Critical Review

Etiology of Three Recent Diseases of Citrus in São Paulo State: Sudden Death, Variegated Chlorosis and Huanglongbing

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Summary

The state of São Paulo (SSP) is the first sweet orange growing region in the world. Yet, the SSP citrus industry has been, and still is, under constant attack from various diseases. In the 1940s, tristeza quick decline (T-QD) was responsible for the death of 9 million trees in SSP. The causal agent was a new virus, citrus tristeza virus (CTV). The virus was efficiently spread by aphid vectors, and killed most of the trees grafted on sour orange rootstock. Control of the disease resided in replacing sour orange by alternative rootstocks giving tolerant combinations with scions such as sweet orange. Because of its drought resistance, Rangpur lime became the favourite alternative rootstock, and, by 1995, 85% of the SSP sweet orange trees were grafted on this rootstock. Therefore, when in 1999, many trees grafted on Rangpur lime started to decline and suddenly died, the spectre of T-QD seemed to hang over SSP again. By 2003, the total number of dead or affected trees was estimated to be over one million. The new disease, citrus sudden death (CSD), resembles T-QD in several aspects. The two diseases have almost the same symptoms, they spread in time and space in a manner strikingly similar, and the pathological anatomy of the bark at the bud union is alike. Transmission of the CSD agent by graft-inoculation has been obtained with budwood inoculum taken not only on CSD-affected trees (grafted on Rangpur lime), but also on symptomless trees (grafted on Cleopatra mandarin) from the same citrus block. This result shows that symptomless trees on Cleopatra mandarin are tolerant to the CSD agent. Trees on rootstocks such as Sunki mandarin or Swingle citrumelo are also tolerant. Thus, in the CSD-affected region, control consists in replacing Rangpur lime with compatible rootstocks, or in approach-grafting compatible rootstock seedlings to the scions of trees on Rangpur lime (inarching). More than 5 million trees have been inarched in this way. A new disease of sweet orange, citrus variegated chlorosis (CVC), was observed in 1987 in the Triângulo Mineiro of Minas Gerais State and the northern and north-eastern parts of SSP. By 2000, the disease affected already 34% of the 200 million sweet orange trees in SSP. By 2005, the percentage had increased to 43%, and CVC was present in all citrus growing regions of Brazil. Electron microscopy showed that xylem-limited bacteria were present in all symptomatic sweet orange leaves and fruit tissues tested, but not in similar materials from healthy, symptomless trees. Bacteria were consistently cultured from twigs of CVC-affected sweet orange trees but not from twigs of healthy trees. Serological analyses showed the CVC bacterium to be a strain of Xylella fastidiosa. The disease could be reproduced and Koch’s postulates fulfilled, by mechanically inoculating a pure culture of X. fastidiosa isolate 81.1.b into sweet orange seedlings. The genome of a CVC strain of X. fastidiosa was sequenced in SSP in the frame of a project supported by FAPESP and Fundecitrus. X. fastidiosa is the first plant pathogenic bacterium, the genome of which has been sequenced. Until recently, America was free of huanglongbing (HLB), but in March 2004 and August 2005, symptoms of the disease were recognized, respectively in the State of São Paulo (SSP) and in Florida, USA. HLB was known in China since 1870 and in South Africa since 1928. Because of its destructiveness and its rapid spread by efficient psyllid insect-vectors, HLB is probably the most serious citrus disease. HLB is caused by a phloem sieve tube-restricted Gram negative bacterium, not yet available in culture. In the 1990s, the bacterium was characterized by molecular techniques as a member of the alpha proteobacteria designated Candidatus Liberibacter africanus for the disease in Africa, and Candidatus Liberibacter asiaticus for HLB in Asia. In SSP, Ca. L. asiaticus is also present, but most of the trees are infected with a new species, Candidatus Liberibacter americanus.

