INVITED REVIEW

HUANGLONGBING: A DESTRUCTIVE, NEWLY-EMERGING, CENTURY-OLD DISEASE OF CITRUS[1]

J.M. Bové

Laboratoire de Biologie Cellulaire et Moléculaire, Institut de Biologie Végétale Moléculaire (IBVM), Unité Mixte de Recherche en Génomique, Développement et Pouvoir Pathogène, Centre de Recherche INRA de Bordeaux, 71, Ave. Edouard Bourlaux, 33883 Villenave d'Ornon Cedex, France, and Fundecitrus, 201, Av. Dr. Adhemar Pereira de Barros, Araraquara S.P., Brazil

SUMMARY

A detailed account is given of the history, aetiology, biology, epidemiology, detection, geographical distribution, and control of huanglongbing (HLB), a destructive disease of citrus that represents a major threat to the world citrus industry, and is slowly invading new citrus-growing areas. HLB, whose name in Chinese means "yellow dragon disease", was first reported from southern China in 1919 and is now known to occur in next to 40 different Asian, African, Oceanian, South and North American countries. The agent is a phloem-restricted, non cultured, Gram-negative bacterium causing crippling diseases denoted "greening " in South Africa, "mottle leaf" in the Philippines, "dieback" in India, "vein phloem degeneration" in Indonesia. The HLB bacterium belongs to the genus *Candidatus* Liberibacter, three species of which are currently known, *Candidatus* Liberibacter asiaticus, occurring in Asian countries and, to a lesser extent, in Brazil and the USA (Florida), *Candidatus* Liberibacter africanus with its subspecies "capensis", recorded from African countries, and *Candidatus* Liberibacter americanus present in Brazil. The suggestion is that each liberibacter species has evolved in the continent after which it is named. HLB symptoms are virtually the same wherever the disease occurs. Infected trees show a blotchy mottle condition of the leaves that results in the development of yellow shoots, the early and very characteristic symptom of the disease. Trees are stunted, declining and bear a few, small-sized, and deformed (lop-sided) fruits, that are poorly coloured (greening) and with coloration starting at the peduncular end (colour inversion). HLB can be transmitted by grafting from citrus to citrus and by dodder to periwinkle. The psyllids *Trioza erytreae* and *Diaphorina citri* are natural vectors. Two different types of HLB are known: the heat-sensitive African form transmitted by *T. erytreae*, which develops at temperatures of 22-25°C, and the heat-tolerant Asian form, transmitted by *D. citri*, which stands temperatures well above 30°C. Although the HLB pathogen can be identified by electron microscopy, other laboratory methods are used for routine detection. ELISA with monoclonal antibodies is not recommended. Better systems are dot blot hybridization with a DNA probe, and various PCR formats (one-step, nested, multiplex) using species-specific primers based on 16S rRNA or *rpl*KAJL-*rpo*BC operon sequences. Because no curative methods of HLB are available, control is preventive and largely based on inoculum elimination by removal of infected trees and chemical treatments against vectors. Strict quarantine measures must be implemented to impair further international spread of HLB agents and their vectors.

Key words: Citrus, huanglongbing, liberibacter, psyllid vectors, diagnosis, control.

INTRODUCTION

Huanglongbing (HLB), a destructive disease of citrus, 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. Another destructive affection of citrus is tristeza disease, due to *Citrus tristeza virus* (CTV), which induces quick decline and death of citrus trees grafted on sour orange (*Citrus aurantium* L.) rootstock. Control of tristeza disease is achieved by replacing sour orange by rootstocks giving tolerant combinations with the scions. Many important citrus regions, where CTV is endemic, have learned to live with it. For HLB however, no control is known, except preventing the trees from becoming infected. Therefore, HLB is probably the most serious disease of citrus, much more serious than tristeza, and it represents a dangerous threat for regions still free of the disease, such as the Mediterranean basin, Western Asia,

Corresponding author: J.M. Bové
Fax: +33.557.122369
E-mail: joseph.bove@wanadoo.fr

[1] This review is dedicated to the memory of Professor S.P. Capoor (1912-1993), one of the most eminent phytopathologists of India, who carried out most of his work on virus and virus-like diseases of citrus and other crops at the Virus Research Center in Poona, Maharashtra, India.

Australia 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, Brasil, and in Florida, USA, two of the largest citrus growing regions in the world. This discovery has given a renewed interest in HLB, as witnessed by the organisation of an international workshop on HLB in November 2005 in Florida.

Even though HLB is newly emerging in America, it is probably one of the oldest known diseases of citrus. Reviews on HLB in Asia and Africa have appeared (Garnier and Bové, 1993; da Graça, 1991; da Graça and Korsten, 2004; Halbert and Manjunath, 2004). In this review, America will be covered as well. Until 1995, the disease was generally known under the South African name "greening". Today, the official designation is "huanglongbing", the most appropriate Chinese name (see below). Here, the abbreviation "HLB" will be used throughout as the generic name of the disease, regardless of the country where it occurs.

The mycoplasmal agent of citrus stubborn disease (Igwegbe and Calavan, 1970; Laflèche and Bové, 1970b) and the bacterial agent of HLB (Laflèche and Bové, 1970a) were discovered by electron microscopy (EM) in the same year, 1970. The stubborn agent was available in culture by 1971, characterized as *Spiroplasma citri* by 1973 (Saglio *et al*., 1973), and its genome sequence completed by 2005. Even today, the HLB bacterium has not yet been obtained in culture. This explains why characterisation of the HLB agent has not progressed as fast as that of *S. citri*. Molecular techniques had to become available to finally characterise the organism at the phylogenetic and taxonomic level, and this review will focus in particular on the nature of the HLB bacteria and their detection for HLB identification and confirmation. As the phytoplasmas and the HLB liberibacters have not yet been cultured, Koch's postulates could not be fulfilled. However, from overwhelming but indirect evidence, it is assumed that they are the causal agents of the diseases with which they are associated. This assumption will be followed here.

HISTORICAL BACKGROUND

Huanglongbing in China. Reinking (1919), evaluating diseases of economic plants in southern China, used English to report, in 1919, on "yellow shoot" of citrus, a disease which he thought to be of little importance in those days. However, later surveys showed that by 1936 the disease had spread to become a serious problem. The most extensive work on HLB in southern China was to be conducted from 1941 to 1955 by Lin Kung Hsiang (Fig. 1A). Born in 1910 in Cunxia, within the Fujian citrus belt, he obtained his Ph.D. at Cornell University, USA, in the late 1930s (see Lin Kung Shun* *et al.*, 1996). On his return to China, he joined the Christian University of Lingnan in Guangzhou, later to become the South China Agricultural University, and devoted most of his time to the study of HLB. Between 1941 and 1955, he carried out several surveys and field visits (Fig. 2). For instance, in 1943 he visited Taiwan, which he found affected by HLB, or rather "likubin", the name for HLB in the island. From the discussions he had with the farmers of the Chaozhou county in Guangdong province (see Fig. 2), he learned that HLB had been there since the 1870s, and on the basis of various observations he estimated that HLB in South China originated from that area.

In the Chaozhou district, the name given by the farmers to the disease was "huang long bing", "bing" standing for disease, "huang" meaning yellow, and "long", dragon, hence: yellow dragon disease. However, in other districts, different names were used. Lin considered that "huang long" was the most appropriate name, because "huang long", *i. e.* yellow dragon or yellow shoot, is the name given by the farmers to the new flush of growth on infected trees, and represents a characteristic, early symptom of the disease. Fig. 6 shows such yellow shoots or dragons. The most significant result obtained by Lin was the demonstration, by precise experimental work, that HLB is a graft-transmissible, infectious disease, and should neither be attributed to physiological disorders such as mineral deficiencies or water logging, nor to soil-borne diseases such as nematode infestation or *Fusarium* infection (Lin, 1956). In Lin's experiments, transmission of the HLB pathogen was rightly obtained by graft-inoculation. Previously, Chen Chi-bao (1943) had also obtained "transmission", but only by graft- propagation of HLB-affected shoots. As pointed out by Lin (1956), Chen failed to provide evidence for the systemic, infectious nature of HLB.

Lin's work was carried out during difficult times in China. The results were published in 1956 (Lin, 1956). It is often said that Lin's results remained practically unknown to the scientific community out of China. This is probably so, but there was at least one "Western" phytopathologist, Prof. Antonio Ciccarone, who not only knew the work of Lin, for having visited the Guangzhou area in 1956, but who also tried to make it known by publishing a note on "yellow shoot" in the Italian journal *Rivista di Agrumicoltura* 2: 45-50, 1957, in which he gave the reference to Lin's 1956 publication. In his note, Antonio Ciccarone pointed out rightly that in Lin's transmission experiments, which were all done in the open, some of the uninoculated control plants showed HLB symptoms. Lin was well aware of this situation, and this prompted him to undertake, early 1951, a second trans-

* Lin Kung Shun is the brother of Lin Kung Hsiang.

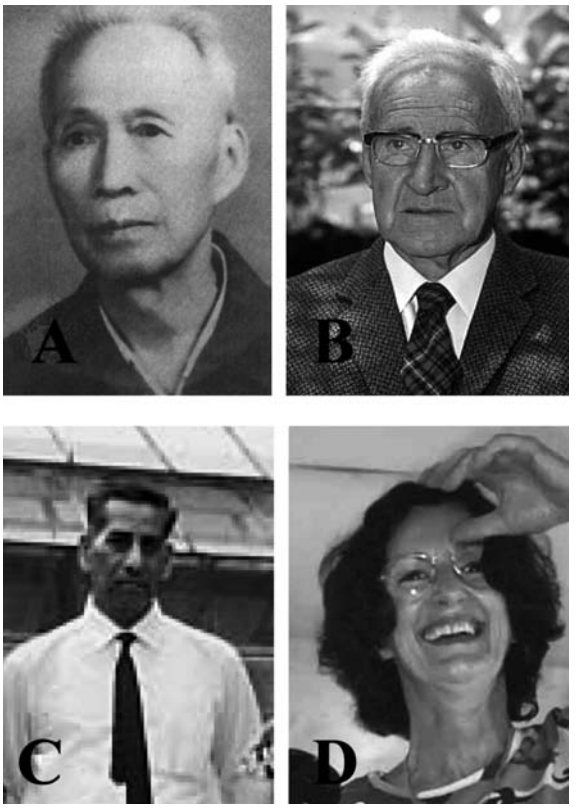

Fig. 1. Pioneering studies on HLB were conducted by: (**A**) Lin Kung Hsiang (1910-1986) in China (Ph.D. from Cornell University, USA), (**B**) A.P.D. McClean (1900-1995) in South Africa (Ph.D. from Natal University, South Africa), (**C**) S.P. Capoor (1912-1993) in India (Ph.D. from London University, UK), and (**D**) Monique Garnier (1949-2003) in France (Ph.D. from Bordeaux 2 University, France).

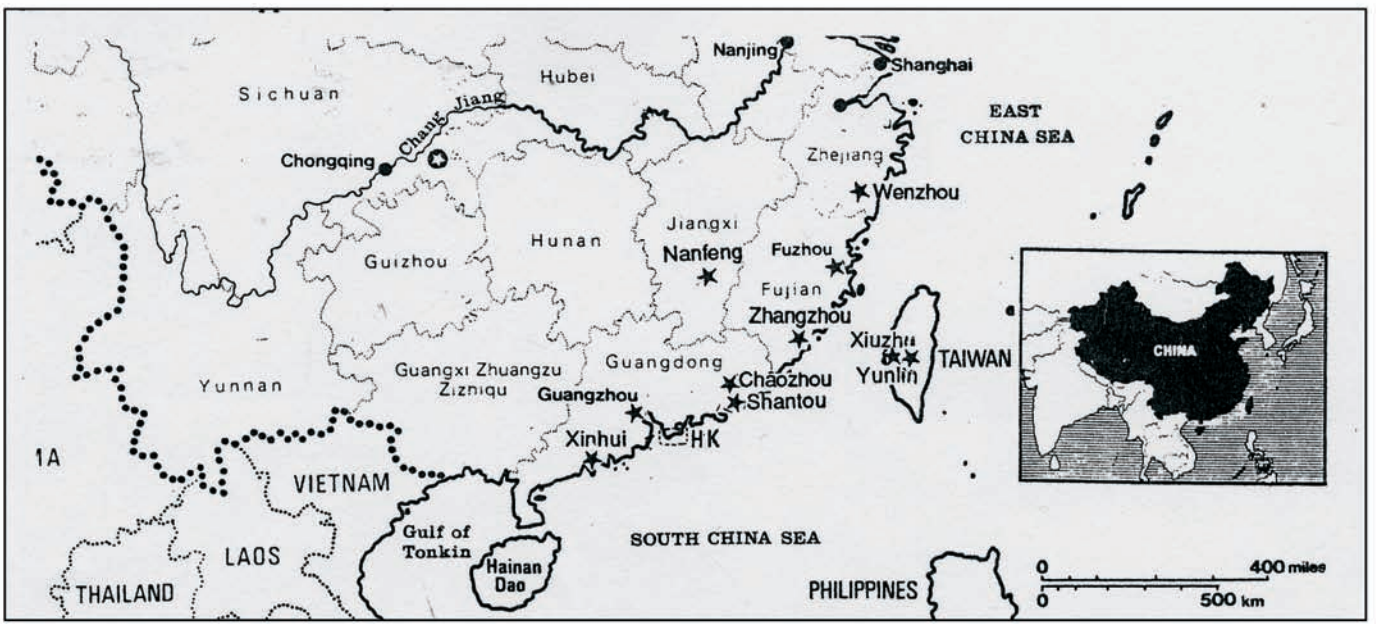

Fig. 2. Places visited by Lin Kung Hsiang between 1941 and 1955 in southern China and Taiwan. Found affected by HLB: (*), with no clear evidence for HLB: (o).

mission experiment involving a Tankan mandarin block and a Ponkan mandarin block. At the end of 1954, in the Ponkan block for instance, none of the 112 uninoculated control trees and none of the 50 uninoculated guard row trees were symptomatic, while as many as 83 of 94 inoculated trees showed HLB symptoms, and only 4 of 22 uninoculated guard row trees were symptomatic. These data clearly demonstrated transmission of the HLB pathogen by graft inoculation. In addition, in order to explain the fact that some uninoculated trees became affected, Lin assumed vector transmission to occur.

It has become evident that Lin was the first to demonstrate, by graft inoculation, the infectious nature of the disease, for which he appropriately used the name "huanglongbing". For these reasons, the International Organization of Citrus Virologists (IOCV) proposed in 1995 at the 13th conference of the Organization in Fuzhou (Fujian, China), that the official name of the disease be huanglongbing (HLB), and this proposal was accepted. Today, HLB is widely used for the African, American, and Asian forms of the disease.

Greening in South Africa. A disease similar to HLB, was observed in 1928 under the name "yellow shoot" in the western Transvaal, while the name "greening" prevailed in the eastern Transvaal.(Oberholzer *et al.*, 1965). However, the true nature of the disease was not immediately recognized, and, in the first description of "greening" in 1937, the problem was still assumed to be mineral toxicity (van der Merwe and Andersen, 1937). It was the collaboration between a phytopathologist, A.P.D. McClean (Fig. 1B), and a horticulturist, P.C.J. Oberholzer, that resulted in 1965 in the demonstration that greening was transmissible by graft inoculation (McClean and Oberholzer, 1965a) as well as by the African citrus psylla, *T. erytreae* (McClean and Oberholzer, 1965b). The infectious nature of "greening" was thus established, however ten years after that of "huanglongbing".

