Mixed Infections of Ca. Liberibacter asiaticus and Citrus Tristeza Virus Affecting Calamansi (*Citrofortunella microcarpa* Bunge) in Oriental Mindoro, Philippines

Alliah Czarielle N. Mañugo¹, Julie Ann Mae G. Bolo¹, Julianne G. Camacho¹, Jamil de Guzman, Edna A. Aguilar², and Filomena C. Sta. Cruz.*¹

¹Institute of Weed Science, Entomology and Plant Pathology, College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna 4031, Philippines
²Institute of Crop Science, College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna 4031, Philippines

*Author for correspondence; Email: fctsacruz@up.edu.ph

Received: March 16, 2022/ Revised: September 16, 2022/ Accepted: October 11, 2022

Huanglongbing (HLB) and tristeza are the most destructive diseases affecting citrus crops worldwide, including calamansi (*Citrofortunella microcarpa* Bunge Wijnands) in the Philippines. Oriental Mindoro is the largest calamansi-producing province contributing to about 33% of the country’s production. This study was conducted to determine the current status of the occurrence of HLB and tristeza diseases affecting calamansi in the province through visual observation of symptoms in field surveys and detection of the causal pathogen. Both HLB and tristeza were found in all the nine villages surveyed located in the municipalities of Naujan, Pola, Roxas, and Victoria. Plants were showing the HLB symptom of blotchy mottle accompanied by interveinal chlorosis similar to zinc deficiency, defoliation, and twig die-back. Leaves were small, narrow, thick, and corky with enlarged veins and erect in appearance. Among the total of 155 plants tested from the surveyed farms, 150 were positive for HLB disease. Polymerase chain reaction using the LAS606F and LSSR primer pair that amplifies a 500 bp fragment of the *Candidatus Liberibacter asiaticus* 16S rDNA has detected the HLB causal pathogen in symptomatic samples with blotchy mottle. Likewise, 154 out of 155 plants were also positive for tristeza by enzyme-linked immunosorbent assay. HLB and tristeza occurred mostly in mixed infections in seven farms while 83% and 90% in the other sites. HLB symptoms appeared to be dominantly expressed.

Keywords: Huanglongbing, citrus greening, tristeza, calamansi, *Citrofortunella microcarpa*, *Candidatus Liberibacter*, citrus tristeza virus

INTRODUCTION

Huanglongbing (HLB) and tristeza diseases greatly affect the citrus industry worldwide. Huanglongbing, meaning “yellow dragon disease,” was first described in China in 1919 and given various names such as “greening” in South Africa, “leaf mottling” in the Philippines, “dieback” in India, “vein phloem degeneration” in Indonesia, and “citrus greening” in the Americas (da Graça 1991; Bove 2006; Lee 2015). Later, “huanglongbing” was considered as the official name of the disease during the 13th Conference of the International Organization of Citrus Virologists (Van Vuuren 1996). The disease is distributed in Asia, Africa, and the Americas (Bove 2014). HLB is caused by the phloem-limited, gram negative bacteria belonging to the alpha subdivision of Proteobacteria consisting of the Asiatic form, *Candidatus Liberibacter asiaticus* (Las), the African form Ca. L. africanus (Laf), and the American form Ca. L. americanus (Lam) in Brazil (Garnier et al. 1984; Jagoueix et al. 1994; Teixeira et al. 2005a; Teixeira et al. 2005b; Lee 2015). The Asiatic, African, or American forms are transmitted in a persistent manner by the Asian citrus psyllid *Diaphorina citri*, which is the most efficient vector. The *Trioza erytreae,*
which occurs mostly in Africa, transmits the African and Asiatic forms (Bove 2006; Lee 2015). HLB is also transmitted through vegetative propagation using infected budwoods, and the use of infected planting materials can be responsible for the establishment of the disease and its spread into new areas. Infected trees develop yellow shoots while other parts remain symptomless, thus symptoms show a sectoral appearance (Gottwald et al. 2007). Leaves develop blotchy mottle characterized by a pattern of dark and green areas lacking clear limits between the colors and appear asymmetric on the two halves of the leaf. At later stages, leaves also display interveinal chlorosis similar to zinc deficiency symptoms and become erect in appearance, followed by defoliation and twig dieback (da Graca 1991; Gottwald et al. 2007; Lee 2015; Gabriel et al. 2020). Infected trees produce few small and lopsided fruits that remain green at the stylar end, which give it the name “citrus greening” disease (da Graca 1991; Bove 2006; Gottwald et al. 2007). Fruits often drop prematurely, resulting in significant yield reduction from 30–100% depending on the proportion of the canopy affected and the age of the trees when infected.

