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Huanglongbing (HLB): An unresolved citrus greening disease caused by bacteria vectored by insect, Psyllids with future perspective of research

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Abstract
Citrus production in the world declined continuously due to 'Huanglongbin' (HLB) diseases, caused by phloem sap localized bacteria, *Candidatus Liberibacter asiaticus* (CLas) that was inoculated by Asian Citrus Psyllid (ACP), *Diaphorina citri* during stylet feeding. CLas was a phloem-limited, and gram-negative proteobacterium. Once inoculated by insect stylet probing, phloem environment favored aerobic respiration to 'CLas' resulting in rapid proliferation which caused blockage source-sink relationship, callose deposition, and cell collapse in the phloem tissue. The conventional control measure focused on reducing CLas titer by chemical spray, planting disease-free nursery stock, removing infected trees, and promoting root health etc. Unfortunately, these management practices evidenced as inadequate control measure to restrict HLB epidemics. The resistant plants produced a wide spectrum of defense compounds such as terpenoids, alkaloids, nonprotein amino acids, benzoxazinoids, cyanogenic glucosides, and phenolic compounds. But the mechanisms underpinning citrus tolerance to CLas has not been pinned yet. Cracking the 'mystery' was essential for sustainable development of CLas mediated citrus greening disease. This review lighted on future research perspectives which could be potentially explored in future.

Keywords: 'Huanglongbin' diseases, *Candidatus Liberibacter asiaticus*, Asian citrus psyllid, *Diaphorina citri*

Introduction
Citrus fruit production was recorded more than 100 million metric tons globally (Wang and Trivedi, 2013) [44]. Citrus fruits are enriched with vitamins, dietary fiber, minerals, flavonoids and carotenoids. Unfortunately, HLB diseases was a constant threat to the citrus production. The HLB disease was caused by phloem sap restricted bacteria., inoculated by ACP feeding. *Candidatus Liberibacter*, responsible for citrus greening was characterized by phloem-limited, and gram-negative bacterium. Three species of *Candidatus Liberibacter* are associated with citrus greening: *Candidatus Liberibacter asiaticus* (CLas) (Asia, North America, and Brazil), *Candidatus Liberibacter africanus* (CLaf) (Africa), and *Candidatus Liberibacter americanus* (CLam) (Brazil). The Asian Citrus Psyllid (ACP), *Diaphorina citri* (Kuwayama) vectored both CLas and CLam, while the African citrus psyllid, *Trioza erytrea* transmitted CLaf (Wang and Trivedi, 2013; Jianga *et al*., 2019) [44, 29].
Successful acquisition and transmission of CLas by *D. citri* started with ingestion of the bacteria from the infected citrus through the stylet feeding as psyllid tapped phloem sap from infected trees through slender and long stylet (Ghanim *et al*., 2016; Ammar *et al*., 2013) [4]. CLas mobilized through long stylet and mobilized into the gut lumen, from where it again reached to the hemolymph through the gut wall. Therefore, CLas was also traffic to the salivary glands from hemolymph. During phloem sap ingestion from sieve element, *D. citri* easily transmitted CLas to a new susceptible citrus plant thorough stylet salivation (Ammar *et al*., 2011; Ammar *et al*., 2017).
HLB was identified for enormous economic loss to citrus industry globally since long ago (Bove, J. M., 2006; McClean & Oberholzer, 1965). For example, HLB had been reported in China in 100 years back (Chen., 1943). In USA, the HLB disease was identified in 2005 in Florida. The HLB disease caused loss of citrus production from 750,000 acres and 170 million boxes to 520,000 acres and less than 80 million boxes in 2015–2016 in Florida only (Neupane *et al*., 2016). Therefore, only Florida citrus industry experienced over 50% loss of citrus cultivation area, and production (United States Department of Agriculture, 2015). Severe HLB

disease symptom was characterized by blotchy mottling with green islands on leaves. Infected shoots are stunted, and the branches gradually die as the disease progresses. Fruit from diseased trees was smaller in size and lopsided, with poor coloration which was very unattractive to consumers.