Mottle leaf in the Philippines. The disease was described in 1921, and thought to be related to zinc deficiency (Lee, 1921). It became a serious problem in the late 1950s. In 1966, Salibe and Cortez (1968) and Martinez and Wallace (1968) stressed the similarities between the symptoms of "mottle leaf" and those of HLB in China and Taiwan, and "greening" in South Africa. The major contribution of the Philippines to HLB was the demonstration, in 1967, that "mottle leaf" could be transmitted by the Asian citrus psylla, *D. citri* (Martinez and Wallace, 1967). In the same year, it was also reported in India that HLB could be transmitted by *D. citri* from trees affected by "citrus dieback".

Dieback and HLB in India. India is apparently another country where HLB seems to have a long history. As indicated by Capoor (1963), citrus in India has been known to suffer seriously from certain disorders resulting in low production, twig dieback, slow death and even sudden wilting. These symptoms had been attributed to "dieback", a disease that was first observed by Roghoji Bhonsale (cited by Capoor, 1963) in the 18th Century in the Central Provinces, soon after the introduction of citrus in India. It was also observed by Bonavia (1888) in Assam. However, the problem with Indian "dieback" comes from the fact that, as pointed out by Asana (1958), "dieback in citrus is not a specific disease". Similarly, for Capoor (1963), dieback was merely a symptom picture, and many factors, including soil disorders, nutritional deficiencies, twig fungi, and viruses, were evoked to account for it. In some cases, the major cause was CTV. Indeed, in the State of Bombay, it was clearly proven by the Virus Research Center at Poona that some type of dieback was due to CTV (Vasudeva and Capoor, 1958; Capoor, 1963). In other cases, HLB might have been involved. This was most certainly the case in the Coorg region, north of Mysore. As indicated by Asana (1958), in that area "the most pronounced symptom of the disease is the characteristic mottling of leaves". This was a very pertinent observation, as mottling is indeed, as known today, the most characteristic symptom of HLB. Several years later, positive dot blot hybridisation confirmed HLB in mottled Coorg mandarin leaves (Varma *et al.*, 1993), and members of the 12th conference of the IOCV in India in 1992 were able to see very severe HLB on the "Coorg" mandarin trees and other citrus cultivars in Gonicoppal and Chitalli.

In 1966, further support to the HLB hypothesis came from the work of Fraser and co-workers (Fraser *et al.*, 1966; Fraser and Singh, 1968). Questioning tristeza as the general cause of dieback, they indicated that CTV was not invariably present in dieback-affected trees. Many of the citrus species affected in India are tolerant to tristeza in other countries. Moreover, deficiencies of zinc and other minor elements were often implicated in dieback on a symptom basis, but applications of these elements failed to cure the dieback condition, although sometimes slight and temporary improvements were obtained.

These observations suggested that HLB might have been involved in dieback, and a survey for "dieback" in all major citrus areas of India was made. A striking feature of dieback was observed during the survey, namely the fact that, in early stages of the disease, leaf symptoms were often restricted to one or a few limbs. This symptom had already been described for "greening" in South Africa by McClean and Oberholzer (1965), and for "huanglongbing" by Lin (1956) in China, but Lin's work had remained unknown, as mentioned above. It was thus concluded that dieback was "caused by the virus responsible for greening disease of citrus in South Africa". However, this conclusion was based only on symptom observations, and was not confirmed by experimental work.

Unquestionable proof for the presence of HLB in India was eventually obtained at the virus Research Center at Poona by Capoor (Fig. 1 C) and co-workers, when they succeeded in transmitting the HLB pathogen by the Asian psylla, *D. citr*i (Capoor *et al.*, 1967). With this technique at hand, they were able to show that trees with dieback symptoms invariably proved positive for HLB. Among the trees positive for HLB, some carried CTV others did not. Finally, in 1971, Bové and co-workers were able to detect the HLB bacterium in a Musambi sweet orange seedling, which had been experimentally infected by *D. citri* nymphs with the "Poona" strain of HLB (and was thus free of CTV), and had been kindly provided by Prof. Capoor in 1969 (Laflèche and Bové, 1970b; Bové and Saglio, 1974). Many results on HLB in the Bordeaux laboratory have been obtained with the Poona strain.

Phloem necrosis and Vein phloem degeneration in Indonesia. In Indonesia, HLB is a major problem (Aubert *et al.*, 1985; Bové *et al.*, 2000b). Interestingly, the disease is called "Vein phloem degeneration" (Tirtawidjaja *et al.*, 1965).

In an anatomical study of greening-affected sweet orange shoots from South Africa, Schneider (1968) found localized pockets of necrotic phloem scattered throughout the vascular system of mature leaves, that blocked the translocation stream. Other anatomical aberrations were observed and believed to be reactions to the blockage, i.e. massive accumulation of starch in the plastids, disordered cambial activity with excessive formation of phloem, soon to become necrotic. Leaf mottle symptoms associated with HLB are very probably the consequence of these alterations.

Destruction of citrus by HLB in Thailand. HLB first appeared in Thailand in the 1960s and was so severe that the length of time between the onset of the disease and debilitation of the entire tree was about two years (Schwarz *et al.*, 1973). Roistacher (1996) has stressed the seriousness of the disease and its destruction of citrus in Thailand. HLB destroys 10 to 15% of tangerine trees each year and, in the northern regions, many citrus areas have gone out of production. In addition to being spread by the Asian psyllid vector, *D. citri*, the disease is also propagated by infected nursery productions. Indeed, Thailand was known as one of the rare countries in the world to use marcotting (or air layering) on a large scale to produce nursery trees.

ELECTRON MICROSCOPY AND THE BACTERIAL NATURE OF THE HLB AGENT

The HLB pathogen is a Gram negative bacterium. By 1967, at a time when the name "HLB" was not yet used, it became established that "greening" was transmissible by graft inoculation as well as by the two citrus psyllids. These results suggested that the pathogen causing greening was a virus, the only plant agent known at that time to be transmitted in these ways, and it became fashionable to speak of the "greening virus". For the same reasons, the expression "stubborn virus" entered the literature (see for instance Fraser and Singh, 1968; Salibe and Cortez, 1968). It was even thought that greening and stubborn, which had some common symptoms, were caused by different strains of the same virus.

In 1967, mycoplasma like organisms (MLOs) were found to be associated with plant diseases, the aetiologies of which were previously thought to be of virus nature (Doi *et al.*, 1967). Mycoplasmas are special bacteria, which lack a cell wall and are surrounded only by a single cytoplasmic membrane. Most of the "MLO-diseases" were of the "yellows" type, and their symptoms resembled those of greening and/or stubborn. For these reasons, search for such MLOs in greening-, and stubborn-affected sweet orange leaves was initiated by electron microscopy (EM), first in Versailles, and later in Bordeaux, France.

In both cases, micro-organisms were detected in the phloem sieve tubes of symptomatic leaves, but not in those of healthy leaves. In the case of stubborn, the micro-organism was soon obtained in culture, and turned out to be a new type of mycoplasma, one with helical morphology and motility, and named *Spiroplasma citri* (Saglio *et al.*, 1973). In the case of the greening organism, which could not be obtained in culture, it was initially thought that it was also a mycoplasma (Laflèche and Bové, 1970a, b), but the agent was soon found to be enclosed by a 25-nm-thick envelope, which was much thicker than the 7 to 10-nm-thick cytoplasmic membrane envelope, characteristic of mycoplasmas, including *S. citri* (Fig. 3 E) (Saglio *et al.*, 1971; Garnier *et al.*, 1976). These properties suggested that the HLB organism possessed, in addition to its cytoplasmic membrane, a bacterial cell wall. This was indeed found to be the case, and it could be shown that the cell wall was of the Gram negative type, composed of a membrane and a peptidoglycane layer (Garnier and Bové, 1977; Garnier *et al.*, 1984a). Thus, the HLB agent was a Gram negative bacterium (Garnier *et al.*, 1984b). As shown by Garnier and Bové (1977), in classic specimen fixation procedures for EM, the cell wall of the HLB bacterium appears as a characteristic, electron-dense layer surrounding the bacterial cell (Fig. 3 B, D), and this suffices for HLB identification. With special fixation methods, cell membranes reveal their triple layered ultrastructure, and the HLB bacterium is seen as surrounded by two such triple layered membranes (Fig. 3 C), i.e. the inner, cytoplasmic membrane, and the outer membrane, as part of the Gram negative cell wall (Garnier and Bové, 1977; see also Moll and Martin, 1974). Under

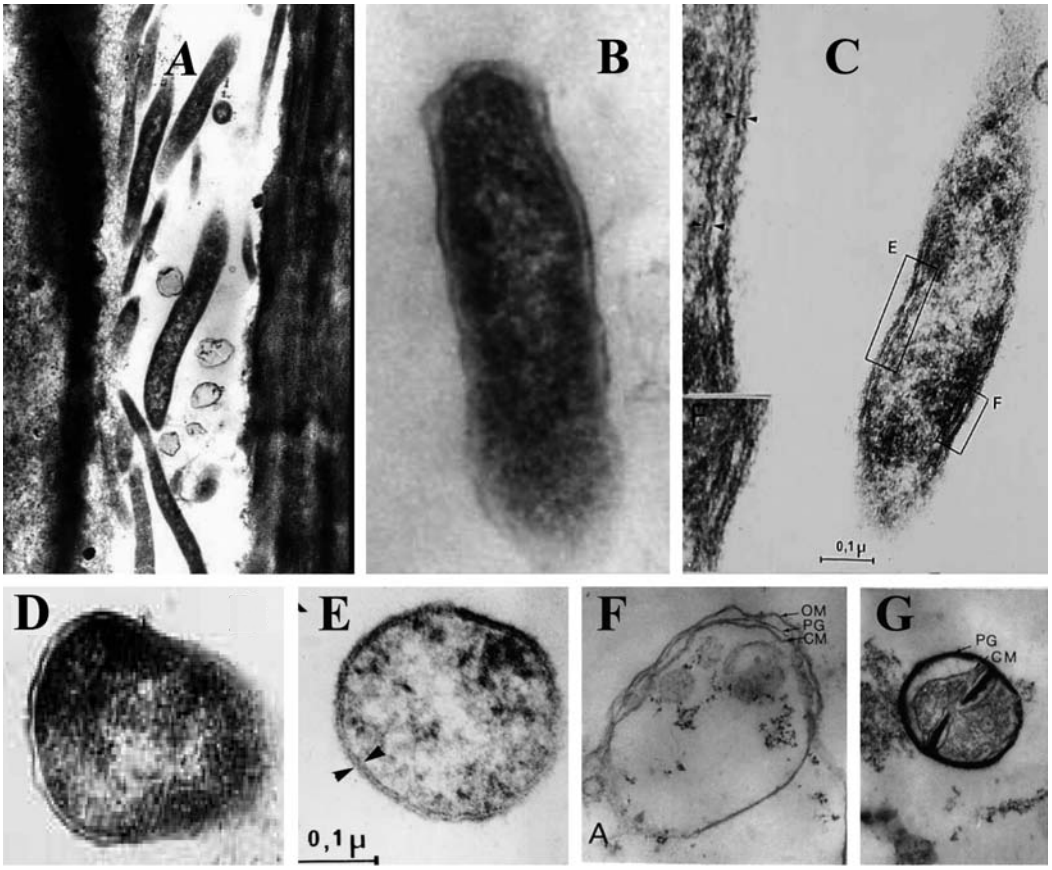

Fig. 3. Electron micrograph of liberibacter cells in a sieve tube of sweet orange leaf in Saudi Arabia (A). At higher magnification, the cells are seen surrounded by a cell wall, which shows up as an electron dense layer (B, D). At even higher magnification and with special techniques, one sees the triple-layered ultrastructure of both the outer cell wall membrane and the inner cytoplasmic membrane (C: → ←), while mycoplasmas, such as *Spiroplasma citri* seen here (E: → ←) have no cell wall, and are surrounded by a cytoplasmic membrane only. With special techniques, the peptidoglycane layer of the Gram negative cell wall can de visualized (PG on F) in between the inner membrane cytoplasmic membrane (CM on F) and the outer cell wall membrane (OM on F). The cell wall of Gram positive bacteria has no membrane, but only a heavy layer of peptidoglycane (PG on G), surrounding the cytoplasmic membrane (CM on G). All electron micrographs are from M. Garnier.

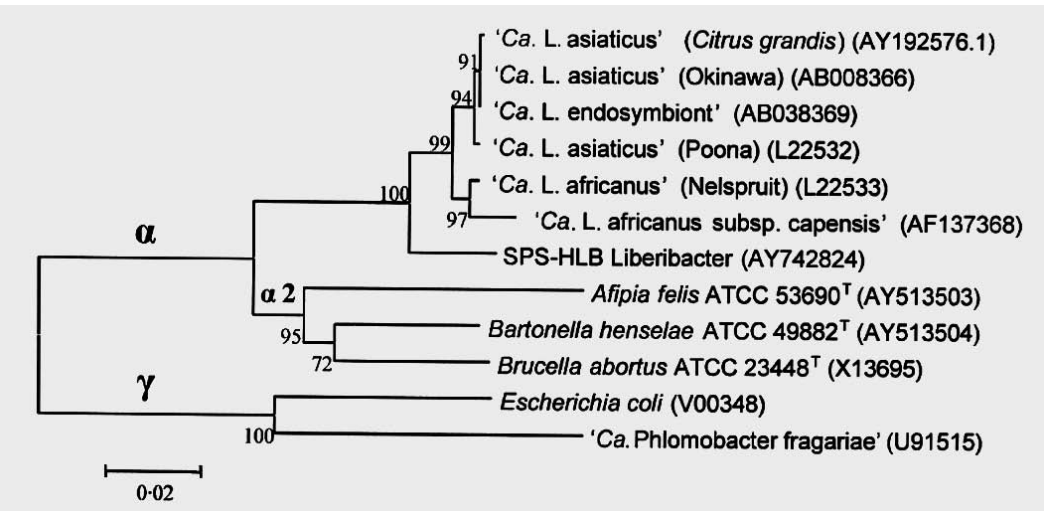

Fig. 4. Phylogenetic tree constructed with MEGA version 2.1 using 16S rDNA sequences (GenBank accession numbers in parentheses).

these conditions, the peptidoglycane layer (PG) is generally not seen, but sometimes, the inner zone of the cell wall membrane is somewhat thicker, more electron-dense than the outer zone, and is reminiscent of a PG. EM detection of PG (Fig. 3 F, G) required the use of cytochemical techniques involving papain treatments to separate the PG from the cell wall membrane, and lysozyme treatments to digest the PG and prove that it is indeed peptodoglycane (Garnier *et al*., 1984a, b). In control experiments, identical results were obtained with the Gram negative bacterium *Escherichia coli*, but not with the Gram positive bacterium *Staphylococcus aureus* (Fig. 3 G).

