Challenges for Managing Candidatus Liberibacter spp. (Huanglongbing disease pathogen): Current Control Measures and Future Directions

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ABSTRACT

Huanglongbing (HLB; "citrus greening" disease) has caused significant damages to the global citrus industry as it has become well established in leading citrus-producing regions and continues to spread worldwide. Insecticidal control has been a critical component of HLB disease management, as there is a direct relationship between vector control and Candidatus Liberibacter spp. (i.e., the HLB pathogen) titer in HLB-infected citrus trees. In recent years, there have been substantial efforts to develop practical strategies for specifically managing Ca. Liberibacter spp.; however, a literature review on the outcomes of such attempts is still lacking. This work summarizes the greenhouse and field studies that have documented the effects and implications of chemical-based treatments (i.e., applications of broad-spectrum antibiotics, small molecule compounds) and non-chemical measures (i.e., applications of plant-beneficial compounds, applications of inorganic fertilizers, biocontrol, thermotherapy) for phytopathogen control. The ongoing challenges associated with mitigating Ca. Liberibacter spp. populations at the field-scale, such as the seasonality of the phytopathogen and associated HLB disease symptoms, limitations for therapeutics to contact the phytopathogen in planta, adverse impacts of broad-spectrum treatments on plant-beneficial microbiota, and potential implications on public and ecosystem health, are also discussed.
INTRODUCTION

Huanglongbing (HLB), also known as “citrus greening”, has become the most destructive citrus disease worldwide. It is caused by the psyllid-transmitted, phloem-limited bacteria Candidatus Liberibacter asiaticus, Ca. Liberibacter americanus, and Ca. Liberibacter africanus (Bové 2006). These organisms were confirmed as the causative agents of HLB based on reinfection studies because Koch’s postulates cannot be fulfilled due to the inability to culture them ex planta (Jagoueix et al. 1996; do Carmo Teixeira et al. 2005). Infection by any one of these organisms, which were named for their geographic distributions, causes similar symptoms of fruit malformation and altered host phenotype (da Graca and Korsten 2004; Jagoueix et al. 1996; do Carmo Teixeira et al. 2005). Diseased trees develop leaves with characteristic blotchy mottle (i.e., asymmetric leaf yellowing) and produce undersized, lopsided, and greenish-colored fruit that often drops prematurely (Martinelli and Dandekar 2017). HLB-induced host limitations for photoassimilate transport and nutrient uptake, and the associated dieback of canopy and fibrous roots, lead to tree death, typically, within a decade of initial symptom development (Bové 2006). All varieties of cultivated citrus species are susceptible to HLB, though to varying degrees (Folimonova et al. 2009; Gottwald et al. 2012). Moreover, the economic impacts of the disease have been severe. For example, in the United States, damages to the Florida citrus industry are estimated to have exceeded $4.5 billion since Ca. Liberibacter asiaticus was first detected in the state in 2005 (Hodges and Spreen 2012). HLB has already spread to other important citrus growing states as well (e.g., California, Texas) (Kumagai et al. 2013; Kunta et al. 2012), where it is likely to have similar consequences. With Ca. Liberibacter spp. having
become established in over 40 countries (Croxton and Stansly 2014), and considering that citrus is one of the most important fruits in the world in terms of its commercial production market, processing, and global trade (Liu et al. 2012), there is an urgent need for the development of effective strategies to mitigate HLB.

Despite the presence of HLB-like symptoms dating back over a century (in China) and the ongoing efforts to control the disease, there is still a lack of treatment options that are technically feasible, sustainable, and environmentally safe (Grafton-Cardwell et al. 2013; Martinelli and Dandekar 2017). Controlling the insect vector that transmits Ca. Liberibacter spp. has been a primary strategy for HLB management, yet it may be limited to only slowing the spread of disease and lessening the severity of consequences in areas that HLB has become well-established (Monzo and Stansly 2017). Although this form of management is essential, especially considering the severity of HLB, it alone cannot mitigate the long-term consequences of the disease. Thus, additional efforts have been made to develop novel strategies to manage HLB. This literature review summarizes the major treatment options for managing Ca. Liberibacter spp. populations and controlling HLB disease symptoms, including the use of (a) broad-spectrum antimicrobials, (b) Ca. Liberibacter asiaticus-specific antimicrobials, (c) thermotherapy, and (d) compounds that stimulate plant growth and/or boost host defenses. We also discuss the citrus-associated microbiome and implications for citrus health, including what is known about the core citrus microbiome and prospects for implementing microbial biological control to manage Ca. Liberibacter spp. Strategies for developing genetic resistance to the phytopathogen that are on the horizon are described as well. We conclude with a discussion of ongoing challenges for
managing Ca. Liberibacter spp. and mitigating HLB symptoms at the field-scale (e.g.,
resiliency of the phytopathogen, mode of therapeutic delivery, antimicrobial resistance).
Because evaluating treatment effects depends on accurate, reliable pathogen detection
methods, we begin our discussion there.

EVALUATING TREATMENT EFFECTS: PHYTOPATHOGEN DETECTION

The HLB pathogens cannot be cultured for laboratory study, despite recent
advancements made based on comparative genomics that may provide insights to how
this may be possible in the future (Fagen et al. 2014). Thus, with regard to monitoring
HLB and evaluating for potential treatment effects on populations of Ca. Liberibacter
asiaticus, Ca. Liberibacter americanus, and Ca. Liberibacter africanus, several nucleic-acid-based tests are available for detection and quantification of the (viable) pathogen in
plant tissue samples (e.g., leaf, root). Polymerase chain reaction (PCR) is traditionally
used to detect the DNA of any one of these phytopathogens in a sample, indicating
presence, and quantitative PCR (qPCR) is often used to measure the number of copies
of a specific DNA sequence of the phytopathogen, corresponding to its titer (Bové 2006;
Zhang et al. 2010). Based on qPCR estimates of gene copy number, the number of
cells of Ca. Liberibacter spp. per gram of plant tissue sample can be predicted, as
described in the work of Zhang et al. (2011). From these values, or based on the Ct
values obtained from qPCR, the relative change in phytopathogen population in
response to treatment can be quantified. That is, for studies that only reported Ct values
(and not population densities) of Ca. Liberibacter spp. when assessing the impacts of
control measures on HLB (Zhang et al. 2013a; Shin et al. 2016), the relative change in
phytopathogen titer in response to treatment can still be determined with the $2^{-\Delta Ct}$
method (Livak and Schmittgen 2001). Moreover, reverse-transcriptase qPCR (RT-qPCR), which can quantify the abundance of gene transcripts (i.e., reflecting transcriptional activity indicative of the cell’s viability), is also used for the detection and/or quantification of the HLB pathogens, as described in works that have focused on the viability of Ca. Liberibacter spp. in response to chemical treatments (Gardner et al. 2016; Pagliai et al. 2014). In addition, the relative abundance of Ca. Liberibacter spp. within a microbial community (i.e., the proportion of the amount of Ca. Liberibacter spp. to that of all bacteria in the community) can be inferred from the abundances of 16s rRNA genes that are detected in a sample and assigned to different OTUs. Taxon abundances can be estimated based on DNA microarrays (e.g., Phylochip) (Zhang et al. 2013a), clone library sequencing (Sagaram et al. 2009), and high-throughput amplicon sequencing (e.g., Illumina) (Blaustein et al. 2017). Overall, the various studies that have reported on the effects of control measures on Ca. Liberibacter spp. have used a variety of detection methods, and the interpretation of results is dependent on the respective method used. Throughout this review, we present data from these studies in the context of relative changes in Ca. Liberibacter spp. in response to the treatment, as computed from changes in population densities that have been reported for the phytopathogen, when available, or from raw data for Ct values using the Livak and Schmittgen (2001) method.

