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Bové, J M

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Autobiography

From spinach chloroplasts to endogenous bacteria causing diseases in citrus: an autobiography of Joseph Marie Bové

JM Bové

Emeritus Professor, University of Bordeaux and National Institute for Agricultural Research (INRA), Laboratory for Cellular and Molecular Biology, INRA Research Center, 33883 Villenave d'Ornon, France.

(with the input of P Moreno and N Duran-Vila. Centro de Protección Vegetal y Biotecnología. Instituto Valenciano de Investigaciones Agrarias)

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The present autobiography was begun by Joseph Marie Bové (Josy for his family and friends) under the request of the Journal of Citrus Pathology to prepare an autobiography that would summarize his scientific contributions. Unfortunately, Josy passed away on June 2 of 2016, when only a partial draft had been prepared. The kind cooperation of his son Hugue allowed us to recover a draft from his computer and use it to complete the present autobiography. Obviously, the present text will necessarily differ from the one that Josy would have prepared. However, we have tried to follow closely the outline that he had prepared and have added those details that we considered important to fully understand his huge contribution to our present knowledge and control of citrus diseases, mainly those induced by endogenous bacteria.

JM Bové wanted to dedicate his autobiography to his great-grandfather Nicolas Bové (1802-1842) who was always a source of admiration/inspiration.

Introduction

Joseph Marie Bové was born in the city of Luxembourg in 1929, far away from the citrus growing areas. As he remarked "As a child, I would get a couple oranges only for Christmas". From 1936 to 1940, he lived with his parents in Belgium, where he learned Dutch and French, and then, because of the Second World War, they moved in 1940 to Bordeaux, France, where his parents had a vineyard. His inclination for agriculture likely came from his family, in which his father, grandfather and other family members were horticulturists/botanists. Josy felt particular admiration for his great-great grandfather (Nicolas Bové, 1802-1842), who was in charge, in Cairo, of the farms of Muhammad Ali, the viceroy of Egypt, from 1829 to 1832. In this period, Nicolas collected plants, not only in Egypt, but as well in Palestine, Syria, Saudi Arabia and Yemen. He was the first to describe the flora of the Sinai Peninsula before he moved to Algeria in 1834, where he worked on the Algerian flora until he died of malaria in 1842.

Joseph Marie Bové met Colette Dumeau (1927-2014) in 1948 in Bordeaux and they married in 1952. She had studied mathematics and physics and became a high-school teacher. He left Bordeaux for Paris in 1950 to learn agronomy at the French National School of Agronomy and Biology at the University of Paris. He became interested in plant pathology and had a good relationship with Georges Viennot-Bourgin, the professor of phytopathology at the

school, with whom he would later share different scientific missions after Viennot-Bourgin was retired.

When in 1953, he had to look for his first job, he was still a citizen of Luxembourg and thus he was not eligible for a position at French national research institutes such as INRA (National Institute for Agricultural Research) or Universities: it was necessary to be a French citizen! Therefore, being interested in research and on the advice of Viennot-Bourgin, he decided 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 (today: part of CIRAD at Montpellier, France). Being a French citizen was not required to work at IRFA. He 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 California and Arizona, USA. This is how he got into citrus and citrus diseases. Shortly afterwards, Colette also joined IRFA to work on citrus and since that time both would always work together in the same laboratories.

Being aware that they were lacking experience in research, with the help of IRFA, both Josy and Colette got scholarships to work from September 1956 to March 1959 at the University of California in Berkeley. They worked in some of the most prestigious laboratories there: (i) DI Arnon on photosynthesis (Bové et al. 1963); (ii) PK Stumpf on enzymology (Bové et al. 1959); (iii) EE Conn on metabolism of aromatic compounds (Bové and Conn

1961); and (iv) RL Steere on viral RNA. Finally, in the Berkeley Virus Laboratory, they came across the nascent field of molecular biology (Bové and Raacke 1959).

Before leaving for California, Josy had worked part time with George Morel (the “father” of virus-free shoot-tip cultures) in his laboratory at INRA, Versailles, where he became acquainted with plant tissue culture techniques, particularly with citrus tissues. This experience would be useful in his future work. At that time, some scientists at INRA studied the effect of light of different wavelengths on photosynthesis, but they worked with whole plants, whereas in Arnon’s laboratory they worked on isolated chloroplasts and the Warburg’s system. Josy was most impressed by the efficiency of this system that allowed multiple experiments to be performed in the same day with high precision.

While in California, he attended the first “Conference on Citrus Virus Diseases” at the Citrus Research Center in Riverside (November 18 to 22, 1957), at which the International Organization of Citrus Virologists (IOCV) was founded. Many subjects were covered, including the stubborn disease, but not South African greening nor huanglongbing in China, even though in 1956 Lin Kung Hsiang had achieved the first transmission of huanglongbing by graft-inoculation, thus proving the infectious nature of huanglongbing (HLB). Lin published his work in *Acta Phytopathologica* (Lin 1956) and in this publication “huanglongbing” was used to name the disease and in 1995, at the 13th IOCV conference in Fuzhou, China, it became the official name of the malady, even though the name “citrus greening” is still used.

IRFA, which wanted Josy to work on the stubborn disease, was also interested in HLB because of its similarities to stubborn! Indeed, stubborn and HLB had similar symptoms, particularly fruit symptoms, and therefore their agents were thought to be related: they were supposed to be different strains of the same virus. Why virus? Because in those days only viruses were known to be graft-transmissible infectious agents of plants and every graft-transmissible agent was believed to be a virus.

The years at INRA, Versailles (1959 to 1971)

On his return to France in 1959, after the Berkeley years, and since his “tropical” employer, IRFA, had no laboratory facilities in continental France, Georges Morel offered him lab space in his department at INRA with the assignment to study the etiology of citrus stubborn disease and thus, ipso facto, HLB. Indeed, the importance of HLB was increasing at that time, since diseases similar to the South African citrus greening were present in Asia: HLB in China, likubin in Taiwan, leaf-mottling in the Philippines, and vein phloem degeneration in Indonesia.