Indirect evidence for the Gram negative nature of the HLB bacterium had already been obtained prior to the above EM experiments, by studying the effect of penicillin on HLB affected trees. Penicillin inhibits a late step (transpeptidation) in the biosynthesis of peptidoglycane. The antibiotic was supplied to glasshouse-grown sweet orange plants through the roots (Bové *et al*., 1980), and to field-grown sweet orange trees on Troyer citrange in Reunion island by trunk injections (Aubert and Bové, 1980). In both cases, penicillin had a beneficial effect on HLB-affected citrus. Penicillin-treated citrus plants outgrew symptoms, had a much better root system, and produced larger symptomless shoots and leaves than untreated controls. No HLB bacteria could be seen in the sieve tubes of new, symptomless leaves. However, the bacteria and the symptoms reappeared when the treatments were discontinued. As expected, penicillin had no effect on *Spiroplasma citri*-affected citrus, since mycoplasmas, lacking a cell wall and having no peptidoglycane, are insensitive to penicillin.

The HLB bacterium was detected by EM for the first time in sweet orange leaves infected with South Africa "greening" (Laflèche and Bové, 1970a), but soon similar organisms could also be seen by EM in leaves infected with Reunion "greening", India "dieback", Taiwan "likubin", and Philippines "mottle leaf" (Laflèche and Bové, 1970b; Bové and Saglio, 1974). These data were confirmed for likubin by Chen *et al*. (1971) and Tanaka and Doi (1973), and for mottle leaf by Tanaka and Doi (1973). In 1979, the same organisms were also found to be associated with HLB in Southern China (Chen *et al*., 1979a, b; Ke *et al*., 1979). Finally, the HLB bacterium was also seen in *T. erytreae* (Moll and Martin, 1973) and in *D. citri* (Chen *et al*., 1973).

The HLB bacterium is probably the first walled, sieve tube-restricted bacterium seen in plants. Organisms similar to the HLB organism occur in plants other than citrus, and are involved in more than 20 diseases. Papaya bunchy top (Davis *et al*., 1996), watermelon yellow vine (Bruton *et al*., 1998), strawberry marginal chlorosis (Zreik *et al*., 1998), and low sugar syndrome of sugar beet (Gatineau *et al*., 2002) are such diseases. The associated bacteria are restricted to the phloem sieve tubes, none of them has been obtained in culture, and they are transmitted by leafhoppers or psyllids.

The publication on HLB as a mycoplasma-like organism appeared in 1970 (Laflèche and Bové, 1970a, b). However, as shown above, Bové and his group questioned the mycoplasma nature of the HLB agent as early as 1971 (Saglio *et al*., 1971), and provided many arguments against the mycoplasma hypothesis during the 1970s, until the Gram negative nature of the HLB was finally demonstrated in 1984 (Garnier and Bové, 1984a, b). Nevertheless, the erroneous term "mycoplasma-like" was still in use in several publications until 1980.

Electron microscopy: first laboratory technique for identification and confirmation of HLB. Specific HLB symptoms do not exist. Some symptoms, such as yellow shoots, leaf blotchy mottle, and lopsided fruits with color inversion and aborted seeds, are characteristic, but they do not always occur together on the same tree, they can be distorted or masked by symptoms of other diseases, or induced by causes other than HLB.

For instance, HLB-induced zinc deficiency is indistinguishable from genuine zinc deficiency, yellow shoots can be the result of phytophthora gummosis, and removal of a ring of bark (girdling) for air-layering produces mottle on the leaves. Stubborn and HLB have common symptoms. No countries are known where the two diseases occur together, but this situation may change in the future. For these various reasons, a technique for unequivocal identification of HLB was required.

In our laboratory, and in the hands of Monique Garnier (1949-2003) (Fig. 1 D), EM detection of the HLB bacterium has fulfilled this purpose for many years (see for instance Aubert *et al*., 1988; Bové and Garnier, 1984; Catling *et al*., 1978; Garnier and Bové, 1996). The reliability and specificity of EM is based on two properties of the HLB bacterium: its exclusive location in the sieve tubes (Fig. 3 A), and the presence of a cell wall (Fig. 3 B, D). In citrus, no bacteria other than the HLB agent fit these properties. For EM detection of the HLB bacterium, leaf midribs are used. If the number of bacteria per sieve tube is low, it is recommended to use longitudinal sections of sieve tube cells.

Several years of experience with EM detection of Asian and African HLB have shown that the number of bacteria in sieve tubes is higher in leaves with strong mottle than in those with mild mottle. Therefore, leaves with strong mottle are to be preferred. Symptomless leaves are useless. For indisputable identification of HLB by EM, it is necessary that at least one bacterium in one section shows the electron dense cell wall layer surrounding the cell. Most often, the layer is seen only in certain parts of the cell (Fig. 3, B, D). Sometimes, several sections have to be examined before a bacterium with a "good" cell wall is seen (Fig. 3, B, D). Finally, based on many EM examinations, no morphological differences

could be found to distinguish the Asian HLB bacterium from his African counterpart.

VARIOUS FORMS OF HLB: ASIAN, AFRICAN, AND AMERICAN

Symptoms of citrus stubborn disease and HLB are influenced by temperatures at which affected trees grow. Symptoms of stubborn are severe in hot, dry areas of California, Arizona, Morocco, Iran, and Iraq. In South Africa, HLB and the African psyllid vector, *T. erytreae*, occur in the cool, "highveld" areas of Transvaal and Swaziland, but not in the hotter," lowveld" areas of Swaziland. However, HLB and *T. erytreae* are present in the Cape Town region (Garnier *et al*., 2000a), where the southern latitude compensates for the low altitude, and is responsible for a temperate climate.

In Madagascar, HLB and *T. erytreae* only occur on the central, high plateau (1200 to 1500 m a.s.l.), but not on the low lying coastal areas. In Kenya, HLB and *T. erytreae* are not seen below an altitude of 600 to 700m. On the contrary, in Asia, HLB and the Asian psyllid vector, *D. citri*, are found in hot low altitude areas. For instance, in Indonesia, in 1984, HLB and *D. citri* were present in the whole of Java coastal lowlands, but many trees in orchards located between 800 and 1200 m a.s.l. were still healthy (Aubert *et al*., 1985). In North Bali, HLB was commonly present below an altitude of 650 m, but was rarely seen above 1000 m, and its distribution reflected that of *D. citri* (Bové *et al*., 2000b). Similarly, in the Ningnan county of Sichuan (South China), 100% of trees were affected by HLB in orchards located at altitudes of 1090 to 1200 m, where *D citri* was abundant, while between 1385 and 1620 m, the percentage went down to 3% and no psyllids could be found (Zhao, 1981).

In the frame of an international cooperation experiment started in 1969, the influence of temperature on HLB symptoms was also studied under phytotron conditions in two temperature controlled chambers: a "cool" chamber (24°C with a 16-hr light period and 22°C with a 8-hr dark period) and a "warm" chamber (32°C with a 16-hr light period and 27°C with a 8-hr dark period) (Bové *et al*., 1974). Sweet orange plants graft-inoculated with the following HLB sources were used: South Africa (Nelspruit) "greening", India (Poona) "dieback", and Philippines (Lipa City) "mottle leaf". The presence of HLB bacteria in inoculated plants was established by EM.

With South African HLB, severe symptoms were obtained after 30 weeks in the cool chamber, and the plants averaged 35 cm in height; no symptoms developed in the warm chamber, and the plants reached 190 cm. When, after 40 weeks in the cool chamber, severely affected plants were transferred to the warm conditions, they quickly produced new, vigorous growth, recovered, and remained symptomless during the remaining 10 months of the experiment. With both Indian HLB and Philippine HLB, symptoms were as pronounced at 22-24°C as at 27-32°C. After 30 weeks in the warm chamber, symptomatic seedlings infected with Indian HLB and Philippine HLB measured 25 and 40 cm, respectively, while healthy controls had grown to 180 and 140 cm.

This experiment as well as the field observations show that HLB in Africa is heat-sensitive, and occurs only in cool areas, with temperatures remaining below 30-32°C. Similarly, the African psyllid vector, *T. erytreae*, thrives only in cool environments, and is also sensitive to high temperature combined with low relative humidity (Catling, 1969c). On the contrary, HLB in Asia is heat-tolerant, and symptoms occur even when temperatures are well above 30°C. The Asian psyllid vector, *D. citri*, has similar properties, and is also heat-tolerant.

The South African Nelspruit strain, and the Indian Poona strain of the HLB bacterium could be transmitted to periwinkle (*Catharanthus roseus*) plants (see below). Temperature experiments conducted with infected periwinkle plants gave the same results than those obtained above with HLB-affected citrus seedlings: with the Indian HLB bacterium, symptom expression occurred at both 25 and 32°C, but only at 25°C with the South African strain (Garnier and Bové, 1983). The effect of temperature on symptom expression is thus the same in periwinkle and citrus. Therefore, the temperature effect is due to the HLB bacterium and not to the plant, the African HLB bacterium being heat sensitive and the Asian HLB bacterium, heat tolerant. This biological difference indicates that the African and Asian HLB bacteria are not identical. Indeed, as shown below, they represent different bacterial species: *Candidatus* Liberibacter africanus and *Candidatus* Liberibacter asiaticus .

The fact that the African HLB bacterium and *T. erytreae* are both heat-sensitive, and the Asian HLB bacterium and *D. citri,* both heat-tolerant, seems to be a good example of adaptation between vector and pathogen. It has to be remembered however, that, experimentally at least, each one of the two vectors can transmit each one of the two HLB bacterial species (Massonié *et al*., 1976; Lallemand *et al*., 1986).

In the State of São Paulo, Brazil, *Ca* L. asiaticus has been found in less than 10% of the HLB-affected trees. Indeed, a third bacterial species has been discovered: *Candidatus* Liberibacter americanus (see below), which affects more than 90% of the trees. In São Paulo State, the Asian psyllid vector, *D. citri*, was reported as early as 1942, and transmits not only *Ca* L. asiaticus, but probably also *Ca* L. americanus. Indeed, the new liberibacter could be detected by PCR within *D. citri* psyllids collected on *Ca* L. americanus-infected trees. Furthermore, in the glasshouse, sweet orange seedlings, graft-inoculated with *Ca* L. americanus, showed severe HLB leaf mottle at both cool (22-24°C) and warm (27-32°C) conditions (Teixeira

et al., 2005c). These observations suggest that the South American (Brazil) HLB is of the heat-tolerant form.

In Florida, the presence of *D. citri* was reported in 1998 (Halbert, 1998), HLB has been observed in the Southern part of the State in August 2005, and only *Ca* L. asiaticus has been detected in HLB-affected trees. Therefore, the North American (USA) HLB is probably also of the heat-tolerant form.

TRANSMISSION OF THE HLB BACTERIUM FROM CITRUS TO PERIWINKLE BY DODDER

In citrus, the HLB bacterium is present only in small numbers, but in dodder (*Cuscuta campestris*) the organism reaches high titers (Ghosh *et al*.,1977). Periwinkle (*Catharanthus roseus*) is a host plant in which also many mycoplasma-like organisms multiply actively in sieve tubes. These observations suggested to attempt transmission of the HLB bacterium from citrus to periwinkle through dodder. Positive transmissions were indeed obtained (Garnier and Bové, 1983). In the case of Indian HLB, one of four periwinkle plants connected via dodder to a HLB-affected sweet orange seedling developed peculiar yellowing symptoms, and contained HLB-like bacteria in sieve tubes, some of which were particularly rich in bacteria. Shoots from the symptomatic periwinkle were top-grafted onto healthy periwinkle plants, which developed the characteristic symptoms after 3 months at 25°C. In this way, large numbers of symptomatic, HLB-infected periwinkle plants were produced. Similar results were obtained with South African, Chinese, and Philippine strains of the HLB organism. It must be stressed that periwinkle plants naturally infected with the HLB agent have never been observed. Citrus psyllids dislike periwinkle, are unable to transmit the HLB agent to this plant, and die when forced to stay on it.

Ultrathin sections from HLB-infected periwinkle plants were used in EM to demonstrate the Gram negative nature of the bacterium (Garnier *et al*., 1984a, b) (see above). As the HLB bacterium is not available in culture, great numbers of infected periwinkles have been used as convenient sources for the phylogenetic and taxonomic characterization of the HLB bacterium, the production of monoclonal antibodies, and the development of molecular detection techniques (see below).

Transmission of the HLB bacterium to tobacco (*Nicotiana tabacum* Xanthi) by dodder has also been obtained (Garnier and Bové, 1993).

PHYLOGENETIC AND TAXONOMIC CHARACTERIZATION OF THE AFRICAN AND ASIAN HLB BACTERIA

16S rDNA. To determine the phylogenetic position of the HLB bacterium, the 16S ribosomal DNAs (16S rDNA) of the South African Nelspruit strain and the Asian Poona strain were obtained from total DNA of HLB-infected periwinkle plants, by polymerase chain reaction (PCR)-amplification, using the universal PCR-primers f-D1/r-P1 for amplification of prokaryotic 16S rDNA (Weisburg *et al*., 1991). In these experiments, care must be taken to prevent interference by periwinkle mitochondrial and chloroplast 16S rDNAs. This can be achieved by cutting mitochondrial 16S rDNA with endonuclease *Bcl*1 before PCR amplification. The amplified DNA, about 1500 base pairs (bp) in size, contains bacterial as well as chloroplast 16S rDNA. The presence of bacterial DNA can be ascertained by *Eco*RI, which cuts it into two fragments (~650bp and ~850bp), while chloroplast DNA is untouched. The 16S rDNAs were cloned from the ~1500bp amplified products, and sequenced. Hybridization and PCR experiments performed with oligonucleotides specific for the amplified sequences revealed that the DNAs obtained were indeed the 16S rDNAs of the HLB bacteria, and not the DNA of a contaminating organism (Jagoueix *et al*., 1994).

Comparisons with 16S rDNA sequences obtained from the GeneBank database showed that the African and Asian HLB bacteria belonged to the α subdivision of the class *Proteobacteria*. Even though their closest relatives were members of the α-2 subgroup, the HLB bacteria were distinct from this subgroup, as the level of 16S rDNA sequence identity was only 87.5%. Therefore, the two HLB bacteria represented a new lineage in the α subdivision of the class *Proteobacteria*. As the members of this class are Gram negative bacteria, these results confirmed the Gram negative nature of the HLB bacterium, a property which had already been deduced from earlier EM studies by Garnier *et al*. (1984a, b).

The α subdivision of *Proteobacteria* is a diverse group of microbes that includes both plant pathogens or symbionts with some distinctive properties (*Agrobacterium tumefaciens, Bradyrhizobium* spp.) and human pathogens (*Rochalimea* spp., *Bartonella baciliformis, Brucella abortus, Afipia* spp., etc). Organisms in this group live in intimate association with eukariotic cells and, in many cases, have acquired the ability to survive and grow within an arthropod vector. The HLB organism fits this description remarkably well. Indeed, it grows in a specialized niche in its eukariotic plant host, the phloem sieve tubes, and it is transmitted by two arthropod vectors, the psyllids *T. erytreae* and *D. citri*, in which it multiplies in the hemolymph and within the salivary glands.

***Rpl*KAJL-*rpo*BC operon and DNA probes.** Using total DNA from periwinkle plants infected with the Asian Poona (India) strain of the HLB bacterium, several DNA fragments of the bacterial genome could be obtained by random cloning (Villechanoux *et al*., 1992).

