Chapter 18

Bacterial pathogens of citrus: Citrus canker, citrus variegated chlorosis and Huanglongbing

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18.1 Introduction

Commercial citrus groves are invariably planted as dense monocultures comprising a few commercial citrus varieties covering large tracts of land, providing little to no natural variation in resistance to any pest/vector, pathogen, or a combination of both with capacity to exploit it. The majority of bacterial pathogens need no insect vector at all, for example, the xanthomonads causing citrus canker, although disease incidence can be dramatically accelerated by leafminers. These xanthomonads enter through stomata or wounds, actively attacking mesophyll cells from the outside and directly affecting citrus cell gene expression by injection of eukaryotic transcription factors. Nevertheless, as discussed in detail below, there is remarkably little variation in resistance among commercially grown citrus genera, species, and varieties to the Asiatic strains of the disease.

Liberibacters are literally injected by their psyllid vectors directly into living phloem cells. Psyllids free of Liberibacters may be introduced into a citrus growing region free of Huanglongbing (HLB), resulting only in the direct damage caused by the psyllid. Independent of vector introduction, Liberibacters may be introduced by planting infected and asymptomatic citrus into a previously uninfected region, but the pathogen cannot spread without an insect vector with host range on citrus. As discussed in some detail below, the excessively long incubation period required before HLB symptoms become recognizable can prevent or frustrate timely detection of such introductions, even with inspections. Epidemics can result from the intersection of these two independent introduction events, as appears to have occurred relatively recently in Florida with HLB. Of course, both events can be concurrent. Again, however, there is remarkably little variation in resistance among commercially grown citrus varieties to Liberibacter infections.

It is useful to distinguish between parasitism and pathogenicity. A parasite is capable of deriving nutrition from, and reproducing on or within, its host(s), and is not always associated with the disease. For example, a Liberibacter-infected pear tree is nearly asymptomatic (Raddadi et al., 2011). A pathogen causes disease, but not always on all hosts. Pathologists typically pay attention only to pathogens on commercially important crops, but pathogens can have a much wider parasitic host range than expected if they cause no disease or cause the disease on weeds. A recurring theme in this chapter involves the worldwide increase in commerce and in transcontinental dispersal of many pathogens and their vectors. The fact that there is often overlap of parasitic, if not pathogenic, host ranges of multiple bacterial species, and the fact that apparent host range limitations of a given pathogen can be disguised by the host range limitations of its insect vector can result in emerging and sometimes surprisingly severe disease threats. In general terms, if a vector is introduced into a geographic region with an unusually wide host range that may include weed or arboreal crops, it is conceivable that the vector may facilitate exposure of previously isolated bacterial populations to one another, thereby facilitating horizontal gene transfer events, either by cell–cell conjugation or by phage transduction within either the insect vector or plant host. Transcontinental dispersal of pathogens and vectors dramatically increases the opportunities for horizontal gene transfer and allows increased recombination frequencies between pathogenic variants of a species and even across genera.

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Xylella strains are injected by their various vectors directly into (dead) xylem vessels. In the case of citrus variegated chlorosis (CVC), despite the fact that sharpshooters feeding on both oranges and other citrus varieties are abundantly available, it is therefore perhaps surprising that host range on citrus appears limited to *Citrus sinensis*. Why only sweet orange? One can speculate that since coffee plantations with coffee leaf scorch disease are in close proximity to citrus groves with CVC in Brazil and since both diseases are caused by the same *X. fastidiosa* subspecies pauca (Xfp), the opportunity arose for horizontal gene transfer or recombination of host range factors that allowed emergence of CVC. Recombination is documented between Xf subsp. morus strains pathogenic to mulberry and blueberry in the United States (Almeida and Nunney, 2015), and Xfp strains from coffee (Jacques et al., 2016). The wide variation in *Xylella* pathogenicity and host range illustrates the need for a better understanding of the genetic factors determining both. In contrast with both citrus canker and HLB, there is remarkably strong variation in resistance among commercially grown citrus genera.

In all the three cases, each involving very different pathogen disease cycle and reproductive strategies, the causal bacterial genera exhibited a wide host range, pathogenicity, and genome variations that indicate horizontal gene transfer of pathogenicity factors. Selection in citrus monocultures does the rest. In addition to the three bacterial pathogens most damaging to commercial citrus that are the focus of this chapter, other bacteria causing notable citrus diseases of worldwide importance include *Burkholderia andropogonis* (bacterial brown leaf spot) and a few Mollicute (lacking a cell wall) species such as *Spiroplasma citri* (citrus stubborn disease) and *Phytoplasma* species (witches' broom disease of lime and HLB-like diseases). Currently the worldwide incidence and importance of these bacteria are minor.

18.2 Citrus canker

18.2.1 History and background

Asiatic citrus canker (ACC) is an important bacterial disease of citrus with severe yield, trade, and quarantine implications. The ACC is also referred to as "Canker A" and is by far the most widespread and severe form of the disease. The disease is caused by *Xanthomonas citri* (*ex* Hasse 1915) (Gabriel et al., 1989) subsp. *citri* (Schaad et al., 2006) pathotype A (XccA). Historical synonyms for XccA are *X. citri, Xanthomonas axonopodis* pv. *citri*, and *Xanthomonas campestris* pv. *citri* "strain" A). ACC is believed to have originated in Southeast Asia-India based on herbarium specimens from India and Java in the 1830–1840s. During the 1900s it was found throughout Asia, including Japan, as well as South and Central Africa, Middle East, Australia, New Zealand, Pacific Islands, South America and Florida, USA. Another form of citrus canker, "Cancrosis B," with nearly identical symptoms as ACC (erumpent, hyperplastic lesions on leaves, stems, and fruit are the key diagnostic symptoms) emerged in South America (Carrera, 1933) with host range limited to lemons, Mexican lime, sour orange, and pummelo (Stall and Seymour, 1983). Cancrosis B is also referred to as "False canker" or "Canker B" and is caused by a different and phylogenetically quite distinct species, *Xanthomonas fuscans* subsp. *aurantifolii* (Xfa) (Schaad et al., 2006) pathotype B (XfaB). Cancrosis B is prevalent on lemons in Argentina, Paraguay, and Uruguay, but is less aggressive than ACC and is largely under control (Canteros et al., 2017). Since it is in a separate species, XfaB (Cancrosis B) strains can be easily differentiated by microbiological, serological, genetic, and host range inoculation tests from XccA (ACC) strains.

What do the two species, *X. citri* and *X. fuscans*, have in common to allow their respective subspecies, Xcc and Xfa, to elicit very similar symptoms on citrus? Both encode and inject nearly identical eukaryotic transcriptional activator-like (TAL) protein effectors into citrus cells. Pathogenicity gene *pthA* was the first member of the *avrBs3/pthA* gene family demonstrated to function for pathogenicity and is required for both growth and pathogenicity of XccA strains on citrus (Swarup et al., 1991, 1992). *pthA* is capable of conferring ability to cause canker-like symptoms to strains that cannot otherwise cause canker, such as *X. aurantifolii* pv. *citrumelo*. A very close homolog, *pthB*, is similarly required for pathogenicity of XfaB (Cancrosis B) strain B69 and is found on a self-mobilizing plasmid that can transfer itself between these two species (El-Yacoubi et al., 2007). All canker-causing strains of *X. citri* subsp. citri tested carry *pthA* homologs (Cubero and Graham, 2002; Mavrodieva et al., 2004; Munoz-Bodnar et al., 2017). When *pthA* is transiently expressed in citrus using either *Agrobacterium tumefaciens* or particle bombardment, small canker-like lesions are elicited (Duan et al., 1999). Therefore, *pthA* and its homologs encode the primary causal effectors of the citrus canker disease phenotype.