From 1959 onward, in the framework of IRFA, Josy was active in establishing the citrus experiment station at San Giuliano in Corsica, France. This station was created in 1958 to maintain and increase the experience of IRFA on citrus culture, after the expected independence of Morocco, where the former citrus station was located. He

and Robert Vogel (1929-2002), his friend and colleague at the station, introduced indexing procedures for virus and virus-like diseases of citrus, discovered a new graft-transmissible disease, “cristacortis”, widespread in the Mediterranean basin, and used shoot-tip grafting to supply nurseries with disease-free budwood, which eventually resulted in a budwood certification program.

At this time, studies on stubborn were limited to experiments on graft-transmission and on the sensitivity/resistance of different citrus varieties. A new approach to gain a deeper insight on the disease seemed necessary. However, since stubborn and HLB, as well as all other graft-transmissible diseases of plants including citrus, were supposedly caused by viruses, Josy and Colette felt that they should learn more about viruses and got permission from IRFA to develop a basic research program in virology, in addition to the more applied project on stubborn and HLB. They were very lucky that the general director of IRFA, Richard Guillerme, understood the need to perform basic research even at an institute so far only devoted to applied research. This is also the reason why he hired Colette who knew nothing about plants, but had a strong background in mathematics, physics and chemistry. Josy developed a PhD thesis on a virus (*Turnip yellow mosaic virus*, TYMV) that had nothing to do with citrus, banana or pineapple, the three main crops studied by IRFA. Under the direction of George Morel and the advice of Jacques Monod, he studied the enzymes involved in the virus RNA synthesis, following the approach learned at the Arnon laboratory, that is, using subcellular fractions (chloroplasts, mitochondria and nuclei) instead of plant tissues to isolate the proteins involved in its synthesis. For the first time, he was able to isolate and characterize in detail the viral replicase of TYMV. It is ironic that he got into virology as a way to understand diseases that he showed later to be caused not by viruses but by bacteria: stubborn, HLB, witches’ broom disease of lime, and citrus variegated chlorosis!

An important development occurred in Japan in 1967 when mycoplasmas, i.e. bacteria lacking a cell wall, were discovered to be infectious disease agents of plants (Doi et al. 1967). Mycoplasmas were essentially unknown in plant pathology, but they had been recognized as disease agents in humans and animals. In plants, they were restricted to the phloem sieve tubes. Josy, who had attended this meeting to present a communication, immediately related the symptoms observed in the mycoplasma-infected plants (yellowing, witches’ broom, and stunting) with those of citrus affected by stubborn or HLB. Indeed, at that time stubborn and HLB were thought to be caused by two strains of the same virus. So then, could stubborn and/or HLB be caused by a mycoplasma rather than a virus? Josy and co-workers answered these questions between 1969 and 1973 when they demonstrated that stubborn was associated with mycoplasma, whereas HLB was associated with bacteria. However, both organisms were localized in the phloem, specifically in the sieve tubes.

The stubborn agent

Electron microscopy (EM) clearly showed the stubborn agent to be a mycoplasma. This result was obtained simultaneously in Riverside, USA (Igwegbe and Calavan 1970) and Versailles, France (Lafèche and Bové 1970a). Furthermore, the stubborn mycoplasma could be cultured independently in the same two laboratories (Fudl-Allah et al. 1971, 1972; Saglio et al. 1971a). This was the first time a plant mycoplasma had been cultured. Characterization of the stubborn mycoplasma took place in Bordeaux to where Josy and his group moved in 1971 (see below). The organism was found to be motile and to have an unexpected helical morphology, and was described in 1973 as *Spiroplasma citri*, a new species and a new genus within the Mollicutes (Saglio et al. 1973).

HLB in South Africa

The first encounter of Josy with HLB in the orchard was in 1968 in South Africa where research on “citrus greening” (the name of the disease in South Africa) was most active. He was accompanied by Jacques Cassin, a citrus horticulturist of IRFA stationed in Corsica. In South Africa they learned that HLB was severe in the cool, moist upland citrus areas at an altitude above ~600 m, where the African citrus psyllid *Trioza erytraeae*, was abundant (Schwarz 1967). There was a strong correlation between the incidence of HLB and high populations of *T. erytraeae* (Schwarz 1967). The psyllid was known to be sensitive to high temperatures (32 °C and higher), especially when combined with low relative humidities (25% and lower) (Van der Merwe 1941; Catling 1969). In Swaziland, the situation was identical, with HLB present in the “Highveld” zone (Mbabane: ~1300 m) and the “Middleveld” zone (Malkerns: ~800 m), but absent in the “Lowveld” zone (Tambukulu: ~250 m; Big Bend: ~150 m).

In addition, in 1968, at Woodhouse (South Africa) located at ~800 m, HLB-infected sweet orange trees were grown experimentally in fiber-glass cages. In these cages, (i) temperatures were found to be 8 to 10 °C higher than the outside ambient temperatures and (ii) fruit symptoms of HLB were drastically decreased (Schwarz and Green 1972). This important result needed confirmation by experiments conducted under controlled temperature conditions.