Fragment In-2.6 (2.6 kbp), when used as a probe in Southern- or dot-hybridisations, hybridised at high stringency with all tested strains of the Asian HLB bacterium, but not with the African strain. At low or intermediate stringency, some hybridisation was also seen with the African strain. The Asian strains that gave positive hybridisation signals with probe Poona In-2.6 were those from Poona (India) (homologous strain), Nakhom Pathom (Thailand), Lipa city (Philippines), Fujian (China), Taiwan, and Bali (Indonesia).

By sequencing, In-2.6 was found to be part of the *rplKAJL-rpo*BC gene cluster, the well-known, bacterial β-operon, which codes for ribosomal proteins K, A, J, and L, and RNA polymerase subunits β and β' (Villechanoux *et al*., 1993). From the sequence of In-2.6, two PCR primers were designed, f-1898 and r-1897, and used to amplify part of the *rpl*KAJL-*rpo*BC operon of the African Nelspruit HLB bacterium (Planet *et al*., 1995). A clear DNA band of about 1700 bp was obtained. Upon cloning and sequencing, the DNA from the African strain (1676 bp) was indeed found to correspond to part of the expected β-operon. It was called AS-1.7. The AS-1.7 DNA hybridised at high stringency with DNA from periwinkle or citrus plants infected with the African Nelspruit strain, but no hybridisation was observed in the case of the Asian strains tested (Planet *et al*., 1995). As indicated above, opposite results were obtained when In-2.6 was used as the probe: no hybridisation with the African strain, but strong hybridisation with all Asian strains tested. The overall nucleotide identity between In-2.6 and AS-1.7 was 74.2%. This relatively low homology for similar organisms explains why no hybridisation was observed between In-2.6 and DNA from periwinkle plants infected with the African Nelspruit HLB bacterium, and vice versa. This low homology also suggested that the African strains of the HLB bacterium and the Asian strains were members of two different species of the same genus (Jagoueix *et al*., 1994; Planet *et al*., 1995).

Monoclonal Antibodies. Since 1987, only thirteen different monoclonal antibodies (MA) specific for the HLB bacteria have been produced (Garnier *et al*., 1991; Gao *et al.*, 1993). This low number is explained by the fact that the HLB bacteria are not available in culture. The first ten MAs were raised using as immunogen homogenates of phloem tissue from HLB-affected periwinkle plants. Of these MAs, two (including MA 10A6) were against the Indian Poona strain, five against a strain from China (Fujian), and three against the South African Nelspruit strain. The use of these MAs for the detection of HLB-bacteria has shown that each MA is very specific for the strain used for immunisation and, therefore, they cannot be used for generalized diagnosis of HLB (Garnier *et al*., 1991).

In attempting to produce antibodies recognizing most or all strains of the HLB bacterium, an antigenic protein of the Indian Poona strain was purified by immunoaffinity-chromatography using MA 10A6 directed against this protein, and used for *in vitro* immunisation of spleen cells (Gao *et al*., 1993). Three MAs were obtained, one of which (1A5) recognized all Asian strains tested except the Chinese one, whereas the other two recognized most of the Asian, but not the Chinese strain. None of the three MAs reacted with the South African strain. These results agreed with those obtained with DNA probes, confirming that Asian and African HLB bacteria are members of two different bacterial species.

MA 10A6, coupled to CNBr-activated sepharose 4B, has also been successfully used to purify the Poona BLO by immunoaffinity (Villechanoux *et al*., 1990). In this way, purified cells of the HLB bacterium could be observed for the first time in the electron microscope

***Candidatus* genera and species: *Candidatus* Liberibacter africanus and *Candidatus* Liberibacter asiaticus.** Bacteriologists have had a conservative attitude when it came to give Latin binomial names to non-cultured organisms. However, with the development of DNA amplification by PCR and DNA sequencing, it became possible to characterise such organisms at the molecular and phylogenetic level. On the basis of such considerations, Murray and Schleifer (1994) proposed the "*Candidatus*" designation as an interim taxonomic status, to provide a proper allocation of sequence-based potential new taxa at the genus and species level. One of the first non-cultured bacteria to benefit from the *Candidatus* proposal was the HLB bacterium, shown by 16S rDNA sequence comparisons to be the first member of a new subgroup in the α subdivision of the *Proteobacteria* (see above). The trivial name liberobacter (Jagoueix *et al*., 1994), later replaced by liberibacter (Garnier *et al*., 2000b) (from the Latin liber [bark} and bacter [bacterium]), was given to organisms in this new subgroup.

As indicated above, the HLB liberibacter strains from Africa can be distinguished from those in Asia on the basis of temperature sensitivity (Bové et al., 1974), DNA hybridizations and genomic properties (Villechanoux *et al*., 1992, 1993), and serology (Garnier *et al*., 1991; Gao *et al.*, 1993). For these reasons, they represent two different species. Therefore, following the *Candidatus* proposal of Murray and Schleifer, the HLB liberibacters from Asia should be denoted *Candidatus* Liberibacter asiaticus, and the HLB liberibacters from Africa *Candidatus* Liberibacter africanus (Jagoueix *et al*., 1994; Garnier *et al*., 2000b).

The sequence variability of the region between the 16S rRNA gene and the 23S rRNA gene in the ribosomal operons is useful for differentiating species within genera. This 16S/23S intergenic region was obtained for two Asian strains of the HLB liberibacter, the Indian Poona strain and a strain from Fuzhou, Sichuan (China), as well

as for the African Nelspruit liberibacter (Jagoueix *et al.*, 1997). The intergenic regions of the two Asian liberibacters had 100% sequence identity, even though they belong to different serotypes and were isolated from geographically distant areas. However, the sequence identity between an Asian liberibacter strain and the African liberibacter was only 79.46%, confirming the fact that the African liberibacter and the Asian liberibacter belong to two different species. Similar results have also been obtained by Subandiyah *et al.* (2000).

***Candidatus* Liberibacter africanus subsp. capensis**. A third liberibacter was detected by PCR in an ornamental rutaceous tree, Cape chestnut (*Calodendrum capense*), in the Cape region of South Africa. Leaves of the affected tree showed characteristic mottling. The new liberibacter was characterized by serology and the sequences of its 16S rDNA, the intergenic 16S/23S rDNA region, and ribosomal protein genes of the β operon. Phylogenetic analysis showed the new liberibacter to be more closely related to *Ca.* L. africanus than to *Ca.* L. asiaticus, and a subspecies status was assigned to it: *Candidatus* Liberibacter africanus subsp. capensis (Garnier *et al.*, 2000b).

16S rDNA-based phylogeny tree. Fig. 4 shows the 16S rDNA-based phylogeny tree of liberibacters. The tree was constructed from 16S rDNA sequences obtained from GenBank. It can be seen that all four *Ca.* L. asiaticus strains cluster together, and the "asiaticus" cluster is close to the "africanus" cluster. The "asiaticus" cluster and the "africanus" cluster form the "asiaticus"/"africanus" liberibacter group. The new liberibacter from São Paulo State, *Ca.* L americanus (see below), is not part of this group, but forms a separate branch, indicating that it is a species different from *Ca.* L. asiaticus and *Ca.* L. africanus. All liberibacters are members of the α subdivision of the class *Proteobacteria*, and their closest relatives are members of the α2 subgroup. The tree also shows two members of the γ subdivision.

Recently, a phylogenetic tree was constructed from the *omp* gene sequences of the African and Asian liberibacters, and confirmed the phylogeny based on 16S rDNA sequences (Bastianel *et al.*, 2005). The *omp* gene sequences were also used to study the diversity of several strains of *Ca.* L. asiaticus. Each strain could be characterised by a specific PCR-RFLP profile.

IDENTIFICATION OF HLB BY DETECTION OF THE AFRICAN AND ASIAN LIBERIBACTERS

Until 1992, electron microscopy visualization of the walled HLB organisms in the sieve tubes of citrus leaves showing blotchy mottle was the only reliable method of detection, and was widely used (Garnier and Bové, 1996). However, the technique was heavy, and unable to distinguish between Asian and African liberibacters. This differentiation has now become possible with the development of molecular techniques such as DNA hybridization and PCR. As mentioned, monoclonal antibodies are too specific to be used for diagnosis.

DNA Hybridisation. In dot hybridisation, probe In-2.6 (see above) gives positive hybridisation signals with DNA isolated from citrus leaves infected with Asian liberibacter strains, while probe AS 1.7 reacts positively with African liberibacter strains (Villechanoux *et al.*, 1992, 1993; Planet *et al.*, 1995)**.** These probes can also be used very efficiently to detect liberibacters in psyllid insect vectors. Individual insects are crushed onto a nylon membrane and the membrane or "crush-blot" is submitted to hybridisation with one or the other probe (Bové *et al.*, 1993) Non-radioactive probes have been developed (Hocquellet *et al.*, 1997).

PCR. Two PCR systems have been used. The first is based on the amplification of a 1160 bp fragment of liberibacter 16S rDNA (Jagoueix *et al.*, 1996). 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. In countries were the two liberibacter species are known or suspected to be present, it is advisable to use the two forward primers, OI1 + OA1, and the common reverse OI2c primer in the same PCR mixture. Sequence analysis shows that the rDNA amplified from the Asian liberibacter has one *Xba*1 restriction site, and yields, upon *Xba*1 treatment, two fragments 520 bp and 640 bp. in size, respectively. The rDNA amplified from the African liberibacter has an additional site, and yields three fragments with a size of 520 bp, 506 bp, and 130 bp. Hence, by *Xba*1 treatment of the amplified DNA, it is easy to identify the liberibacter species present in a given sample (Jagoueix *et al.*, 1996).

The second PCR system is based on the sequence of the *rpl*KAJL-*rpo*BC operon, which is slightly different from one liberibacter species to the other. In particular, the intergenic region between genes *rpl*A and *rpl*J is 34 bp larger in the Asian than in the African liberibacter. With forward primer f-*rpl*A2, selected in the *rpl*A gene, and reverse primer r-*rpl*J5 from the *rpl*J 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 (Hocquellet *et al.*, 1999). Finally, a PCR assay was developed, based on the β operon sequence and specific for the detection of Candidatus Liberibacter

africanus subsp. capensis (Garnier *et al*., 2000b).

By the use of these molecular techniques, the presence of HLB has been clearly established in several African and Asian countries (Bové *et al*., 1993; Bové *et al*., 1996; Bové *et al*., 2000b; Doe Doe *et al*., 2003; Garnier and Bové, 1996; Garnier and Bové, 2000; Garnier *et al*., 2000a; Korsten *et al*., 1996; Regmi *et al*., 1996; Varma *et al*., 1993). The presence of both liberibacter species, sometimes in the same trees, was confirmed in Reunion and Mauritius islands (Garnier *et al*., 1996).

HLB IN SÃO PAULO STATE, BRAZIL: OLD AND NEW LIBERIBACTERS

Even though HLB was reported in São Paulo State (SPS) only in 2004, the Asian psyllid vector of HLB, *D citri*, has been present in Brazil at least since 1942, when it was reported for the first time (Lima, 1942). Specimens of *D. citri* from Brazil were present at the British Museum in 1969 (Eastop, 1969, personal communication to Catling, 1970). In certain years, high populations of the insect could be seen in SPS in sweet orange groves.

Discovery of a new liberibacter: *Candidatus* Liberibacter americanus. In March 2004, symptoms of HLB were observed in sweet orange trees near the city of Araraquara in SPS (Anonymous, 2004; Coletta-Filho *et al*, 2004; Ayres *et al.*, 2005; Teixeira *et al*., 2005a, b). 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. Leaf symptoms of HLB in SPS were very similar, if not identical, to those in Africa and Asia, with characteristic and pronounced blotchy mottle, as described by McClean and Schwarz (1970), on both small and large leaves (Fig. 6, 7). Fruits were small and lopsided, exhibited strong colour inversion, seed abortion, and brown/orange-stained vascular bundles (Fig. 8, 9). These fruit symptoms were more similar to those seen in China than to those observed in South Africa. A survey conducted in September 2004, only six months after the disease was recognized, showed HLB to be already present in 46 municipalities of SPS. A few orchards had more than 50% of affected trees. These observations suggested that the disease had been present for almost ten years, but without being properly diagnosed. History repeats itself…

In April 2004, the 16S rDNA-based PCR technique with forward primers OA1+OI1 and reverse primer OI2c, described by Jagoueix *et al*. (1996), was applied to sweet orange leaves with strong mottle from suspicious trees in SPS 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 (see for instance Bové *et al*., 1996, 2000b; Garnier and Bové, 1996; Garnier *et al*., 1996). Whenever leaves with the classic blotchy mottle symptoms were used, positive PCR reactions were obtained, and yielded the characteristic 1160 bp amplicon. Unexpectedly in SPS, only negative PCR reactions were obtained from mottled leaf samples from 43 affected trees, many of which had also severe fruit symptoms. Under the same testing conditions symptomatic control citrus leaves infected with *Ca*. L. asiaticus or *Ca*. L. africanus from the HLB collection in Bordeaux, gave positive PCR reactions (Teixeira *et al*., 2005a, b). However, at the same time, and using practically the same PCR technique, *Ca*. L. asiaticus was detected in 2 of 10 leaf samples by other workers (Coletta-Filho *et al*., 2004). Because of the repeatedly negative PCR reactions, the presence of a new bacterial pathogen in many of the symptomatic, blotchy mottle leaves from SPS was suspected and investigated.

Evidence for the presence of a new HLB bacterium (SPS-HLB bacterium) was obtained by PCR amplification with universal primers fD1 and rP1 for prokaryotic 16S rDNA amplification. 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 HLB-leaf samples testing negative for *Ca*. L. africanus and *Ca*. L. asiaticus. Most leaf samples (98%) were infected with the SPS-HLB bacterium, the remaining samples (2%) carried *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 (Teixeira *et al*., 2005c).

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. In particular, in the 16S rDNA phylogenetic tree (Fig. 4), 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 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 *Candidatus* Liberibacter americanus, sp. nov. (Teixeira *et al*., 2005a, b, c). This designation refers to

the fact that the new liberibacter species was detected for the first time in the American Continent, and that it represented the major *Ca.* Liberibacter species associated with HLB in the affected SPS region. The designation is in line with the other *Ca.* 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 was obtained, and EM obervations showed the American liberibacter to be restricted to sieve tubes (Teixeira *et al*., 2005c; Tanaka *et al.*, 2004).

PCR detection of *Ca.* L. americanus and *Ca.* L. asiaticus in citrus leaves. For the specific PCR detection of *Ca*. L. americanus, forward primer GB1 and reverse primer GB3 were designed from the 16S rDNA sequence of the new liberibacter (accession number AY742824). These primers, as well as those specific for *Ca*. L. africanus and *Ca*. L. asiaticus (OA1+OI1/OI2c), were used for detection of the three liberibacters in each leaf sample according to Teixeira *et al*. (2005b). A first aliquot of the DNA from a leaf sample was used for the detection of *Ca*. L. americanus with primers GB1/GB3, yielding an amplicon of 1027 bp, and a second aliquot served for the detection of *Ca*. L. africanus and *Ca*. L. asiaticus with primers OA1+OI1/OI2c, giving an amplicon of 1160 bp.