INTRODUCTION: FROM TRISTEZA-QUICK DECLINE TO CITRUS SUDDEN DEATH

The State of São Paulo (SSP) grows some 212 million sweet orange trees on some 659 thousand hectares of land, produces 13.4 million tons of sweet oranges for the juice industry, and exports orange juice for a value of 2.0 billion US$, representing 63% of the orange juice production in the world, and giving jobs to some 400,000 people in the State.
These figures explain why SSP is the first citrus growing region in the world. Yet, the SSP citrus industry has been, and still is, under constant attack from various diseases. Since 1989, Brazil and France have joined efforts on the etiology of three of these diseases: Citrus Sudden Death (CSD), Citrus Variegated Chlorosis (CVC), and Huanglongbing (HLB).

In the 1940s – 1950s, nine million sweet orange trees, out of a total of eleven million, were destroyed by citrus tristeza-quick decline disease (T-QD), and the SSP citrus industry was almost wiped out. The total number of trees killed in South America was around 25 million, and reached 100 million world-wide. As the result of intensive research, in Brazil and abroad, the cause of the disease and its pathogenicity mechanism were elucidated. A graft-transmissible virus, citrus tristeza virus (CTV), insect-transmitted by the brown citrus aphid, *Toxoptera citricida*, was found to kill citrus trees when they were grafted on sour orange (*Citrus aurantium* L.) rootstocks. Sour orange as a rootstock had been discovered and developed in Spain in the 1850s, and had become the leading rootstock, not only in the Mediterranean area, but also in South America, including Brazil and SSP. The new rootstock was a true factor of progress, but only as long as CTV was absent. Unfortunately, CTV was introduced with infected citrus plants from South Africa into Argentina in the 1930s and, spread by *T. citricida*, invaded Brazil in 1937, and killed all sweet orange trees grafted on sour orange. Fortunately however, the pathogenicity mechanism of tristeza as a bud union disease having been understood, control of the disease was at hand: replacement of the sour orange rootstock by rootstocks giving tolerant combinations with sweet orange scions. This is why, soon, the Paulista citrus orchards became grafted on such alternative rootstocks as Rangpur lime, Volkamer lemon, Cleopatra and Sunki mandarins, Orlando tangelo, *Poncirus trifoliata*, citranges, citrumelos, rough lemon, and the SSP citrus industry resurrected.

Because of its drought resistance and its compatibility with CTV, and in spite of its sensitivity to other diseases, one of these rootstocks, Rangpur lime (*Citrus limonia* Osb.), had become, by the 1990s, the rootstock of 85% of all sweet orange trees grown in SSP. After the sour orange era, most of the Paulista citrus industry found itself, once again, grafted on one single rootstock!

In view of the importance gained by Rangpur lime in SSP, the discovery in 1999 and dying, came as a shock (1). The disease, Morte Subita dos Citros or Citrus Sudden Death (CSD), started to kill hundred of thousands of trees in the Triângulo Mineiro of Minas Gerais State, and soon moved into northern SSP. The spectre of tristeza-quick decline seemed to hang over SSP again, even though the incriminated rootstock, Rangpur lime, was known for its compatibility with CTV. On the basis of extensive surveys by Fundecitrus, the total number of dead or affected trees was estimated to be over one million in June 2003. CSD has been observed on all sweet orange varieties grown in the affected areas: Valencia, Pera, Hamlin, Natal, Westin, Rubi and Pineapple.

**CITRUS SUDDEN DEATH (CSD)**

**CSD, a Graft-transmissible, Tristeza-like, Bud Union Disease**

Symptoms of CSD Resemble those of Tristeza-quick Decline. Symptoms of CSD in the affected region of Brazil were found to be very similar to those of T-QD as seen in Florida in the early 2000s (2). In particular, in both diseases fruits and dried leaves remain attached to the trees when sudden death occurs. However, the yellow discoloration (see 2), very characteristic of Rangpur lime bark from CSD-affected trees, and of great diagnostic value, does not occur in the sour orange bark from TQD-affected trees. Also, symptoms of T-QD develop as early as 6 – 9 months after exposure to infection by the aphid vectors in the field, while the yellow discoloration of the Rangpur lime rootstock bark takes at least 2 years to show up in the field.