In the Philippines, leaf mottling was first observed in Bataan in 1921 (Lee 1921) and subsequently Batangas in 1957. The disease also has spread in other growing areas such as Laguna, Quezon, Rizal, Cavite, Bicol, Mindanao, Mountain Province, and Mindoro (Cortez and Celino 1972) and caused the decline of the citrus industry in the country. It was believed that the disease spread to other parts of the country by shipment of planting materials from Batangas to the Bicol Region (Altamirano et al. 1976). Symptoms were described as leaf mottling, premature leaf drop, twig dieback, and undersized fruits (Salibe and Cortez 1966; Salibe and Cortez 1968; Martinez and Wallace 1969). For a long time, the leaf mottling disease was thought to be caused by a virus since it was graft transmissible. However, it was transmissible by the Asian citrus psyllid D. citri but not by T. citricida, thus it was not considered as tristeza (Salibe and Cortez 1966; Salibe and Cortez 1968; Martinez and Wallace 1967a; Martinez and Wallace 1967b; Martinez and Wallace 1969). Furthermore, it was thought to resemble the yellow shoot disease in China and Likubin in Taiwan and the greenening disease in South Africa. The disease was proposed to be called “citrus greening” by Martinez and Wallace (1969). Later, the disease, now known as “huanglongbing”, was found to be related with HLB affecting citrus in other countries. In 1996, Ochasan and co-workers reported the prevalence of HLB affecting almost all citrus species in the surveyed areas located in the highlands of Northern Philippines. Molecular analysis by polymerase chain reaction (PCR) has detected the presence of Ca. L. asiaticus in citrus samples from Baguio City (Harakava et al. 2000). In 2010, the Ca. L. asiaticus was identified to be the causal agent of HLB through graft transmission study and PCR detection in mandarin oranges, pummelo, kumquat, and calamansi grown in Southern Luzon (Sta. Cruz et al. 2010). Later, the nationwide survey conducted by Ochasan et al. (2016) found that 17% of the samples tested were HLB positive by PCR assay. To date, the presence of HLB has been reported in Laguna, Batangas, Quezon, Cavite, Rizal, Baguio, Camarines Sur, Cotabato, Cebu, Mountain Province, Nueva Ecija, Oriental Mindoro, and Mindanao (Salibe and Cortez 1966, Salibe and Cortez 1968; Cortez and Celino 1972; Martinez and Wallace 1967; Martinez and Wallace 1969; Altamirano et al. 1976; Harakava et al. 2000; Sta. Cruz et al. 2010; Ochasan et al. 2016).

Tristeza is caused by Citrus tristeza virus (CTV) belonging to genus Closterovirus, family Closteroviridae with genome consisting of a single-stranded positive sense RNA which forms thread-like particles with a length of 2000 nm and 10 – 12 μm in diameter (Kitajima et al. 1964; Lee 2015; Fuchs et al. 2020). The virus is transmitted in a semi-persistent manner by several aphid species, but the main vector is Toxoptera citricida (Xu et al. 1988; Lee 2015). It is also transmitted through propagation of infected buds. Tristeza symptoms vary depending on the CTV strains (Atta et al. 2012; Dawson et al. 2013). Occurrence of tristeza in the country was first observed affecting calamondin in Camarines Sur in 1958 (Bigornia and Calica 1961) and later in Batangas, Laguna, Mindanao, Mountain Province, and Mindoro based on the observation of stem pitting symptoms and transmission to indicator plants (Cortez and Celino 1968). Presence of tristeza was confirmed through detection of CTV by ELISA (enzyme-linked immunosorbent assay) in mandarin, pummelo, grapefruit, and calamansi from Davao, Batangas, and Los Baños, Laguna (Herradura et al. 1996) and in sweet orange and mandarin from Benguet, Mountain Province, and Ifugao (Ochasan et al. 1996). Tristeza symptoms are stem pitting in mandarin; vein corking, stem pitting, and stunting in pummelo; vein clearing in lime and calamansi (Herradura et al. 1996); and stem pitting and stunting in sweet orange and mandarin (Ochasan et al. 1996). Both HLB and tristeza remain a serious problem affecting all the citrus crops including calamansi, and it is believed that both are widespread in the country.