**EFFECTS OF CHEMICAL TREATMENTS**

Antimicrobials have played an important role in plant agriculture for controlling a variety of bacterial phytopathogens. The most common examples are chemical therapeutics with active ingredients of streptomycin or oxytetracycline, which have been
marketed and used for over 50 years in the USA to mitigate *Erwinia amylovora*, the
pathogen responsible for fire blight of apple, pear, and related ornamental trees
(Stockwell and Duffy 2012). In fact, it has been suggested that without incorporating
antimicrobial treatments into certain apple and pear production operations to prevent
and/or combat fire blight, several popular cultivars and even entire orchards would have
been abandoned due to excessive losses (McManus et al. 2002). These compounds, as
well as gentamicin and oxalic acid, are also approved for use in several countries in
Europe, the Middle East, Central America, and Mexico in order to control bacterial
diseases of fruit and vegetable crops caused by members of *Erwinia*, *Pectobacterium*,
*Pseudomonas*, *Ralstonia*, and *Xanthomonas* genera (McManus et al. 2002; Stockwell
and Duffy 2012). Commercial usage of these treatments varies by location and is
subject to extensive regulation based on potential efficacy and associated risk (e.g., fate
of the chemicals in the environment, selective pressures associated with evolution and
spread of antibiotic-resistant pathogens). In 2016, streptomycin sulfate,
oxotetracycline hydrochloride, and oxytetracycline calcium complex were approved for
use as foliar sprays to treat HLB in Florida, USA; however, the potential benefits of
these compounds are still unclear (Wang et al. 2017a). While judicial use of narrow-
spectrum antimicrobials (e.g., small molecules) may be desirable for broadening the
toolset of pro-active solutions for managing Ca. Liberibacter spp., the use of antibiotics
that are closely related to those used in human and veterinary medicine (e.g.,
streptomycin, oxytetracycline, and other broad-spectrum compounds) should be closely
evaluated for the balance of potential benefits and also risks to public health (Chang et
al. 2015; Franklin et al. 2016).
**Broad-spectrum Antimicrobials**

When HLB was hypothesized to be of bacterial origin in the 1970s, antibiotic trunk injections, using mainly tetracycline-based compounds, were tested throughout Asia and Africa for their impacts on disease progression and severity (Chiu et al. 1979). However, due to limited success, along with phytotoxic effects and labor costs for annual applications of these compounds in order to limit reinfection (i.e., tetracyclines are bacteriostatic not bactericidal), these treatments were not considered feasible at the time and were abandoned (Zhang et al. 2013). However, due to the substantial impacts that HLB has had on leading citrus production industries during the past 10-15 years, the development of antimicrobial-based strategies to mitigate *Ca. Liberibacter* spp. is, once again, gaining interest. Several recent greenhouse and field studies have documented the responses of the titer of *Ca. Liberibacter asiaticus* within leaves of HLB-diseased trees to antibiotic foliar sprays, root drenches, and/or trunk injections (Hu and Wang 2016; Shin et al. 2016; Yang et al. 2016; Zhang et al. 2011a; Zhang et al. 2013). Within these studies, broad-spectrum compounds within antibiotic classes that have varying activities, such as aminoglycosides (streptomycin, kasugamycin), tetracyclines (oxytetracycline), $\beta$-lactams (penicillin), and sulfonamides (sulfadimethoxine, sulfathiazole), were utilized (Table 1). We note that since *Ca. Liberibacter* spp. may demonstrate seasonality as it is capable of moving throughout citrus trees from sources to sinks, upward from roots and downward from canopy, at different times of the year (Zhang et al. 2013a; Wang et al. 2017a) relatively long-term trials (i.e., 1+ years) are needed to distinguish antimicrobial effects from noise that may be associated with natural seasonality in phytopathogen populations.
Penicillin G trunk injections (1.0 and 6.0 g per tree) were recently tested on 2-year-old Ray Ruby grapefruit seedlings in the greenhouse and 7-year-old Ray Ruby grapefruit trees in the field (Shin et al. 2016). Within 24 hours, the chemical became distributed throughout the canopy and roots of the seedlings, yet only throughout the canopy, but not roots, of the mature trees (Shin et al. 2016). This suggests possible limitations for thorough chemical transport through the vascular system of citrus trees in the field. According to qPCR analyses from the field study, at 3 months following the trunk injections, the mature leaves from trees receiving the low and high rates of penicillin G had, on average, a 6-fold and 12-fold decreases in Ca. Liberibacter asiaticus titer, respectively, compared to those of the untreated control trees. Alternatively, the phytopathogen was more than 15-fold lower in the new flushes of trees receiving the higher dose of treatment than within those of control trees ($p<0.05$) (Shin et al. 2016). Furthermore, the authors reported the chemical applications to have little or no side effects on native bacterial populations or on the proliferation of penicillin-resistant bacteria, as measured by a crude assessment of the bacteria that could grow on generic growth media. Thus, despite the benefits of penicillin G slowing disease progression and promoting no resistance over the 3-month period (Shin et al. 2016), in the absence of long-term studies, the world-wide concern associated with the spread of virulent β-lactam resistant bacteria (Stockwell and Duffy 2012) may limit the feasibility of commercial applications of this β-lactam at the field-scale.

The effects of oxytetracycline hydrochloride trunk injections on 5-year-old HLB-infected Hamlin trees (2 g per tree) were also recently documented (Hu and Wang 2016). The titers of Ca. Liberibacter asiaticus (as measured by qPCR) within both
leaves and roots were reported to have dropped precipitously during the month following treatment, varying between 1-3 log unit decreases from the starting density of more than 3 x 10^6 cells per gram tissue, depending on the amount of trunk injection ports used (Hu and Wang 2016). The population density of Ca. Liberibacter asiaticus remained significantly lower in treated trees than untreated controls over the course of the 9-month study. However, during months 2-9, there was about 1-log of phytopathogen regrowth, perhaps a reflection of the bacteriostatic activity of the antibiotic and/or re-infection of aboveground tissues from Ca. Liberibacter asiaticus present in reservoirs such as roots. It is possible that the phytopathogen moves through different plant compartments seasonally, resulting in such observed titer fluctuations. In addition, although improvements in HLB symptoms in response to treatment were described (i.e., no HLB symptoms were present on new growth, so the overall canopy appeared healthier), there were also moderate side effects (i.e., brown discoloration to “leaf burn”) on young leaves (Hu and Wang 2016). The concentration-dependent phytotoxicity of oxytetracycline has been reported in several other works (Zhang et al. 2011a; Zhang et al. 2012; Zhang et al. 2014). Therefore, although oxytetracycline was able to suppress Ca. Liberibacter asiaticus and help alleviate the progression of HLB symptoms, the longevity of these impacts remains unclear because of the phytopathogen regrowth, and an optimal application rate must be used in order to limit phytotoxicity.