In this way, from August 2004 to September 2005, 1525 leaf samples gave positive PCR reactions. Each sample came from a single tree with leaves showing blotchy mottle, ranging from mild to strong. Of 1525 samples, 1411 (92.5%) were found to be infected with *Ca*. L. americanus, 82 contained *Ca*. L. asiaticus (5.4%), and both liberibacter species were found in 32 samples (2.1%). *Ca*. L. africanus was not detected. Leaf samples for liberibacter detection and identification were collected from many citrus farms representing all 79 HLB-affected SPS municipalities. For these reasons, the results obtained are meaningful, and indicate that the major liberibacter in SPS is *Ca*. L. americanus, and that both *Ca*. L. americanus and *Ca*. L. asiaticus can be found in the same tree. Even though as many as 79 municipalities host HLB-affected trees, 95% of these trees are in only 10 municipalities, indicating that most municipalities have a low rate of infection, with less than 0.1% diseased trees in the affected farms.

In another set of assays, of 216 leaf samples infected with *Ca*. L. americanus, 208 were from sweet orange trees ('Chamout', 'Hamlin', 'Lima', 'Natal', 'Pera', Valencia', and 'Westin'), 5 from 'Ponkan' mandarin trees, 1 from a 'Murcott' tangor tree, and 2 from 'Cravo' mandarin trees. These proportions reflect essentially the fact that sweet orange is by far the major cultivar in SPS, but they also indicate that HLB is not restricted to sweet orange.

In the above experiments, blotchy mottled leaves from HLB-affected trees, i.e. trees showing leaf and fruit symptoms, never failed to give positive PCR reactions, which emphasizes the importance of using symptomatic leaves for HLB diagnosis.

The presence of *Ca*. L. americanus and/or *Ca*. L. asiaticus in symptomless leaves has also been investigated. Symptomless leaves were collected from symptomless parts of infected trees, from symptomless trees adjacent to symptomatic trees, and from trees in a region not affected by HLB. As expected from previous results with *Ca*. L. africanus and *Ca*. L. asiaticus, all symptomless leaves tested PCR-negative. This result is not due so much to lack of sensitivity of PCR, as similar results were also obtained with the more sensitive nested PCR, but probably reflects the uneven distribution of liberibacters in recently infected trees, and the difficulty of sampling liberibacter-infected leaves when no symptoms are present to guide the choice. For the same reasons, indexing by PCR symptomless nursery or orchard trees for HLB presence seems meaningless.

Several new developments in diagnosis have taken place. Thus, "multiplex" PCR is being developed for routine detection of all three liberibacters in one test tube with a single reaction mixture containing the two sets of primers described above: (i) f-GB1 and r-GB3 for 16S rDNA amplification of *Ca*. L. americanus, and (ii) f-*rpl*A2 and r-*rpl*J5 for *rpl* gene amplification of *Ca.* L. asiaticus and/or *Ca.* L. africanus (Teixeira D. do Carmo, unpublished results). Nested PCR is also being developed (Li and Ke, 2002; Ding Fang *et al*., 2004, 2006; Li *et al*., 2005). Hartung *et al.* (2005a) have compared several methods for the detection of the Asian liberibacter.

PCR detection of *Ca.* L. americanus and *Ca.* L. asiaticus in *Diaphorina citri* psyllids. By the end of 2004, *Ca*. L. americanus was present in 46 municipalities of SPS, and one year later, the number had increased to 79, indicating a rapid spread of the liberibacter. As mentioned, the psyllid vector of *Ca*. L. asiaticus, *Diaphorina citri*, was established in Brazil since the 1940s. The rapid spread of *Ca*. L. americanus suggested that a vector was involved, and that it might be *D. citri*. Thus, psyllids were collected in August 2004 on three Pera sweet orange trees with severe symptoms of HLB and shown by PCR to be infected only with *Ca*. L. americanus. The insects were subdivided into 22 batches of 10 individuals each. The 22 batches gave negative PCR reactions with the primers specific for *Ca*. L. africanus and *Ca*. L. asiaticus. However, 6 batches gave positive PCR signals with the primers specific for *Ca*. L. americanus (Teixeira *et al*., 2005b).

In an additional experiment, psyllids were collected in a severely HLB-affected sweet orange orchard from: (i) symptomatic branches of symptomatic trees (S/S psyllids); (ii) asymptomatic branches of symptomatic trees

(AS/S psyllids), and (iii) asymptomatic trees (AS/AS psyllids). The psyllids were subdivided in batches of 10 insects. *Ca*. L. americanus was detected in 5 of 36 batches of AS/AS psyllids, 13 of 36 batches of AS/S psyllids, and 27 of 76 batches of S/S psyllids. *Ca*. L. asiaticus was detected in only 2 batches of S/S psyllids. These data indicate that in a severely affected orchard (~50% HLB trees), symptomless branches of symptomatic trees carry as many liberibacter-infected psyllids as symptomatic branches, probably because of psyllid movement, and that infected psyllids, probably coming from symptomatic trees, can be found on trees with no symptoms. Also, *Ca*. L. americanus was found in a total of 45 psyllid batches, and *Ca*. L. asiaticus in 2. This proportion is quite similar to the ratio of Ca. L. americanus-infected trees to *Ca*. L. asiaticus-infected trees in SPS.

Finally, the American liberibacter was also detected in one batch of 10 psyllids collected on a *Murraya paniculata* plant close to a HLB-affected citrus orchard. In China, Ca. L. asiaticus has also been reported to infect *D. citri* (Li and Ke, 2002).

These results as a whole, clearly suggest that *D. citri* is a vector of *Ca*. L. americanus in SPS.

PCR detection of *Ca.* L. americanus in *Murraya paniculata* leaves. *M. paniculata* (jasmin orange), an ornemental rutaceous shrub or tree, has a wide distribution throughout SPS, and represents the preferred host of *D. citri*. *M. paniculata* plants with suspicious foliar symptoms have been observed in HLB-affected citrus orchards in SPS. *Ca*. L. americanus was detected in symptomatic leaves from 3 of 13 such plants, but not in asymptomatic leaves of the same plants (Lopes *et al*., 2005). In China, *Ca*. L. asiaticus was reported to infect also *M. paniculata* by Li and Ke (2002) but not by others (see for instance Hung *et al*., 2000).

In view of these conflicting results, it is essential to confirm if *M. paniculata* is a host of *Ca*. L. americanus and *Ca*. L. asiaticus. Should this be confirmed, *M. paniculata* would become an additional source of liberibacter inoculum available for psyllids, thus calling for eradication of *M. paniculata* plants present in citrus farms. Removal of such plants from public parks, streets and avenues, as well as house gardens, must also be seriously discussed. Planting and growing new *M. paniculata* plants should probably be prohibited. Regarding other citrus relatives, *Severinia buxifolia* (Chinese box orange) and *Limonia acidissima* (wood apple) (Hung *et al*., 2000), and *Clausena lansium* (Chinese wampee) (Ding *et al*., 2006) have been shown to be hosts of *Ca*. L. asiaticus.

DIAPHORINA CITRI AND HLB IN FLORIDA

The Asian HLB-vector, *D. citri*, was detected in Palm Beach County, Florida, in June 1998 in backyard plantings of *M. paniculata*, and by 2001, it was found in as many as 31 counties. (Halbert, 1998, 2005; Halbert *et al*., 2003, 2004). It is believed that the psyllid had been present in South Florida 6 to 12 months prior to its discovery, so that eradication was no longer feasible at the time of detection. Today, the insect is throughout Florida, essentially as a result of distribution of *M. paniculata* plants, largely through discount stores (S. Halter, personal communication).

HLB was discovered in Miami-Dade County, Florida, on two samples of a pummelo [*Citrus grandis* (L.) Osb.] tree in August 2005, seven years after detection of the vector in the same region (Halbert, 2005). By mid-October, as a result of additional surveys, the disease was found in many residential properties stretching northwards over 250 km from Miami-Dade County to St. Lucie County. Several commercial citrus farms were also affected in Palm Beach and Hendry Counties. In view of the large area already affected, it appeared that the disease had probably been present in Southern Florida since quite some years, a situation excluding its eradication.

PCR was used to confirm HLB, and *Ca*. L. asiaticus was detected by PCR in pummelo, grapefruit, sour orange, sweet orange, Key lime, lemon, kumquat, and calamondin, as well as in *D. citri* psyllids, but not in *M. paniculata* (Sutton *et al*., 2005).

WORLD DISTRIBUTION OF HLB: NATURE OF THE LIBERIBACTERS AND THE PSYLLID VECTORS INVOLVED

Asia, Southeast Asia, and Oceania. *D. citri* is the vector, *Ca*. L. asiaticus is the HLB agent, and both are heat tolerant (Asian form of HLB, see above). Asian regions or countries where *Ca*. L. asiaticus has been detected by EM, DNA-hybridisation and/or PCR include: the Indian subcontinent (India, Pakistan, Nepal, Bhutan, Bangladesh, Sri Lanka), Indochina (Myanmar, Thailand, Malaysia, Cambodia, Laos, Vietnam), South-eastern China, Taiwan, Southern Japan (Ryukyu islands, Okinawa) (Miyakawa and Tsuno, 1989), Philippines, Indonesia (Java, Sumatra, Eastern Kalimantan, Southern Sulawasi, Bali), East Timor, Papua New Guinea.

Africa and Madagascar island. *T. erytreae* is the vector, *Ca*. L. africanus is the HLB agent, and both are heat-sensitive (African form of HLB). East and South African regions and countries where *Ca*. L. africanus has been detected by EM, DNA-hybridisation and/or PCR include: South Africa, Zimbabwe, Malawi, Burundi, Kenya, Somalia, Ethiopia. In West Africa, only Cameroon is involved.

Arabian Peninsula. In Saudi Arabia, HLB is present

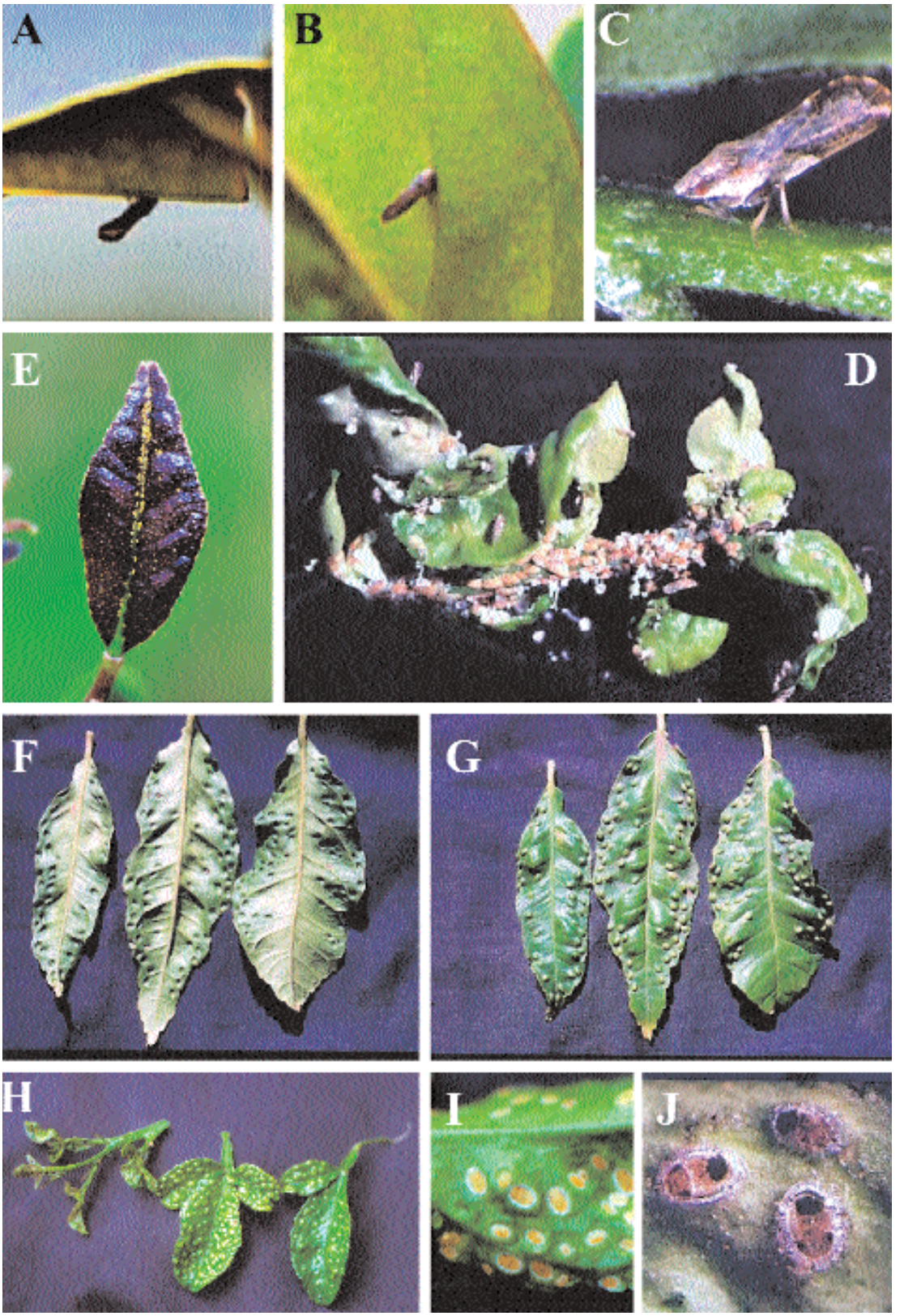

Fig. 5. Psyllid vectors of HLB. *Diaphorina citrri* feeding on the lower side of a sour orange leaf in Peshawar, Pakistan (**A**), sweet orange leaf in Guangxi, China (**B**), and citrus shoot in Reunion island (**C**, courtesy of B. Aubert). Notice position of insect at a 45° angle. Heavily curled citrus leaves infested by *D. citri* nymphs and adults (**D**). Yellow eggs of *Trioza erytreae* along the midrib and on the edges of a lemon leaf (**E**). *T. erytreae* nymphs develop on the underside of the leaves, becoming embedded in pits or nests, which look like bumps on upper side of the leaves (**G**). When adult psyllids emerge from nymphs, the pits are left empty (**F**). Bumps on the upper side of citrange leaves (**H**). Nymphs on the underside of citrange leaves (I.). *T. erytreae* nymphs parasitized by *Tamarixia dryi*, that emerges through a hole (**J**).

along the Red Sea region from Mecca to Najran, close to the Yemeni border. The vector is *D. citri*. HLB occurs in hot oases, and is, for that reason, of the heat-tolerant form. In Yemen, the vector is *T. erytreae*, and HLB is only seen in cool highlands. Therefore, Yemeni HLB is of the heat-sensitive form (Bové and Garnier, 1984).

North of the Saudi/Yemeni border, in the Abha-Khamis Mushayt area, both vectors have been found (Bové and Garnier, 1984), and the two forms of HLB are probably present. This situation can be explained in the following way. In Yemen, African HLB and *T. erytreae* have undoubtedly been introduced from nearby Ethiopia, across the Southern Red Sea, through the narrow "Bab al Mandab" strait, and have moved northwards. In Saudi Arabia, Asian HLB and *D. citri* have entered the Arabian Peninsula, probably with pilgrims to Mecca, and have moved southwards. Eventually, the two vectors, as well as the African and Asian HLB, have met in the Abha-Khamis Mushayt region. Further movement of *T. erytreae* up North will be hindered by hot climate, but movement of *D. citri* southwards towards Yemen would not be impaired by climatic conditions.