Not surprisingly, variant pathotypes within both Xcc and Xfa species have emerged that are primarily distinguished on the basis of host range and determined by apparently minor differences in virulence factors. A third citrus canker type, described as Cancrosis C or "Canker C," was discovered in Brazil on Mexican lime by Namekata (1971) with an even more restricted host range than XfaB (Cancrosis B) strains (Stall and Seymour, 1983). Cancrosis C is also caused by Xfa, and strains of this group (XfaC) are primarily limited to Mexican lime, although sour orange can also be a host. XfaC strains are currently distinguished from XfaB strains by pathogenicity tests and from ACC strains by species-level tests.

Variants of *X. citri* subsp. *citri* (Xcc) have also emerged more recently. These also elicit ACC-like lesions but with less aggressive symptoms and with host range largely limited to Mexican lime: XccA* and XccA^{w} (Gordon et al., 2015). XccA* strains were discovered in Oman, Saudi Arabia, Iran, and India with host range limited to Mexican lime (Vernière et al., 1998). The XccA^{W} pathotype is also characterized by host range restricted to Mexican lime and lemon and has to date only been found in Florida and Texas (Munoz-Bodnar et al., 2017). Grapefruit (*Citrus paradisi*), resistant to both XccA* and XccA^{W} strains, may serve to differentiate the two groups, since XccA^{W} strains elicit a strong hypersensitive response (HR) in grapefruit, while XccA* strains do not (Brunings and Gabriel, 2003). A single avirulence gene was found to restrict an XccA^{w} strain from virulence on grapefruit, but this gene was not detected in XccA* strains tested (Rybak et al., 2009). Interestingly, XfaC strains also cause an HR on grapefruit, while XfaB strains do not (Brunings and Gabriel, 2003). Minor genetic differences in one or a few virulence factors may account for the differences from XccA in the restriction of these pathotypes to lime and from each other. The restricted host range variants of Xcc (XccA* and XccA^{W}) and Xfa (XfaB and XfaC) are of limited worldwide distribution and economic consequence and therefore further discussion will be limited to ACC, caused by XccA.

18.2.2 Symptoms and epidemiology of ACC

ACC is primarily a leaf and fruit spotting disease characterized by erumpent lesions (cankers) on fruit, foliage, and young stems (Fig. 18.1) of susceptible cultivars of citrus (Schubert et al., 2001; Timmer et al., 1996). Most commercial citrus varieties are moderately to highly susceptible to the disease, although grapefruit is the most susceptible fruit. When the disease is severe, defoliation, dieback, and fruit drop can occur and infected fruit that remain are less valuable or entirely unmarketable (Graham and Gottwald, 1991). ACC is spread by rain splash combined with wind and is greatly exacerbated by tropical storms and hurricanes (Gottwald et al., 2002). ACC epidemics are aggravated by the Asian citrus leaf miner, *Phyllocnistis citrella*, introduced to Florida in 1993 and to Brazil in 1996. Where the leaf miner and citrus canker coincide, canker has increased significantly as a consequence of the insect's feeding activities which create wounds that expose leaf mesophyll tissues to splashed inoculum, thus greatly expanding the probability of infection (Gottwald et al., 1997, 2007).

During seasons when spring and summer rains are combined with wind speeds in excess of 8 m/s damage from the disease can range from nominal to significant (Serizawa et al., 1969; Serizawa and Inoue, 1974). When lesions on leaves are wetted for only 1–2 min, the bacteria ooze to the surface in great numbers and this inoculum release can persist for several hours, continuously providing inoculum for dispersal throughout prolonged storm events (Bock et al., 2010a,b). Distance of spread due to bacterial aerosols is directly related to rain splash and wind speed, from a few cm during gentle rains without wind to several km during tropical storms and hurricanes (Gottwald and Irey, 2007; Bock et al., 2011). For moderate windblown rain events, natural windbreaks such as conifers or hardwood trees with interlocking canopies can effectively reduce wind speed and trap windblown inoculum, thereby reducing infection. Citriculturists effectively control ACC in Argentina



FIG. 18.1 Asiatic citrus canker (ACC) lesions on leaves, fruit, and young stems of grapefruit.

by judicious use of such natural windbreaks effectively surrounding plantings 1–5 ha in area (Stall and Seymour, 1983). ACC is highly contagious. Spread is often human mediated by contaminated clothing, vehicles, equipment (e.g., pruners, hedgers, mowers, ladders, harvesters), or animals either in commercial or residential citrus. When the foliage is wetted, the bacteria ooze from the hyperplastic lesions that have broken the foliar surfaces (for a physiological process description, refer Brunings and Gabriel, 2003) and can be transferred when the mechanical device, human, or animal come in contact with the bacterial ooze and inadvertently transfer it to susceptible foliar tissues on adjacent or distant trees.

18.2.3 ACC control through eradication

ACC currently exists in the majority of commercial citrus producing regions of the world and in the Americas has plagued multiple States in the Southeastern US, as well as Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay (Braithwaite et al., 2002; Koizumi, 1985; Gottwald et al., 2007). The approach to controlling ACC depends on whether the disease is newly introduced or if it has become endemic. If it is newly introduced, by far the best approach is eradication. ACC infestations have most significant impact on commerce and trade due to the governmental regulatory actions that result in restricted interstate and international transport and sale of fruit originating from known infested areas.

The consequences of ACC introduction to citriculture must be examined through a historical, economic, and sociopolitical lens to fully comprehend its impact. If the disease is recently introduced and in low incidence, eradication has often been attempted to return the area to a disease-free status, thereby avoiding the cost of quarantines and unfavorable trade impediments. Conceptually, eradication is quite simple. Diseased plants must be removed more rapidly than they become diseased. The more rapidly and efficiently diseased individuals are removed, the less chance of continued pathogen spread, and the quicker the disease will be extinguished. In practice, however, eradication is complex. It is not always easy to recognize newly infected plants. A several week-long incubation period between infection and disease expression is quite common. Thus, some infected members of the plant population are "subclinical"; that is, infected but not yet expressing disease. For ACC the subclinical infections can persist for 10–14 days without expressing symptoms and even then, these symptoms are not always easily seen. The average time of detection in residential areas was estimated to be 108 days postinfection providing ample time for polycyclic reproduction and spread (Gottwald et al., 2001a,b).

Additionally, diseased plants are not always recognizably aggregated into convenient or contained groups for easy eradication. The disease is often somewhat considerably diffuse among an otherwise healthy population. The combination of lengthy incubation periods and diffuse patterns of disease necessitates that to achieve eradication, large radii around recognized infections of both diseased and asymptomatic but potentially latently infected plants must be removed or "culled" as well (Gottwald et al., 2001a,b, 2002, 2007; Schubert et al., 2001). For ACC epidemics, the resulting eradication programs have at times exceeded the social tolerance of commercial growers and additionally residential homeowners who resist the removal of their apparently healthy but potentially asymptomatically affected trees in support of the commercial citrus industry.