Calamansi (Citrofortunella microcarpa Bunge), also known as the Philippine lemon, is indigenous and one of the major fruit crops of the Philippines (DA-PRDP 2012).
It is mainly consumed fresh for beverages and condiments, and also processed for fruit juices. Calamansi is also used for bleaching agents and essential oils, and for some medicinal purposes (DA-PRDP 2012; Rodeo 2016). The crop is mainly grown in Central Luzon, Zamboanga Peninsula, CALABARZON, and MIMAROPA. In MIMAROPA, Oriental Mindoro produces more than 97%, contributing to 33% of the country’s total calamansi production (PSA 2017). Thus, the province is aptly called the “Calamansi King” of the Philippines (DA-PRDP 2012). However, calamansi production in Oriental Mindoro has been affected by HLB and tristeza. In 1968, tristeza was first reported affecting calamansi in the province (Cortez and Celino 1968) while HLB was detected in 2016 (Ochasan et al. 2016). However, the areas surveyed were limited and diagnosis was based mostly on symptomatology and limited laboratory testing. The present study was conducted to determine the current status of the occurrence of HLB and tristeza in Oriental Mindoro through visual assessment of disease symptoms, serological detection of CTV, and molecular detection of the Ca. L. asiaticus. Information on the occurrence of these diseases are important for the calamansi rehabilitation program in the province.

**MATERIALS AND METHODS**

**Field Survey and Sample Collection**

Field surveys were conducted from February to April 2019 to determine the occurrence of HLB and tristeza affecting calamansi in the major growing areas in Oriental Mindoro. A total of nine farms planted with more than 20-year-old calamansi trees located in the villages of Naujan in Paniquian; Matututlata in Pola; Leido, San Antonino, Sampaguita, and San Cristobal in Victoria; and nursery farms in Pacquias, San Isidro in Victoria and Tanauan in Roxas were surveyed. Occurrence of HLB was determined by visual observation of symptoms such as blotchy mottle and interveinal chlorosis, while tristeza with leaf yellowing and tree decline. In each farm, samples were collected from 10 – 30 plants with each sample consisting of shoots carrying leaves with various symptoms such as blotchy mottle, interveinal yellowing on mature leaves, and interveinal yellowing on young leaves. The presence of the disease was confirmed through detection of Ca. L. asiaticus by PCR and CTV by ELISA.

**DNA Extraction**

Total DNA was extracted following the Dellaporta method (Dellaporta et al. 1983) from leaf samples with various symptoms. About 100 mg leaf tissue was homogenized with liquid nitrogen in a 1.5 ml microcentrifuge tube using a Kontis pestle, then 500 µl Dellaporta extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, and 10 mM B-mercaptoethanol) and 30 µl 20% sodium dodecyl sulfate (SDS) was added and then vortex-mixed for 2 min. The tube was incubated at 65°C for 10 min. Then, 160 µl of 5M potassium acetate (0.3 volume of KoAC) was added and mixed for 2 min and centrifuged at 14000 rpm for 10 min. The supernatant (450 µl) was mixed with 225 µl of isopropanol and centrifuged at 14000 rpm for 10 min. The nucleic acid pellet was washed with 500 µl 70% ethanol and centrifuged at 14000 rpm for 5 min. The supernatant was removed and the pellet was air-dried and suspended in 50 µl nuclease-free water.

**Detection Ca. L. asiaticus by Polymerase Chain Reaction**

The PCR components and conditions followed the protocol for DreamTaq Green PCR Master Mix (ThermoScientific) in a 25 µL reaction volume using 100 ng template DNA, 0.4 µM Las606F/LSSR primer pair (forward primer Las606F [5′-GGA GAG GTG AGT GGA ATT CCG A-3′] and reverse primer LSSR [5′-ACC CAA CAT CTA GGT AAA AAC C-3′]) (Fujikawa and Iwanami 2012). The Las606F/LSSR primer pair targets a specific part of Las 16S ribosomal DNA. Thermal cycling conditions were denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec, extension at 72°C for 1 min, and a final extension of 72°C for 1 min. Total DNA extracted from leaf samples of infected and healthy calamansi plants were used as positive and negative controls, respectively. Presence of amplified product was visualized by gel electrophoresis in 1% agarose gel using Bio-Rad electrophoresis chamber, stained with GelRed® nucleic acid gel stain (Biotium), and viewed in a gel documentation system, AlphaImager® Mini- Alpha Innotech.