Treating HLB with multiple antibiotics at the same time has also been attempted. Using combination therapy was suggested in a greenhouse study that investigated the effects of drenching the roots of HLB-infected Ray Ruby grapefruit seedlings with
penicillin G (1.0 g L\(^{-1}\)), streptomycin (0.1 g L\(^{-1}\)), or both of the antibiotics these rates

(Zhang et al. 2011a). At 3 months after treatment, the population density of Ca. Liberibacter asiaticus (as estimated based on qPCR) had decreased by about 2-4 log units, depending on treatment, from the starting value around 5 x 10\(^5\) cells per gram of leaf tissue, while that of the untreated controls remained relatively stagnant. However, by 6 months, the reduced level of Ca. Liberibacter asiaticus was only maintained in the leaves of seedlings that had received the combination treatment, while, alternatively, the seedlings that were treated with only one compound had leaves with the number of Ca. Liberibacter asiaticus cells that was comparable to that at pre-treatment (Zhang et al. 2011a). Thus, synergistic effects of two or more compounds may allow for prolonged antimicrobial activity against the phytopathogen.

A field study on the effects of penicillin and streptomycin via trunk injections on 7-year-old HLB-infected Lee mandarin x Orlando tangelo trees (5 g penicillin G + 0.5 g streptomycin per tree; 10 g penicillin G + 1 g streptomycin per tree) was also described in Zhang et al. (2011). The lower rate did not demonstrate any phytotoxicity, and compared to untreated control trees, those receiving this rate experienced a 3.5-5 log unit reduction in Ca. Liberibacter asiaticus within leaves, as measured by qPCR (Zhang et al. 2011a). However, there were still trends for up to 2 log units of increase in Ca. Liberibacter asiaticus titer in treated trees between 4 and 14 months following treatment, which suggested that continued chemotherapy with these compounds would be necessary in order to manage HLB over the long-term. Moreover, Zhang et al. (2013a) built on that field work and attempted trunk injections of penicillin G and streptomycin (5 g penicillin G + 0.5 g streptomycin per tree), as well as trunk injections
of oxytetracycline and kasugamycin (2 g oxytetracycline hydrochloride + 1.0 g kasugamycin per tree) on other HLB-diseased trees at the same site. The titer and relative abundance of Ca. Liberibacter asiaticus in leaves were monitored bimonthly for up to 14 months with qPCR and Phylochip analyses, respectively. Although Ca. Liberibacter asiaticus was suppressed by combination therapies, being anywhere from 3- to 30-fold lower in leaves of trees receiving treatment than in untreated controls, the phytopathogen demonstrated seasonality and regrowth over time (Zhang et al. 2013). For example, even though the titer was lower in leaves of trees receiving kasugamycin and oxytetracycline than it was in untreated controls during the fall months of the study, all trees experienced increases in titer by about 10-fold between spring and fall months (Zhang et al. 2013a). Such increases may have been driven by Ca. Liberibacter asiaticus re-growth and, at least partially, by the seasonality of the phytopathogen or that of the insect vector (i.e., since this was a field study, psyllid feeding could have re-introduced inoculum). Moreover, both types of combination treatments induced a change in leaf-associated bacterial populations, as the numbers of bacterial OTUs detected in the midribs of leaves from treated trees comprised only 79.7% of all OTUs that were detected in the midribs of leaves from control trees (Zhang et al. 2013a). Thus, applications of penicillin with streptomycin and applications of oxytetracycline with kasugamycin were able to suppress the HLB pathogen in a season-dependent manner, which coincided with subtle adverse effects on native microbiota.

In addition to β-lactams (e.g., penicillin G), tetracyclines (e.g., oxytetracycline), and aminoglycosides (e.g., streptomycin, kasugamycin), there are a few other broad-spectrum antibiotics that have been used to treat HLB. Yang et al. (2016) reported root
drenches of sulfonomides (i.e., 1.0 g sulfathiazole sodium, or 1.0 g sulfadimethoxine sodium) to have moderate effects on the relative abundance, but not the titer, of Ca. Liberibacter asiaticus in leaves of HLB-infected Ray Ruby grapefruit seedlings in a recent greenhouse study. Specifically, the average relative abundance of the phytopathogen was about 7-9% lower, yet the average titer was 1.5-2 times higher, in leaves of treated seedlings than in those of controls (Yang et al. 2016). This may be explained by considering an increase in the numbers of all bacteria within the community, along with the detected increases in numbers of Ca. Liberibacter asiaticus. That is, if the cumulative number of bacteria within leaf communities increased, which could have occurred in response to the antibiotics disrupting a key taxon or taxa that may have kept others in check, then the content of the phytopathogen among the broader community could have still slightly decreased even though the numbers of Ca. Liberibacter asiaticus nearly doubled. Accordingly, there was up to a 35% decline in total bacterial OTUs within leaves following treatments; several bacterial populations other than Ca. Liberibacter asiaticus, possibly those associated with the fitness of the phytopathogen, may have been selected for during treatment (Yang et al. 2016). Furthermore, the authors noted enhanced effects of both sulfonamides against Ca. Liberibacter asiaticus when they were used in combination with thermotherapy (i.e., when the seedlings were placed in a growth chamber at 40°C or 45°C for 1 week prior to the root drench treatment). The lack of impacts of sulfonamides on the HLB pathogen at room temperature (i.e., thermotherapy less logistically feasible in the field), along with subsequent adverse effects on native microbiota, make them relatively ineffective and not a feasible option for controlling Ca. Liberibacter asiaticus.
Some of the aforementioned treatment studies were performed following screening tests for a variety of broad-spectrum compounds that were hypothesized to demonstrate activity against Ca. Liberibacter spp. (Zhang et al. 2010; Zhang et al. 2011a; Zhang et al. 2012; Zhang et al. 2013b; Zhang et al. 2014). These screens were done by soaking either cuttings or budsticks/scions from HLB-infected plants in a solution containing antimicrobials and then rooting and planting the cuttings or grafting the budsticks/scions onto a healthy stock and monitoring the outcomes. For example, using the grafting approach, Zhang et al. (2014) screened 31 compounds from a variety of antibiotic classes (i.e., aminoglycosides, ansamycins, β-lactams, cefalosporins, glycopeptides, lincosamides, oxazolidinones, polypeptides, quinolones, sulfonamides, and tetracyclines) for effectiveness and phytotoxicity. From the diverse set of compounds tested, ampicillin, carbenicillin, penicillin, cefalexin, rifampicin and sulfadimethoxine were reported to be most effective in eliminating or, at least, suppressing HLB, based on Ca. Liberibacter asiaticus titers measured (by qPCR) in leaves both the treated scion and the inoculated rootstock (Zhang et al. 2014). This method was also implemented to screen for potential impacts of chemical treatments on citrus-associated microbiota. Zhang et al. (2013b) soaked budsticks taken from HLB-diseased lemon trees in ampicillin- or gentamycin-containing solutions and graft-inoculated healthy grapefruit seedlings. The leaf microbiota were then monitored over six months with PhyloChip analyses. Although the ampicillin treatment eliminated Ca. Liberibacter asiaticus, it also induced a 15% reduction in community richness, compared to the diseased control (Zhang et al. 2013b). Alternatively, while the gentamycin treatment did not impact Ca. Liberibacter asiaticus, it, interestingly, caused
the communities to become less stable as a plethora of low abundance OTUs appeared (Zhang et al. 2013b). Disruption of keystone species in response to antimicrobial solution treatment may have caused that spike in diversity. In summary, the screening methods have also provided insight to the efficacy and potential side effects of several antibiotics that could be considered for use in treating HLB.

Overall, although the usage of antibiotics to control Ca. Liberibacter spp. has had promising developments, challenges and consequences associated with using broad-range compounds include seasonality of the pathogen and re-establishment over time, minimal inactivation of the pathogen in older flushes, phytotoxicity, and adverse impacts on richness and diversity of native microbiota (Hu and Wang 2016; Shin et al. 2016; Zhang et al. 2011a; Zhang et al. 2013a). Optimization of field treatments with compounds that may suppress Ca. Liberibacter spp. or combinations of such antibiotics warrants further investigation.

