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Half a century on huanglongbing: learning about the disease, trying to control it.

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Born in 1929 in Luxemburg from a family of horticulturists and myself a graduate of the School of Agronomy in Paris as well as a biologist of the University of Paris, I married in 1952 Colette Dumeau, a professor of Physics and Mathematics.

When, in 1953, I looked for my first job, I could not expect, as a citizen of Luxembourg, a position at a French university or a national research institute such as INRA (National Institute for Agricultural Research). You had to be a French citizen to apply! Therefore, being interested in research, I chose to join the French Institute for Citrus and Tropical Fruit Research (IRFA), a semi-private organization for overseas research on citrus, banana, and pineapple crops. I was asked by IRFA to consider working on citrus stubborn, a disease prevalent in the Mediterranean Region, the Near East and the Middle East, as well as in the USA. This is how I got into citrus and citrus diseases. Shortly after me, Colette also joined IRFA to work on citrus. From now on, we would always work together in the same laboratories on the same subjects.

However, being aware that we were lacking experience in research, both Colette and I managed to get, with the help of IRFA, a scholarship to work, from September 1956 to March 1959, at the University of California in Berkeley, in the laboratories of (i) DI Arnon on photosynthetic phosphorylation, (ii) PK Stumpf on enzymology, (iii) EE Conn on aromatic compounds, and (iv) RL Steere on viral RNA. We are very much indebted to them!

While in California, we attended the first "Conference on Citrus Virus Diseases" (Riverside, November 1957) at which the International Organization of Citrus Virologists (IOCV) was founded. Stubborn disease was on the program, but not huanglongbing (HLB)/greening, even though Lin Kung Hsiang had achieved, in China in 1956, the first transmission of HLB by graft-inoculation, proving in this way the infectious nature of HLB. He published his work in *Acta Phytopathologica Sinica* in 1956. In this publication "huanglongbing", meaning "yellow shoot disease", was used to name the disease and became in 1995, at the 13th IOCV conference in Fuzhou, China, the official name of the malady.

Our institute, IRFA, was interested in HLB because of citrus stubborn. Indeed, stubborn and HLB have similar symptoms, fruit symptoms in particular, and therefore their agents were thought to be related. They were even thought to be different strains of the same virus! Why virus? Because only viruses where known in the late 1950s / early 1960s to be infectious agents of plants! Therefore, after our return to France in 1959, we developed a virus laboratory to study Stubborn/HLB! Our laboratory was housed within the department of Georges Morel (the "father" of virus-free shoot-tip cultures) at the INRA Research Center in Versailles, where we stayed until our departure for Bordeaux in 1971.

In Versailles, with the green light of IRFA, we were working essentially on the replication of turnip yellow mosaic virus RNA. But then, in 1967, an important development occurred in Japan: mycoplasmas, i.e., bacteria lacking a cell wall, were discovered as infectious disease agents of plants by Doi et al. Mycoplasmas were practically unknown in plant pathology, but they were already recognized as disease agents in humans and animals. In plants, they were restricted to the phloem sieve tubes. So then, could stubborn and/or HLB be caused by a mycoplasma rather than a virus?

Electron microscopy (EM) clearly showed the stubborn agent to be a mycoplasma. This result was obtained in 1970, simultaneously in Riverside, USA, by Igwegbe and Calavan, and Versailles, France by Laflèche and Bové. Furthermore, the stubborn mycoplasma was cultured independently in the above two laboratories. This was the first time a plant mycoplasma had been obtained in culture. Finally, characterization of the stubborn mycoplasma took place in Bordeaux to where we had moved in 1971. The organism was found to be motile and to have an unexpected helical morphology, and was described in 1973 by Saglio et al. as *Spiroplasma citri*, a new species and a new genus within the Mollicutes.

In addition to studying the stubborn agent, EM was also used to examine the HLB agent, first in Versailles by Dominique Laflèche, later in Bordeaux by Monique Garnier. EM detected sieve tube restricted bacteria in HLB from both Africa and Asia. We first thought that the bacteria were mycoplasmas, but soon, in comparison with

the true stubborn mycoplasma, lacking a cell wall and only surrounded by an approximately 7 nm thick cytoplasmic membrane, we discovered that the HLB bacteria had a cell envelope with a thickness of approximately 20 nm, indicating that they had a cell wall in addition to the cytoplasmic membrane. The cell wall was eventually shown by Garnier et al. in 1984 in Bordeaux to be of the Gram-negative type. Thus, the stubborn and HLB agents were not viruses and they were clearly different, one being a wall-less bacterium, i.e. a mycoplasma, and the other a bacterium with a wall of the Gram-negative type, but both being restricted to the phloem sieve tubes. Contrary to the stubborn spiroplasma, which could be cultured as early as 1970, the HLB bacterium has not yet been obtained in permanent culture.