Several early eradication programs were successful in South Africa, Australia, the Fiji Islands, Mozambique, New Zealand, and the United States (Koizumi, 1985). In Northeast Argentina, ACC was first recognized in 1928 and eradication was attempted in 1976 but subsequently failed in part due to grower's concern and reluctance to remove the large proportions of commercial trees to achieve eradication (Canteros et al., 2017; Hogg, 1985; Rodriguez, 2012). In Australia ACC has been introduced and successfully eradicated three times: 1918, 1984 and a third time in Queensland in 2004 (Gambley et al., 2009). In Brazil ACC was first detected in 1957 and eradication programs were intermittent over the next 60 years eventually leading to establishment of ACC; by 2009 the disease was declared endemic and the eradication program halted (Behlau et al., 2016).

ACC was first detected in Florida in 1910 and it was eradicated by the 1930s after massive removal of infected trees (Schubert et al., 2001). ACC was discovered a second time in Florida in 1986 and was declared eradicated in 1992 but was discovered a third time in multiple residential areas in Dade County in 1995 within a 14 mile^2 area south of the Miami International Airport (Gottwald et al., 2001a,b). This discovery prompted an intensive eradication campaign by a joint state/federal citrus canker eradication program (CCEP). The program was based on an epidemiological study of ACC spread that resulted in an ACC-eradication protocol that required the removal of all "exposed trees" within a 579-m radius of a known infected tree (Gottwald et al., 2001a,b). Over the next 10 years, the canker eradication program cost approximately $1 billion dollars, the largest plant disease eradication program worldwide to date. The program was plagued by legal conflicts between the CCEP and residential homeowners who felt that the regulatory actions being taken protected the citrus industry at too high a cost to residential citrus tree owners. The litigation of numerous injunctions and appeals led to a discontinuous and sporadic eradication program. As the legalities of eradication were debated, the disease continued to spread, and outbreaks in new commercial and residential areas erupted across Florida.

Hurricanes and tropical storms in Florida have been associated with long-range dissemination and increased local incidence of ACC (Gottwald and Irey, 2007; Irey et al., 2006). In 2004, three hurricanes and a major tropical storm crossed the Florida peninsula and exacerbated preexisting ACC infections and caused overwhelming dispersal of the pathogen over the commercial industry. Statistical models developed to predict the spread of ACC due to hurricanes and tropical storms led to numerous discoveries of new ACC outbreaks and more effective and rapid eradication (Irey et al., 2006). However, hurricane Wilma struck Florida in the Fall of 2005 and caused such massive additional spread of the disease that the regulatory agencies, citrus industry, and research scientists alike concluded that the disease had likely become ineradicable, resulting in the termination of the 10-year, $1 billion canker eradication program (Gottwald and Irey, 2007). As a consequence, ACC has greatly increased and is now found throughout the majority of Florida's commercial groves.

18.2.4 Endemic control/mitigation of ACC

Where ACC is endemic, mitigation requires integration of appropriate cultural practices including windbreaks and leafminer control, with frequent applications of copper (copper hydroxide and copper oxychloride) sprays (Leite Jr et al., 1987). Leafminer control via insecticides is particularly important for young trees and certain cultivars that have more frequent and longer duration of growth flush. Copper sprays reduce infection somewhat. Fruit are most susceptible for 90 days after petal fall when copper is most efficacious. Grapefruit is highly susceptible to ACC requiring more sprays to provide acceptable control compared to orange or mandarins which are much less susceptible. Antibiotics such as gentamicin, kasugamycin, and streptomycin also have activity for canker control if properly formulated. However, the possibility of eliciting bacterial resistance and human and animal health issues raise concerns about wide scale antibiotic use in agriculture (Graham et al., 2006; Behlau et al., 2012). Recently the control of ACC via the use of nano-formulated zinc oxide was shown to exceed the cuprous oxide and cuprous oxide/zinc oxide bactericides, although deployment has not been wide scale (Graham et al., 2016).

18.2.5 Implications of citrus canker quarantines and trade

The risk of introduction of citrus canker bacteria to new, unaffected citrus producing areas is a major concern for those citrus industries attempting to remain free of citrus canker. If canker is introduced, quarantines and restrictions could ensue that would severely limit or deny access to domestic and international markets. Various regulatory agencies such as USDA (United States Department of Agriculture), APHIS (Animal and Plant Health inspection Service) initiate extensive Pest Risk Assessments and Risk Management Analysis to evaluate the potential for processed fruit as a pathway for ACC introduction. Based on the assessments and analyses, regulatory agencies develop regulations under which fruit may be moved interstate or intercountry from quarantined areas. To address fruit as a pathway, a series of studies were conducted with citrus fruit as a potential pathway for XccA to enter and become established in these areas, including: (1) survival of XccA in disease lesions on fruit and on fruit surface after disinfectant treatment as used by commercial packing houses; (2) survival of XccA in culled fruit that were discarded in cull piles, and (3) dissemination of XccA and disease establishment in simulated rainstorms. Taken as a group, this series of studies on canker bacteria viability in and on fruit surfaces, and bacteria dissemination, demonstrated that harvested and packinghouse-disinfested citrus fruit are extremely unlikely to be a pathway for canker to reach and become established in canker-free areas (Gottwald et al., 2009). These studies have helped to diminish the need of quarantines and maintain open markets and trade.

18.3 Citrus variegated chlorosis

18.3.1 History and background

Xylella fastidiosa (Xf) is a Gram-negative, xylem-limited, insect vectored and often difficult to culture bacterium that causes some of the most serious plant diseases worldwide and in a wide variety of hosts. Its five (to date) described subspecies tend to have strong host range preferences that may overlap, although individual strains appear to have a highly restricted host range. Xf appears to have originated in the Americas, and causes production-limiting diseases including: (1) Xf subsp. *fastidiosa* (Xff), which causes Pierce's disease (PD) of grapevines in the United States; (2) Xf subsp. *pauca* (Xfp), causes CVC in sweet orange and coffee leaf scorch in Brazil (Nunney et al., 2014); (3) Xf subsp. *sandyi* (Xfs) causes leaf scorch of oleander; (4) Xf subsp. *morus* causes leaf scorch of mulberry and blueberry in the United States, and (5) Xf subsp. *multiplex* (Xfm) causes leaf scorch diseases in North America on a wide range of tree species, including almond, peach, oak and more recently associated with quick decline of olive trees in Italy (Almeida and Nunney, 2015).

CVC is transmitted by sharpshooters and has so far been found limited in host range only to sweet orange varieties. The first trees affected by the disease were observed in 1984 in Argentina, where it was named "pecosita" or "falsa mancha grasienta" (Contreras, 1992), and in 1987 in Brazil within the main citrus growing belt of the country in the northern region of the state of São Paulo (Rossetti et al., 1990). Currently, CVC is present in all major Brazilian citrus producing areas. Nevertheless, in the last 30 years of known disease outbreaks in Brazil, despite the wide distribution on many citrus varieties and occurrence of large populations of the species of sharpshooters known to transmit the pathogen (Yamamoto and Gravena, 2000), no other citrus type was found affected by the disease. This indicates that the host range limitation to sweet orange is determined by the Xfp genome.