Indian Ocean islands: Reunion and Mauritius. The two vectors and the two forms of HLB occur in Reunion and Mauritius (Garnier *et al*., 1996). Distribution of the two psyllid vectors according to temperature/altitude is remarkable. *D. citri* is present on the coastal areas up to an altitude of ~500 m., while *T. erytreae* is prevalent above this altitude. *D. citri* has also been detected in Rodrigues island, East of Mauritius (J.Bové and M. Garnier, unpublished). Both *Ca*. L. africanus and *Ca.* L. asiaticus are present. Some trees proved to be simultaneously infected with the two liberibacters, a situation also observed in São Paulo State with *Ca.* L. americanus and *Ca.* L. asiaticus.

These islands are crossroads, and have been populated by immigrants from both Africa and India. In Reunion, French, African, Malagasy, Chinese, Pakistani and Indian ethnic groups are present. In Mauritius, Indo-Mauritians represent 68%, Creoles, 27%, and Sino-Mauritians, 3%. African HLB has probably been introduced from Eastern Africa and/or Madagascar, and Asian HLB, from the Indian subcontinent.

South America. SPS in Brazil has been the first American region to report HLB, in 2004. The vector is *D. citri*, present in SPS for almost 70 years. Two liberibacters occur. The major HLB agent is the new liberibacter species, *Ca*. L. americanus, present in 92% of trees. *Ca*. L. asiaticus affects 6% of trees, and the two liberibacters occur in 2% of trees (see above).

North America. Florida, U.S.A., has been the second American region to report HLB, in 2005. *D. citri* was recorded 7 years earlier. The liberibacter is *Ca*. L. asiaticus.

CITRUS REGIONS WITH PSYLLID VECTORS BUT NO HLB

The examples of SPS and especially Florida show that once a psyllid vector is present, HLB is not very far. Therefore, the following regions and countries must be seriously on the alert, and scout for HLB, with the hope to catch it early enough to try eradication. In SPS and Florida, several years probably elapsed between the moment the disease was correctly recognized and properly diagnosed, and its first occurrence.

Atlantic ocean islands: **Saint Helena, Madeira, and Tenerife.** The three islands harbor *T. erytreae*. The insect was reported in 1994 from Madeira, and in 2002 from Tenerife (Canary islands). Madeira is located North of Tenerife, both islands being West of Morocco. It is believed that the African citrus psyllid was introduced from Madeira into the Canary island by the dominant North-South trade winds (Gonzales Hernandez, 2003).

Middle East: Iran. A very small number of *D. citri* psyllids were seen in December 1997 in Iran, close to the border with Pakistan (Bové *et al*., 2000a). By 2005, large populations of the insect occurred in the major lime [*Citrus aurantifolia* (Christm.) Swing] growing region, North of Bandar Abbas (Alimorad Sarafrazi, personal communication).

Americas. Halbert and Nuñez (2004) have described the distribution of *D. citri* in South, Central and North America, and the Caribbean basin.

South America: Brazil, Argentina, Venezuela. In Brazil, as of November 2005, 79 municipalities in SPS and 1 adjacent municipality in Minas Gerais are contaminated with HLB, the vector being *D. citri* for *Ca*. L. asiaticus, and probably also for *Ca*. L. americanus. Other Brazilian regions grow citrus, but they do not (yet) have HLB, even though *D. citri* is present. These regions are at great danger of becoming contaminated with HLB. In 1997, *D. citri* was found on citrus in Corrientes, Argentina. The infestation was minor, the populations being probably controlled by local natural ennemies. *D. citri* was reported for the first time in Venezuela in 1999 in the "Peninsula de Paraguana" on *C. aurantifolia*, *C. reticulata*, *C. latifolia*, and *M. paniculata*.

Central America, West Indies, Caribbeans. *D. citri* was found in Guadeloupe, Bahamas, Cayman islands, Cuba and Dominican Republic, and Puerto Rico in 1998, 1999, 2000, 2001, and 2002, respectively (Halbert and Nuñez, 2004). *D. citri* was intercepted on citrus from Belize in a baggage at Houston, Texas, in October 2002.

North America: Texas, Yucatan. *D. citri* is present in

Fig. 6. Sweet orange trees in young, high density orchard (1000 trees per hectare) showing conspicuous symptoms of "yellow shoot" in the Guilin-Yangshuo region, Guangxi, China. Because of HLB, trees do not live longer than 10 years. High tree densities compensate for short orchard life.

the Rio Grande Valley of Texas, U.S.A. (French *et al*., 2001). It was accidentally introduced in the spring of 2001 on potted *Murraya* plants from Florida. *D. citri* was reported in April 2002 in Cancun, Mexico, and samples were collected in November 2003 (Halbert and Nuñez, 2004).

CITRUS REGIONS FREE OF PSYLLIDS AND HLB

The Mediterranean basin, most of Western Asia (Near and Middle East), Australia, and North- and South-Pacific islands are still free of HLB as well as of the psyllid vectors of the disease. However, for these regions, HLB is a serious threat. The Mediterranean basin could become contaminated with *T. erytreae,* well established in the Portuguese Madeira island and the Spanish Canary islands, not very far from the Moroccan coast. It must be remembered that the most efficient aphid vector of citrus tristeza, *Toxoptera citricida*, reported in Madeira island together with *T. erytreae* in 1994, is now present in Northern Portugal and Spain. (It is however not firmly established that Madeira was the source of contamination of the Iberian Peninsula with the tristeza vector).

The Near and Middle East regions are endangered by *D. citri*, which is well established in South-eastern Iran and in southern Saudi Arabia along the Red Sea. *T. erytreae* is in Yemen. Both Saudi Arabia and Yemen, but not yet Iran, host HLB in addition to the psyllid vectors.

Australia has two northern neighbours contaminated with both *D. citri* and HLB: East Timor and Papua New Guinea.

The North- and South-Pacific islands have not reported HLB nor psyllid vectors. They must be aware of the situation in Papua New Guinea. During a recent survey for HLB in French Polynesia, no evidence of its presence was found (Bové and Duran-Vila, unpublished observations). Similarly, no evidence was found for the presence of HLB in four Pacific island countries (Cook islands, Fiji islands, Samoa and Tonga) (Davis *et al*., 2005b).

ORIGIN OF LIBERIBACTERS

Hitherto, *Ca*. L. africanus has only been found in Africa, and *Ca*. L. asiaticus, only in Asia (Garnier and Bové, 1996). This is probably not due to the fact that the psyllid vector *T. erytreae* is only present in Africa, and the psyllid vector, *D. citri*, only in Asia, because both insects have the ability to transmit both liberibacters, at least under experimental greenhouse conditions (Massonié *et al*., 1976; Lallemand *et al*., 1986). Therefore, it seems as if *Ca*. L. africanus originated in Africa, and *Ca*. L. asiaticus in Asia. After having spread initially within these continents, disease and vector have spread later to neighbouring regions. In the SPS in Brazil, the situation is more complex. The Asian psyllid, *Diaphorina citri*, has been present, undisturbed, since the 1940s. In these early years, the insect was probably free of an HLB agent, as HLB occurs in SPS only since about 1995. The presence, in SPS, of *Ca*. L. asiaticus, a very minor component of Brazilian HLB, is probably the result of a relatively recent, uncontrolled introduction from Asia.

If, as suggested above, *Ca*. L. africanus and *Ca*. L. asiaticus have originated in their respective continents, the same could apply to *Ca*. L. americanus, supposed to having arisen in SPS. *Ca*. L. americanus is not a recent "mutation" of *Ca*. L. asiaticus, because the sequence differences in the 16S rDNA and the RIR of the two liberibacters are far too great, and because there is very little sequence variation between isolates of *Ca*. L. asiaticus, including the SPS isolate. The sequence of the 16S rDNA of *Ca*. L. americanus seems to be quite stable too.

The hypothesis proposing that different liberibacter species have evolved in different continents implies that *Ca*. L. americanus is native to SPS, and has not been introduced from known HLB-affected regions. The countless PCR assays made in Africa and Asia for detection of *Ca*. L. asiaticus and *Ca*. L. africanus, have given no indications of the occurrence, in these regions, of a liberibacter different of *Ca*. L. asiaticus and *Ca*. L. africanus. Several hundreds of PCR reactions have been carried out in Africa and Asia, and have always detected either *Ca*. L. asiaticus or *Ca*. L. africanus. However, the number of these PCR assays might not have been sufficiently large to draw conclusions. Hence, in order to confirm or infirm the above hypothesis, search for *Ca*. L. americanus, in Asia in particular, should be continued and intensified, especially since PCR tools for specific detection of the various liberibacters are available.

It has recently been suggested that the liberibacters have Gondwanan origins (Beattie *et al*., 2005). Indeed, until 160 million years ago, Australia, Africa, South America, Madagascar, India, and Antartica were part of the single "super continent" Gondwana. Speciation of the putative Gondwanan liberibacter ancestor could have occurred after dislocation and fractionation of the super continent, isolating the African liberibacter lineage within Africa, the American lineage within South America, and the Asian lineage within India, with subsequent eastward spread to China. A similar hypothesis might account for the distribution of the *Diaphorineae.* However, if so, it is difficult to explain why *D. citri* is apparently not native to South America, and seems to have been introduced recently. In any case, the Gondwanan hypothesis opens an interesting avenue for academic and applied research.

RECOMMENDATIONS FOR HLB IDENTIFICATION

These recommendations apply to small HLB outbreaks, with eradication of the disease as the objective, as well as to situations were HLB is established, the goal being to live with the disease. They are meant to facilitate quick diagnosis and confirmation of HLB.

Search and monitoring for citrus psyllid vectors. Halbert and Manjunath (2004) have recently reviewed the literature on *D. citri.* Catling (1969a, b, c; 1970a, b) has carried out pioneering work on *T. erytreae.*

The psyllids can feed on many citrus species, but preferred hosts of *D. citri* are *M. paniculata* (Orange jasmin, mock orange)*, M. exotica* (Chinese box), and *C. aurantifolia*, followed by *Bergera koenigii* (Curry leaf), and *Clausena* spp.. These plants should be favoured for monitoring the insects. Lemon (*Citrus limon*) is a good host of *T. erytreae*. Psyllids need young, actively-growing foliage (flush) for development, and their populations peak at flush periods. In southern Florida, peaks of *D. citri* occur in May, August, and October through December, and this coincides with the new flush growth of *M. paniculata*. This citrus relative has a more continuous flushing pattern, and serves as an alternative host when citrus is not in flush. Psyllid numbers go down after heavy rains. In regions with clear-cut dry and humid seasons, psyllids will peak at their highest level after the first growth flushes of the new wet season following dry weather (Davis *et al*., 2005a).

Eggs of *T. erytreae* are seen mostly along edges of young leaves (Fig. 5 E). Those of *D. citri* occur on tips of growing shoots, on and between unfurling leaves. Similarly, *T. erytreae* nymphs develop on the underside of leaf blades, while *D. citri* nymphs are found on young stems and petioles. The major difference between the two psyllids concerns precisely the nymphs. The *T. erytreae* nymphs are embedded in pits or nests on the underside of the leaves (Fig. 5 F) which look like bumps on the upper side (Fig. 5 G, H). After an adult has emerged from a nymph, the nest is empty, but the bump remains. Hence, the presence of even one single bump on one single leaf is proof of *T. erytreae* occurrence. No such bumps are produced by *D. citri*. Leaves with bumps may be severely distorted. With *D. citri*, leaves are heavily curled (Fig. 5 D), and may be covered with honeydew. Characteristically, *D. citri* adults form a 45° angle with the plant tissue on which they feed (Fig. 5 A, B, C).

Symptoms of HLB. It is generally thought that it is difficult to diagnose HLB on the basis of symptoms, as none of them is specific, and citrus trees are often affected by additional problems. However, some symptoms are highly characteristic, and those will be emphasized here. They apply to most species of citrus, since nearly all citrus seedling trees and scion-roostock combinations are sensitive.

Yellow shoots and blotchy mottle leaves. A tree, which begins to show symptoms in an orchard in early stages of infection, can be identified, among still many symptomless neighbouring trees, by the presence of one or several yellow shoots. These shoots stand out against the green canopy of the tree (Fig. 6). These conspicuous "yellow shoots", or "huang long" in Chinese, gave the disease its name. A "yellow" shoot is seen as resulting from a shoot where psyllids have made a successful infection, as, indeed, HLB liberibacters can easily been detected in the symptomatic leaves of these shoots. A yellow shoot is thus an early symptom of the disease. On some trees, only one yellow shoot is present, and, with time, this affected shoot grows into a larger yellow branch, or "yellow dragon", since "long" means also "dragon". This yellow shoot or yellow branch is a very characteristic aspect of HLB. Often, more than one yellow dragon "wrap" themselves up around a tree. Eventually, in late stages of the disease, the yellow branches have taken over and canopy the whole tree, which has now become totally infected.

Under cool weather, new flushes may be pale green/yellowish, and zinc deficiency also induces yellow flushes. In these cases, practically all trees in an orchard show the problem. With HLB outbreaks, only some trees carry symptomatic shoots.

The yellow shoots on HLB affected trees look yellow because their leaves are partly yellow, partly green, with several shades of yellow, pale green and dark green, blending into each other, with no sharp limits between the various shades of colour (Fig. 7, 8). Such leaves show what is called "blotchy mottle". This symptom was described in particular by Lin (1956) in China (Fig. 7 A), and McClean and Schwarz (1970) in South Africa (Fig. 7 B, C). It is the most characteristic symptom of HLB wherever the disease occurs, be it in Asia, Africa or America, and whatever the citrus species affected. Also, as seen on Fig. 7 and 8, the blotchy mottle pattern on one side of the leaf midrib is not symmetrical to that on the other side. This asymmetry distinguishes blotchy mottle from zinc (Fig. 8 D), manganese, magnesium, calcium, and iron deficiency symptoms. With time, the whole leaf blade may ultimately turn uniformily yellow. In addition to blotchy mottle and yellowing, the leaves may become thicker, and leathery. Midribs and lateral veins are sometimes enlarged, swollen, and corky. In early stages of infection, blotchy mottle might be the only leaf pattern to be seen. In later stages, foliar symptoms of zinc deficiency will eventually develop, and such leaves have in general an upright growth, making a close angle with the shoot on which they are attached. Eventually, defoliation and dieback occur. With trees in early stages of disease, as observed in SPS, blotchy mottle is seen to affect large, well developed leaves. The same trees, one year later, had produced new mottled leaves, but smaller in size than those from the previous year.