**Antimicrobials that Specifically Target Ca. Liberibacter spp.**

Developing novel therapeutics that may target Ca. Liberibacter spp. is challenging due to difficulties associated with identifying molecular targets, since the phytopathogens cannot be cultured. Efforts have still been made to search for compounds with activity against Ca. Liberibacter asiaticus based on the information from the pathogen’s sequenced genome (Duan et al. 2009) and, also, by using close phylogenetic relatives as model organisms (i.e., *Agrobacterium tumefaciens*, *Liberibacter crescens*, and *Sinorhizobium meliloti*) in culture-based experiments (Gardner et al. 2016; F. a. Pagliai et al. 2014; Akula et al. 2012).
Several small molecule compounds that may target molecular pathways essential to the survival of Ca. Liberibacter asiaticus have been identified (Gardner et al. 2016; F. a. Pagliai et al. 2014; Akula et al. 2012). For example, SecA is an essential component of the type II bacterial secretion system that moves proteins from the cytosol across or into the cellular membrane (Manting and Driessen 2000). SecA interacts with the SecB chaperone to target SecYEG, functioning as a peripheral ATPase when bound to the complex to facilitate ATP hydrolysis-dependent protein translocation (Economou and Wickner 1994; van den Berg et al. 2004). Akula et al. (2012) identified twenty small molecule compounds that could inhibit SecA activity of the HLB pathogen via molecular docking in silico and then confirmed the antimicrobial activity of five of these compounds in vitro using A. tumefaciens as a culturable model. In addition, Pagliai et al. (2014) characterized a regulon of Ca. Liberibacter asiaticus involved in cell wall remodeling, which contains a transcription factor (LdtR) upstream from a transpeptidase (LdtP) that is essential to its survival. LdtP is needed for cell-wall remodeling under osmotic stresses, which are constantly imposed upon Ca. Liberibacter spp. since it lives within the phloem tissue of infected trees (Pagliai et al. 2014), and LdtR has since been determined to be a global transcriptional regulator of several hundred Ca. Liberibacter asiaticus genes (Pagliai et al. 2017). Small molecules that bind and inactivate LdtR were identified with thermal shift assays and their activity was validated with DNA binding assays (Pagliai et al. 2014). Several of these compounds (i.e., benzbromerone, hexestrol, phloretin) demonstrated the ability to down-regulate the expression of LdtR and LdtP in culturable surrogates of Ca. Liberibacter asiaticus (i.e., L. crescens and S. meliloti) and adversely impact the surrogate strains in vitro under artificial osmotic stress conditions (i.e., on
growth medium containing relatively high concentrations of NaCl or sucrose) (Pagliai et al. 2014). The authors further determined that the compounds inhibit the viability of Ca. Liberibacter asiaticus (measured with RT-qPCR) *in planta* in leaf-soaking assays, suggesting their applicability for future HLB treatment studies (Pagliai et al. 2014).

Moreover, another Ca. Liberibacter asiaticus transcription factor (PrbP) with implications for survival of the phytopathogen was recently characterized (Gardner et al. 2016). PrbP shares homology with CarD (Gardner et al. 2016), an essential RNA polymerase accessory protein in members of the *Mycobacterium* genus that is involved in pathogenesis, persistence, cell viability, and environmental stress resistance (Srivastava et al. 2013; Stallings et al. 2009). Since PrbP was putatively essential for Ca. Liberibacter asiaticus survival, compounds that may target the protein were investigated and identified *in vitro* using *L. crescens* and a model γ-proteobacterium *Escherichia coli* (Gardner et al. 2016). One of these compounds (i.e., tolfenamic acid) was also shown to inhibit the viability of Ca. Liberibacter asiaticus (measured with RT-qPCR) *in planta* in leaf-soaking assays (Gardner et al. 2016). Tolfenamic acid was further tested for activity against Ca. Liberibacter asiaticus in HLB-infected Valencia seedlings in a long-term greenhouse experiment (100 µM tolfenamic acid applied by foliar spray and root soaking) (Table 1). Based on RT-qPCR analyses, at 11 months following treatment, there had been approximately an 80-95% reduction in the expression of Ca. Liberibacter asiaticus genes *rplJ* and *gyrA* in 75% of the treated seedlings, suggesting that the compound can lead to substantial inactivation of the metabolically active phytopathogen (Gardner et al. 2016). Importantly, the authors noted that the plants responded to the treatment with substantial improvements in fibrous root
growth/development and enhanced foliage appearance. Thus, the target specificity of
tolfenamic acid, as well as that of other small molecules that have been suggested to
would be an interesting avenue of research for future field studies. Compared to broad-
spectrum antibiotics, small molecule compounds may be able to more effectively control
HLB and minimize adverse ecological impacts (i.e., phytotoxicity, disruption of native
microbiota).

ADDITIONAL MEASURES TO CONTROL Ca. Liberibacter spp.

Thermotherapy

Managing Ca. Liberibacter asiaticus with approaches other than antimicrobial
treatment has also been the focus of several studies (Table 2). For example,
thermotherapy appears to be somewhat effective at suppressing phytopathogen titer, at
least in greenhouse/growth chamber settings (Fan et al. 2016; Hoffman et al. 2012;
HLB-infected Ray Ruby grapefruit seedlings to temperatures of 40°C for 24 h or to 42°C
for 19 h, followed by 30°C for 5 h, in growth chambers for only two consecutive days
resulted in the titer of Ca. Liberibacter asiaticus dropping by more than 40-fold to a level
below the detection limit of qPCR within two months. Importantly, although it was noted
that a 45°C exposure caused severe plant tissue damage, any heat stresses imposed
by the 40°C or 42°C treatment were overcome by the plants (Hoffman et al. 2012).
Alternatively, Zhang et al. (2016) reported thermotherapy of HLB-infected Ray Ruby
seedlings via exposure to 40°C or 42°C for 8h a day for 1 week to be ineffective at
lessening the titer of Ca. Liberibacter asiaticus in leaves, while exposure to 45°C for 8h
a day for 1 week resulted in greater than 5-fold decreases in phytopathogen titer by the 2-month time point. In another study, in which HLB-infected tangerine seedlings were exposed to 45°C or 48°C one day a week for 4h for three consecutive weeks, the titer of Ca. Liberibacter asiaticus within leaves declined by about 30% and 55%, respectively, while that of the controls increased by over 300% (Fan et al. 2016). Moreover, Yang et al. (2016) performed thermotherapy on HLB-infected Ray Ruby seedlings in growth chambers that were set for a 12 h exposure of 40°C or 45°C per day for one week, and the higher temperature treatment resulted in a decrease in titer of Ca. Liberibacter asiaticus within leaves by more than 3 log units to a level that was below the qPCR detection limit. The pathogen titers remained below detection throughout ten months of monitoring post-treatment (Yang et al. 2016). We note that the efficacy of thermotherapy depends on both the aboveground and belowground components of the plant being properly treated. Although thermotherapy is an extensively time-consuming and expensive process in the field, there have been efforts to develop commercial equipment that could make it more feasible at the larger scale (Trotochaud and Ehsani 2016).