**Epidemiology: Spread of CSD in Time and Space.** Extensive surveys carried out by Fundecitrus showed that CSD spreads in time and space in a manner strikingly similar to the spread of CTV, under conditions where the aphid vector of CTV was *Toxoptera citricida* but not any other aphid vector (3). Also, for spatial autocorrelation analyses of proximity patterns, the results for CSD and CTV were remarkably similar. Because of these similarities, CSD could not be an abiotic disease, and it was suggested that CSD was caused by an insect-vector pathogen, such as a new strain of CTV, if not a virus different of CTV (2).

**CSD: A Graft-transmissible, Bud Union Disease.** Transmission of the causal agent of CSD by graft-inoculation was successfully achieved by Fundecitrus (4). Graft-transmitted agents belong to viroids, viruses, and endogenous, phloem- and xylem-restricted bacteria. Search for endogenous, as well as exogenous, bacteria, has produced negative results (3), and no viroids were detected in CSD-affected trees (2). Thus, the causal agent of CSD was probably a virus.

Positive grafted-transmissions of the CSD agent were obtained not only with budwood-inoculum taken on 14-year-old CSD-affected sweet orange trees (grafted on Rangpur lime), but also with budwood-inoculum taken on 22-year-old asymptomatic trees (grafted on Cleopatra mandarin). Both inoculum-donor trees were from the same citrus block. These results indicated that the causal agent of CSD was present not only in CSD-affected trees, but also in symptomless trees. The fact that symptomless trees on Cleopatra mandarin carried the CSD agent showed that trees on Cleopatra mandarin were tolerant to CSD. This result is in agreement with the fact that, in the CSD-affected region, trees on Cleopatra mandarin, next to affected trees on Rangpur lime, have never shown
symptoms of CSD. The same is true for trees on Sunki mandarin, Swingle citrumelo and *Poncirus trifoliata*, which are tolerant too. However, from 2003 to 2005, 3- to 11-year-old trees on Volkamer lemon and rough lemon rootstocks have shown typical symptoms of CSD, including the characteristic yellow discoloration in the rootstock bark, but progress and severity of the disease are less intense than with Rangpur lime.

**Pathological Anatomy of Rangpur Lime Bark at the Bud Union.** It was shown in the 1950s that the sour orange bark below the bud union of T-QD-affected trees shows characteristic histological symptoms (5). The functioning phloem (FP) is greatly reduced, and is characteristically affected by necrosis, collapse and obliteration of sieve tubes and companion cells, as well as by the presence of chromatic parenchyma cells. There is an excessive amount of non-functioning phloem with conspicuous necrotic areas. Medullar rays are hypertrophic and hyperplastic. Very similar anatomical alterations were seen in Rangpur lime and Volkamer lemon bark below the bud union of CSD-affected trees (2). However, in CSD, the amount of FP was not as drastically reduced as in T-QD. This is probably the reason why trees on Rangpur lime begin to show symptoms of CSD only after about two to three years in the field, while trees on sour orange decline after about 6–9 months.

**Control of CSD: Inarching**

Being a bud union disease, CSD control consists, in the CSD-affected region, of replacing Rangpur lime, and eventually Volkamer lemon and rough lemon, with compatible rootstocks. This operation takes however several years, and in the meantime, trees on Rangpur lime continue to decline and die. Fortunately, a control measure that can be applied immediately has been developed: inarching (2).

In general inarching consists of: (i) planting one or two citrus rootstock seedlings next to a grafted tree, and (ii) approach-grafting them onto the scion, above the bud union line. CSD being a bud union disease, affected trees, on Rangpur lime, should recover when inarched with compatible seedlings such as those of Cleopatra and Sunki mandarins or Swingle citrumelo, but they should not recover when inarched with Rangpur lime seedlings. This is precisely what has been observed.

Inarching with the above compatible rootstock seedlings has given exceptionally good results and close to 5 million trees have been inarched since 2002. Inarching serves two purposes: (i) recovery of adult CSD-affected trees, and (ii) preventing CSD symptoms to develop.

(i) CSD-affected trees up to 10-years-old could be recovered when inarched with two seedlings. Pruning of the trees before inarching adjusts the canopy size to the deteriorated and reduced root system, extends the life of the trees by one or two years, and gives the farmers more time to carry out the inarching procedure. However, pruning can also be done immediately after inarching.