These experiments could be performed near Paris within a newly built phytotron, in the frame of an international cooperation between France (Bové), USA (Calavan), India (Capoor), the Philippines (Cortez), and South Africa (Schwarz). Groups of Hamlin sweet orange seedlings were graft-inoculated with a South African HLB strain, and two Asian HLB strains from India and the Philippines, respectively; and grown for symptom expression under cool and warm phytotron environments (See Table). Seedlings inoculated with the Asian HLB strains showed symptoms not only in the cool chamber but also in the warm chamber while those inoculated with the South African HLB strain showed symptoms only in the cool chamber. These results indicated that African HLB was heat-sensitive, confirming the field experiments, but

they showed also, for the first time, that Asian HLB was heat-tolerant (Bové et al. 1974).

| Hamlin sweet orange seedlings inoculated with HLB agents | Cool chamber | | Warm chamber | | Results |
|--|------------------------|-------------------------|------------------------|-------------------------|----------------|
| | Day 16 hrs 24 °C | Night 8 hrs 22 °C | Day 16 hrs 32 °C | Night 8 hrs 27 °C | |
| South African HLB | | + | | - | Heat-sensitive |
| Asian HLB (India) | | + | | + | Heat-tolerant |
| Asian HLB (the Philippines) | | + | | + | Heat-tolerant |

Similar results were later obtained with periwinkle (*Catharanthus roseus*) plants infected experimentally with the HLB agent via dodder (*Cuscuta campestris*) (Garnier and Bové 1983).

HLB in Madagascar

After South Africa, Josy and Jacques Cassin went to examine HLB in the “big” island of Madagascar in east Africa. The island has a high plateau extending from North to South. The presence of *T. erytraeae*, but not HLB, had already been established in 1965 (Brenière and Dubois 1965). They found the psyllid and also HLB on the plateau, between ~600 m and ~1550 m, but not below 600 m, a situation characteristic of African, heat-sensitive HLB (Bové and Cassin 1968a). Josy and Colette Bové went back to Madagascar in October 2011 and collected samples of citrus leaves with characteristic HLB blotchy mottle. Analyzed in Bordeaux, they were found to be infected, as expected, with *Candidatus Liberibacter africanus* (Laf) (Bové 2014; Nelson et al. 2015), confirming 43 years later their 1968 conclusion based on HLB distribution and the presence of *T. erytraeae*: HLB in Madagascar was of the African, heat-sensitive type.

HLB in Reunion and Mauritius islands

After South Africa and Madagascar Josy and Jacques Cassin went to Reunion Island, east of the “big” island. Sylvio Moreira, from Brazil on a mission for the Food and Agriculture Organization, had detected HLB in Reunion in 1967 without mentioning the psyllid situation (Moreira 1967). They detected not only the African citrus psyllid, *T. erytraeae*, above ~500 m, but also the Asian citrus psyllid, *Diaphorina citri*, below ~500 m. However, symptoms of HLB were present above as well as below ~500 m (Bové and Cassin 1968b). In 1996, when both *Candidatus Liberibacter asiaticus* (Las) and *africanus* (Laf) had been characterized and could be detected by PCR, it could be

shown that, in the *D. citri* zone, below ~500 m, the HLB agent was Las, while in the *T. erythrae* zone, above ~500 m, Laf was involved (Garnier et al. 1996). This was the first time that African HLB, with *T. erythrae* and Laf, and Asian HLB, with *D. citri* and Las, occurred in the same region, but at different altitudes. Later, it was found that the situation in Mauritius, an island close to Reunion, was very similar to that in Reunion. The fact that, in Reunion and Mauritius, *D. citri* is restricted to the zone between sea level and ~500 m, does not mean that the Asian psyllid is always restricted to a 0 to 500 m zone. For instance, in Nepal where only Asian HLB occurs, *D. citri* can be found up to ~1400 m (Bové 2014).

In conclusion, distribution of HLB in South Africa, Madagascar, Reunion and Mauritius islands, confirmed the previous phytotron results, namely that African HLB is heat-sensitive and Asian HLB is heat-tolerant.

Learning more about the stubborn and huanglongbing agents: the Bordeaux years (1971 to 1998)

In 1968, Josy had become a French citizen and was appointed director of research at INRA, and in 1971, he moved from Versailles to Bordeaux to become head of the laboratory for cellular and molecular biology at the INRA/University of Bordeaux campus, where he and his group carried out most of their research on the etiology of citrus diseases. Although in vitro culture of spiroplasma and the discovery of the HLB bacterium had started at Versailles, the acquisition of an electron microscope and an analytical ultracentrifuge for the new laboratory would greatly facilitate future work. In the Bordeaux laboratory, the spiroplasmas were always a model system to study plant mycoplasmas, particularly for those discovered in 1967 in Japan. While spiroplasmas can be cultured in vitro and they have a helical morphology and show motility, the organisms found in Japan, now called phytoplasmas, are non-helical mycoplasmas and so far nobody has succeeded with growing them in vitro. The phytoplasmas are more important in plant pathology and agriculture than spiroplasmas, being responsible for several hundred diseases, whereas only a few diseases are caused by the three phytopathogenic spiroplasmas presently known: *Spiroplasma citri*, the causal agent of the stubborn disease of citrus, *S. kunkelii*, the causal agent of corn stunt, and *S. phoeniceum*, found by Josy in Syria affecting periwinkle, albeit its natural host is unknown.

Spiroplasmas are also found in insects and other arthropods. Most of the ~40 spiroplasma species that have been reported are transmitted by and/or live in insects and ticks. For example, two important bee diseases are caused by *S. melliferum* and *S. apis*, respectively. The latter was characterized by Josy's group in Bordeaux.

The stubborn agent has been the subject of many molecular and cellular studies not only during Josy's years of activity, but also after his retirement when his co-workers (J Renaudin, C Saillard, P Carles, X Foissac, to cite only a few) took over. They developed the necessary tools to study the molecular biology and genetics of *S. citri*:

cloning vectors for transformation, transposons for insertional mutation, mutation by gene disruption and others. They obtained spiroplasma mutants that enabled finding the genes involved in pathogenesis and in spiroplasma transmission by insect vectors. These studies culminated in determining the complete sequence of the spiroplasmal genome in 2005. Obviously, these studies were accomplished with *S. citri* because it could be grown in vitro and an array of molecular techniques were available. Presently, similar experiments cannot be performed with phytoplasmas, since they are not culturable and molecular techniques are difficult to apply.