### Non-antimicrobial Compounds that Stimulate Plant Growth and/or Boost Host Defenses

Other non-antimicrobial HLB treatments include applications of compounds that may boost plant growth and stimulate plant defenses. Canales et al. (2016) reported bimonthly foliar sprays of a brassinosteroid (i.e., 0.084 μM epibrassinolide) over the course of one year to Valencia orange trees in the field to induce the expression of several key plant defense genes and lower Ca. Liberibacter asiaticus titers in leaves by
approximately 7-fold at the end of that year. The impacts of the routine applications of
the plant steroid on seedlings in the greenhouse were even greater, as Ca. Liberibacter
asiaticus titer was reported to have reduced from about 10^6 cells g\(^{-1}\) to 10^4 cells g\(^{-1}\) leaf
tissue in as soon as three months (Canales et al. 2016). In addition, spray applications
of several plant defense regulators (i.e., ascorbic acid [AA], β-aminobutyric acid [BABA],
2,1,3-benzothiadiazole [BTH], 2-deoxy-D-glucose [2-DDG], and 2,6-dichloroisonicotinic
acid [INA]) were reported to somewhat slow down the proliferation of Ca. Liberibacter
asiaticus in leaves of 7-year-old HLB-infected Navel orange trees (Li et al. 2016). While
the numbers of Ca. Liberibacter asiaticus cells per gram of leaf tissue from treated trees
were always within 1 log unit from that of untreated controls, these values were still
sometimes significantly different (p<0.05) at time points of 1 year or beyond. In other
words, the phytopathogen titer in all trees increased over time, but the rates of increase
were sometimes slightly lower in treated trees than controls. The treatments were also
described to suppress HLB symptom severity by up to 30% and have positive impacts
on fruit yield and quality (Li et al. 2016). Moreover, spray applications of several other
plant-beneficial compounds, including L-arginine and 6-benzyl-adenine (i.e., a synthetic
cytokinin) combined with gibberellins, were tested on HLB-infected Valencia trees in
greenhouse and field studies (Martinelli et al. 2016). RT-qPCR analyses of plant mRNA
indicated that the treatments had positive impacts on the abundances of citrus gene
transcripts involved in biotic stress responses, starch metabolism, and systemic
acquired resistance. Alternatively, the treatments did not have inhibitory effects on Ca.
Liberibacter asiaticus or HLB symptom progression (Martinelli et al. 2016). The authors
suggested that while certain plant-beneficial treatments can modulate the expression of
key citrus defense genes, they might only be able to have mitigating effects against HLB if they are applied frequently before or immediately at the onset of visible HLB symptoms, which was not the case in their study (i.e., HLB in the trees was already at a more progressed stage) (Martinelli et al. 2016). In summary, it appears that plant-beneficial compounds can somewhat help with slowing HLB progression by having positive effects on citrus defenses and growth; however, these forms of treatment may not have strong deleterious impacts on Ca. Liberibacter spp.

Furthermore, since micronutrient deficiencies, especially zinc deficiency, are often associated with HLB symptoms, it can be speculated that applications of zinc-based compounds may help alleviate disease symptoms. However, a recent greenhouse study demonstrated that treating HLB-infected Ray Ruby grapefruit seedlings with zinc sulfate heptahydrate (i.e., $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; commercially available fertilizer) or zineb (i.e., zinc salt of a bis-dithiocarbamate; USA EPA approved agriculture pesticide) actually induced an increase in titer of Ca. Liberibacter asiaticus within leaves within 4 months; this increase was 1.5x greater than the increase experienced in untreated controls during the same time period (Zhang et al. 2016).

Thus, receiving these zinc-based compounds had expedited proliferation of the HLB pathogen, which may be, at least partially, attributable to the functional high-affinity zinc uptake system encoded in the Ca. Liberibacter spp. genome (Vahling-Armstrong et al. 2012; Zhang et al. 2016). In a separate field study that attempted treating HLB-diseased citrus trees with micronutrients, spray applications of phosphite combined with zinc-metalosate, manganese-carbonate, manganese-metalosate, or copper-metalosate, and injections treatments of soluble copper or silver mixed with a polymer were reported to
have no significant effects on Ca. Liberibacter asiaticus titer, fruit yield, or juice quality (Gottwald et al. 2012). Collectively, these studies suggest that micronutrient amendments cannot mitigate Ca. Liberibacter spp., at least when used alone. Although enhanced nutrient programs may promote citrus tree growth for asymptomatic trees or for those in groves with poor soil fertility at early stages of HLB infection, they lack significant effects on HLB-diseased trees that have developed advanced stages of infection (Wang et al. 2017a). Moreover, we note that zinc-based nanoparticles with potential antimicrobial activity (e.g., Zinkicide™ and ZnO-nCuSi gel) have been gaining attention for use to combat citrus pathogens, including Ca. Liberibacter asiaticus, and warrant further attention (Commerford et al. 2016; Graham et al. 2016; Young et al. 2017).

Overall, with regard to non-antimicrobial approaches to control HLB, thermotherapy appears to be the most effective against Ca. Liberibacter spp., though this has only been confirmed in small-scale settings. Applications of plant growth or defense regulator compounds appear to be beneficial for supporting overall tree health and might be able to slow disease progression; however, they do not lessen the existing pathogen titer. Micronutrient amendments also appear to be generally inconsequential for treating the HLB pathogen. Utilizing some these holistic approaches for phytopathogen control in combination with antimicrobial treatment would be an interesting avenue for future research.

PLANT MICROBIOTA IMPLICATIONS FOR CITRUS HEALTH

Plant microbiota play critical roles in plant development by providing support for disease control and stress tolerance, among other mechanisms (Andreote et al. 2014;
Schlaeppi and Bulgarelli 2015). A growing number of studies have focused on understanding how plant-associated microbial communities change in richness and diversity during disease development (Bulgari et al. 2011; Li et al. 2014; Rastogi et al. 2012; Schreiner et al. 2010; Xu et al. 2012; Zhang et al. 2011b), including that of HLB (Blaustein et al. 2017; Trivedi et al. 2010; Trivedi et al. 2012; Sagaram et al. 2009; Zhang et al. 2017). Harnessing the beneficial potential of native microbiota may be one of the few logistically and economically feasible solutions for controlling certain phytopathogens that are otherwise difficult to manage, such as Ca. Liberibacter spp.

Core Citrus Microbiota

Commonly occurring organisms across similar microbiomes comprise a core microbial community that is hypothesized to play key roles in ecosystem functioning within that type of microbial habitat (Gopal et al. 2013; Shade and Handelsman 2012). In a broad field study that incorporated 16S rRNA gene amplicon sequencing, we demonstrated differential associations between the core members of microbial communities of citrus trees and various factors, including HLB disease symptom severity, location (across Florida, USA), cultivar, season, and time (Blaustein et al. 2017). The relative abundance of Ca. Liberibacter spp. among leaf microbiota (i.e., full leaf samples containing all epiphytes and endophytes) positively correlated with HLB symptom progression and negatively correlated with microbiota alpha diversity (Blaustein et al. 2017). Interestingly, Ca. Liberibacter spp. was detected in leaves of almost all trees that were asymptomatic in appearance (n=27/29), albeit at low population densities, in addition to all of the HLB-symptomatic trees (n=41/41) at relatively higher population densities (Blaustein et al. 2017). Thus, while certain
changes in microbiota structure may have been induced by changes in physiology of
the host trees, there may have been specific changes that were a pre-condition to the
proliferation of Ca. Liberibacter spp. and the associated establishment and progression
of disease. Moreover, citrus-health-associated microbiota of leaves (e.g.,
*Methylobacterium, Sphingomonas*) and roots (i.e., fibrous root samples containing all
epiphytes and endophytes) (e.g., *Bradyrhizobiaceae, Burkholderiaceae, Kaistobacter, Pseudomonas, Rhodanobacter, Sinobacteraceae*) were identified based on having
differential relative abundances in microbial communities of healthy and diseased trees
(Blaustein et al. 2017), several of which were previously described in additional studies
microbiota that stably associate with citrus may provide functions involved in: (a)
competing with Ca. Liberibacter spp. for nutrient resources, limiting its ability to grow
and spread, (b) antibiosis towards the pathogen or other disease-associated bacteria,
(c) assistance with host nutrient acquisition, which could help alleviate disease
symptom-related nutrient deficiencies, or (d) induction of host-plant signaling pathways
that stimulated plant defense responses (Blaustein et al. 2017). Alternatively, it is
possible that some of the aforementioned associations between microbiota and citrus
health may be partially attributable to certain microbial taxa simply thriving on relatively
healthier plants and/or to the microbiome responses to the potential suppression of
plant defenses by Ca. Liberibacter spp. (Wang et al. 2017a) and changes in plant
hormone levels that can occur during vector feeding (Casteel et al. 2012).
Characterization of the directional interactions between native microbiota, Ca.
Liberibacter spp., and citrus hosts are needed for formulating novel strategies to
manipulate plant-associated microbial communities to mitigate HLB.

