in March 2014; 103 trees were removed comprised of 31 of HLB-infected tree (black color); 1 with 100% HLB symptom observed on tree (red color); 29 with 70% HLB symptoms observed on the trees (pink color); 35 with 50% HLB symptom observed on the tree (yellow color) and 7 non-HLB symptomatic trees (gray color). One of potential HLB pathways is infected Asian citrus psyllids from natural movement. The Asian citrus psyllid vector of HLB has a wide host range, can achieve high populations at citrus vegetative flush, can be spread over long distances, and its control demands both continuous inspection and regular insecticide applications. In Florida's Indian River region, an area with a high incidence of HLB, 20 trees of each cultivar were rated for visual HLB symptoms and leaves. Although incidence and severity of HLB varied considerably among the groves, scion-specific differences were apparent (Stover and Greg, 2011).

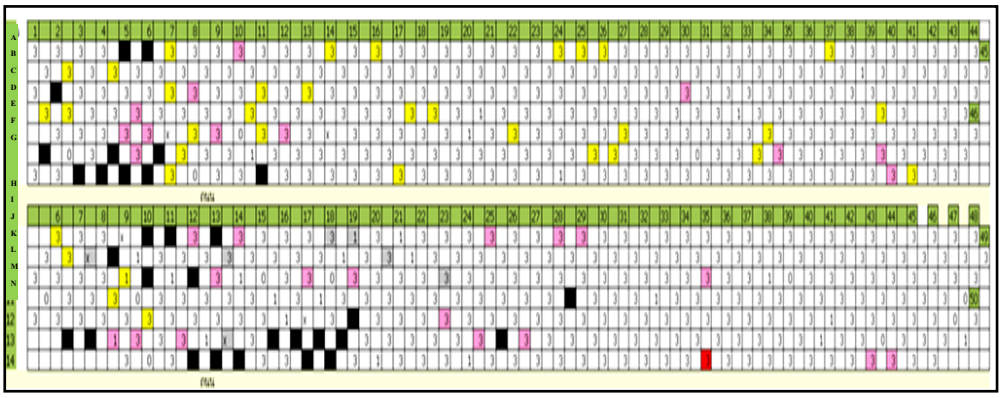

Figure 5. The dispersions model of HLB infection in 589 tangerine trees (*Citrus reticulata*) grown from disease-free nursery stock in Chiang Mai, Thailand in 2012-14; 103 trees were removed comprised of 31 of HLB-infected tree (black color); 1 with 100% HLB symptom observed on tree (red color); 29 with 70% HLB symptoms observed on the trees (pink color); 35 with 50% HLB symptom observed on the tree (yellow color) and 7 non-HLB symptomatic trees (gray color).

Conclusion

In 2012-14, the occurrence of *Huanglongbing* (HLB) disease after infection by the Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psyllidae) was monitored in an experimental planting of 589 tangerine trees (*Citrus reticulata*) grown from disease-free nursery stock in Chiang Mai, Thailand. Observation of HLB symptom and polymerase chain reaction (PCR)

indicated that there is a high incidence of HLB-infected trees at the edges of the plantation. The results showed an increase in disease incidence overtime. The percentage of affected plantation increased from an average of 0.84% in 2012 to 11.54% in 2014.

Acknowledgments

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