In 1970, Josy Bové and his co-worker, Dominique Laflèche, discovered that huanglongbing (HLB), still called greening in these early days, was also associated with a bacterium. As indicated above, both the stubborn and the greening agents were restricted to the sieve tubes in the phloem tissue, but they turned out to be completely different, as shown by EM, response to antibiotic treatments, serology and different molecular techniques (see below).

Electron microscopy

The stubborn agent was identified as a mycoplasma restricted to sieve tubes by EM in 1970 (see above). At the same time, EM was also used to examine the situation with HLB and Josy and co-workers detected bacteria associated with sieve tubes in plants infected with both the African and the Asian HLB. They first thought that these bacteria were similar to the stubborn mycoplasmas (Laflèche and Bové 1970a, 1970b), but soon realized that, in contrast with the true stubborn mycoplasma which are surrounded by a ~70Å thick cytoplasmic membrane, the HLB bacteria had a cell envelope with a thickness of ~200Å, indicating that they had a cell wall in addition to the cytoplasmic membrane (Saglio et al. 1971b).

Monique Garnier, who became in charge of the new electron microscope, was able to show, conclusively by sophisticated EM techniques, that the HLB bacterium did have a cell wall and this wall was of the gram-negative type (Garnier et al. 1984a, 1984b). In contrast to the stubborn organism, the HLB bacterium has not been cultured in vitro and the characterization work had to be performed on ultra-thin sections in situ.

In the hands of Monique Garnier, EM became the first laboratory technique to detect and identify the HLB bacterium. In this way, they confirmed the disease in many African and Asian countries including Bhutan, Cambodia, India, Indonesia, Laos, Malaysia, Mauritius, Nepal, Reunion island (France), Saudi Arabia, South Africa, Vietnam, Yemen and Zimbabwe. HLB-detection laboratories were set up in Kathmandu (Nepal), Batu (Eastern Java, Indonesia) and Thimphu (Bhutan) (Bové 2014). This extensive work showed 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.

Effect of antibiotics

Antibiotics are effective against bacteria, not against viruses! As soon as Josy's group had discovered the HLB bacterium by EM, they telephoned Ralph Schwarz at Nelspruit (South Africa) to suggest antibiotic experiments using tetracycline hydrochloride, an inhibitor of bacterial protein synthesis, against HLB by injection into sweet orange trees. Trunk injection of the antibiotic solution in water (250-1000 ppm) was tried by gravity or under pressure. Even though remission of symptoms was observed, tetracycline injections did not result in a practical treatment. The treatment was expensive and symptom remission was only temporary, tetracycline being bacteriostatic, not bacteriocidal. Treated trees produced small fruits and showed phytotoxic effects occurring in vascular bundles at the injection site, and high levels of residues were found in the fruits of treated trees (Schwarz and Van Vuuren 1971; Schwarz et al. 1974; Moll and Van Vuuren 1977; Moll et al. 1980; Buitendag and Bronkhorst 1983; Buitendag and Von Broembsen 1993). Tetracycline treatments were also conducted in Reunion Island (Aubert and Bové 1980), Taiwan (Su and Chang 1976; Chiu et al. 1979) and Indonesia (Supriyanto and Whittle 1991).

The effect of penicillin, an inhibitor of peptidoglycan synthesis in the bacterial cell-wall, was 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 (Bové et al. 1980) as well as on field-grown trees in Reunion Island (Aubert and Bové 1980). The beneficial effect of penicillin on HLB-affected plants was evidence for the presence of peptidoglycan in the cell-wall of the HLB bacterium. This antibiotic had no effect on stubborn-affected sweet orange plants as the stubborn agent was found to be a mycoplasma, i.e. a bacterium without a cell-wall, and thus, without peptidoglycan.

Characterization of the HLB bacterium with monoclonal antibodies

Thirteen monoclonal antibodies (MAs) against the African or Asian HLB bacterium were produced in Josy's lab in Bordeaux; ten were obtained by in vivo immunization of mice and three by in vitro immunization of spleen cells. They were evaluated for the detection of the HLB bacterium by immunofluorescence in thin sections (Martin-Gros et al. 1987; Garnier, Iskra, et al. 1987, Garnier, Martin-Gros, et al. 1987, Garnier et al. 1991; Gao et al. 1993). None of the three MAs against the African HLB bacterium (Nelspruit strain) detected the 11 Asian strains and none of the 10 MAs against two Asian strains [Poona (India) and Fujian (China)] detected the African strain, suggesting the existence of two different species of HLB bacteria. MAs obtained by in vitro immunization against an Asian strain from Poona (India) recognized most other Asian strains, but not the African strain (Gao et al. 1993). The MAs obtained by in vivo immunization were quite specific to the strain used for immunization and, therefore, were not suitable for generalized diagnosis of HLB (Garnier et al. 1991).

MAs have been used for purification of the HLB bacterium by immunoaffinity chromatography followed by immunogold labeling. This was the first time purified, individual HLB bacteria were seen out of the sieve tubes (Villechanoux et al. 1990).

Taxonomy of the African and Asian HLB Bacteria

With the HLB bacteria not having been cultured, 16SrDNA of an Asian strain (India, Poona strain) and 16SrDNA of an African strain (South Africa, Nelspruit strain) were obtained from infected leaves by PCR-amplification and cloning. The 16SrDNAs were sequenced and the two sequences were compared with 16SrDNA from the GenBank data base. The comparison revealed that the two HLB bacteria 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 (India) strain and the African (South Africa) strain represented two different species, respectively "*Candidatus Liberibacter asiaticus*" (Las) and "*Candidatus Liberibacter africanus*" (Laf), confirming previous work on heat sensitivity of HLB, effect of penicillin and serology with monoclonal antibodies (Jagoueix et al. 1994, 1997).