Xylem colonization of Xfp starts when the insect vector carrying the pathogen feeds on the xylem sap of a healthy tree. Xylem colonization is enhanced by enzymes that degrade the xylem pit membranes, which would otherwise occlude movement of objects as large as Xf (Roper et al., 2006). Xfp colonization ultimately leads to the obstruction of sap flow (Queiróz-Voltan and Paradela, 1999; Garcia et al., 2012), with infected trees expressing symptoms characteristic of the disease first on leaves and later on fruits. Symptoms may be elicited by multiple pathogenicity factors that may not correlate with ability to colonize the xylem (Zhang et al., 2015). CVC-affected leaves show small spots distributed throughout on both the surfaces (Fig. 18.2). As the infection progresses, orange gum appears in the center of the spot present on the lower surface of the leaf. In later infection stages, the yellow spots coalesce and become necrotic. Highly affected leaves usually abscise, leaving behind tufts of small leaves at the tips of affected branches, which commonly exhibit symptoms resembling zinc deficiency. The fruits that appear on the affected branches usually do not drop, as happens in the case of trees affected by HLB. Instead they mature earlier and are smaller (Fig. 18.2) and much harder. The fruit are also richer in sugar but more acidic than the fruit from asymptomatic branches of infected or healthy trees (Laranjeira and Palazzo, 1999). The smaller size causes direct losses since larger numbers of affected fruits are needed to fill a typical commercial box of 40.8 kg (Ayres, 2000). The harder fruits can slow commercial juice production, while the smaller size also makes the fruits less acceptable to consumers in fresh markets.

The disease progress of CVC is faster in younger than older trees, and in trees exposed to seasonal water deficits, as happens more frequently in the northern regions of the state of São Paulo. Under these conditions, tree development is highly affected by the disease, with the decrease in fruit production reaching up to 75% of that in healthy or asymptomatic trees (Ayres, 2000).

18.3.2 Disease management, control, and mitigation

In Brazil, CVC management has relied on the use of preventive measures, particularly eradication of symptomatic citrus, to reduce inoculum sources and the chances of recurrent infections. Although weeds and some arboreal plants may harbor Xfp, the apparently low bacterium titer and short life of weedy hosts greatly limit their importance as sources of CVC (Lopes



FIG. 18.2 Mature leaves and fruits of sweet orange severely affected by citrus variegated chlorosis (CVC). Note the raised, gummy pustules on the abaxial leaf surface in (A). The two green fruits at the top right of (B) are unaffected.

et al., 2003). Correct identification of diseased trees is an important step in this process which, due to constant appearance of new symptomatic trees as a result of constant infections, requires frequent and careful tree inspections. Under certain conditions, the health of diseased trees may be restored by drastic pruning of symptomatic branches. However, for pruning to be successful the tree must be over 2.5 years of age and the symptoms present at the tip of only one secondary branch (Rodas, 1994). Recently, based on the high levels of resistance to Xfp infection observed for the Cravo Rangpur lime rootstock (Garcia et al., 2012), a new mitigation measure was developed (Lopes, 2019). Since the bacteria do not move downwards below the graft union into these rootstocks, new healthy scions can be generated by removing all the infected scion, even from highly affected sweet orange trees, and grafting with healthy budwood onto these rootstocks. Unfortunately, the immunity to CVC exhibited by the rootstocks does not transfer to the scion. However, the new shoots that develop on the rootstock trunk produced new fruits faster and at lower costs than those on the new regularly planted nursery trees.

Since the removal of infected trees is not uniformly followed by all growers, the inoculum pressure from neighboring groves may be high in some areas. Therefore, and as for HLB, aggressive control of the insect vector is a critical component of CVC control. Currently, such control consists in treating younger trees to be used for replants with systemic insecticides applied to the trunk (Yamamoto, 2008). In the state of São Paulo, young trees for planting are produced inside screened nurseries, where they are protected from infected sharpshooters. Clean nursery tree production is a key aspect of CVC control, and has been mandatory in the state of São Paulo since 2003. Systemic insecticide treatments of nursery stock prior to field planting will protect the trees for the next 2–3 months. New applications of systemic and regular spray of contact insecticides must be carried out for the following 2 years after field planting (Yamamoto, 2008), a period when the trees constantly flush with new shoots and become attractive to the vectors. After this period, the effectiveness of systemic insecticide applications is reduced, and the trees must be protected with contact insecticide only, which should be applied more frequently during flushing.

18.3.3 Recent discoveries and advances

In the last three decades, new research and field observations have revealed important aspects of the CVC pathosystem which have contributed to improved cultural practices and strategies to control CVC. The Xfp CVC strain 9a5c was the first plant pathogen to have its genome completely sequenced (Simpson et al., 2000). Functional genomic analyses allowed the development of testable hypotheses that revealed many new aspects of the mechanisms of Xf pathogenicity and bacterial adaptation to the xylem sap. It also revealed important and previously unknown vulnerabilities of these pathogens. For example, while most bacterial pathogens carry multiple redundant copies of TolC, a critical bacterial efflux pump component is needed to efflux toxic phytoanticipins, phytoalexins, and/or antibiotics; Xf strains carry only one copy of TolC and inactivation of this gene is lethal for growth in xylem sap (Reddy et al., 2007). The biology, migration patterns, feeding behavior, and pathogen transmission by the insect vector were also elucidated. New experimental procedures allowed for more precise evaluation of the responses of the distinct citrus varieties and species to pathogen infection (Lopes et al., 2005), and the impact of the environment on disease progress and severity was determined.

During this period of intensive research activity and implementation of integrated pest management strategies, CVC incidence initially continued increasing, reaching 34%–44% of the average 200 million orange trees grown in state of São Paulo from 1999 to 2012. However, the incidence dropped from 37.6% in 2012 to 6.8% in 2015. A further decrease was observed in the 2016 and 2017 surveys, and currently <3.0% of the trees express CVC symptoms in the main citrus belt of Brazil (http://www.fundecitrus.com.br/comunicacao/revistas). The losses attributed to the disease, estimated in the range of $100–120 million per year in the period of high incidence (Bové and Ayres, 2007; Lopes et al., 2004), were in 2017 negligible. Several technical advances help explain the level of control achieved, but other cultural factors were equally important, including: (1) the removal of large areas of abandoned groves, which must have significantly contributed to decrease sources of inoculum; (2) an aggressive inspection and eradication program, and (3) the increased rigor and wider efforts to control the insect vector. Grower attitudes toward stronger and more cooperative integrated control measures were greatly stimulated by the report and fast spread of an even more destructive and incurable insect transmitted citrus disease, as described below.

18.4 Huanglongbing

18.4.1 History and background

HLB, also known as "citrus greening," is caused by uncultured, phloem-restricted alpha-proteobacteria in the genus *Candidatus* Liberibacter. These bacterial plant pathogens cause severe disease symptoms on citrus, but nevertheless are



FIG. 18.3 Classic symptoms of "yellow shoot," "yellow dragon," or Huanglongbing (HLB).

well adapted, circulative, and systemic colonizers of the psyllids that vector them into phloem cells. Three species are currently well described as causing HLB in citrus—*Ca*. L. asiaticus (Las), *Ca*. L. africanus (Laf), and *Ca*. L. americanus (Lam)—and each species appears to have independently evolved in the continent reflecting its name (Beattie et al., 2005; Nelson et al., 2013). HLB is currently the most significant bacterial disease of citrus worldwide. HLB is found in Asia, the Middle East, Africa, and the Americas. It is spread by the psyllid vectors *Diaphorina citri* and *Trioza erytreae*. Other well-described *Ca*. Liberibacter species with different vectors are known to cause serious diseases on solanaceous crops (Liefting et al., 2009; Munyaneza et al., 2009), less serious problems on celery (Teresani et al., 2014), and almost no symptoms at all on pear (Raddadi et al., 2011); these will not be further discussed here.