In the skilled hands of Monique Garnier in Bordeaux, EM became the first laboratory technique to detect and identify the HLB bacterium. In this way, we rigorously confirmed the presence of the disease in many African and Asian countries. This extensive work demonstrated that leaves with blotchy mottle symptoms had the highest titers of the HLB bacterium and were the material of choice for EM identification of HLB. In addition, we demonstrated, under phytotron conditions, that the African HLB agent in citrus plants was heat-sensitive, while the Asian HLB agent was heat-tolerant. In the field, these heat effects were particularly well documented for African HLB in South Africa, Ethiopia and Madagascar, and for both African and Asian HLB in Reunion and Mauritius islands. Heat sensitivity of African HLB was the result of the heat sensitivity of both the African HLB agent and the African citrus psyllid, *T. erytreae*, shown in South Africa in 1965 to be the vector of the HLB agent in Africa. Similarly, heat tolerance of both the Asian HLB agent and the Asian citrus psyllid, *Diaphorina citri*, known since 1967 to transmit Asian HLB, explained the heat tolerance of Asian HLB.

A soon as, by EM, we had identified the HLB agent as a bacterium, the possibility occurred to control HLB by injection of antibiotics into HLB-affected sweet orange trees. This study was taken up in South Africa by Ralph Schwarz with tetracycline hydrochloride. Even though remission of symptoms was observed, no appreciable results with the tetracycline treatment were obtained. The treatment was expensive and remission was only temporary; phytotoxic effects occurred at the injection site and in vascular bundles; fruits of treated trees were small and contained high levels of residues. Tetracycline treatments were also conducted in Reunion, Taiwan, and Indonesia. The effect of penicillin, an inhibitor of peptidoglycan synthesis in the bacterial cell-wall, has been studied mainly to gain information on the nature of the cell-wall of the HLB bacterium. Penicillin was found to have a strongly positive effect on HLB-affected, glasshouse-grown sweet orange plants in Bordeaux as well as on field-grown trees in Reunion. The beneficial effect of penicillin on HLB-affected plants was further evidence, in addition to the EM results, for the presence of peptidoglycan in the cell-wall of the HLB bacterium.

Even though EM allowed rigorous confirmation of HLB symptoms for more than 20 years, techniques less heavy and time-consuming were required. Therefore, in the 1980s and early 1990s, thirteen monoclonal antibodies (MAs) against the African or Asian HLB bacterium were produced for the first time and evaluated by Garnier et al. for the detection of the HLB bacterium by immunofluorescence on thin sections. Unfortunately, the MAs obtained were quite specific to the strain used for immunization and, therefore, were not suited for generalized diagnosis of HLB. MAs have been more successful for purification of the HLB bacterium by immunoaffinity chromatography followed by immunogold labeling. This was the first time that purified, individual HLB bacteria were seen outside of the sieve tubes and found to be elongated, filamentous cells.

When the DNA-based PCR technique became available in the late 1980s, early 1990s, not only a new detection method of the HLB agent emerged, but also, for the first time, the very taxonomical identification of the agent could be envisaged. Indeed, 16SrDNA of the HLB agent was obtained from infected leaves by PCR-amplification and cloning. The 16SrDNA was sequenced and the sequence was compared with 16SrDNA from the GenBank data base. The comparison revealed that the HLB bacterium represented a new genus, "*Candidatus* Liberibacter", in the alpha class of the phylum Proteobacteria (Gram negative bacteria), "Candidatus" indicating that the bacterium had not been cultured and "Liberibacter" coming from the Latin "*liber*" (live bark) and "*bacter*" (bacterium). The Asian HLB bacterium and the African HLB bacterium represented 2 different species, respectively "*Candidatus* Liberibacter asiaticus" (Las) and "*Candidatus* Liberibacter africanus" (Laf), as reported by Jagoueix et al. in 1994.

PCR amplification of 16SrDNA, as reported by Jagoueix et al. in 1996, was the first DNA-based technique to detect the liberibacters. For both Laf and Las, an amplicon of 1660 bp was obtained. To identify Laf or Las, the amplicon had to be treated with restriction enzyme Xba1. In the case of Las, 2 restriction fragments (520 bp and 640 bp) were obtained; with Laf, 3 restriction fragments (520 bp, 506 bp, and 134 bp) were seen. To avoid restriction with Xba1, a second PCR technique was developed, based on the sequence of the *rplKAJL-rpoBC* operon (β-operon). Part of this operon had been obtained in 1992/1993 from Las (India, Poona strain) as a 2.6 kb sequence, named In-2.6, and in 1995 from Laf (South Africa, Nelspruit strain,) as a 1.7 kb sequence, named 1.7-AS. PCR amplification of these sequences with specific primers A2/J5 yields amplicons of 667 bp and 701 bp, respectively for Laf and Las.