**Detection of CTV by Enzyme Linked Immunosorbent Assay**

The presence of CTV was tested in leaf samples with symptoms of blotchy mottle, interveinal chlorosis on young leaves and old leaves by triple antibody sandwich (TAS) ELISA following the manufacturer’s protocol from Agdia (Elkhart IN, USA). Threshold value was computed as twice the average absorbance value (OD reading at 405 nm) of four healthy control samples. Sample with absorbance greater than the threshold value was considered positive to CTV.
RESULTS AND DISCUSSION

The presence of HLB and tristeza diseases which occur as mixed infections were detected in nine villages located in the municipalities of Naujan, Pola, Roxas, and Victoria.

Symptoms of HLB and Tristeza

Plants showed the HLB diagnostic blotchy mottle symptom characterized by a pattern of yellow and green areas lacking clear limits between color and appear asymmetric on each half of the leaf. The young and developing leaves were pale green without (Fig. 1a and Fig. 1b) or with mild interveinal chlorosis (Fig. 1c and Fig. 1d). Older leaves on the same shoot have interveinal chlorosis or blotchy mottle. The blotchy mottle was observed mostly on older leaves (Fig. 1e). Older leaves of the same plant had chlorosis similar to zinc deficiency symptoms characterized by yellow discoloration advancing from the tip while leaving the veins and margins green in color (Fig. 1f) or severe interveinal yellowing of leaves. Leaves were reduced in size, narrow, thick, corky with enlarged veins, and erect in appearance. Signs of psyllid feeding on the shoots are seen as “pinching-like” distortion on the edges of the leaves (Fig. 2). The appearance of yellow shoots were also observed. Infected trees were defoliated and had yellow shoots and twig dieback. The fruits from infected plants were reduced in size; however, a lopsided appearance, which is a typical symptom, was not evident. Instead, the fruits were of a uniform green or light green color (Fig. 3). Symptoms were predominantly of the HLB disease, similar to those previously described in calamansi (Sta. Cruz et al. 2010). The typical tristeza symptoms of seedling yellows and tree decline were not evident, although presence of stem pitting was not examined.

Presence of Ca. Liberibacter asiaticus and CTV in Mixed Infections

The presence of HLB and tristeza was confirmed through detection of Ca. L. asiaticus by PCR and citrus tristeza virus by ELISA in symptomatic samples. HLB was detected in all the nine farms surveyed located in the four municipalities of Oriental Mindoro (Table 1). Among the total of 155 calamansi plants tested, 150 were positive for Ca. L. asiaticus. The pathogen was detected in all (100%) samples from Paniquian in Naujan (6/6); Matulatula in Pola (24/24); Tanauan in Roxas (19/19); Pacquias (5/5); Sampaguita (10/10); San Antonino (28/28), San Cristobal (24/24), and San Isidro (10/10) in Victoria while only 83% (24/29) from Leido. Samples with symptoms of blotchy mottle were positive for the presence of Ca. L. asiaticus by PCR using the primer pair Las606F/LSSR. The expected PCR product of 500 bp was amplified as shown by representative samples in Figure 4. This result also confirmed that Ca. L. asiaticus is the species causing HLB in calamansi in Oriental Mindoro.
Fig. 3. Calamansi fruits harvested from trees with mixed infections of huanglongbing and tristeza showing reduction in size with no evident lopsided appearance. The fruits shown in this figure have symptoms of scab disease characterized by raised irregular scabby or wart-like outgrowths.

Previously, the presence of Ca. L. asiaticus has been detected in PCR tests from limited samples in Oriental Mindoro (Ochasan et al. 2016).