Pathogen

The genome sizes of CLas was 1.23 Mb which was much lower than the closely related bacteria to '*Candidatus Liberibacter*' *spp*. In comparison, the genome size was recorded 3.4 Mb in *Agrobacterium sp*. H13-3, 5.7 Mb in *Agrobacterium tumefaciens* C58, 6.3 Mb in *A. vitis* S4, 6.5 Mb in *Rhizobium etli* CFN 42, 6.7 Mb in *Sinorhizobium meliloti* 1021, and 7.3 Mb in *A. radiobacter* K84 (Leonard *et al*., 2012) [33]. The reduced genome size as well as low GC content in CLas was due to the result of evolutionary association with the stable and nutrient-enriched environments (Moran *et al*., 2008; Moya *et al*., 2008; Wernegreen, 2002) [35, 36, 45]. Interestingly, CLas was lacking of complete restriction-modification system for defense (Duan *et al*., 2009; Lin *et al*., 2011) [13, 34]. Thus, CLas was vulnerable to prophage integration, as several phage-derived gene sequences was also evidenced in its genome. This characteristic enhanced rate of recombination which again enhanced rate of new genome evolution in CLas (Lin *et al*., 2011) [34]. Bastianel *et al*., 2005 [6] explored *omp* based PCR-restriction fragment length polymorphism to record the number of CLas isolates and strikingly it was estimated 23 CLas variants with different symptoms behavior only in seven provinces in China (Hu *et al*., 2011) [26].

Asian Citrus Psyllid

The Asian citrus psyllid (ACP, *Diaphorina citri*) was identified as the primary insect vector of CLas (Grafton-Cardwell *et al*., 2013; Ammar *et al*., 2013) [22, 4]. HLB or citrus greening disease was first reported a hundred years ago in Asia. It was the most serious threat to the citrus-growing industry worldwide for the complexity, destructiveness, and difficulties in management. The HLB was caused by three species of CLas, CLam, and CLaf. All were unable to culture in synthetic media but proliferated in the phloem environment (Bove, J. M., 2006; Bove & Ayres, 2007; Jagoueix *et al*., 1994). A psyllid can acquire the pathogen during nymph and adult stages from the infected plant through slender stylet (Capoor E *et al*., 1974; Inoue *et al*., 2009; Pelz-Stelinski *et al*., 2010; Xu *et al*., 1988) [9, 28, 37, 46]. Acquisition efficiency by nymphs was higher (60 to 100%) as compared to adults (40%) even after 5 weeks of feeding on CLas-infected plants under controlled environment and field conditions (Pelz-Stelinski *et al*., 2010) [37]. Further, it was also recorded that ACP nymph could acquire CLas in 15 min (Capoor E*et al*., 1974) [9]. Inoue *et al*. (2009) [28] suggested that multiplication within psyllids was essential for efficient transmission of CLas. The transmission of CLas from parent to offspring was also evidenced. Interestingly, seed mediated CLas transmission did not evidenced till now.

Phloem environment for CLas proliferation

A number of insects and pathogens nourished from the phloem tissue which was enriched with sucrose. Being a part of the plant vascular system, phloem was essential for long-distance transportation of photosynthates and other molecules from source tissues (e.g., mature leaves) to sink (e.g., buds, flowers, seeds and roots). Phloem content represented a unique ecological niche for a variety of pathogens and insects. In fact, insects and pathogens evolved to acquire nutritionally enriched phloem sap and caused significant economic losses in many crop plants. For instance, citrus greening disease ('huanglongbin' or HLB) was estimated to cause an economic loss of as much as 418 million dollars per year in Florida alone. It is important to note that oxygen concentration was maintained in the phloem environment. *Ricinus communisis* maintained O_2 levels in phloem from 21% (vol/vol) at the surface to 7% (vol/vol) in the vascular region to 15% (vol/vol) toward the hollow center of the stem (van Dongen *et al*., 2003) [42]. Thus, phloem could support aerobic respiration of 'CLas'for its rapid multiplication.

Disease symptoms and severity

HLB disease symptom was characterized as yellowing of the leaf veins and adjacent tissues, premature defoliation, dieback of twigs, ultimate death of infected whole citrus plant (Bové., 2006) [8]. The infected tree showed retarded growth, off season flowering, unattractive bearing, smaller fruits with bitter taste. Outbreak of HLB has lowered down fruit quality and quantity (Gottwald., 2010) [21]. Interestingly, the causal organism proliferated in phloem-tissue environment but not culturable in any synthetic media (Albrecht and Bowman, 2008; 2009) [2, 3]. Most commercial citrus cultivars are susceptible to HLB. HLB enabled in shortening the trees' lifespan and reduced fruit quality, such as total soluble solids (TSS) content, acidity, and the TSS/acidity ratio (Bassanezi *et al*., 2009; Dagulo *et al*., 2010; Tsai *et al*., 2008) [5, 12, 41]. Moreover, HLB could potentially reduce 30 to 100% yield loss. The HLB-diseased trees were more vulnerable to adverse weather exposure like extremes of temperature and moisture.