The above Las In-2.6 and Laf 1.7-AS sequences, labeled with (32Pα)dCTP by random priming, have also been used as DNA probes for dot blot hybridization. Probe In-2.6 detected all Asian Las strains tested (India, Thailand, Philippines, Indonesia, China, and Taiwan), but not the South African Laf strains. Inversely, probe 1.7-AS recognized South African Laf but not Asian Las. DNA

hybridization has been used in particular to detect and confirm HLB in India, South Africa, Nepal and Vietnam. The probes were also used to detect the liberibacters in psyllids in Malaysia.

Using symptomatology and detection of Laf and/or Las by EM, PCR amplification of 16SrDNA and β-operon genes, and/or DNA/DNA hybridization with β-operon derived probes, HLB has been examined in 10 African and 17 Asian countries. Only heat-sensitive HLB, with Laf and *T. erytreae*, was found in Africa and Madagascar, and only heat-tolerant HLB, with Las and *D. citri*, was detected in Asia. Both African and Asian HLB were found on the Arabian Peninsula in Saudi Arabia and Yemen. Reunion and Mauritius islands also harbored both African and Asian HLB.

It was proposed previously that Laf and Las were of Gondwanan origin, the speciation of Laf occurring on the African East coast, while the speciation of Las took place on the Indian tectonic plate moving north to its present position. It has been suggested by Nelson et al. in 2013 that Las acquired its heat-tolerance while the Indian plate was crossing the hot equatorial zone.

I went on retirement in 1998, but was encouraged by Fundecitrus to continue devoting part of my time in São Paulo State (SPS) to (i) citrus variegated chlorosis, a disease which we had shown to be caused by *Xylella fastidiosa*, as reported by Chang et al. in 1993, and (ii) citrus sudden death, a tristeza-like bud union disease of sweet orange trees grafted on Rangpur lime. But, since 2004, HLB in SPS has been my major source of worry.

HLB symptoms were reported for the first time in America near Araraquara (SPS, Brazil) in March, 2004. Two liberibacters were detected by the end of 2004 as reported by Teixeira et al. in 2005: Las, the known Asian liberibacter, in a small percentage of symptomatic sweet orange trees (less than 10%) and *Candidatus* Liberibacter americanus (Lam), a new liberibacter species, in more than 90% of the affected trees. However, from 2005 on, the incidence of Lam-infected trees decreased, while the incidence of Las-infected trees increased. Today, most trees are infected with Las, and Lam-infected trees are rare. Both liberibacters, Las as well as Lam, were found to be transmitted by *D. citri* as reported by Yamamoto et al. in 2006. *Murraya paniculata* was found to be a good host of Lam as reported by Lopes et al. in 2005 and 2006. A diagnostic laboratory for detection of Las and Lam for citrus growers (free of charge) and researchers was developed by Fundecitrus. The complete genome sequence of Lam was determined by NA Wulff, Fundecitrus, in collaboration with University of Bordeaux/INRA and University of Florida (Dean Gabriel) and reported in 2014. This analysis yielded a genome size of 1,195,201 bp, and has revealed in particular that Lam is missing genes related to lipopolysaccharide (LPS) biosynthesis. LPS is a constituent of the outer membrane of Gram-negative bacteria.

An unexpected discovery was made in 2007! In northern SPS, sweet orange trees with characteristic leaf and fruit symptoms of HLB tested negative for liberibacters! They were found to be infected by a sieve tube restricted phytoplasma of 16Sr group IX, as reported by Teixeira et al. in 2008. The HLB phytoplasma has also been detected in *Crotalaria juncea* (Sunn hemp), a cover crop plant widely distributed throughout SPS. The leafhopper, *Scaphytopius marginelineatus*, frequently found in sweet orange orchards, was shown to efficiently acquire the HLB phytoplasma from affected sunn hemp plants and to transmit the phytoplasma to sweet orange, though rarely, as reported by Wulff et al. in 2015. Even though the HLB phytoplasma is widely distributed throughout SPS, the number of trees affected by the phytoplasma in citrus farms is small, the disease incidence ranging from 0.1% to 1.8% affected trees. Also, most of the affected trees are distributed randomly and, in 80% of cases, the minimum distance between affected trees is 100 m, suggesting that primary infection of citrus trees is a rare event and that secondary infections from citrus to citrus do not occur.

As soon as HLB was reported and confirmed in SPS, it became clear that, without management, all citrus would be destroyed soon or later, but it could not be predicted whether management, if practiced, would be successful or not, as no data were available on large scale control of the disease.