In this study, the method used for the detection of HLB pathogen followed the existing protocol but with some modifications in DNA extraction. Extraction of DNA from citrus samples from the previous studies followed the cetyltrimethylammonium bromide (CTAB) method (Lodhi et al. 1994; Sta. Cruz et al. 2010). Dellaporta DNA extraction used in this study is a simple, faster, and more economical method. It involves the use of fewer reagents such as extraction buffer, sodium dodecyl sulphate, and potassium acetate while avoiding the use of organic solvents such as chloroform. The procedure can be completed in almost two hours with fewer steps and uses fewer reagents compared to the CTAB method. In previous studies, leaf samples with blotchy mottle were used for PCR detection of the HLB pathogen (Harakava et al. 2000; Sta. Cruz et al. 2010; Ochasan et al. 2016). Our study showed that samples with blotchy mottle were 96% (149/155) positive to HLB in PCR test, indicating that blotchy mottle can be reliably used as the diagnostic symptom for HLB in calamansi. In a separate test, it was shown that samples with interveinal yellowing on older leaves were also positive to HLB (10/10). The disease was also detectable with interveinal chlorosis on young leaves, albeit in fewer samples (4/10) and not on asymptomatic leaves taken from an infected plant.

Likewise, the presence of tristeza was detected in all the nine farms surveyed (Table 1). Among the total of 155 symptomatic plants tested, 154 were positive for CTV. Samples from eight out of nine surveyed farms were 100% CTV-positive, while samples were 90% CTV-positive in San Isidro. Positive samples have ELISA absorbance values that ranged from 0.169 – 1.801 higher than the threshold value of 0.136. The presence of CTV was detected using samples collected from the plant that were tested for HLB. In the previous survey conducted by Cortez et al. (1968), the presence of tristeza was determined by symptomatology, particularly the presence of stem pitting. In this study, the presence of tristeza was detected by ELISA. HLB and tristeza occurred mostly in mixed infections in seven sites, and 83% and 90% in the other two farms (Leido and San Isidro) (Table 1). Testing for HLB and tristeza diseases from the same samples allowed the identification of mixed infections. To our knowledge, this is the first

Table 1. Occurrence of huanglongbing and tristeza in the municipalities of Oriental Mindoro.

<table>
<thead>
<tr>
<th>Growing Areas/ Village</th>
<th>No. Tested</th>
<th>No. of Positive</th>
<th>Percentage of Positive Plants (%)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>ELISA Absorbance Value&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLB</td>
<td>Tristeza</td>
<td>HLB Alone</td>
<td>HLB + Tristeza</td>
</tr>
<tr>
<td>Paniquian</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Matulatula</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Tanuan</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Leido</td>
<td>29</td>
<td>24</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>Pacquias</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Sampaguita</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>San Antonino</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>San Cristobal</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>San Isidro</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>155</td>
<td>150</td>
<td>154</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Number of plants tested positive for Candidatus Liberibacter asiaticus by polymerase chain reaction using LAS606F/LSSF primer pair, and positive for citrus tristeza virus by enzyme-linked immunosorbent (ELISA).

<sup>2</sup>Percentage of plants infected with huanglongbing alone, tristeza alone, and with mixed infection of huanglongbing and tristeza.

<sup>3</sup>Enzyme-linked immunosorbent assay (ELISA) absorbance value is the OD reading at 405 nm wherein all values presented indicate positive reactions which were higher than the threshold value of 0.136 computed as twice the mean absorbance value of four healthy control samples.

Absorbance values: CTV positive control (1.30), healthy control (0.068).
CONCLUSION

Huanglongbing and tristeza are present and widely distributed in the calamansi growing areas of Oriental Mindoro. The HLB pathogen, \textit{Ca. L. asiaticus}, and the citrus tristeza virus were detected by PCR and ELISA, respectively in calamansi leaf samples collected from nine villages located in the municipalities of Naujan, Pola, Roxas and Victoria. HLB and tristeza occur mostly in mixed infections. The observed symptoms were predominantly HLB blotchy mottle and interveinal chlorosis. Identification of HLB and tristeza as major diseases affecting calamansi in Oriental Mindoro as well as determining the specific areas affected by these diseases will help in disease management and rehabilitation programs of the calamansi industry in the province.

ACKNOWLEDGEMENT

The study was conducted under the project “Upgrading the Calamansi Value Chain Towards Improving the Calamansi Industry of Oriental Mindoro. The authors are grateful for the funding support provided by the Department of Agriculture - Bureau of Agricultural Research (DA-BAR), and to Dr. Pedcris M. Orencio and Ms. Anna Gale C. Vallez of the Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA) who led and coordinated the project implementation.

REFERENCES CITED


VAN VUUREN SP. 1996. Huanglongbing the official name for greening disease of citrus. ITSG Info Bull, p.5-6.