**Prospects for Organic Amendments and Biocontrol**

Implementing biological control, either alone or in combination with other disease
management strategies, such as the use of organic amendments, tolerant/resistant
cultivars, or pathogen-free certified planting material, may be feasible for treating
emergent woody plant diseases that have proven challenging to control otherwise (e.g.,
HLB) (Cazorla and Mercado-Blanco 2016). Perhaps Ca. Liberibacter spp. may be
treatable in ways similar to how fungi, phytopathogen-specific viruses, and beneficial
bacteria and secondary metabolites have been used to treat *Fusarium oxysporum* (i.e.,
causative agent of bayoud disease) in date palm, *Heterobasidion annosum* (i.e.,
causative agent of root rot) in conifers, and multiple pathogens of grapevine (Compant
Moreover, endophytic microbes that possess biocontrol capabilities offer an especially
interesting potential for the development of novel agricultural biotechnologies (Cazorla
and Mercado-Blanco 2016). For example, inoculating stems of citrus variegated
chlorosis (CVC)-diseased *Catharanthus roseus* with *Curtobacterium flaccumfaciens*,
which was a biocontrol agent originally isolated from asymptomatic citrus, reduced CVC
symptoms over time (Lacava et al. 2007). This effect was suggested to have been
mediated by (1) the induction of systemic resistance of the host by *C. flaccumfaciens*
and/or (2) antagonistic interactions between *C. flaccumfaciens* and *Xylella fastidiosa*
(i.e., the xylem-limited CVC pathogen), which have been previously demonstrated *in
that direct and/or indirect interactions between *X. fastidiosa* and the biocontrol bacterium *Methylobacterium mesophilicum* may also affect CVC progression in citrus.

Strategies to manage the phloem-limited HLB pathogen with beneficial microbes are an interesting avenue for future research. It was recently reported that *Bradyrhizobium* and *Burkholderia* may have implications for beneficial citrus plant-microbe interactions after they were identified as the most dominant genera to be enriched between the citrus rhizosphere and rhizoplane that were adversely impacted by HLB (Zhang et al. 2017).

In fact, inoculating citrus seedlings with *Burkholderia* strains isolated from healthy citrus root microbiota was shown to trigger the expression of citrus genes involved in systemic acquired resistance and limit, though not prevent, *Ca. Liberibacter asiaticus* infection (Zhang et al. 2017). Also, the mutually exclusive relationships between *Ca. Liberibacter* spp. and members of the *Burkholderiaceae*, *Micromonosporaceae*, and *Xanthomonadaceae* that have been identified, based on a network analysis of time series data for citrus leaf-associated microbial community transitions, present an interesting avenue for future research (Blaustein et al. 2017). These interactions were inferred from negative correlations (*p*<0.05) that were elucidated from a network analysis of 1-year time series data for bacterial relative abundances in citrus leaf microbiota of HLB-affected trees (Blaustein et al. 2017). Additionally, phage-based therapies have demonstrated efficacy in controlling other citrus diseases caused by bacterial phytopathogens (i.e., citrus canker, citrus bacterial spot) (Balogh et al. 2008) and may have interesting applications for biocontrol of *Ca. Liberibacter* spp. if the delivery method can be optimized.
Aside from applications of plant beneficial microbiota, using organic amendments to promote the growth and diversity of resident microbiota are another form of biological control. It was reported that the diversity of microbiota in soils of citrus groves increased when using a fertilizer regime that incorporated a mix of composted manure with lower rates of the inorganic fertilizers that were traditionally used at the site, compared to using only the full rate of inorganic fertilizers (Joa et al. 2010). The microbial diversity of soils may be important for combatting HLB due to implications that native microbiota may have for citrus health.

**STRATEGIES FOR DEVELOPING HLB-RESISTANT CITRUS**

Since all varieties of cultivated citrus are susceptible to infection by *Ca. Liberibacter* spp. (Hao et al. 2016), breeding HLB-resistant citrus has fundamental challenges. Alternatively, creating transgenic citrus that overexpresses the *Arabidopsis thaliana* *NPR1* gene, which is involved in regulating systemic acquired resistance (SAR), or that which overexpresses genes involved in production of thionins with antimicrobial activity has been reported to enhance HLB resistance (Dutt et al. 2015; Hao et al. 2016). On the horizon, there are prospects for utilizing an engineered version of citrus trietza virus (CTV) *in planta* to attack *Ca. Liberibacter* spp. or to produce a spinach defense gene that may be effective against HLB (Ledford 2017). Researchers are also attempting to use the CRISPR-Cas9 gene editing tool to create citrus cultivars that are less susceptible to *Ca. Liberibacter* spp. and/or express genes that may prevent vector transmission (Ledford 2017; Wang et al. 2017a; Zheng et al. 2016). Similar genome editing methods were previously used to confer citrus resistance to another important phytopathogen, *Xanthomonas citri*, the causative agent of citrus canker (Jia et
al. 2016). However, these novel CTV-based and gene editing approaches are still several years away from producing desired results and, ultimately, meeting regulatory requirements that would make them feasible and marketable for commercial production operations (Ledford 2017). Thus, controlling the HLB pathogen is still largely limited to the methods described in previous sections of this review.

ONGOING CHALLENGES ASSOCIATED WITH FIELD-SCALE TREATMENT

Despite ongoing efforts made by growers to modify components of citrus production operations (e.g., fertilizer regime, irrigation schedule, usage of pesticides to manage Ca. Liberibacter spp. and D. citri) to control HLB, the phytopathogen has spread to infect the vast majority of citrus groves. While chemical compounds with activity against Ca. Liberibacter spp. are becoming recognized (Hu and Wang 2016; Gardner et al. 2016; Pagliai et al. 2014; Zhang et al. 2013a), there are a number of factors that continue to make treatment at the field-scale relatively challenging.