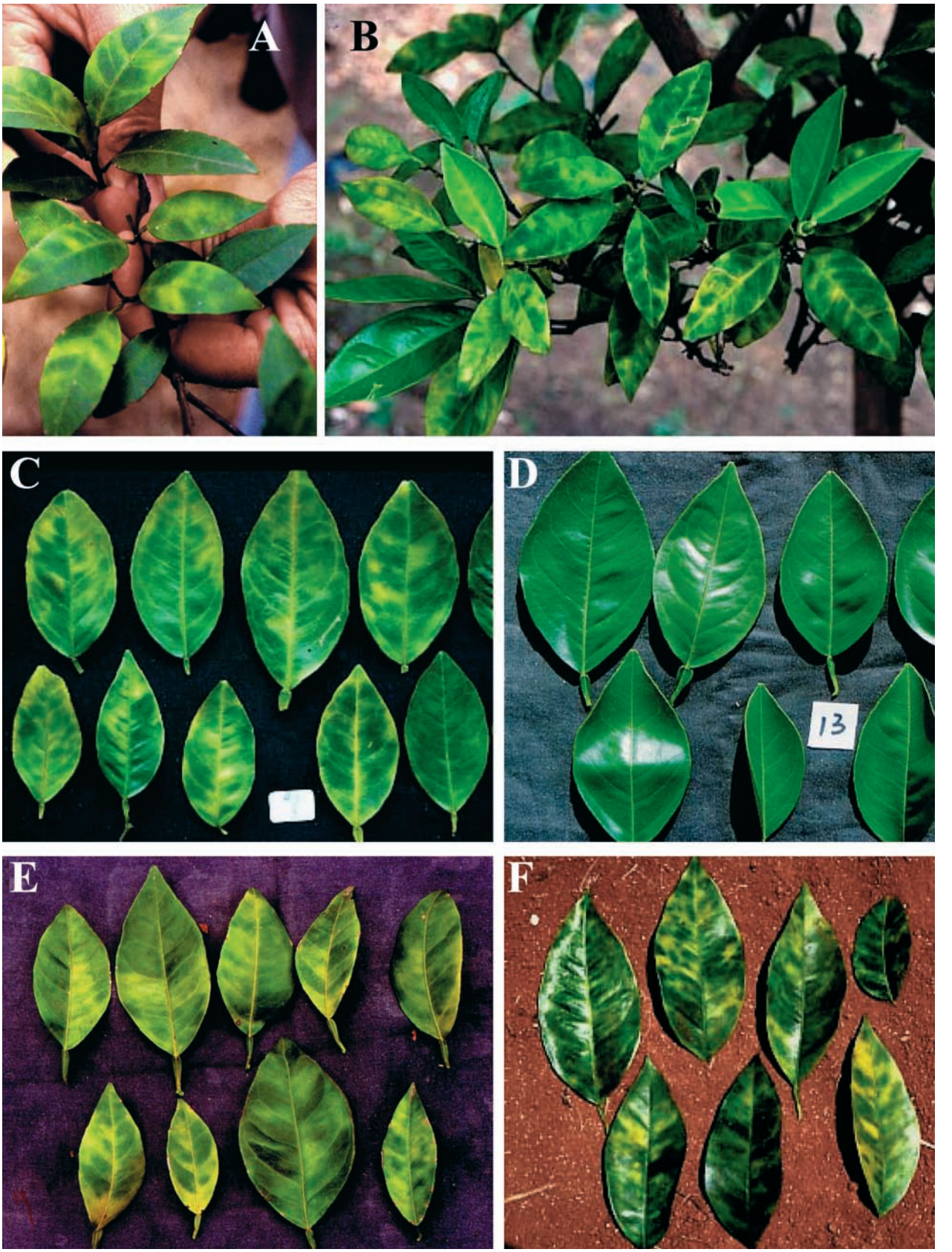

Fig. 7. Blotchy mottle-affected sweet orange leaves from China (**A**), South Africa (**B**, **C**), São Paulo State, Brazil (**E**), and Madagascar (**F**). Healthy sweet orange leaves in **D**.

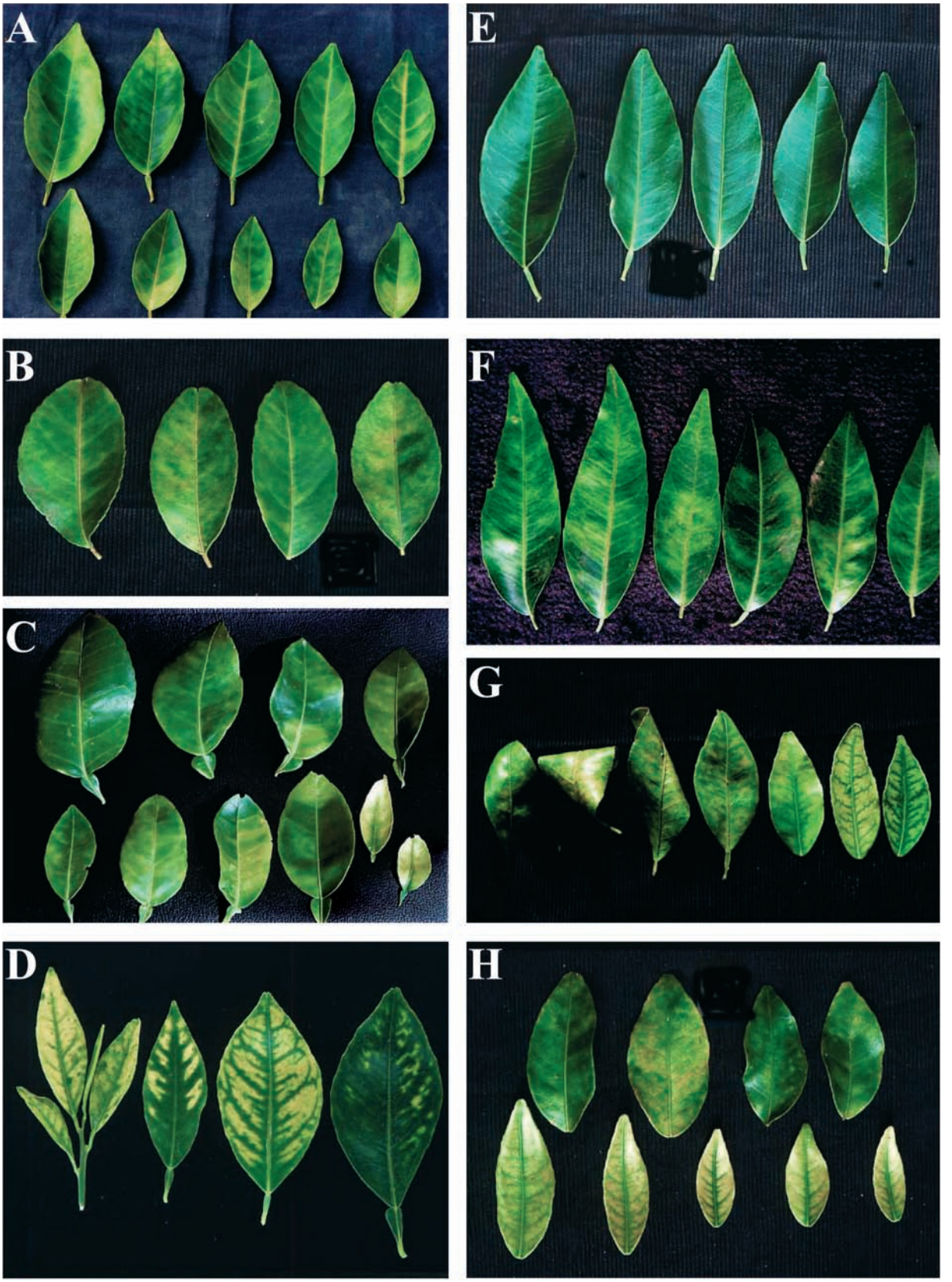

Fig. 8. Blotchy mottle-affected leaves of sweet orange from São Paulo State (**A**), rough lemon (**B**) and grapefruit (**C**) from Poona India, clementine from South Africa (**F**), and mandarin from Coorg, India (**G**, **H**). Healthy clementine leaves (**E**). Sweet orange leaves showing zinc deficiency symptoms with increasing severity from right to left (**D**).

In certain situations, blotchy mottle is difficult to find. In 1996 in North Bali, Indonesia, HLB was severely affecting almost 100% of young, 4-years-old Tejakula mandarin trees in many orchards of the coastal plain. The trees were uniformly yellow. Some leaves showed zinc deficiency symptoms, but leaves with clear-cut blotchy mottle were rare. These trees had practically no new flushes. PCR tests did however confirm HLB as the cause of decline in all these orchards. At the same time in some orchards at higher altitudes, HLB was just starting, yellow shoots occurred, and leaves had strong blotchy mottle.

Blotchy mottle is probably best observed on sweet orange trees, but most citrus species and varieties show it, including some mandarin varieties such as Tejakula in Bali and clementine in South Africa (Fig. 8 F) Other mandarin varieties, such as the Coorg mandarin in India, do not present good, classic blotchy mottle. The colour patches of yellow, dark green and pale green are not as large as on sweet orange leaves, and rather produce a mosaic pattern (Fig. 8 G, H). Also, in the orchard, affected mandarin leaves tend to be more curled than sweet orange leaves (Fig. 8 G). However, in the greenhouse, leaves of affected sweet orange trees are frequently curled.

Fruit symptoms. HLB induces also very characteristic fruit symptoms, seen especially well on sweet oranges and mandarins, and sometimes pummelos. Again, these symptoms are non specific, as similar symptoms are associated with stubborn disease. Fruits with HLB symptoms should be looked for on yellow shoots or branches. Symptomatic fruits are small, asymmetric, lopsided, with a bent fruit axis. At the time when the fruit changes colour, from green to yellow/orange, HLB-affected fruit shows colour inversion: the peduncular end of the fruit turns yellow/orange, while the stylar end is still green, whereas on normal fruit the coloration starts first at the stylar end, and moves only later to the peduncular end (Fig. 9 A, D, F to J). In addition, when the peduncle of a fruit with colour inversion is carefully removed, the resulting circular scar is stained orange, while on a normal fruit the scar is pale green. Colour inversion can be very pronounced in China, where it is called "red nose". In South Africa, HLB-affected fruit colours also unevenly (Fig. 9 C), and this symptom was responsible for calling the disease "greening". Sometimes, when one presses such fruit with the thumb, a silvery "finger mark" results (Fig. 9 E).

To further observe the symptoms, fruits should be cut in half. When the sectioning is made perpendicular to the fruit axis, it is easy to see the small, brownish/black aborted seeds, highly characteristic of HLB, but also present in stubborn affected sweet oranges. Cutting a lopsided fruit through the fruit axis, reveals its asymmetry, and some aborted seeds can also be seen (Fig. 10 A, B, C). In addition, the vascular bundles within the fruit axis at the peduncular end have a strong brownish stain (Fig. 10 B). As in stubborn-affected fruit, the albedo is sometimes thicker at the peduncular end than at the stylar end (Fig. 10 B).

In summary, when a tree shows one or several yellow shoots with blotchy mottled leaves, and small, lopsided fruits with colour inversion and aborted seeds, it is highly likely that the tree is affected by HLB. To confirm the HLB diagnosis, PCR detection of the HLB liberibacters should be made.

PCR confirmation of HLB diagnosis. For PCR detection of the HLB liberibacters blotchy mottled leaves should be used. Such leaves almost never fail to give strong, positive PCR reactions when the symptoms are due to HLB. Therefore it is untrue to state, as it is sometimes written, that it is difficult to detect liberibacters. For detection of *Ca*. L. africanus and *Ca*. L. asiaticus two sets of primers can be used: primers f-OA1 and r-OI2c for amplification of 16S rDNA, and primers f-*rpl*A2 and r-*rpl*J5 for amplification of ribosomal protein genes (*rpl*). In the case that the presence of both African and Asian liberibacters is suspected, the primer pair f-(OA1+OI1) / r- OI2c is recommended. *Ca*. L. americanus is detected with PCR primers f-GB1 and r-GB3. Simultaneous detection of the three liberibacters by multiplex PCR has been developed with the following two primer pairs in the same reaction mixture: (i) f-*rpl* A2 / r-*rpl* J5 and (ii) f-GB1 / r-GB3.

As mentioned above, initially in Brazil, most PCR reactions gave negative results, even though leaves with strong symptoms of blotchy mottle were used. This was later shown to be due to the fact that a new liberibacter species, *Ca*. L. americanus, was involved (Teixeira *et al*., 2005a, b, c). If a similar situation is encountered elsewhere, search for a new liberibacter should be sought.

In some cases, when HLB symptoms are not immediately recognized, and are attributed to some other causes, such as mineral deficiencies, the relevant trees might be in relatively late stages of the disease, and mottled leaves may be lacking. In such cases, leaves with zinc-deficiency symptoms should be used.

For PCR detection, HLB-affected leaves, infected with either one of the three liberibacters, should be available as positive controls. If not, DNA preparations from such leaves, obtainable from several laboratories, can be used. Healthy citrus leaves are used as negative controls.

CONTROL OF HLB

There are no curative methods to control HLB. When HLB was found to be a bacterial, rather than a viral disease, antibiotic control by trunk injections of tetra-

cyclines was tried in several countries, including South Africa, Indonesia, and Taiwan, but soon abandoned, because of ecological reasons, but essentially because tetracycline is bacteriostatic, rather than bactericidal, and the treatment had to be repeated each year. Thus, control is by preventing trees from becoming infected. If HLB is not yet present, quarantine measures should be enforced to keep it out. The most serious situation is when HLB has entered a region previously HLB-free. The first immediate measure is to survey and determine the extent of the outbreak. The next objective is to prevent as many trees as possible from becoming infected. This can only be done: (i) by eliminating as much as possible liberibacter inoculum, *i. e*. by removing infected trees, and (ii) by keeping psyllid populations as low as possible. Without these control measures, HLB evolution in an affected orchard is fast. In China, it has been stated repeatedly that, without control, it takes only about five years for an orchard to reach 100% infection. Similar situations seem to occur in SPS. In the Reunion island, the time needed to reach 100% infection was estimated to be 13 years. Average time is eight years.

Quarantine measures. If HLB is still absent from a given region, do not introduce it! The biggest quarantine threat is transport, by people, of citrus and citrus relatives. These plants may be infected with liberibacters and may carry psyllid eggs and/or nymphs. *M. paniculata*, an ornemental citrus relative, is a preferred host of *D. citri*, and if further work confirms that it is also a host of the liberibacters, it deserves special quarantine attention. Also, it must be remembered that the liberibacters cannot be detected in symptomless leaves, even though their detection in mottled leaves is straightforward. Symptomless leaves are either healthy, and will never give positive PCR reactions, or they are already infected, but the liberibacter titer is still too low to be detected by the PCR methods available today. For these reasons it is meaningless to use conventional HLB PCR techniques with symptomless plant material.

Eliminating inoculum by removal of infected trees. If all infected trees were symptomatic, it would be easy to spot them, on the basis of their symptoms, and remove them. Unfortunately, there is a latency period during which recently infected trees do not show symptoms. This period extends from the moment the tree becomes infected by the psyllids up to the moment it expresses symptoms. The length of this period may vary from tree to tree, but is generally assumed to be 6 to 12 months long, if not longer. Therefore, removal of all symptomatic trees will not result in removal of all infected trees. In practice, several surveys are required to remove as many infected trees as possible. Some infected trees, which were still symptomless at survey # n, will show symptoms at survey # n+1, and can be removed; other trees will only be removed at survey # n+2. Also, within the time period between surveys n and n+1, or n+1 and n+2, psyllids (if not well controlled), may infect additional trees, and so on. Obviously, the time period between successive surveys is important. In particular, if this period is too long, some symptomatic trees will have become severely affected before they are removed, and serve as new sources of inoculum. In SPS, experience seems to show that a period of about three months is a good compromise.