Detection of the African and Asian Liberibacters PCR-amplification of 16SrDNA

PCR amplification of 16SrDNA was the first DNA-based technique to detect the liberibacters (Jagoueix et al. 1996a, 1996b). 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 *Xba*I: while in the case of Las the amplicon possessed only one *Xba*I restriction site and yielded two restriction fragments (520 bp and 640 bp), the Laf amplicon had two sites and gave three restriction fragments (520 bp, 506 bp and 134 bp).

PCR amplification of ribosomal protein genes from the β -operon

To avoid restriction with *Xba*I, a second PCR technique was developed, based on the sequence of the *rplKJL-rpoBC* operon, also named β -operon, which codes for proteins K, A, J and L of the large ribosomal subunit (50S) and proteins B and C of the bacterial RNA-polymerase. Josy's group had been able, previously, to clone and sequence part of the β -operon from Las (India, Poona strain) as a 2.6 kb sequence, named In-2.6 (Villechanoux et al. 1992, 1993). Using the sequence of In-2.6 from Las, they defined primers for PCR amplification of the equivalent β -operon genes of Laf (strain Nelspruit, South Africa); an amplicon of 1.7 kb was obtained and named 1.7-AS (Planet et al. 1995). Two primers, A2 and J5, were defined from the comparison of the In-2.6 and 1.7-AS sequences. Primer A2 is at the 3' end of gene *rplA* and primer J5, at the 3' end of gene *rplJ*; PCR amplification with these primers yield amplicons of 667 and 701 bp for Laf and Las, respectively. The 34 bp difference between

the two amplicons comes from the fact that the intergenic region separating *rplA* from *rplJ* is 34 bp larger in Las than in Laf (Hocquellet et al. 1999).

The titer of HLB liberibacters are much higher in periwinkle than in citrus (Garnier and Bové 1983). Periwinkle plants infected with Las or Laf by dodder transmission from infected sweet orange plants, were used to develop the above PCR detection techniques.

DNA/DNA hybridization with β -operon derived probes

The above Las In-2.6 and Laf AS-1.7 sequences, labeled with (32 P)dCTP by random priming, were used as DNA probes for dot blot hybridization. Probe In-2.6 detected all Asian Las strains tested (India, Thailand, the Philippines, Indonesia, China, Taiwan), but not the South African Laf strains. Inversely, probe AS-1.7 recognized South African Laf but not Asian Las (Villechanoux et al. 1992; Planet et al. 1995). DNA hybridization has been used in particular to detect and confirm HLB in India (Bové et al. 1993; Varma et al. 1993), South Africa (Korsten et al. 1996), Nepal (Regmi et al. 1996), and Vietnam (Bové et al. 1996). The probes were also used to detect the liberibacters in psyllids in Malaysia (Bové et al. 1993).

Distribution of Laf and Las before ~2000. Origin of Laf and Las

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 was examined in 10 African and 17 Asian countries. Only heat-sensitive HLB, with Laf and *T. erytrae*, 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 (Bové 2014). Reunion and Mauritius islands also harbored both African and Asian HLB.

It was proposed previously that a Laf and Las ancestor was 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 was proposed that Las acquired its heat-tolerance while the Indian plate crossed the hot equatorial zone (Nelson et al. 2013).

Additional activities during the Bordeaux period (1971 to 1998)

Although endogenous bacteria, namely spiroplasmas, phytoplasmas and liberibacters, were the main objective of Josy's team in Bordeaux, they also developed some activities, mainly cooperative activities with other laboratories, on citrus viruses and viroids. As indicated above, Josy participated actively in the development of the citrus experiment station at San Giuliano in Corsica (France), created by IRFA in 1958, and later collaborated with this station during his full active life. This collaboration, particularly with Robert Vogel, resulted in the discovery of three new graft-transmissible diseases of

citrus: (i) *cristacortis*, a disease spread in the Mediterranean citrus industry characterized by the presence of large pits and sometimes with gum at the bottom of the pits in different mandarin and hybrid varieties, as well as in sweet and sour oranges (Vogel and Bové 1968); (ii) a disease affecting rough lemon with sweet orange scions inciting bud-union crease with indentations and wood staining (Grimm et al. 1955; Mc Lean 1974); and (iii) the first strain of *Citrus tristeza virus* (CTV) causing no decline on sweet orange trees propagated on sour orange rootstock and no symptom on indicator seedlings of Mexican lime [*Citrus aurantifolia* (Christ.) Swing.] (Bové et al. 1988). This latter finding made it clear that CTV detection by biological indexing could be unreliable with some CTV isolates and that EM, serology, or RNA-based detection of the virus should be used in those cases. Josy's lab also was able to obtain one of the first genomic libraries of the CTV genome. In cooperation with the Spanish virology group of IVIA, this DNA library was used later to examine genetic variation of CTV isolates and to demonstrate that aphid transmission often alters the population structure of CTV isolates (Albiach-Martí et al. 1996, 2000), thus helping to explain in part the biological diversity observed among CTV isolates from the same citrus area.

After 1985, spiroplasmas, phytoplasmas and liberibacters became the only research subject of Josy's group, albeit the HLB bacterium served as a model to discover Phlomobacter, the pathogenic agent of a new disease called "strawberry marginal chlorosis", found in the Bordeaux region and affecting about 60% of the strawberry-growing surface. This is also a non-cultured bacterium, but different from the liberibacters. While the latter is an alpha-proteobacterium, the phlomobacter causing marginal chlorosis of strawberry is a gamma-proteobacterium (Zreik et al. 1998).

Strawberry culture comprises two steps separated in time and space: the nurseries for plant propagation and the field for strawberry production. Marginal chlorosis was observed in plants of both steps, but while the phlomobacter was readily detected by molecular methods (Foissac et al. 2000) in the production fields, the same methods were unable to detect it in affected nursery plants. This puzzling result was explained some weeks later when they discovered that a well-known phytoplasma affecting tomato was able to infect nursery strawberry plants and induce symptoms identical to those incited by phlomobacter in the production fields. Phlomobacter is vectored in the field by the planthopper *Cixius wagneri* (China) (Danet et al. 2003).