Upon transmission to citrus trees, Ca. Liberibacter spp. proliferates within and translocates throughout phloem tissue, reaching high, unevenly distributed numbers in leaves and roots (e.g., more than $10^6$ cells per gram leaf tissue) (Tian et al. 2014; Hu and Wang 2016). Thus, in order for antimicrobials to be effective against HLB, they must come in contact with Ca. Liberibacter spp. by entering the phloem and becoming widely dispersed throughout the vascular system. Although foliar sprays may be the most logical way to deliver treatment at the field-scale, this mode of delivery may not allow for chemical absorbance into phloem due to physical barriers of waxy leaf surfaces and the robustness of plant cell walls. Alternatively, although applying treatment via trunk injection may provide direct transfer of antimicrobials into the plant
vascular system, this form of delivery is highly labor intensive in the field. Even with trunk injections, there are still limitations with chemical transport throughout trees to reach the roots (Shin et al. 2016). For example, antimicrobials that are applied above rootstock grafts (e.g., via foliar spray or trunk injection) cannot eliminate Ca. Liberibacter spp. within citrus roots unless they are transported downward to the root system at effective inhibitory concentrations. In addition, while relatively low doses of antimicrobials may be ineffective at suppressing pathogens, higher doses of at least some of the tested antimicrobials are known to have phytotoxic effects (Zhang et al. 2013). Thus, with inherent challenges for chemical dispersal within trees, the added issue of phytotoxicity when using broad-range antibiotics makes phytopathogen control even more difficult.

There is a latency period between infection and early symptom expression (e.g., blotchy mottle on leaves), during which time infected trees appear asymptomatic (Stansly et al. 2014). While HLB treatments are expected to have highest efficacy if administered at the onset of infection (Martinelli et al. 2016), this timing is not easily anticipated. By the time HLB is recognized and treatments are implemented, Ca. Liberibacter spp. populations may already be well established and capable of, at least partial, withstanding of antimicrobial treatment effects. For example, when chemical treatments suppress Ca. Liberibacter spp. within diseased trees, populations of residual Ca. Liberibacter spp. that survive the treatment have been reported to regrow over time (Hu and Wang 2016; Zhang et al. 2013a). Thus, the usage of antibiotics may need to continue over time in order to control HLB over the long-term, which introduces problems associated with operation costs and potentially adverse environmental effects.
Although it remains largely unknown, there may also be issues associated with resilient Ca. Liberibacter spp. populations and native microbiota becoming resistant to antibiotics that are used, which is concerning with regard to the dissemination of antibiotic resistant bacteria and associated genes through the environment.

Treatments with broad-spectrum antibiotics can have adverse effects on native microbiota (Yang et al. 2016; Zhang et al. 2013a; Zhang et al. 2013b), which are known to play an important role in supporting tree health. Under the right circumstances, the plant microbiota can bolster plant productivity by providing protection against phytopathogens, improving tolerance of environmental stresses, priming immune response signaling pathways, and assisting with acquisition of nutrients from soil, among other mechanisms (Andreote et al. 2014; Schlaeppi and Bulgarelli 2015).

Finding a treatment with minimal effects on native microbiota, or even developing strategies for biological control (Wang et al. 2017b), may be needed for improving field-scale HLB management.

Although antimicrobials can suppress Ca. Liberibacter spp. within infected trees, high levels of pathogen inoculum are still widespread in areas where HLB has become endemic. For example, in Florida, over 130,000 acres of land previously used for commercial citrus production have become unmanaged or abandoned due to logical issues associated with maintaining groves infected with HLB (Pelz-Stelinski et al. 2016). These areas remain reservoirs for Ca. Liberibacter asiaticus and D. citri. Therefore, even if the phytopathogen is able to be managed within a specific field site, there remains the possibility for re-introduction via vector migration. Thus, efforts to control populations of D. citri (i.e., the psyllid vector of Ca. Liberibacter asiaticus and Ca.
Liberibacter americanus) and *Trioza erytreae* (i.e., the psyllid vector of *Ca. Liberibacter africanus*), which were recently reviewed by Grafton-Cardwell et al. (2013), are critical for field-scale control of *Ca. Liberibacter* spp. Overall, although vector control is the primary strategy for HLB management as it may slow the spread of disease and lessen its severity (Monzo and Stansly 2017), there is an urgent need to augment and reduce the dependency on insecticides by identifying feasible measures to mitigate *Ca. Liberibacter* spp. and associated HLB symptoms (e.g., via applications of antimicrobials, applications of plant-beneficial compounds, biological control). Integration of these approaches with insecticidal controls may be essential for improving the efficacy and sustainability of HLB disease management.
LITERATURE CITED


Croxton, S. D., and Stansly, P. A. 2014. Metalized polyethylene mulch to repel Asian...
Article 1630.


Schreiner, K., Hagn, A., Kyselkova, M., Moenne-Loccoz, Y., Welzl, G., Munch, J. C. and


Table 1. Antimicrobials that have been tested against HLB infection in studies that incorporated quantification of the phytopathogen.