Reports on the occurrence of HLB as "yellow shoot" disease (Fig. 18.3) date back to 1919 in Southwest Asia, from China to Pakistan, 1928 in Africa, and 2004 in the American continent, although references to maladies similar to HLB are even older. HLB was first called Greening in South Africa, Mottle Leaf in the Philippines, Dieback in India, and Vein Phloem Degeneration in Indonesia; in the Americas, it is most commonly called HLB or citrus greening (Bové, 2006; da Graça, 1991).

The most characteristic symptom of HLB is the appearance of asymmetric, blotchy mottles on the leaves, which is a diagnostic symptom. Before the development of DNA-based diagnostic tests allowing the detection of specific Liberibacters, the occurrence of HLB was confirmed mainly by two tests: a thin layer chromatographic test (Schwarz and van Vuuren, 1970) and a starch test to demonstrate its high abundance in symptomatic samples (Su, 2008). However, both methods lacked sufficient specificity to be used routinely in diagnosis. Although transmission electron microscopy (TEM) was used to identify the presence of walled bacteria in phloem cells, it is time consuming and laborious and does not distinguish bacteria at species level.

In several old descriptions of the occurrence of symptoms resembling HLB, the authors mentioned the presence of psyllids and damage caused by psyllid feeding. It was not until the 1960s that *T. erytreae* in South Africa (McClean and Oberholzer, 1965) and *D. citri* in India (Capoor et al., 1967) were demonstrated to vector the disease. In 1970 bacterial cells were found in phloem sieve tubes (Laflèche and Bové, 1970), characterized as Gram-negative bacteria (Garnier et al., 1984) and later determined to be a species of alpha proteobacteria (Jagoueix et al., 1994). Two species were then proposed: Las for the disease in Asia and Laf for the disease in Africa. In 2004, when HLB was seen for the first time in the Americas (in São Paulo State, Brazil), both Las and a new species, Lam, were proposed as causal agents (Teixeira et al., 2005b).

Koch's postulates have yet to be completed for any Liberibacter, and none of the pathogenic Liberibacters have been cultured, likely because they have lost certain key genes to make them capable of axenic growth (Jain et al., 2017b). The only extant isolates of *Liberibacter crescens* (Lcr) were cultured after isolation from diseased mountain papaya in Puerto Rico (Leonard et al., 2012; Fagen et al., 2014) but none of these could be transmitted back to papaya or any other plant or insect host, despite numerous efforts. Lcr may be nonpathogenic. Although the causal relationship between Liberibacters

and HLB is technically associative, strong and accumulating evidence leaves little doubt that Liberibacters are the primary etiological agent of HLB. Much of this conclusion rests upon diagnostic molecular tests, primarily polymerase chain reaction (PCR), which is currently the gold standard for detection and diagnosis. Metagenomics were used to sequence multiple genomes of Las (Duan et al., 2009; Lin et al., 2013; Katoh et al., 2014), Lam (Wulff et al., 2014), and Laf (Lin et al., 2015). Molecular techniques and other tools have been used to help elucidate the insect-Liberibacter as well as the plant-Liberibacter interactions.

18.4.2 Symptoms

Las growth *in planta* is phloem limited (Fig 18.4A), causing perturbed assimilate partitioning, leaf chlorosis, loss of productivity, and eventual decline and death of infected trees after several years. By contrast, the bacterium is systemic and circulative in its psyllid host, causing only localized apoptosis in the midgut tissue and accumulating on the outside of the gut under a biofilm (Fig. 18.4B) but without causing overt disease symptoms (Ghanim et al., 2016). Field diagnoses of HLB affected trees are initially determined visually by trained human inspectors. HLB in Florida was initially discovered by spotting the appearance of "yellow shoot" symptoms (Fig. 18.3). No field deployable device has yet been validated by PCR results as reliably allowing field use for diagnoses of Las infection of symptomatic or asymptomatic trees. However, replicated field studies have indicated that canines can detect Las- and Laf-infected plants with >99% accuracy and preliminary results indicate canines will alert trained human handlers to Lam-infected plants as well (Gottwald et al., 2017). The main symptoms used for field diagnosis is blotchy mottle of the leaves (Fig. 18.5), and also *asymmetric* chlorosis along the axis of the leaves, frequently delimited in color from green to yellow by veins. A similar—but symmetrical—yellowing of the leaves is characteristic of zinc deficiency, and this close similarity can cause some confusion and doubt, even to trained scouts looking for symptomatic trees in areas where HLB was previously unknown.

Within one to multiple years after HLB has been identified in a citrus grove, nearly 100% of the trees typically become infected, and often without apparent symptoms. The severity of the symptoms is correlated with the time that a plant has been infected. After a plant is first infected, a surprisingly lengthy (usually no shorter than 6 months) incubation period is required before any symptoms start to develop (Lee et al., 2015). The first symptoms are a pale or mild leaf mottle, which only slowly progresses to more characteristic leaf mottle symptoms. Symptom progression is usually faster during the fall and winter seasons. Leaf curling, reduced leaf size, and thickening of the midveins or raised corky veins are additional symptoms (Fig. 18.6) often, but not always, found. Twig dieback is also observed but takes longer to develop. Fruits developing from infected branches can be lopsided (Fig. 18.6), harbor aborted seeds, and have discolored columellas (Fig. 18.7). Fruit color inversion is sometimes seen, with yellowing starting from the peduncular end and the stylar end remaining green (these symptoms gave rise to the common name "greening"). A yellow to orange abscission zone is seen in the peduncular scar of the fruits, even in the absence of color inversion (Bové, 2006). Leaf and fruit drop are common characteristics, and infected groves experience very high levels of premature fruit drop, exacerbated by drought conditions. Root systems are