(ii) Young trees begin to show CSD symptoms only after having been in the field for at least two years. Therefore, enough time is available to inarch young trees, and one inarched seedling is enough to prevent CSD from setting in. In the case of T-QD, young trees show severe symptoms much earlier, and this is probably the reason why inarching has not been used, in the 1940s, as a method to control T-QD. Interestingly, it has been observed that with inarched trees, the initial Rangpur lime rootstock does not die, and continues to provide at least mechanical support to the trees. The rootstock seedlings used to inarch the trees on Rangpur lime are not as drought resistant as the initial Rangpur lime rootstock. However, as inarching keeps the Rangpur lime rootstock alive, inarched trees might show some drought-resistance. This possibility should not be overlooked.

The epidemiology studies have shown that CSD spreads very much like CTV, and that aphids such as *T. citricida* could be responsible for this spread. Hence, it was to be expected that CSD would continue to increase within the affected region, as well as beyond the affected region and invade further regions of the Paulista citrus belt. This is indeed what the surveys of 2002 (June to September) and 2003 (September to December) have shown. However, since 2004, CSD has not continued to progress out of the affected region, and seems to be restricted to the ‘marginal’ citrus lands of the Triangulo Mineiro and northern/northwestern SSP. The number of trees that have become affected or have died in Minas Gerais and São Paulo States has been estimated to be over 2 million.

**Etiology of CSD**

At this time, the causal agent of CSD is not yet known. All CSD-affected trees are infected with CTV, as this virus is endemic all over Brazil, including SSP. In 2005, Maccheroni et al. reported the discovery of a second virus in association with CSD (6). The CSD-associated virus (CSDaV) is a *Marafivirus* (Tymoviridae family). There was a 99.7% correlation between disease symptoms and presence of the virus. As mentioned above, the epidemiology studies (3) have indicated that CSD is very probably spread by an aphid vector such as *Toxoptera citricida*. As expected, the CSDaV could be detected in three aphid species feeding on CSD-affected trees and known to be CTV-vectors: *T. citricida*, *Aphis gossypii*, and *Aphis spiraecola* (6). CSDaV is very probably transmitted by these aphids, even though *Marafivirus* are usually transmitted by leafhoppers, and no *Tymoviridae* viruses are known to be aphid-transmitted. This suggests that CSDaV is cotransmitted with CTV, needing CTV as a helper virus (2).

In collaboration with Fundecitrus, it was shown that CSDaV is present in citrus plants graft-inoculated with budwood-inoculum from CSD-affected trees (on Rangpur lime) as well as from symptomless, tolerant trees (on Cleopatra mandarin) in the same block, and carrying the CSD agent (6).
This demonstrates that CSDaV is graft-transmissible, and is present not only in symptomatic trees (on Rangpur lime) but also in symptomless trees (on rootstocks such as Cleopatra mandarin). However, none of these interesting results proves that CSDaV is the causal agent of CSD. Further research efforts are necessary to identify CSDaV, CTV, or the two viruses together, as the cause of CSD.

**Conclusion**

Today, after eight years of work, guided by tristeza-inspired hypotheses, ways to efficiently control CSD have been developed for the short term (inarching) and the long term (tolerant trees), even though the causal agent of CSD is not yet identified. It is now unlikely that CSD will wipe out the Paulista citrus industry as did T-QD some 60 years ago. However, by losing Rangpur lime and eventually Volkamer lemon, as drought resistant rootstocks, the industry might be forced to turn to irrigation, and this might be the major, if not the most beneficial consequence of CSD on the long term. Continued efforts remain indispensable. CSD should not be neglected because an even more serious disease has reached the forefront in March 2004: huanglongbing (see below).