International cooperation

There were two traits in the personality of Josy Bové that are important to fully understand his scientific career as well as his personal autobiography. The first is his permanent interest in crop plant problems and his sharp observation capacity in the field, even when his original background had been mostly oriented to biochemical and molecular biology, as the proper tools to study plant

diseases. His deep involvement in IOCV activities since its foundation in the Riverside meeting in 1957 and his close friendship with Victoria Rossetti (Instituto Biológico, São Paulo, Brazil), who invited him to her laboratory and showed him the main diseases affecting citrus in São Paulo. This probably fostered his interest for real field problems. The result was an outstanding and relatively uncommon synthesis between laboratory and field approaches by which, surveying citrus plantings Josy was one of the best experts in disease symptom identification, while he was also the main reference for the use of molecular tools for disease characterization and control. The second trait was his clear and well-organized mind and his ability to make the most complex problems into simple questions. This together with his capacity and willingness to explain his views and share his knowledge with anyone who was really interested, made him an ideal collaborator. Let us complete the picture by saying that his open mind free of prejudices was always ready to cooperate with anyone having an important problem to solve, no matter the country, religion or political system of the partner.

As a result of these traits from 1981 to 1993, acting as a consultant of the Food and Agriculture Organization, Josy surveyed many countries in the Near East, the Middle East and Western Asia, for diseases of citrus (Bové, 1995).

This is how he and his co-workers discovered: (i) that the leafhopper-vector of stubborn disease in Morocco, Turkey and Syria was *Circulifer haematoceps* and not *C. tenellus*, the main vector of the disease in California (Bové et al. 1988); (ii) the presence of HLB in Saudi Arabia, Yemen and Somalia (Bové and Garnier 1984; Bové 1986; Bové 1995); and (iii) a new disease of citrus in Oman, witches' broom disease of lime trees (WBDL), caused by a non-cultured mycoplasma, *Candidatus Phytoplasma aurantifolia*, and vectored by the leafhopper, *Hishimonus phycitis* (Bové et al. 1988; Garnier et al. 1991), as later confirmed in Iran (Salehi et al. 2002). WBDL is also severely affecting citrus in the United Arab Emirates and Iran.

He had important input in the development of the citrus industry in countries like Nepal and Bhutan in which citrus was still propagated as seedlings, where he encouraged the implementation of grafted trees and the need to develop citrus nurseries (Regmi et al. 2010). His many visits in the region prompted a fascination for Buddhism and Hinduism that was enhanced by the fact that they were considered a "philosophy of life" and not a religion. Many characteristic items including the Buddhist flags were part of his home.



From 1989 to 1993, Josy and his team, in collaboration with Victoria Rossetti (Brazil) and CG Chang (University of Georgia), demonstrated by electron microscopy and serology that citrus variegated chlorosis (CVC), a disease affecting more than 50% of all sweet orange trees in São Paulo State, was caused by the well-known plant bacterium, *Xylella fastidiosa*, the xylem-restricted agent of a most severe disease of grapevine, Pierce's disease. Collaboration with Brazil continued and strengthened after Josy's retirement (see below). In October 2015, he even followed *X. fastidiosa* when it was reported as damaging and killing the very old olive trees in Apulia (Southern Italy).



Particularly intense and prolonged in time were the cooperative and friendship relationships of Josy with several research groups in Spain, particularly with plant pathologists at IVIA. Some of them received training on various subjects at Josy's laboratory in Bordeaux, and Josy was invited many times to Valencia to attend conferences and participate in different courses on citriculture. Scientific cooperation formally started in 1987 after Josy organized a laboratory workshop inviting eight IVIA scientists to exchange ideas and projects with his own group, and it would continue with variable intensity until retirement or death of the members of each group, including Josy. Results of this cooperation were: (i) surveys to collect *S. citri* and phytoplasma isolates from Spain to be analyzed in Bordeaux; (ii) introduction of molecular biology approaches at the Virology Laboratory of IVIA and the use of the cDNA library of the CTV genome obtained in Bordeaux to study genetic variation of CTV in Spain (Albiach-Martí et al. 1996); (iii) setting up a joint experiment to test the effect of different viroids on several scion/rootstock combinations under field conditions (Vernière et al. 2004, 2006); (iv) anatomical study of bud-union samples from plants affected by citrus sudden death (CSD) by the team of Mariano Cambra at IVIA (Roman et al. 2004), after Josy was asked to study this problem of the Brazilian citrus industry (see below); and (v) a series of lectures and a detailed survey of the Northern and Northwestern coast of Spain to assess the incidence and movement of the South African HLB vector, recently introduced in the Iberic Peninsula (Duran-Vila et al. 2014; Duran-Vila and Bové 2015; Bové and Duran-Vila 2016).

The input of Josy in terms of encouraging collaboration between IVIA and INRA-CIRAD in Corsica was essential to define the effect of single viroids and viroid combinations in the performance of citrus in the field. Following a first contact with his friend R Vogel, a successful collaboration with his team that lasted many years, was undertaken to establish a field plot and evaluate the performance of inoculated clementine trees grafted on *Poncirus trifoliata* rootstocks, which was the major rootstock scion combination in commercial groves in Corsica. The experience of Josy in the field was critical in terms of symptom evaluation and the overall results confirmed that: (i) *Citrus exocortis viroid* (CEVd) induced exocortis symptoms on trifoliolate orange; (ii) only the cachexia variant of *Hop stunt viroid* (HSVd) induced cachexia symptoms; (iii) CEVd, HSVd, or *Citrus bark cracking viroid* (CBCVd, formerly CVd-IV) induced bark cracking symptoms on the trifoliolate orange rootstock; and (iv) additional information regarding dwarfing and yield reduction as well as changes in the expected performance as a consequence of synergistic and antagonistic viroid interactions were also identified (Vernière et al. 2004, 2006). However, since in many citrus growing areas the major rootstocks are Citrange hybrids, additional field assays were also established in Spain to define the performance of viroid infected trees grafted on such rootstocks following Josy's advice and cooperation (Murcia et al. 2015).