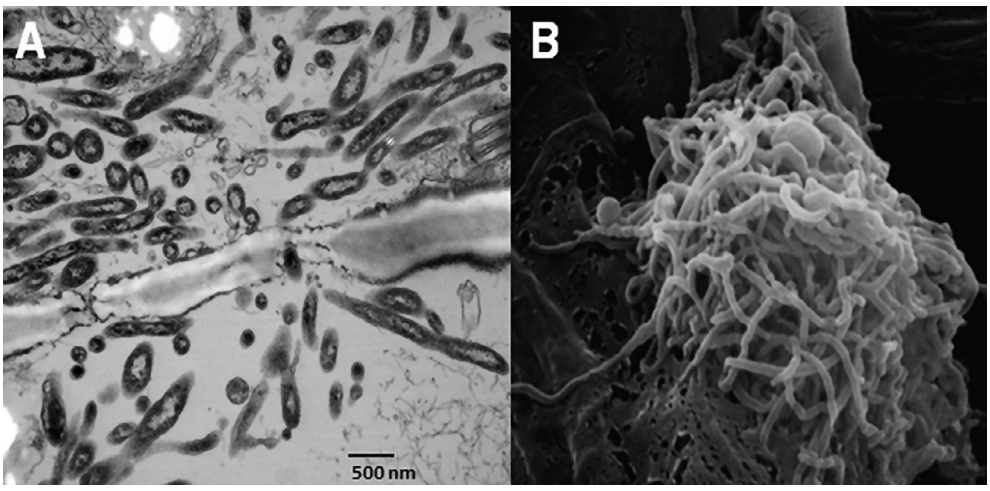


FIG. 18.4 Transmission electron micrographs of (A) *Ca*. Liberibacter americanus (Lam) packed in a sieve tube element and moving through a sieve pore in plant host (periwinkle) phloem cells, and (B) Las in insect host (psyllid), on outer gut surface following bacterial penetration through the gut and growth on the *outer* gut surface, bathed in hemolymph. *(A: Photo courtesy of Dr. Brigitte Batailler, UMR-1332 de Biologie du Fruit et Pathologie, INRA, Université Bordeaux, Villenave d'Ornon, France. Bar = 500 nm; B: Photo courtesy of Prof. Mike Davis, University of Florida.)*

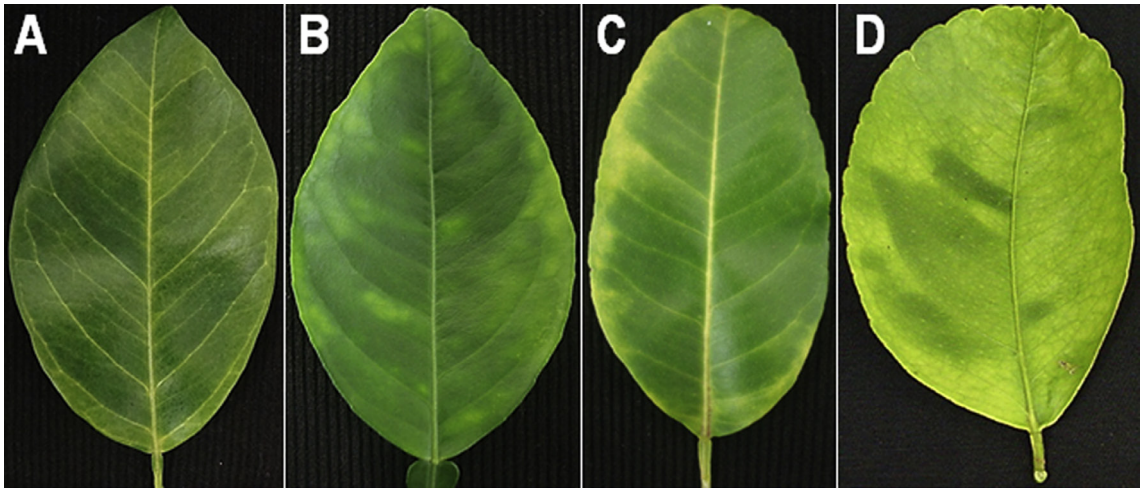


FIG. 18.5 Blotchy mottle symptoms of Huanglongbing (HLB) caused by *Ca*. Liberibacter asiaticus (Las) in sweet orange (A and B), Rangpur lime (C), and Tahiti lime (D).



FIG. 18.6 HLB symptoms of corky veins and misshapen fruit in field grown grapefruit.



FIG. 18.7 HLB symptoms in Valencia fruit (aborted seeds, and misshapen, discolored columellas).

also affected by severely reduced numbers of fibrous roots, and increased susceptibility to *Phytophthora* (Graham et al., 2013). With time, symptoms become severe, branches die and tree decline is evident; skeletonized trees in groves with long-standing HLB becomes all too commonplace (Fig. 18.8).

18.4.3 Etiology and diagnosis

Liberibacters were first found in citrus affected with HLB using TEM (Laflèche and Bové, 1970). Up to the 1990s, TEM was the way to prove the association of phloem-restricted bacteria with HLB. With the advance of molecular techniques and characterization of ribosomal protein and 16SrDNA genes, PCR-based diagnostics and the nature of HLB etiology were



FIG. 18.8 Scenes from commercial citrus Hamlin groves in Florida, 2018 (A, still productive) and 2016 (B, abandoned), about 10 years after HLB was found in the groves.

established. The causal bacteria were named *Ca*. Liberibacter spp.; "*Candidatus"* refers to inability to culture the bacteria and "Liberibacter" refers to the liber or sieve elements in which they were found (Jagoueix et al., 1994). Attempts to raise monoclonal antibodies from the infected tissues failed to yield either Las-specific or universal antibodies, largely due to the inability to grow Las in axenic cultures. However, extensive genome sequencing may now provide adequate targets for antigen production and generation of useful antibodies (Ding et al., 2017).

DNA probes for the detection of nucleic acids from Liberibacters in Southern and dot blots were developed (Villechanoux et al., 1992), but were soon surpassed by PCR-based diagnostic tests. PCR is useful for Liberibacter detection and identification, both in plant and in psyllid samples (Jagoueix et al., 1996; Hocquellet et al., 1999; Teixeira et al., 2005a, 2008a; Li et al., 2006; Roberts and Pietersen, 2017). There are many variations in PCR techniques and primers used, with the most common based on the detection of the 16S and ribosomal protein genes. Real time (semi-quantitative, or qPCR) enjoys widespread use. PCR and qPCR are much less effective in evaluating chemical treatment effects, since DNA from dead Liberibacter cells remains in phloem cells (Trivedi et al., 2009). In plant tissues, small pioneer or fibrous roots and seed coats of developing seeds are the organs with highest Liberibacter cell numbers (Achor et al., 2014). qPCR also enabled the quantification of Las and Lam titers in plants and psyllids. Estimated average highest titers in individual psyllids were 6.3×10^8 for Las and 5.6×10^4 for Lam; and in 1 g of tissue of sweet orange and orange jasmine leaves, respectively, 4×10^7 and 1×10^3 for Las, and 3.2×10^6 and 2×10^4 for Lam (Duan et al., 2009; Lopes et al., 2010; Teixeira et al., 2008a).

Fluorescent in situ hybridization (FISH) indicated that Las is able to colonize the filter chamber, midgut, Malpighian tubules, hemolymph, salivary glands, ovaries, and muscle and fat tissues of *D. citri* (Ammar et al., 2011). Loop-mediated isothermal amplification (LAMP)-based diagnostic tests have the advantage of reduced cost of equipment, but the relative lack of specificity and single-target detection are concerns (Okuda et al., 2005; Li et al., 2007). Conventional PCR can be still very useful for limited sample testing, allow multiple pathogen detection; although it is less sensitive than qPCR, but has the advantage of reduced initial costs. For activity related to sampling and eradication programs, where laboratory diagnoses may guide scouting operations, current molecular techniques are most useful for confirmatory purposes.

The vast majority of publications dealing with diagnosis of HLB have established a consensus that Liberibacters are the most frequently encountered causal agent of HLB. Phytoplasmas can also elicit HLB symptoms, but these agents are of marginal occurrence in relation to Liberibacters (Teixeira et al., 2008b; Chen et al., 2009; Wulff et al., 2015). Outside citrus and Rutaceae, the finding of new *Ca*. Liberibacter species is flourishing. As mentioned above, *Ca*. L. solanacearum was found in 2008 infecting Solanaceae and other crops, such as carrots and celery, causing disease in these hosts.