**CITRUS VARIEGATED CHLOROSIS (CVC)**

**CVC in SSP: Extent, Damage and Losses**

A new disease of sweet orange, named citrus variegated chlorosis (CVC) or amarelinho, was observed in 1987 in the Triangulo Mineiro of Minas Gerais State and the northern chlorosis (CVC) or amarelinho, was observed in 1987 in the forefront. However, by losing Rangpur lime and eventually Volkamer lemon, as drought resistant rootstocks, the industry might be forced to turn to irrigation, and this might be the major, if not the most beneficial consequence of CSD on the long term. Continued efforts remain indispensable. CSD should not be neglected because an even more serious disease has reached the forefront in March 2004: huanglongbing (see below).

**Etiology: Xylella fastidiosa, the Agent of CVC**

When, in 1987, CVC was reported for the first time in SSP, the symptoms and the cause of the disease were unknown. Huanglongbing (HLB), caused by sieve tube-restricted bacterium, was another disease, the symptoms of which were unknown in SSP. HLB was considered in Asia and Africa the most severe disease of citrus, and represented a serious threat for Brazil. Hence, quite understandably, it was feared, when the symptoms of CVC were discovered, that they could be those of HLB. As the Bordeaux laboratory was specialized, since many years, in the detection of the HLB bacteria by electron microscopy, a collaboration between Instituto Biologico (Victoria Rossetti, head, plant pathology laboratory) in São Paulo and University of Bordeaux/INRA (Monique Garnier, head, electron microscopy laboratory) in Bordeaux, was decided in 1990. Glutaraldehyde-fixed leaf and fruit specimens were mailed to Bordeaux, and examined by electron microscopy.

No bacteria were found in the sieve tubes of CVC-affected specimens, and thus huanglongbing was not involved, but xylem vessels were filled with bacteria suggestive of *Xylella fastidiosa*. The xylem-limited bacteria were present in all symptomatic sweet orange leaves and fruit tissues tested, but not in similar material from symptomless trees (7).

On several media known to support the growth of *Xylella fastidiosa*, a bacterium was consistently cultured from CVC twigs of sweet orange trees but not from twigs of healthy trees (8, 9). The cells of the CVC bacterium were rod-shaped, 1.4 – 3 \( \mu m \) in length, and 0.2 – 0.4 \( \mu m \) in diameter, with rippled walls. An antiserum against isolate 8.1.b of the bacterium was raised, and gave strong positive DAS-ELISA reactions with other cultured isolates from CVC citrus, as well as with several type strains of *X. fastidiosa*. This result showed the CVC bacterium to be a strain of *X. fastidiosa* (8, 10). The disease could be reproduced, and Koch’s postulates fulfilled, by mechanically inoculating a pure culture of *X. fastidiosa* isolate 8.1.b into sweet orange seedlings (8, 9). These results were later confirmed by others (11, 12). Detection techniques of the CVC bacterium by ELISA (10) or DIBA (13), and by PCR (14) have.
been developed. It could be shown by ELISA that ‘Pecosita’, known in Argentina since 1984, and CVC in Brazil were one and the same disease (10). CVC was also reported in Uruguay in 1998 and in Costa Rica in 2005.

*X. fastidiosa* and other xylem-restricted bacteria as well as phloem-restricted bacteria have been reviewed (15).

**Xylella fastidiosa Genomics and Post-genomics**

In 1998, in the frame of a renewed collaboration between Fundecitrus in Araraquara and University of Bordeaux 2/INRA, *X. fastidiosa* isolate 8.1.b was cloned and yielded triply cloned strain 9a5c. Sweet orange seedlings inoculated with strain 9a5c showed conspicuous CVC symptoms 5 months post inoculation, indicating that strain 9a5c had kept the pathogenicity properties of parent strain 8.1.b. Strain 9a5c is the one that has been used in SSP for the *X. fastidiosa* genome sequencing project, a project initiated and supported by FAPESP, in which Fundecitrus and University of Bordeaux 2/INRA were partners. The project was successfully completed in 2000 (16).