The cooperation with IVIA and the frequent visits Josy paid, gave the impression that for him Valencia was almost his second home.

In Brazil with Fundecitrus on CVC and CSD (1998 to 2004)

Since 1998, when he retired, Josy collaborated actively with Fundecitrus, the foundation for the sanitary control of citrus in São Paulo State (SPS), Brazil, on various citrus diseases including: (i) citrus variegated chlorosis (CVC), a disease which had been shown to be caused by *X. fastidiosa* (Chang et al. 1993); and (ii) citrus sudden death (CSD), a tristeza-like, bud union disease of sweet orange trees grafted on Rangpur lime (Bové 2005; Román et al. 2004).

CSD, a disease never seen before, killed trees grafted on Rangpur lime, with a pathological anatomy at the bud union being very similar to that of tristeza quick decline. In addition to citrus tristeza virus (CTV), endemic in SPS, CSD was found by Allelyx Applied Genomics (Campinas, SPS, Brazil) to be associated with a second virus, a marafivirus (*Tymoviridae*). CSD is transmissible by graft inoculation and by aerial vectors, probably aphids (Coletta-Filho et al. 2010), but the respective roles of the two viruses is not yet understood. The disease was finally controlled by inarching, i.e. approach-grafting seedlings of tolerant rootstocks to the scion trunk of affected trees on Rangpur lime.

In Brazil with *Fundecitrus* on HLB bacteria (2004 to 2016)

Old and new HLB Liberibacters in São Paulo State, Brazil

In March 2004, HLB was identified for the first time in Brazil, namely in Araraquara, SPS. It became the major worry of Fundecitrus and also of Josy who dedicated to it the remaining twelve years of his life.

Two liberibacters were detected by the end of 2004: 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 was found in more than 90% of the affected trees (Teixeira, Ayres, et al. 2005; Teixeira et al. 2005a, 2005b; Teixeira, Eveillard, et al. 2008). However, from 2005 onward, the percentage of Lam-infected trees decreased, while the percentage of Las-infected trees increased. Today, most trees are infected with Las; Lam-infected trees are rare. This is probably because: (i) Lam, like Laf, was found to be heat-sensitive and (ii) the titers of Lam in sweet orange trees were 10 times smaller than those of Las, rendering transmission of Lam by psyllids less efficient (Lopes and Frare 2008; Lopes, Bertolini, et al. 2009; Lopes, Frare, et al. 2009). Both liberibacters, Las as well as Lam, were found to be transmitted by *D. citri* (Yamamoto et al. 2006). *Murraya paniculata* (Jasmin orange) was found to be a good host of Lam (Lopes et al. 2005, 2006). A diagnosis laboratory for detection of Las and Lam for citrus growers (free of charge) and researchers was established. PCR detection of liberibacters kept improving, with increased sensitivity, from conventional 16SrDNA-PCR and β -operon-PCR to nested PCR and RTi-PCR (Teixeira, Danet, et al. 2005; Teixeira, Lopes, et al. 2005; Teixeira, Saillard, et al. 2008).

The genome size of Lam was determined by pulse field gel electrophoresis to reach 1.3 Mbp (Wulff, Eveillard, et al. 2009). In collaboration with Dean Gabriel (University of Florida), the complete genome sequence of Lam was accomplished, yielding a genome size of 1,195,201 bp, and revealing that Lam is missing genes related to lipopolysaccharide (LPS) biosynthesis (Wulff et al. 2014). LPS is a constituent of the outer membrane of Gram-negative bacteria.

A citrus phytoplasma inducing HLB symptoms

In the Barretos region of northern SPS, sweet orange trees with characteristic leaf and fruit symptoms of HLB were discovered, but, surprisingly, they tested negative for liberibacters! PCR-amplification and sequencing of 16SrDNA identified a phytoplasma having 99% 16SrDNA sequence identity with the “pidgeon pea witches’ broom phytoplasma”, a 16Sr group IX phytoplasma. Primers specific for PCR detection of the HLB phytoplasma by 16SrDNA amplification were developed (Teixeira, Wulff, et al. 2008). The HLB phytoplasma also was detected in *Crotalaria Juncea* (Sunn hemp), a cover crop plant widely distributed throughout SPS (Wulff, Teixeira, et al. 2009). *Scaphytopius marginelineatus*, a leafhopper frequently found in sweet orange orchards (Marques et al. 2012) was

shown to efficiently acquire the HLB phytoplasma from affected sunn hemp plants and to transmit the phytoplasma to sweet orange, albeit rarely (Wulff et al. 2015).

Even though the HLB phytoplasma is widely distributed throughout SPS, the number of trees affected by the phytoplasma in citrus farms was small, with a disease incidence ranging from 0.1% to 1.8% affected trees. Also, most of the affected trees were distributed randomly and, in 80% of the cases, the minimum distance between affected trees was 100 m, suggesting that primary infection of citrus trees is a rare event and that secondary infections from citrus to citrus did not occur (Wulff et al. 2015).

Trials to control HLB: Management by the three-pronged system (TPS)

After discussion with different scientists and technical staff of the main citrus growing companies, Josy was able to convince most of them to start farm management by the phytopathologically sound “Three-Pronged System” (TPS) in July 2004, only three 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. Ground applications involved foliar, trunk and drench applications. The TPS is a preventive HLB-management system to reduce more trees from becoming infected.

