Session 2: HLB Survey
2.1 Current situation of citrus Huanglongbing in Cuba

Llauger R.1, Luis M.1, Collazo C.1, Peña I.1, González C.1, Batista L.1, Teixeira D.2, Martins E.2, Perdomo A.3, Casín J.C.4, Pérez J.L.5, Cueto J.R.1 and Bové J.M.6

1Research Institute on Tropical Fruit Culture, Playa, Havana, Cuba; 2Fundecitrus, Araraquara, SP, Brazil, 3Jagüey Grande Citrus Enterprise, Jagüey Grande, Matanzas, Cuba; 4National Center for Plant Health, Plaza, Havana, Cuba; 5Fruit Enterprises Group, Boyeros, Havana, Cuba, 6Centre INRA, Institut National de la Recherche Agronomique and Université de Bordeaux 2, France.

Huanglongbing, one of the most devastating diseases of citrus worldwide, has recently been detected in Cuba. The disease is caused by a Gram negative α Proteobacteria of the Candidatus (Ca.) Liberibacter (L.) genus. It is phloem limited and does not grow in culture. The African type of the disease is caused by Ca. L. africanus, a thermosensitive species of the bacteria that is transmitted by the Tryoza erytreae Del Guercio (Bové, 2006) psyllid. Ca. L. asiaticus is the most severe species; it is heat-tolerant and of the two is the most widely distributed species. Additionally Ca. L. americanus, another thermosensitive species, and a phytoplasma from the group IX 16S rRNA, have been detected and associated with the disease symptoms exclusively in the state of Sao Paulo, Brasil (Teixeira et al., 2005; 2008; Lopes, et al., 2008). Transmission occurs by grafting or is vectored by psyllid insects. Diaphorina citri Kuwayama, a vector of both the Asian and American Liberibacter species, has been present in Cuba since 1999 and has spread all over the country (González et al., 2007). In 2007, Ca. L. asiaticus was reported in Cuba in urban areas of La Habana City. After its detection, surveys were carried out in the western, central and eastern parts of the country, as well as in residential areas of the capital to determine the extent of the disease in different geographic areas.

To confirm the presence of the bacteria in different citrus species, 127 symptomatic trees from the following species: grapefruit (Citrus paradisi Macf.), Persian lime (C. latifolia Tan.), tangerine (C. reshni Hort. and C. tangerina Hort.), C. macrophylla Webs., C. volkameriana Tan. & Pasq, Mexican lime (Citrus aurantifolia Christm. Swing.), pummelo (C. grandis L.) and sour orange (C. aurantium L.) were sampled. The trees had yellow shoots, asymmetric leaf mottling, some with chlorotic or corky veins and, in more advanced stages, nutritional deficiencies (Zn and Mn). In these plants some fruits were lopsided and showed color inversion or rounded diffuse spots, yellow vascular bundles, and aborted brownish seeds. In some trees, symptoms were present in part of the canopy, while in others the whole canopy was affected, showing dieback of the branches, suggesting that infection had occurred some time ago. Presence of Candidatus Liberibacter bacteria was confirmed by transmission electron microscopy.

Leaf DNA was extracted from 500 mg of central veins according to the CTAB protocol of Murray and Thompson (1980). Duplex PCR was performed using rplA2/rplJ5/GB1/GB3 primers to identify Ca. L. africanus, Ca. L. asiaticus and/or Ca. L. americanus (Teixeira, et al., unpublished data). The results showed the 703 pb band characteristic for Candidatus Liberibacter asiaticus in 95.3 % of the samples (Figure 1A). Amplification of the 16S rDNA was carried out using the fOA1+fOI1/roI2C primers (Figure 1B) (Jagoueix et al., 1996). The two-band pattern (640 and 520pb) characteristic of Ca. L. asiaticus obtained by enzymatic digestion.
of the 1160 pb fragment using XbaI (Promega) was found in 121 of the analyzed samples, while for the *Ca. L. africanus* control a three-band pattern was observed (Figure 2).

Fragments of the *rpl*KAJL-*rpo*BC operon amplified with the *rpl*A2/*rpl*J5 primers were cloned, sequenced and compared to the sequences published in international data bases. The highest percentages of identity were observed for the *Ca. L. asiaticus* isolates from Asia and Brazil, ranging from 99-100%.