Phloem blockage

Phloem blockage was one of the major issues in correlation with HLB disease symptom development (Kim *et al*., 2009; Schneider, H. 1968) [31, 39]. HLB-associated phloem was evidenced as complete blockage of sieve pores. Characteristically, HLB bacterial aggregate was not the reason for clogging as CLas did aggregate in citrus vasculature conduit (Kim *et al*., 2009) [31]. This blockage had adverse effect on CLas as vascular blockage restricted CLas viability as well as host cell physiology (Trivedi *et al*., 2009) [40]. Interestingly, higher concentrated CLas was spotted in phloem sieve tubes from pre-symptomatic young flushes but characteristically CLas was not found in highly symptomatic leaf samples (Folimonova and Achor, 2010) [18].

Attenuation of fibrous root

Restricted development as well as loss of fibrous root function were correlated with stress intolerance. Johnson and colleagues (Johnson *et al*., 2012) [30] reported that 4-year-old 'Valencia' orange trees showed 30 and 37% reduction in fibrous root mass density in pre-symptomatic and symptomatic trees, respectively, as compared to the healthy trees (Johnson *et al*., 2012) [30]. Therefore, the HLB disease was correlated with the growth and development of fibrous root.

Sucrose translocation

Sucrose was the primary photo-assimilate which was trafficked through phloem from green leaves to sink organs (Zimmermann and Ziegler, 1975) [48]. Sucrose accumulation in CLas-infected leaves strongly indicated that photo-assimilate translocation was attenuated by CLas infection due to phloem blockage (Fan *et al*., 2010; 2012; Kim *et al*., 2009; Koh *et al*., 20011) [15, 16, 31, 32]. On the other hand, sucrose deficiency was evidenced with restricted fruit size (Gomez-Cardenas *et al*., 2000) [20]. Importantly, CLas-infected trees curtailed carbohydrate content of the fruits (Rosales and Burns, 2011) [38]. Microscopic changes (e.g., callose deposition in sieve elements and phloem cell collapse) were evidenced both infected species but the phloem transport activity in rough lemon was much less affected by HLB than in sweet orange (Fan *et al*., 2010) [15]. However, bark samples and symptomless leaves also enriched with higher levels of starch than healthy controls without visible phloem blockage (Etxeberria *et al*., 2009) [14]. This observation indicated that different mechanisms in addition to phloem blockage might also be involved in HLB disease development. CLas enabled to metabolize sugars such as glucose, fructose, and xylulose but not mannose, galactose, rhamnose, or cellulose (Duan *et al*., 2009) [13]. Characteristically, the concentrations of fructose and glucose were very low in the phloem sap (Chino *et al*., 1991; Flowers and Yeo., 1992) [11, 17]. Therefore, biochemical utilization of fructose by CLas during infection might shift in the host metabolite equilibrium. It was further observed that accumulation of glucose but not fructose in CLas infected citrus was evidenced and suggested that 'CLas' might preferentially utilize fructose, similar to *S. citri* (Fan *et al*., 2010) [15].

Limitation for controlling HLB disease

The reduced genome was very common in phloem-limited pathogenic prokaryotes and even all core metabolic pathways were erased from the genome during evolution. Thus, phloem-associated prokaryotes were obligatory on vasculature environment for the essential nutrients and signals applicable to *phytoplasmas*, *spiroplasmas*, *Candidatus Liberibacters* and *Candidatus*. The in-vitro culturing of phloem-limited pathogens was not successful till now in a synthetic media. The inability to establish pure cultures for the phloem limited pathogens hardly follow Koch's postulate. The inability to culture other phloem-inhabiting prokaryotic pathogens was also linked to the reduced genomes of almost all phloem-limited pathogens. The CLas transient culture was already reported in biofilm-based mixed culture, in which CLas was a minor component. Hence, establishment of successful culture in synthetic media will be primary requirement to advance domain of disease etiology, disease mechanisms, and effective screening of antibacterial compounds. Innovative culturing approaches for the phloem-limited organism will be considered in future research to the study of phloem–pathogen interactions.