<table>
<thead>
<tr>
<th>Broad Antimicrobial Class (Target activity)</th>
<th>Antimicrobial Compound(s)</th>
<th>Field/Greenhouse (Applic. method)</th>
<th>Impact on Ca. Liberibacter asiaticus (Detection method)</th>
<th>Impact on HLB Symptoms</th>
<th>Potential Side Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides (inhibit protein synthesis)</td>
<td>Streptomycin</td>
<td>Greenhouse (root drench)</td>
<td>Reduction in population density in leaves by more than 3 log units within 3 months after treatment, yet re-growth to a level close to the starting concentration by the 6-month time point (qPCR)</td>
<td>Not discussed</td>
<td>No phytotoxicity</td>
<td>(Zhang et al. 2011a)</td>
</tr>
<tr>
<td>β-lactams (inhibit transpedditation/ cell wall modification)</td>
<td>Penicillin G</td>
<td>Field (trunk injection)</td>
<td>Concentration-dependent reduction in titer by 6- to 12-fold in leaves of treated trees compared to untreated controls at the 3-month time point after treatment (qPCR)</td>
<td>Slight increases in canopy size</td>
<td>No phytotoxicity; little or no impact on native bacterial populations and penicillin resistance within populations</td>
<td>(Shin et al. 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Greenhouse (root drench)</td>
<td>Reduction in population density in leaves by more than 3 log units within 3 months after treatment, yet re-growth to a level close to the starting content by the 6-month time point (qPCR)</td>
<td>Not discussed</td>
<td>No phytotoxicity</td>
<td>(Zhang et al. 2011a)</td>
</tr>
<tr>
<td>Sulfonamides (inhibit metabolic pathway for folic acid synthesis)</td>
<td>Sulfadimethoxine</td>
<td>Greenhouse (root drench)</td>
<td>Approximately a 9% lower relative abundance (Phylochip), but about twice as high titer (qPCR), in leaves of treated seedlings than controls at the 2-month time point after treatment</td>
<td>Slightly less chlorosis development in canopy of treated seedlings than in that of controls</td>
<td>Partial deleterious effects on relative abundances of native bacteria</td>
<td>(Yang et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>Sulfathiazole</td>
<td>Greenhouse (root drench)</td>
<td>Approximately a 7% lower relative abundance (Phylochip), but about twice as high titer (qPCR), in leaves of treated seedlings than controls at the 2-month time point after treatment</td>
<td>Slightly less chlorosis development in canopy of treated seedlings than in that of controls</td>
<td>Partial deleterious effects on relative abundances of native bacteria</td>
<td>(Yang et al. 2016)</td>
</tr>
<tr>
<td>Tetracyclines (inhibit protein synthesis)</td>
<td>Oxytetracycline</td>
<td>Field (trunk injection)</td>
<td>Depending on the amount of injection ports used for application, the population density in leaves decreased 1-3 log units within 1-month after treatment (qPCR). It remained lower in treated trees than controls for 9 months, although population re-growth occurred during this time.</td>
<td>New flushes did not display chlorosis, so the overall canopy appeared healthier</td>
<td>Moderate phytotoxicity – brown discoloration to leaf burning on some young flushes</td>
<td>(Hu and Wang 2016)</td>
</tr>
<tr>
<td>Small molecules (inhibit transcription factors produced by L. asiaticus that may be essential for pathogenesis and)</td>
<td>Tolfenamic Acid</td>
<td>Greenhouse (foliar spray; root drench)</td>
<td>Approximately an 80-95% reduction in the expression of L. asiaticus genes rplJ and gyrA in 75% of the treated seedlings, indicating substantial reduction in viable population (RT-qPCR)</td>
<td>Substantial improvements in fibrous root development and foliage appearance</td>
<td>No phytotoxicity</td>
<td>(Gardner et al. 2016)</td>
</tr>
<tr>
<td>Combination treatments including β-lactams, Aminoglycosides, and/or Tetracyclines (see above)</td>
<td>Penicillin G + Streptomycin</td>
<td>Greenhouse (root drench) and Field (trunk injection)</td>
<td>In the greenhouse study, there was reduction in population density by more than 2 log units within 2 months following treatment, which continued to slightly decrease during the 6-month monitoring period (qPCR). In the field study, the population density in leaves of treated trees was anywhere from 3.5-5 log units lower than in those of controls during 14 months of monitoring; however, there were about 2 log unit increases between the 4- and 14-month time points (qPCR).</td>
<td>Not discussed</td>
<td>Slight phytotoxicity in both studies</td>
<td>(Zhang et al. 2011a)</td>
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<tr>
<td>Field (trunk injection)</td>
<td>The titer in leaves of treated trees, which correlated with relative abundance (PhyloChip), was anywhere from 3- to 30-fold lower than in those of controls during 14 months of monitoring; however, seasonal fluctuations indicated re-growth (qPCR)</td>
<td>Fluctuating titer was described to somewhat correlate with symptom appearance</td>
<td>Partial deleterious effects on relative abundances of native bacteria</td>
<td>(Zhang et al. 2013a)</td>
<td></td>
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<tr>
<td>Kasugamycin + Oxytetracycline</td>
<td>Field (trunk injection)</td>
<td>The titer in leaves of treated trees, which correlated with relative abundance (PhyloChip), was anywhere from 3- to 30-fold lower than in those of controls during 14 months of monitoring; however, seasonal fluctuations indicated re-growth (qPCR)</td>
<td>Fluctuating titer was described to somewhat correlate with symptom appearance</td>
<td>Partial deleterious effects on relative abundances of native bacteria</td>
<td>(Zhang et al. 2013a)</td>
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</tr>
</tbody>
</table>
Table 2. Control measures other than antimicrobials that have been tested against HLB in studies that incorporated quantification of the phytopathogen.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment Details</th>
<th>Field/Greenhouse (Applic. method)</th>
<th>Impact on Ca. Liberibacter asiaticus (Detection method)</th>
<th>Impact on HLB symptoms</th>
<th>Side Effects</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermotherapy</td>
<td></td>
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<tr>
<td>40°C</td>
<td>Thermotherapy</td>
<td>Greenhouse</td>
<td>Anywhere from no change to &gt;40-fold reduction in titer (qPCR), depending on the study. About a 9% reduction in relative abundance (PhyloChip)</td>
<td>Some instances of chlorosis-like symptoms being mitigated over time, though some cases of normal symptom progression</td>
<td>Some cases of moderate leaf tissue damage</td>
<td>(Hoffman et al. 2012; Yang et al. 2016; Zhang et al. 2016)</td>
</tr>
<tr>
<td>42°C</td>
<td>Thermotherapy</td>
<td>Greenhouse</td>
<td>Anywhere from no reduction to &gt;40-fold reduction in titer (qPCR), depending on the study.</td>
<td>Some instances of chlorosis-like symptoms being mitigated over time, though some cases of normal symptom progression</td>
<td>Some cases of moderate leaf tissue damage</td>
<td>(Hoffman et al. 2012; Zhang et al. 2016)</td>
</tr>
<tr>
<td>45°C</td>
<td>Thermotherapy</td>
<td>Greenhouse</td>
<td>Anywhere from 5-fold to 1000-fold reduction in titer in response to treatment (qPCR). Over 80% reduction in relative abundance (PhyloChip)</td>
<td>Chlorosis-like symptoms generally mitigated over time</td>
<td>Some cases of severe leaf tissue damage</td>
<td>(Yang et al. 2016; Zhang et al. 2016; Fan et al. 2016)</td>
</tr>
<tr>
<td>48°C</td>
<td>Thermotherapy</td>
<td>Greenhouse</td>
<td>Reductions in titer in leaves of treated trees by about 55%, while that in untreated controls increased by over 300% (qPCR)</td>
<td>Chlorosis-like symptoms mitigated over time</td>
<td>Not discussed</td>
<td>(Li et al. 2016)</td>
</tr>
<tr>
<td>Chemical inducers of plant defenses</td>
<td>AA, BABA, BTH, INA, 2-DDG (used individually or in combination)</td>
<td>Field (spray)</td>
<td>No reduction in titer within leaves; however, the rate of increase was slowed compared to controls (qPCR)</td>
<td>Trees receiving treatment had greater fruit yield and quality than controls</td>
<td>Not discussed</td>
<td>(Fan et al. 2016)</td>
</tr>
<tr>
<td>Plant-regulating compounds</td>
<td>L-arginine; 6-benzyl-adenine + gibberellins</td>
<td>Greenhouse (spray) and Field (spray)</td>
<td>No reduction in titer within leaves of trees receiving treatments (RT-qPCR)</td>
<td>No phenotypic effects; however, genes involved in plant metabolism and immune response were up-regulated</td>
<td>Not discussed</td>
<td>(Martinelli et al. 2016)</td>
</tr>
<tr>
<td>Brassinosteroids</td>
<td>epibrassinolide</td>
<td>Greenhouse (spray) and Field (spray)</td>
<td>Concentration-dependent reduction in titer within leaves by about 160- and 7-fold in greenhouse and field study, respectively (qPCR)</td>
<td>No HLB symptoms on new flushes; genes involved in plant defense response were up-regulated</td>
<td>Not discussed</td>
<td>(Canales et al. 2016)</td>
</tr>
<tr>
<td>Micronutrient-based compounds</td>
<td>Zinc sulfate heptahydrate (ZnSO₄·7H₂O)</td>
<td>Greenhouse (root drench)</td>
<td>Increases in titer (qPCR) and relative abundance (PhyloChip) in leaves</td>
<td>No effects; symptoms progressed</td>
<td>Not discussed</td>
<td>(Zhang et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>Zineb (i.e., zinc salt of a bis-dithiocarbamate; USA EPA approved agriculture pesticide)</td>
<td>Greenhouse (root drench)</td>
<td>Increases in titer (qPCR) and relative abundance (PhyloChip) in leaves</td>
<td>No effects; symptoms progressed</td>
<td>Not discussed</td>
<td>(Zhang et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>Phosphate combined with Mn-carbonate, Mn-, Cu-, or Zn-metalosate; soluble copper or silver combined with a polymer</td>
<td>Field (spray; trunk injection)</td>
<td>No reduction in titer in leaves (qPCR)</td>
<td>No effects; symptoms progressed</td>
<td>Not discussed</td>
<td>(Gottwald et al. 2012)</td>
</tr>
</tbody>
</table>