The CVC strain of *X. fastidiosa* is the first plant pathogenic bacterium, the genome of which has been sequenced. Several putative pathogenicity-related genes have been identified, including genes coding for proteins involved in degradation of plant cell walls. Such proteins could be involved in facilitating the lateral movement of bacteria between adjoining vessels, and promoting systemic invasion of the plant. Interestingly, no genes for a type III protein secretion system have been identified on the *X. fastidiosa* genome. Similarly, *Spiroplasma citri* and *Candidatus Phytoplasma asteris*, the genomes of which have been sequenced recently, also lack type III secretion systems. These results had been predicted, since type III secretion systems system would not be required for endogenous bacteria, since these bacteria enter their plant habitat, the xylem vessels or the phloem sieve tubes, with the aid of insect-vectors. The genome sequence of *X. fastidiosa* has also revealed the presence of an operon closely related to the gum operon of *Xanthomonas campestris*, suggesting that the CVC bacterium is able to synthesize a novel exopolysaccharide, fastidain gum, different from the *Xanthomonas* xanthan gum, and possibly involved in pathogenicity (17).

Functional genomics of *X. fastidiosa* require tools for transformation of the bacterium and production of mutants. Stable transformation of CVC strains by oriC plasmids has been obtained (18), and several mutants have been produced by gene disruption involving one or two crossing overs (19).

**HUANGLONGBING (HLB)**

Huanglongbing (HLB) has been reviewed recently (20). The disease is caused by endogenous, sieve tube-restricted bacteria, named liberibacters, which are transmitted from tree to tree by citrus psyllid insect vectors: *Diaphorina citri* in Asia and America, and *Trioza erytreae* in Africa. Practically all commercial citrus species and cultivars are sensitive, regardless of rootstocks. As indicated above, in the case of tristeza-quick decline or citrus sudden death, control is achieved by replacing respectively sour orange or Rangpur lime by rootstocks giving tolerant combinations with the scions. For HLB however, no control is known, except preventing the trees from becoming infected. For this reason, but also because of its destructive-ness and its rapid spread by efficient vectors, HLB is probably the most serious disease of citrus, much more serious than tristeza-quick decline and CVC, and it represents a dangerous threat for regions still free of the disease, such as the Mediterranean basin, Western Asia, Australia, New Zealand, and Pacific Ocean islands.

Until recently, America was also free of HLB, but in March 2004 and August 2005, symptoms of the disease were recognized, respectively in the State of São Paulo (SSP), Brazil, and in Florida, USA, two of the largest citrus growing regions in the world. Even though HLB is newly emerging in America, it is probably one of the oldest known diseases of citrus, mentioned in southern China since the late 19th century. Until 1995, the disease was generally known under the South African name ‘greening’. Today, the official designation is by the Chinese name ‘huanglongbing’ (yellow shoot disease), as this was the name used when, in southern China in 1956, the disease was reported for the first time to be graft-transmissible (20).

The HLB bacterium has not yet been obtained in culture. After electron microscopy had shown in the 1970s that the HLB agent was a Gram negative bacterium, molecular techniques had to become available in the 1990s to characterize the organism at the phylogenetic and taxonomic levels.

**Characterization of the African and Asian Liberibacters**

In order to determine the phylogenetic position of the HLB bacterium, plants infected with an Asian isolate (Poona, India) and plants infected with an African isolate (Nelspruit, South Africa) were used to prepare total DNA. The 16S rDNAs of the two isolates were obtained from the two total DNA preparations by PCR-amplification, using universal PCR-products fD1 and rP1 for amplification of prokaryotic 16S rDNA (21). Comparisons with sequences of 16S rDNAs obtained from the GeneBank database revealed that the two HLB bacterium were indeed Gram negative, and belonged to the alpha subdivision of the class Proteobacteria. The trivial name ‘liberibacter’ (from the Latin liber [bark] and bacter [bacteria]), was given to the HLB bacteria (22), and replaced the previous ‘liberobacter’ designation (23). Furthermore, the HLB liberibacter isolates from Africa could be distinguished from those in Asia on the basis of temperature sensitivity (24), DNA hybridizations and genomic properties (25, 26), and serology (27, 28). For these reasons, they represented two different species, and the HLB liberibacter from Asia was described as *Candidatus Liberibacter asiaticus*, and the HLB liberibacter from Africa, as *Candidatus Liberibacter africanus*.
The term *Candidatus* indicates that the organism has not been cultured and was characterized essentially on the basis of DNA properties (29).