Ca. L. europaeus was found as an asymptomatic endophyte of pear trees and *Liberibacter crescens* was described in 2012 as the first axenically cultivated member of this genus. Except *L. crescens*, all other Liberibacters—that is, the known pathogenic Liberibacters—are all axenically uncultured to date due at least in part to the loss of the glyoxylase pathway and the presence of phage that are repressed by host factors (Jain et al., 2017a,b; Merfa et al., 2019).

Laf diversity at the subspecies or haplotype level in Africa is noteworthy. Besides having a host range on citrus trees, Laf subsp. capensis (LafC) has been found in cape chestnut (*Calodendrum capense*), while recently, additional rutaceous hosts were described for the following subspecies: Laf subsp. clauseane (LafCl) in *Clausena anisata*, Laf subsp. vepridis (LafV) in *Vepris lanceolata*, Laf subsp. zanthoxyli (LafZ) in *Zanthoxylum capense*, and Laf subsp. tecleae (LafZ) in *Teclea gerrardii* (Roberts et al., 2015, 2017). Additionally, LafCl strains with a wide host range were identified in *Citrus*, with a characteristic pattern of single nucleotide polymorphisms (SNPs) named LafCl ex citrus. Of great importance for diagnostic testing is the fact that the widely used 16S PCR primers used for Las identification (Li et al., 2006) incorrectly identified LafCl ex citrus, and LafCl, LafV, and LafZ were also misidentified as Las or Laf by the same qPCR test. Clearly, additional primer sets are required to distinguish these subspecies. A more general caution is that a great deal of care must be taken when dealing with first occurrences of Liberibacters in unusual places and plant and insect hosts (Roberts et al., 2017).

Currently Las is the prevalent species in HLB samples from Brazil, China, and the United States, where Las is now found in all citrus growing states. In Brazil, only a very limited number of samples harboring groups IX and III phytoplasmas and Lam are now found, where once Lam was the predominant HLB causal agent. One reason for the sharp decrease in Lam incidence in Brazil seems to be its lower titer levels in citrus tissues that reduce the chances of being acquired and transmitted by psyllids (Lopes et al., 2009a). Lam and phytoplasmas would probably show up occasionally in additional places. It should also be noted that since Las and Lam can be dodder transmitted to tobacco and periwinkle, it seems likely that *Ca*. Liberibacter host range may be more limited by the host range of the insect vector responsible for phloem transmission than by any particular pathogenicity or virulence determinants in these pathogens.

18.4.4 Pathogen biology: Phloem restriction in citrus, systemic infection in psyllid, and lack of axenic culture

After the transmission of Las, Lam, and Laf to periwinkle using dodder, the bacteria were found restricted to phloem sieve tubes, as expected. The TEM was also used to characterize uncultured *Ca*. Liberibacters as Gram negative by visualization of the tri-lamellar cell wall envelope (Garnier et al., 1984). Periwinkles were used for the maintenance of multiple isolates *in planta* at INRA in Bordeaux, France for decades by Bové's group (Teixeira et al., 2008a; Wulff et al., 2014). Periwinkles have allowed the maintenance of isolates in a smaller space than citrus and even comparative strain characterizations, such as heat tolerance (Las is exceptionally more heat tolerant than Lam and Laf; Bové et al., 1974; Lopes et al., 2009b), antibody generation (Garnier and Bové, 1993; Ke et al., 1993), and the description of the first Las phage particles (Zhang et al., 2011). Indeed, periwinkle became the source of DNA enriched for genome sequencing of Lam (Wulff et al., 2014). However, transmission from periwinkle to citrus cannot be accomplished because *Diaphorina citri* and *T. erytreae* are restricted in host range to citrus.

Ca. Liberibacter spp. multiply in the sieve tubes of infected plant hosts and there are pockets of sieve cells filled with elongated, electron dense young bacterial cells (Fig. 18.4) while most sieve tubes are free of bacteria. *Ca*. Liberibacters literally squeeze from sieve cell to sieve cell through sieve pores (Su, 2008), apparently without degrading plant cell walls, since there are no cell wall degrading enzymes annotated in any Liberibacter genomes. With maturity, the bacterial cells form less dense spherical bodies of 600–800 nm, eventually plasmolyzing (Su, 2008). As a host response to the spread of the bacterium in the vascular system, an increase in callose deposition occurs and together with bacterial multiplication, blocks the flow of phloem sap between the cells, disrupting the source to sink movement of sugars (Koh et al., 2012). HLB-affected trees are imbalanced in sugars (Fan et al., 2010). Notably, all pathogenic Liberibacters, by stark contrast with *L. crescens*, lack any known mechanism for sucrose import, and appear to rely instead on direct import of ATP from host cells (Jain et al., 2017b).

One consequence of the development of an intracellular lifestyle is the necessity of avoiding (to the greatest extent possible) and suppressing (where avoidance is impossible) the triggering of innate immune responses by any host, plant or insect. This can involve the shedding or downregulation of genes that directly encode such triggers; for example, flagellin, lipopolysaccharides (LPS), or enzymes that synthesize these triggers. Flagellin and LPS are two classic examples of a general group of molecules collectively referred to as "pathogen-associated molecular patterns" (PAMPs), which are recognized by the innate immune systems in eukaryotic hosts of all types (Jones and Dangl, 2006). Although flagella-like appendages have been observed on the surface of *Ca*. L. solanacearum infecting potato (Cicero et al., 2016), flagella have not been observed with any citrus-associated Liberibacter, and the flagellin produced by Las is a PAMP weaker than those

of other plant pathogenic bacteria (Zou et al., 2012). Eukaryotic cells typically are highly sensitive to LPS, which serves as a highly protective bacterial outer membrane defensive barrier against plant antimicrobial compounds such as phytoalexins; interestingly, Lam has no LPS genes at all (Wulff et al., 2014). The severe reduction in genome size observed generally with all intracellular pathogenic bacteria is likely driven, in part, by selection pressure to remove genes encoding PAMPs.

Las actively suppresses innate citrus immune responses to infection, helping to explain why HLB symptoms have such a long incubation period following initial introduction by the psyllid vector (a period spanning multiple months to years). The suppression of citrus defense reactions, particularly generation of reactive oxygen species (ROS) and prevention of apoptosis, is accomplished by a phage-encoded peroxidase (Jain et al., 2015) and two chromosomally encoded peroxiredoxins (Jain et al., 2018, 2019). The peroxidase and one peroxiredoxin are both nonclassically secreted directly into host phloem cytoplasm, resulting in: (a) straight detoxification of host-generated H_2O_2, (b) limited catastrophic chain reaction spread of a lipid peroxidation event in the phloem cell membrane, and (c) dampening of the systemic propagation of H_2O_2-mediated defense signaling in the host plant following the initial colonization event. Las peroxiredoxin may represent a critical secreted effector conserved across all pathogenic Liberibacter species (even in the phageless strains) that functions to suppress host symptoms, a tactic used by most biotrophic plant pathogens.