Management by the phyto-pathologically sound "Three-Pronged System" (TPS) started in July 2004, only 3 months after the disease had been reported in SPS. The TPS is based on: (i) elimination of liberibacter inoculum by identification of symptomatic trees and their removal, (ii) closed, insect-free nurseries for the production of trees free of HLB to replace the trees removed, and (iii) reduction of psyllid (*D. citri*) populations by insecticide treatments of all orchard trees by ground and airplane applications. The TPS is a preventive HLB-management system, trying to prevent as many trees as possible from becoming infected.

HLB management by the TPS has limitations. (i) Insecticide treatments are not 100% efficient: some insects escape. Psyllids tend to accumulate on the border of groves and, thus, more trees become infected and more symptomatic trees are removed on the borders or edges of groves than inside the grove. The border effect requires that psyllid control has to be heavier on the borders than inside the groves. The fewer borders, the fewer psyllids! For the same grove size, a circular or a square grove has mathematically less borders than a rectangular grove. (ii) Removing symptomatic trees does not result in removing all infected trees. Indeed, some trees may show symptoms at a time when other infected trees do not yet. For a given tree, the period between the time of infection by the psyllids and the time at which symptoms appear is the "latency period" and may extend from 6 to 18 months. The problem of having trees that are infected, but still symptomless, can be partly overcome by having several inspections, so that infected trees still symptomless at inspection #n, have become symptomatic and can be identified at inspection #n+1, #n+2,… (iii) Even the best team of inspectors can identify only 60% of symptomatic

trees at each inspection; in other words, approximately 40% of symptomatic trees escape detection. Here again, trees, which escaped inspection #n are likely to be detected at inspection #n+1, #n+2,…(iv) Symptom expression varies during the year; symptoms are more pronounced in autumn/winter and less so in spring/summer. (v) Inspections were first carried out by inspectors on the ground, walking from tree to tree. Inspections have been greatly facilitated by the use, in between rows, of tractor-pulled platforms to accommodate the inspectors. For many adult trees, symptoms begin to appear in the top. Therefore, platforms with 4 inspectors have come into use, the lower 2 inspectors looking at the sides of the trees, the upper 2 at the tops.

The way management by the TPS works in a given farm has to be carefully evaluated by recording the number of symptomatic trees removed at each inspection in the given blocks of the farm. The TPS works well when the total number of trees removed on the farm in a given year is smaller than the number from the previous years and keeps getting smaller in the following years.

The major citrus companies in SPS have shared their data on TPS management with Fundecitrus and other institutions in order to identify factors that make it easier or more difficult to achieve HLB-control in SPS, as reported by Belasque et al. in 2010. Some of these factors cannot be changed to influence management: (i) size and (ii) shape of farm, (iii) age of trees, (iv) HLB-incidence in the region where the farm is located, (v) neighboring, near-by farms with no or poor HLB management ("bad" farms), (vi) HLB-incidence in the farm at first inspection for HLB-affected trees. Other factors are in the hands of the growers and can be modified to improve, if necessary, the situation: (i) time between infection of farm and start of the TPS, (ii) number of inspections with platforms, (iii) number of insecticide treatments, type of insecticide, mode of application, (iv) increased tree density at borders because of "border effect", (v) extra insecticide treatments at borders because of border "effect", (vi) using dormant insecticide treatments.

In conclusion:

- The TPS was applied immediately after HLB was detected in March 2004, a time when HLB-incidence in the region and the farms was still low.
- Practically all the large farms (≥400 ha or 200,000 trees) used the TPS.
- From 2004 to 2014, the TPS has been constantly improved and adapted to changing situations.
- The need to control psyllids AND to remove symptomatic trees has never been denied.
- The major difficulty for well-managed farms is the presence of "bad" farms, i.e., neighboring farms with no or poor HLB-management.
- The TPS has made it possible to keep the HLB-incidence in the farm below 1% affected trees per year, approximately 98% trees being healthy, symptomless and non-infected.
- The acreage of TPS-managed farms with low (≤1%) HLB-incidence amounts to approximately 200,000 ha, almost half of the total citrus acreage in SPS.
- SPS is the only region in the world where the TPS has been successful on a large scale.
- The successful results of the TPS (not predictable in 2004!) have changed the perspectives of the SPS citrus industry as indicated by Bové in 2012.
- The TPS must be maintained on the 200,000 ha of low HLB incidence: there is no substitute for the TPS.
- Thus, by 2020 to 2025, when, hopefully, HLB-resistant, genetically modified citrus (GMC) trees become commercially available, SPS will have 2 long-term options: (i) orchards with HLB-resistant GMC-trees and (ii) "low-HLB" orchards with regular, non-GMC trees, under TPS-management.