For the detection of the bacteria inside the vector, 1-10 *Diaphorina citri* adult specimens were collected in symptomatic citrus plants. Presence of the bacteria was confirmed by PCR. Insect DNA was extracted according to the method described by Yamamoto *et al.*, 2006. In 21 of the 25 samples of insects studied, *Ca. L. asiaticus* was detected by two PCR reactions with the *rpl*A2/*rpl*J5 and fOA1+fOI1/rOI2C primer pairs, using the same protocol as for the leaf samples.

**Figure 1:** Electrophoresis on 1% Agarose gel. **A:** PCR products amplified with *rpl*A2/*rpl*J5, GB1/GB3. MM: 1Kb DNA ladder (Promega), lane 1: Brazilian *Ca. L. asiaticus* positive control; lanes 2-5: samples of symptomatic citrus plants, lane 6: water. **B:** PCR products amplified with OI1/OA1/OI2c. MM: 1Kb DNA ladder (Promega). Lane 1: water, lane 2: negative control from a healthy plant, lane 3: *Ca. L. africanus* positive control, lane 4: *Ca. L. asiaticus* positive control from Brazil, lanes 5-13: symptomatic citrus samples.

**Figure 2:** Electrophoresis on 4% Agarose gel of the *XbaI* enzymatic digestion products of PCR using OI1/OA1/OI2c primers. MM: 1Kb DNA ladder (Promega). Lane 1: water; lane 2: healthy plant negative control; lane 3: *Ca. L. africanus* positive control; lane 4: Brazilian *Ca. L. asiaticus* positive control; lanes 5-7: symptomatic citrus samples.
In the inspections carried out in orchards from different geographical areas of the country, 27% and 25.7% of the predominant symptoms found were intense chlorosis and asymmetric diffuse mottling of the leaves, respectively (Figure 3). In producing-trees, sometimes characteristic symptoms were also observed in the fruits (less than 5%).

**Figure 3** Percentage of plants showing different types of HLB associated symptoms with respect to the total symptomatic plants. C: leaves with intense chlorosis, BM: leaf asymmetric mottling, CV: corky veins, MS: misshapen fruits, MF: fruits with diffuse mottling, CI: fruits with color inversion, AS: fruits with aborted seeds.

Of the provinces surveyed, 58% of the total amount of plants with HLB symptoms were found in the province of Matanzas. A smaller number of symptomatic plants (less than 1%) were found in the provinces of Guantánamo, Santiago de Cuba and Holguín, located in the eastern part of the island, provinces that were farther away from the place where the insect vector was first detected.

Vector biology and behavior were studied by periodically observing young shoots of Valencia orange and Marsh grapefruit in orchards from different citrus areas. It was found that the developmental stages of *D. citri* tend to be distributed in an aggregated way. The highest population densities were observed during periods of new sprouting, with a preference for the leaf bundles, independent of the cardinal point. The highest rates of eggs and nymphs were found in La Habana during May and August; while for Cienfuegos, it was during January and May. *D. citri* had low population levels throughout the year in Isla de la Juventud, with increases in April, May and July. In Matanzas, a higher incidence was observed in January, April and May, with over 60% of nymphs. Climatic factors were found to affect the psyllid population behavior. The presence of natural enemies was studied as well for their effect on the vector. Of the list of biocontrol agents consisting of: *Cycloneda sanguinea* (L), *Chilocorus cacti* (L), *Exochomus cubensis* Dimn, *Scymnus distinctus* Casey, *Chrysopa sp*, *Ocyptamus sp*, *Tamarixia radiata* Waterston e *Hirsutella citriformis* Speare, it was demonstrated that *T. radiata* has the leading role in the natural control of *D. citri* in Cuba, according to its distribution, specificity and effectiveness for parasitism of the nymph stages N3, N4 y N5 (30,72% a 97,26%).

**Figure 4.** Distribution of HLB symptomatic citrus plants in the Cuban provinces. PR: Pinar del Río, H: La Habana, M: Matanzas,
At the present time, work is being carried out to determine HLB incidence in every citrus growing area in the country. Additionally a disease management program is being implemented that includes: periodical surveys in the commercial citrus areas, elimination of infected plants, use of certified material for planting, and control of Diaphorina citri by using insecticides to reduce its populations as well as the use of biological control with hymenoptherous parasites, such as Tamarixia radiata.

References