The extent of Las and Lam multiplication and symptom induction in citrus is quite different. Las multiplies to 10-fold higher titers in citrus than Lam, but Lam elicits much more severe symptoms of HLB, despite lower titer (Lopes et al., 2009a). Las can be transmitted by grafting budwood with efficiencies close to 100%, whereas Lam transmission by grafting is usually lower than 50%. There are no comparable data for Laf. Natural transmission by psyllids is influenced by several factors and bacterium multiplication and temperature are key aspects. Las titer in citrus influences *D. citri* transmission; while Las is readily transmitted from citrus to citrus, Lam is less efficiently transmitted. Interestingly and by contrast, Lam titer in *Murraya exotica* (*M. paniculata*) is higher than that of Las (Lopes et al., 2010). *Murraya* spp. is a good host for Lam acquisition and transmission to sweet orange plants, but this is not true for Las (Gasparoto et al., 2010; Damsteegt et al., 2010).

18.4.5 Genomics and taxonomy

Alphaproteobacteria are quite diverse as a class, and often form associations with eukaryotes. Free-living members have genome sizes over 5 Mbp and include the mutualistic endosymbionts in the Rhizobiaceae family: *Rhizobium, Azorhizobium*, *Bradyrhizobium*, and *Mesorhizobium*, as well as the pathogenic *Agrobacterium* species. Alphaproteobacteria are also well known to adapt an intracellular lifestyle with differing species utilizing independent paths of severe genome reduction. These famously include the mitochondria, as well as the Rickettsiales (including tick-borne diseases in genus *Rickettsia*, and endosymbionts of arthropods in genus *Wolbachia*). Liberibacters form a cluster of closely related alphaproteobacteria in the Rhizobiaceae family with highly reduced genomes as compared with free-living members. Interestingly, they appear much more highly adapted to their insect hosts than plant hosts, spreading inter- and intracellularly across salivary glands, alimentary canal, midgut epithelium and basement membrane, Malpighian tubules, hemolymph, muscle and fat tissues, and ovaries with little or no evidence of pathogenic effects (Ammar et al., 2011).

Only two strains of a single species of *Liberibacter*, *L. crescens* strains BT-0 and BT-1 (NC_019907.1), have been cultured in vitro (Leonard et al., 2012). Both were isolated from the sap of Babaco mountain papaya (*Carica stipulata* × *C. pubescens*) and are not known to be pathogenic, despite numerous efforts in several labs, and they have no known insect host. *L. crescens* has a slightly larger genome size (1.5 Mb) than any of the pathogenic *Ca.* Liberibacters (all about 1.2 Mb). *L. crescens* appears to have diverged from the uncultured, pathogenic *Ca.* Liberibacter spp. early (Nakabachi et al., 2013). The first Liberibacter genome to be completely sequenced was Las strain psy62 (Duan et al., 2009); since then more than nine additional Las genomes have been sequenced. In addition to Las, complete genome sequences were determined for Lam strain São Paulo (Wulff et al., 2014), Laf strain PTSAPSY (Lin et al., 2015), and Lso strain CLso-ZC1 (Lin et al., 2011). All the chromosomes contain three ribosomal operons and genome GC% of 35.4%, while Lam GC% is lowest, at 31.1%.

All pathogenic *Ca*. Liberibacters contain prophage or remnants of prophage not found in *L. crescens* with GC% content of 36% (Zhang et al., 2011). Typically, Las strains have 1–2 complete prophages, but a few strains have only remnants, such as Ishi-1 from Japan (Katoh et al., 2014). Both prophages may be integrated in the chromosome, but one of them typically replicates as an excision plasmid, thus expanding the copy number per cell of the encoded genes. As mentioned above, the peroxidase is encoded on the excision plasmid, can functionally suppress plant defenses and is therefore likely a horizontally acquired lysogenic conversion factor benefitting plant infections (Jain et al., 2015). One of the prophages can be lytic *in planta* and even form particles in citrus (Fu et al., 2015), yet remain as a stable lysogen in psyllids (Zhang et al., 2011). Suppression of Las phage gene expression appears to be critically important in psyllid hosts (Fleites et al., 2014; Jain et al., 2017a). Despite the few horizontally acquired gene additions, comparative genomics and metabolic pathway analyses of

the chromosomal genomes have revealed a trend toward reduction or complete absence of multiple biosynthetic pathways, including glycolysis and glyoxylase, metabolic enzymes, LPS biosynthesis, sucrose import and secretion systems in these genomes, helping to explain the failure to obtain sustainable axenic cultures of the pathogenic *Ca*. Liberibacters to date (Wulff et al., 2014; Jain et al., 2017b).

18.4.6 Epidemiology: Latency and incubation

In the absence of a vector, all *Ca*. Liberibacter species remain confined to their plant hosts. Citrus trees infected with various pathogens are not infrequently brought into commercial citrus growing areas by unsuspecting homeowners. If a Las-infected tree is brought into an area without the vector, both the tree and the Las that infects it can remain alive for many years, due to the surprisingly long incubation period between the initial infection and the appearance of even the slightest symptom. The vectors of HLB are also found in disease-free areas and introduction of Las-free insect vectors usually precedes the discovery of HLB by many years. In sharp contrast to the long incubation period for HLB, the latency period of the transmission of Las from citrus to psyllid and back again is measured in weeks, and the multiplication of infected psyllid vectors is explosive (Lee et al., 2015). The importance and surprising nature of the very short latency period for transmission of Las, coupled with the exceptionally long incubation period for HLB cannot be overemphasized. Las epidemics spread very rapidly in completely asymptomatic trees, making early detection critical (Gottwald, 2010). *T. erytreae* is found in the Mediterranean basin, although the bacteria are not, so the disease is not known to be present (Pérez-Otero et al., 2015; Siverio et al., 2017). The absence of both HLB and its vectors, such as in Australia, is a great advantage to the success and sustainability of citriculture.

Psyllid transmission is the only natural mode of dissemination of Las, Lam, and Laf, although spread by infected budwood during citrus propagation may occur locally. Illicit importation of budwood or entire infected plants will spread the disease and perhaps the vector over long distances including internationally. Strict quarantine measures should be applied to restrict the imports of propagative citrus material which originate only from certified regions or from certified plants free of psyllids and Liberibacters.

18.4.7 Control/management

Since there is no cure for HLB, preventive and mitigation actions must be taken, starting with quarantine regulations, implementation of inspections of nursery trees, and production of budwood in insect-proof nurseries. Although systemic or semi-systemic antibiotics have shown promise with trunk injection (e.g., Bové et al., 1980; Hu and Wang, 2016), there is some controversy over the widespread use of even limited types of antibiotics in agriculture. Psyllid monitoring (Miranda et al., 2017) and control (Miranda et al., 2016) by means of insecticide control in commercial areas is a key aspect to avoid the spread of HLB-infection in orchards (Bassanezi et al., 2013). In order to reduce inoculum, scouting for infected trees and removal of symptomatic trees must be carried out. These three strategies have been used in South Africa, Brazil, and Costa Rica (Bové, 2012; Aubert, 1990) and proven to be effective as a regional management strategy (Bassanezi et al., 2013). Due to the very short latency period for Las transmission, the development of presymptomatic Las detection techniques is a research priority. Due to the severe economic impact of HLB in regions and states where HLB infections have recently been detected, deployment of all available early detection resources, such as the use of canines, should be strongly considered.

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