The TPS would function ideally if: (i) the insecticide treatment would kill 100% of the psyllid vectors; and (ii) if inspections would be able to eliminate all liberibacter-inoculum, i.e. all liberibacter-infected trees. Unfortunately, (i) insecticide treatments are not 100% efficient: some insects escape; and (ii) removing symptomatic trees does not result in removing all infected trees, because some trees already infected may not show symptoms at the inspection time. For a given tree, the period between the time of infection by the psyllids and the time at which symptoms appear (the latency period) 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, would become symptomatic and would be identified at inspection # n+1. It was found that having four inspections per year is a minimum.

Another difficulty comes from the fact that even the best team of inspectors can identify only ~60% of symptomatic trees at each inspection, with ~40% escaping detection. This comes from the fact that the symptoms to be seen by the inspectors are early symptoms on a few shoots only and can be easily missed. Here again, trees, which escaped detection at inspection # n are likely to be detected at inspection # n+1.

The way management by the TPS works in a given farm has to be evaluated carefully by recording the number of symptomatic trees removed at each block of the farm at each inspection. The removed trees should be carefully

marked on the farm map to allow the determination of HLB incidence in the various parts 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. In this way, the TPS really tells what is going on and what needs to be done. This is how the “border effect” was discovered, namely the fact that 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. The border effect requires that psyllid control be heavier on the borders than inside the groves. The less borders, the less psyllids, and the less extra border control. For the same grove size, a circular or a square grove has mathematically less borders than a rectangular grove. Flat, elongated rectangular groves are the worst.

Symptom expression varies during the year; it is most distinctive in autumn/winter, and less visible in spring/summer. Therefore, comparing the number of trees removed in December (summer in Brazil) with the number from the previous July (winter in Brazil) is meaningless!

Inspections were first carried out by inspectors on the ground, walking from tree to tree. Detection was greatly improved by using tractor-pulled platforms to enable the inspectors to look down on trees. It was found that on many adult trees, symptoms begin to appear on the top of the trees. Platforms with four inspectors had come in to use, the lower two inspectors looking at the sides of the trees, the upper two at their top. Some farms had inspectors on horses.

Major Citrus companies in SPS (Agrindus S/A, Branco Peres, Cambuhy, Citrosucco Fisher, Cutrale, Louis Dreyfus Commodities, NovAmerica, Rancho Rey fazenda) shared their data on TPS management with research institutions (Fundecitrus, UNESP, University of Bordeaux & INRA). Factors that make it easier or more difficult to achieve HLB-control in SPS were identified (Belasque et al. 2010) and are indicated in the table opposite.

Notice that factors 1 to 6 cannot be changed to influence management. Only factors 7 to 12 can be modified by growers to improve, if necessary, the management of HLB. Application of the above management measures in the SPS citrus industry allowed the following conclusions:

- The TPS began immediately after HLB had been 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, 200,000 trees) used the TPS.
- From 2004 to 2016, the TPS was constantly improved and adapted to changing situations.
- The need to control psyllids and to remove symptomatic trees was confirmed.

| Factors making it easier or more difficult to achieve HLB-control by the TPS in SPS | Easier | More difficult |
|---|------------------------|----------------------|
| 1. Size of farm | Large (≥ 400 ha) | Small |
| 2. Shape of farm | Square | Long, flat rectangle |
| 3. Type of trees | Adult | Young |
| 4. HLB-Incidence in the region | High | Low |
| 5. Presence of “bad” farms | Far away | Close, adjacent |
| 6. % of HLB-trees at 1st inspection | Low ($< 1\%$) | High ($> 10\%$) |
| 7. 1st inspection after farm became infected | Immediately after | Long after |
| 8. Number of inspections with platforms | 12 / year | 4 / year |
| 9. Number of insecticide treatments (I.T.) | 24 / year | 6 / year |
| 10. High tree-density at borders | Yes | No |
| 11. Extra I.T. at borders | Yes | No |
| 12. Dormant insecticide treatments | Yes | No |
| Quality of control | Excellent | Poor |

- The major difficulty for well-managed farms was the presence of “bad” farms, i.e. neighboring farms with no or poor HLB-management.
- The TPS made it possible to keep the HLB-incidence in the farm below $\sim 1\%$ affected trees per year, $\sim 98\%$ trees being healthy, symptomless and non-infected.
- The acreage of TPS-managed farms with low ($\leq 1\%$) HLB-incidence amounted to $\sim 200,000$ ha, almost half of the total citrus acreage in SPS.
- SPS is the only region in the world where the TPS has been so successful on a large scale.
- The successful results of the TPS (not predictable in 2004!) changed the perspective of the SPS citrus industry (Bové 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 will become commercially available, SPS will have two long-term options: (i) orchards with HLB-resistant GMC-trees; and (ii) “low-HLB” orchards with regular, non-GMC trees, under TPS-management.

In Spain and Portugal looking for HLB and its vector

Josy learned that in summer 2014, the African citrus psyllid was recorded in Galicia (North-Western Spain) and

Northern Portugal (Pérez-Otero et al. 2015). Because this was the first time that one of the HLB vectors was found in countries of the Mediterranean Basin, in spite of his poor health, Josy initiated a survey to confirm its presence and distribution because of the potential of the vector to spread HLB to the nearby citrus growing areas. This survey of January 2016 was Josy’s last trip, which confirmed the presence of the vector but found no HLB-infected plants. As usual he provided farmers and plant pathologists with information and concern, both in Algarve (Portugal) and Andalusia (Spain).



Citrus pathologists lost an excellent professional and friend

As a result of the leukemia he suffered many years, Josy Bové left us on June 2, 2016. He was aware of his increasing weakness but having his brain as sharp as always, when Nuria Duran-Vila visited him at the hospital on May 24 to 25 he told her "...inform our friends...". It has been a very sad request because his friends and colleagues will never forget him. As an honor to him and to his wife Colette, INRA and the Bordeaux University dedicated one of the buildings to them.



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