### PCR detection of the African and Asian Liberibacters

Two PCR systems have been used. The first is based on the amplification of a 1160 bp fragment of liberibacter 16S rDNA (30). The primer pair OI1/OI2c is able to amplify the rDNA of both liberibacter species, while the pair OA1/OI2c amplifies preferentially the African liberibacter rDNA. *Xbal* treatment of the amplified DNA yields two DNA fragments in the case of *Ca. L.* asiaticus, and three with *Ca. L.* africanus, and is used to identify the liberibacter species present in a given sample (30).

The second PCR system is based on the sequence of the rpl/KAJL-rpoBC operon (β operon), in which the intergenic region between genes rplA and rplL is 34 bp larger in the Asian liberibacter than in the African liberibacter. With forward primer f-rplA2, selected in the rplA gene, and reverse primer r-rplL35 from the rplL gene, a 703 bp DNA is amplified from the Asian liberibacter, while a 669 bp DNA is obtained with the African liberibacter. When both liberibacter species are present in the same sample, amplification of the two DNAs is obtained, and upon agarose gel electrophoresis, two DNA bands are seen, the upper (703 bp) corresponding to the Asian liberibacter, and the lower (669 bp), to the African liberibacter (31).

By the use of these PCR techniques, the presence of HLB has been clearly established in several African and Asian countries (32–36). The presence of both liberibacter species, sometimes in the same trees, was confirmed in Reunion and Mauritius islands (37).

### The American liberibacter

In March 2004, symptoms of HLB were observed on sweet orange trees near the city of Araraquara in SSP (38–40). This was the first reported case of HLB from the American Continent. Affected trees could be spotted from a distance because of their conspicuous yellow shoots (huanglongbing!). Leaf symptoms of HLB in SSP were very similar, if not identical, to those in Africa and Asia, with characteristic and pronounced blotchy mottle on both small and large leaves (see 20). Fruits were small and lopsided, exhibited strong color inversion, seed abortion, and brown/orange-stained vascular bundles.

In April 2004, the 16S rDNA-based PCR technique with forward primers OA1+OI1 and reverse primer OI2c (see above), was applied to sweet orange leaves with strong blotchy mottle from suspicious trees to confirm the presence of HLB in the area and identify the liberibacter involved. This PCR method had been assayed in many Asian and African countries for the detection of the two HLB liberibacters. Whenever leaves with the classic blotchy mottle symptoms were used, positive PCR reactions were obtained, and yielded the characteristic 1160 bp amplicon. Hence, blotchy mottled leaf samples were collected in SSP from 43 trees, which had also severe fruit symptoms, and submitted to PCR analysis. Unexpectedly, all PCR reactions were negative (40). Because of these repeatedly negative PCR reactions, the presence of a new bacterial pathogen in the symptomatic, blotchy mottle leaves from SSP was suspected and investigated.

Evidence for the presence of a new HLB bacterium (SPS-HLB bacterium) was obtained by PCR amplification with universal primers ID1 and rPI for prokaryotic 16S rDNA amplification, as was done in the 1990s to characterize the African and Asian liberibacters (see above). The 16S rDNA of the SPS-HLB bacterium was cloned and sequenced, and the sequence was used to design the specific primer pair f-GB1/r-GB3. PCR amplification with these primers made it possible to detect the SPS-HLB bacterium in all 43 HLB-leaf samples, which had previously tested negative with the primers for *Ca. L.* africanus and *Ca. L.* asiaticus. Primer GB3c, complementary to GB3, was used in conjunction with reverse primer 23S1 to amplify, clone, and sequence the 16S/23S ribosomal intergenic region (RIR). In total, a 1479 bp sequence of 16S rDNA (almost the complete 16S rDNA sequence), followed by the complete 583 bp sequence of the RIR, was available for characterization of the SPS-HLB bacterium by comparison with similar sequences of various isolates of *Ca. L.* asiaticus, and the Nelspruit isolate of *Ca. L.* africanus (41). These comparisons clearly showed that the SPS-HLB bacterium was not only a member of the genus *Candidatus* Liberibacter, having all the oligonucleotide signatures of the liberibacters, but was a new species of this genus (41). In particular, on the 16S rDNA phylogenetic tree, all isolates of *Ca. L.* asiaticus clustered together within the *Ca. L.* africanus/*Ca. L.* asiaticus group, but the SPS-HLB bacterium did not, and formed a separate branch. Also, the ribosomal 16S/23S intergenic region (RIR) sequences of different isolates of *Ca. L.* asiaticus were identical or almost identical (99 to 100% identity). However, the RIR of the SPS-HLB bacterium and that of *Ca. L.* asiaticus had only 78% sequence identity. With *Ca. L.* africanus, the sequence identity was even lower: 66%.