Surveys must be carried out carefully. All trees in an orchard, however large it is, should be examined one by one, and enough time, say a few minutes, must be spent at each tree. Experience gained in a large grove (one million trees) in southern China suggests that the scouts in charge of the surveys should work in pairs, so that each tree is examined by the two scouts of a pair, one scout on each side of the row. In the case of orchards with large, adult trees, it is essential to examine the top of the trees. In SPS, relatively high towers have been built onto tractors to permit efficient observation of tree tops. Finally, once affected trees have been identified, they should be removed as quickly as possible.

In SPS, recommendation is that all symptomatic trees be removed, whatever their age or the severity of their infection (Ayres *et al*., 2005). Removing HLB-affected branches by pruning, instead of removing the whole tree, has been discarded because the many sprouts that develop at the site of pruning turn into "yellow shoots" and favour psyllid development. In South Africa, it has been recommended (Buitendag and von Broembsen, 1993) that HLB-affected trees up to 5 years of age be removed. Trees from 6 to 10-year-old, when severely affected, are also removed. With less affected trees, and with trees older than 10 years, only the affected branches are removed.

In SPS, citrus canker is another disease of citrus, the control of which also involves removal of trees. In this case, the infected tree as well as all surrounding trees, within a distance of 15 m, are removed. From the epidemiology studies of HLB (Gottwald, 2005; Bassanezi, 2005), it seems that this strategy is probably inadequate for HLB. Indeed, when HLB vectors move, either naturally in search of new feeding opportunities or when disturbed, they not only move to neighbouring trees (primary foci), but also to trees located at distances of 25 to 50 m (secondary foci). In addition, psyllids can move a distance of at least 1.5 km with wind, and probably much longer distances with hurricanes or tropical storms, such as those in Florida.

When an orchard is too severely affected, with a high percentage of affected trees, it might be best to remove the whole stand. The following figures might help to make the decision. It can be roughly estimated that with Asian HLB, *D. citri* being the vector, an orchard with 10% symptomatic (Sy) trees, has a total of 20% infect-

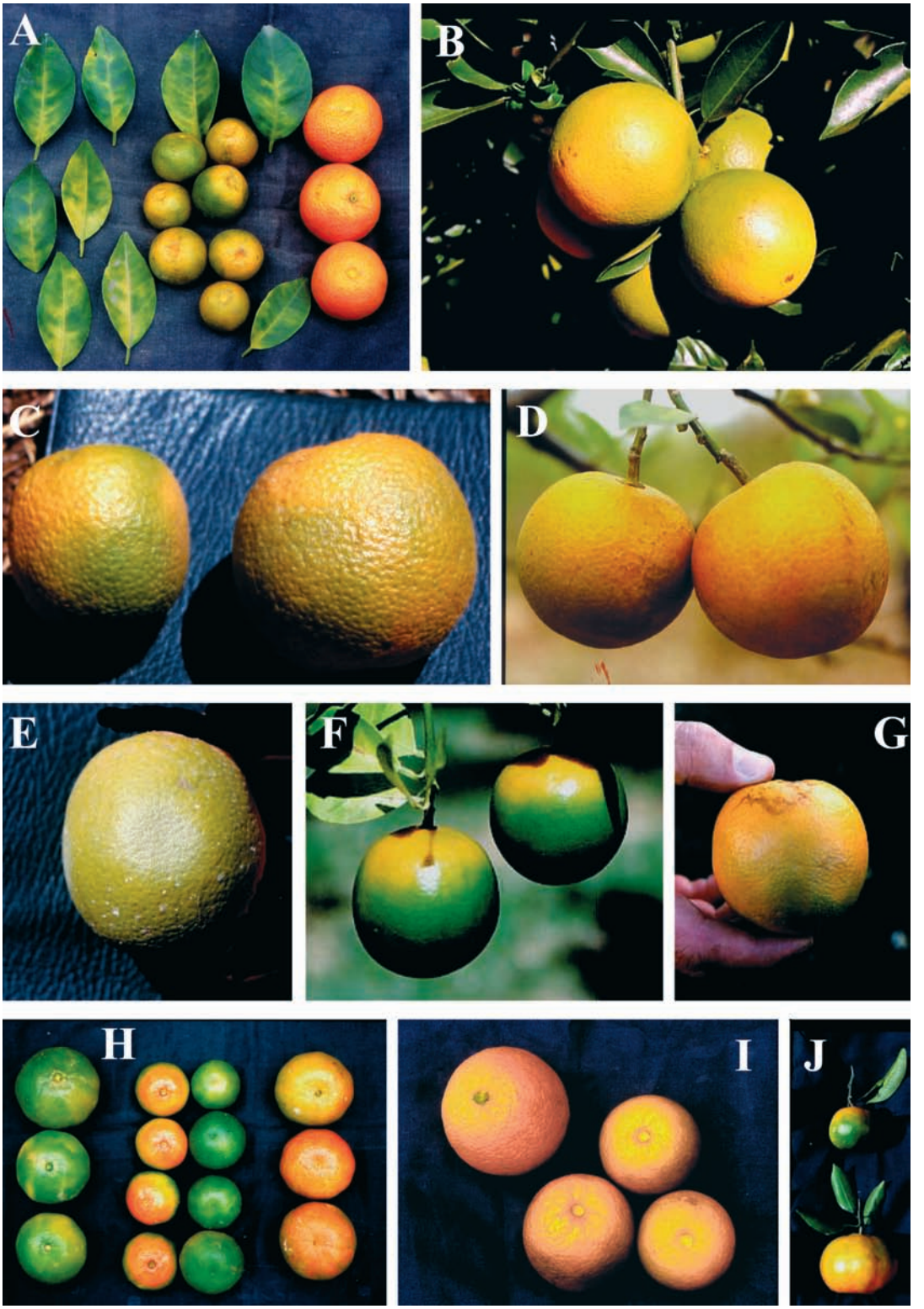

Fig. 9. The two most characteristic symptoms of HLB are leaves with blotchy mottle and fruits with small size and colour inversion (**A**, normal sweet orange on right; Behai, Guangxi, China). Poorly coloured "greening"-affected oranges from South Africa (**C**), which, when pressed with a finger, develop a silvery "finger mark" (**E**). Normal oranges with coloration starting at the stylar end (**B**). Oranges and mandarins with coloration starting at the peduncular end (colour inversion): (i)sweet oranges from China, Guangxi (**D**) and Fujian (**H**, normal fruits with orange stylar ends on far right, and green peduncular ends on far left; fruits with colour inversion, in the middle), and São Paulo State, Brasil (**G**, **I**); (ii) mandarins from North Bali (**F**), and China (**J**, lower fruit: normal, upper fruit: with colour inversion).

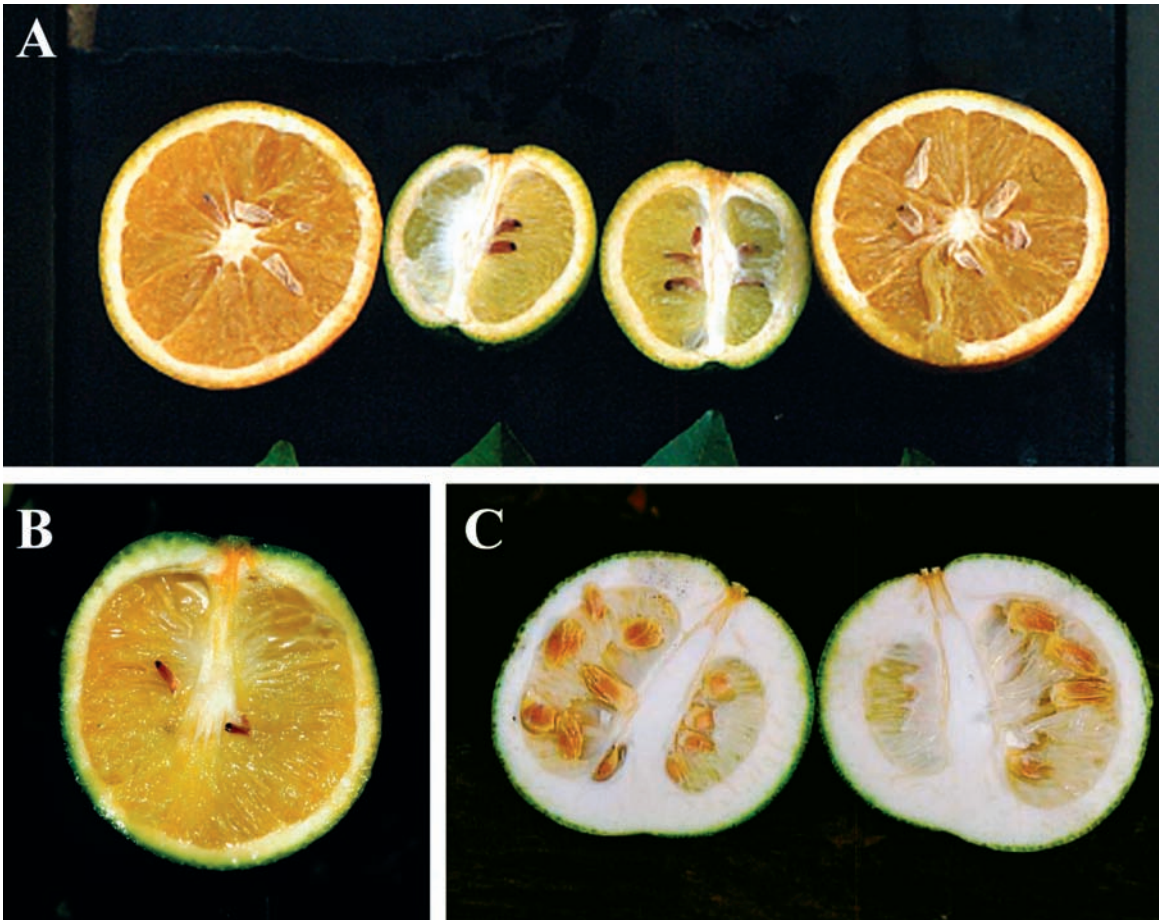

Fig. 10. A. Far left and far right, normal sweet orange halves showing normal seeds; middle, two halves from a lopsided HLB-affected sweet orange from China. Notice asymmetry of the fruit section, and the brownish-black seeds. B. Sectioned lopsided sweet orange from São Paulo State, with brownish-black seeds, thick albedo at the peduncular end, and vascular bundles stained orange-brown at the peduncular end of fruit axis. C. Lopsided pummelo showing vascular bundles stained orange-brown.

ed trees, i.e., symptomatic trees plus asymptomatic trees (Sy+Asy). For 20%, 30% and 50% Sy trees, there will be 36%, 50%, and 70% (Sy+Asy) trees. In other words, in an orchard with 30% symptomatic trees, half of the trees are infected, and will have to be pulled out sooner or later. Is it worthwhile to keep such an orchard? Probably not!

When affected trees have been pulled out, they can be replaced by young, healthy trees. In SPS, by law, all nursery trees have to be produced in covered, insect proof screenhouses, and this obligation guarantees that they are HLB-free.

Even though in SPS removal of HLB-affected trees is compulsory by law, great efforts have been made through the media (articles in newspapers, programs on radio and TV, training courses for growers and technicians) to explain the situation, and convince the growers to remove the affected trees on a voluntary base (Ayres *et al*., 2005). Most of the growers cooperate, and 350 thousand symptomatic trees have been voluntarily eliminated, as of October 2005. Cooperation of the growers is also required for vector control.

Psyllid vector control. Key features of the psyllids, which need to be taken into account in the formulation of control strategies have been reviewed (Buitendag and von Broembsen, 1993; Halbert and Manjunath, 2004; Hall, 2005).

Treatments with contact and systemic insecticides reduce psyllid populations, and thus HLB spread. The efficiency of the treatments is easier to estimate with *T. erytreae* than with *D. citri*, because of the foliar "bumps" produced by *T. erytreae* nymphs. In the large, South African citrus estates, control of *T. erytreae* can be very efficient, as "bumps" are hardly seen. Systemic insecticides, aimed specifically at psyllids (monocro-

tophos), have been applied directly to the tree trunks, and have been very effective. In Asia, a large range of insecticides, mostly organophosphates and pyretroids, are used in very intensive spray programs to kill eggs and nymphs on flush growth. Horticultural and agricultural mineral oils are being developed as alternative treatments, and these are much less damaging to the environment and less disruptive to biocontrol of other pests (Davis *et al*., 2005a).

Reunion island is the only region where biological control of both *T. erytreae* and *D. citri* has been effective (Etienne and Aubert, 1980; Aubert *et al*., 1980). The success is due to the fact that, initially, the two psyllids occurred in the island in the absence of parasites, and thus, hyperparasites. Therefore parasites of the two psyllids were introduced into the island, care being taken not to introduce hyperparasites at the same time. *Tamarixia dryi*, a parasite of *T. erytreae*, and *Tamarixia radiata*, a parasite of *D. citri*, were collected, respectively in South Africa and India, reared in Reunion and released in abandoned citrus orchard having received no insecticide treatments for quite some time. A second reason for success is the fact that *T. dryi* multiplied not only on *T. erytreae*, but also on an additional psyllid, *Trioza eastopi*, not a citrus psyllid, but multiplying on a widely distributed weed, *Litsea chinensis*. Releases of *Psyllaephagous pulvinatus*, introduced from South Africa, were also made, but the insect, much less vigorous than *T. dryi*, disappeared from the island.

In Florida, *T. radiata* has been introduced from Taiwan and Vietnam, and is being established in Florida**.** In China, studies are conducted on *Campylomma chinensis* Schuh, as a potential biocontrol predator of *D. citri* (Wu, 2005).

The Brazilian experience. In SPS, encouraging results have been obtained in the control of HLB by tree removal and insecticide treatments against psyllids (Ayres *et al*., 2005). For instance, in one large citrus orchard (71,000 trees), 10 surveys for identification of HLB-affected trees were carried out from September 2004 until September 2005. The first survey identified 7% affected trees. On the 2nd survey, in December 2004, the percentage went down to 0.01%, but increased again to 1.1% after the 6th survey in May 2005. From there on, the percentages decreased regularly to 0.5% (7th survey, June 2005), 0.15% (8th survey, July 2005), 0.05% (9th survey, August 2005), and 0.03 (10th survey, September 2005). It is hoped that the percentages of affected trees will remain at the level of 0.05%, even with less frequent surveys and tree removals. HLB will not be eradicated, but it will be possible to live with the disease.

To achieve this goal, SPS has a number of favourable factors. Brazil, contrary to Florida, is not affected, year after year, by hurricanes and tropical storms, the role of which in spreading the disease and its vector, might be more significant than thought. A task force of 800 people from both the private sector (Fundecitrus) and the public sector (Government, public research organizations) is involved in HLB control and helps the growers. A good interface has been built between growers, Fundecitrus and the government. Sixty million trees have already been surveyed, 70 million more trees have still to be inspected. The first wide range survey will be completed in February 2006. The surveys for HLB benefit from the year long experience of Fundecitrus in the control of citrus canker. The nursery trees required to replace the removed HLB-affected trees are produced from healthy budwood in covered, insect proof nurseries, a procedure that guarantees that they are free of HLB and all other graft-transmissible diseases (except CTV strains used for cross protection). Even though HLB has been present for several years before it was recognized, most orchards in the affected municipalities have still a low disease incidence.

Very few countries have been able to control Asian HLB. SPS might be one of the first to be successful, and live with the disease until genetically engineered citrus cultivars, resistant to HLB, will eventually become available (Hartung *et al*., 2005b).

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