For the above reasons the SPS-HLB bacterium was denoted a new species: *Candidatus* Liberibacter americanus, sp. nov. (39–41). This designation refers to the fact that the new liberibacter species was detected for the first time on the American Continent, and that it represented the major *Ca. L.* Liberibacter species associated with HLB in the affected SSP region. The designation is in line with the other *Ca. L.* Liberibacter names, which also refer to the continents where they occur: Africa for *Ca. L.* africanus, and Asia for *Ca. L.* asiaticus.

Additional properties of the SPS-HLB bacterium fit those of the other two liberibacters. Transmission to healthy orange seedlings by graft inoculation and to periwinkle (*Catharanthus roseus*) by dodder (*Cuscuta campestris*) was
obtained, and EM observations showed the American liberibacter to be restricted to the sieve tubes (41).

**Two Liberibacter Species in SSP**

While the work on the new liberibacter went on, the group of M. Machado in Cordeiropolis detected for the first time *Ca. L. asiaticus* in a small number of trees (38), a result confirmed by Fundecitrus. Between August 2004 and September 2005, 1525 blotchy mottle leaf samples (all samples from different trees) gave positive PCR reactions, of which 92.5% corresponded to *Ca. L. americanus*, 5.4% to *Ca. L. asiaticus*, and 2.1% to both liberibacters (trees simultaneously infected with the two liberibacters), indicating that a great majority of trees were infected with the American liberibacter (40). However, from the samples analyzed in 2006, it seems as if the proportion of the Asian liberibacter has increased.

**CONCLUSION**

The causal agent of CSD is not yet identified, but is very probably a virus. Whether it is a new strain of CTV, or the Marafivirus associated with CSD, or even an association between CTV and the Marafivirus, remains to be seen. Control of CSD has been developed, and is similar to that of T-QD: replacement of Rangpur lime (and Volkamer lemon) by rootstocks giving tolerant combinations with sweet orange and other scions. However, an additional technique, inarching, has made it possible to extend the longevity of adult trees affected by CSD and to protect young trees against CSD. CSD and T-QD are examples of insect transmitted diseases where control resides in ‘living’ with the causal agents by the use of trees tolerant to the agents.

CVC and HLB are caused by endogenous bacteria: the xylem-restricted *Xylella fastidiosa* of CVC, transmitted by sharpshooters, and the two sieve tube-restricted *Candidatus Liberibacter* species of HLB in SSP, transmitted by the psyllid *D. citri*. The major citrus variety grown in SSP, sweet orange, is sensitive to CVC and HLB, irrespective of rootstocks. Hence, the SSP citrus industry, based essentially on sweet orange for juice production, cannot ‘live’ with CVC or HLB. The sweet orange trees must be prevented from becoming infected with the causal bacteria. This preventive control is based on: (i) insecticide treatments to decrease the insect vector populations, (ii) eliminating, survey after survey, infected plant material to reduce the sources of inoculum on which the insect vectors, when feeding, become contaminated, and (iii) using healthy young trees from screen-house protected nurseries as resets to replace removed trees. This preventive control is expensive and unfriendly to man and his environment. The future of the Paulista citrus industry is conditioned by the development of sweet orange plant material resistant to HLB and CVC, and perhaps on repellent chemicals preventing the insect vectors to contaminate citrus trees.

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