Effective strategies against CLas bacterium in *Citrus* production are still elusive and breeding for resistant citrus varieties was the potent option for an efficient and sustainable strategy development against HLB. But, traditional citrus breeding experienced limitations due to polyembryony, pollen-ovule sterility, sexual and graft incompatibilities, and extended juvenility. Therefore, there was no fruitful option from commercial genetically modified citrus varieties available due to lack of consumer acceptance. Therefore, the development of GMO citrus will definitely consume many years to release an HLB-resistant citrus cultivar. Noticeably, a number of antibiotics like ampicillin, carbenicillin, penicillin, cephalexin, rifampicin, and sulfadimethoxine have been reported as an effective against CLas bacterium (Zhang, *et al*., 2014). It was also reported that oil-in-water and water-in-oil nano-emulsion of antibiotic delivery were also effective. Therefore, the nano emulsions enhanced the efficacy of the antibiotics against CLas bacterium (Yang *et al*., 2015, 2016). Spraying of antibiotics in agricultural practices was restricted as a serious threat to public health might emerged due toenergence of new strain of multiple antibiotic-resistant bacteria.

Gap in Knowledge

At present, there is no cure measure for controlling HLB disease. The only way is to limit the disease by destroying all infected citrus trees and replaced them. Although many studies had been completed on HLB disease but the mechanisms underpinning citrus tolerance to CLas was still in elusive zone. Cracking this mystery is required for new perspective towards development of CLas-tolerant varieties. Some citrus varieties such as *P. trifoliata* which showed tolerant to CLas were also tolerant to citrus tristeza virus (CTV) (Bhattacharyya *et al*., 2002) [7]. Further it was revealed that several antibacterial agents were enriched in the fruit and seed of *P. trifoliata*. Therefore, it was concluded that the phloem sap of CLas-tolerant varieties might enrich with antibacterial compounds.

Phloem-feeding insects and pathogens caused tremendous economic losses worldwide. Current control strategies for diseases and infestations heavily dependent on insecticide and antimicrobial sprays. Additional control strategy like exploring resistant cultivars, biological control and cultural practices practiced in the field level. Current control measure against CLas infection was dependent on chemical spraying for controlling vectors population, planting disease-free nursery stock, removing infected trees, and promoting root health (Harrell, C, 2014) [24]. Unfortunately, these management strategies impaired to limit HLB epidemics.

Novel perspective

The ACP was identified as the primary insect vector of CLas, the bacterial pathogen associated with the development of citrus greening disease (Grafton-Cardwell *et al*., 2013; Ammar *et al*., 2013) [22, 4]. Citrus greening was the most unresolved disease worldwide (Wang and Trivedi P, 2013) [44]. Phloem-feeding insects inoculated pathogenic cocktail along with microbiota whose genome size were very small and devoid of all core metabolic pathways. Most phloem-associated prokaryotes were obligatory in nature and dependent on phloem sap composition for all essential requirements. The inability to culture the phloem-limited prokaryotic pathogens in artificial media was assumed to be attributed by the reduced genomes. The survival and multiplication on nutrient enriched artificial media will be a breakthrough research in the domain of disease etiology, disease mechanisms, and effective screening of antibacterial compounds.

It was a fundamental question with respect to whether a single microbe or a microbial consortium consisting of two or more interacting microbes caused the disease initiation as well as development of disease. Indeed, progression of citrus HLB appeared to be associated with certain compositions of citrus microbiome. Hence, the obligate pathogens with reduced genomes might require "assistance" from other microbes inoculated by insect feeding. Thus, large-scale surveys of phloem-associated microbiota in phloem diseases might be an important future perspective of research area. A comprehensive understanding of phloem defense/susceptible to inoculated microbiota will be a crucial for the development of innovative long-term control measures. Two types of plants should be identified: one type of citrus that promoted psyllid vectors and the second type promoted the inoculated bacterial pathogen multiplication. These two types of citrus had different significance in HLB management. The psyllid vectors had a relatively narrow host range as compared with the wide physiological host range of the bacterial pathogens. Hence, the low vector–pathogen specificity may have potential implications for the disease epidemiology (Gottwald, 2010; Halbert and Manjunath, 2004) [21, 23].

Relevance of CRISPR-CAS

CRISPR was the third generation of genome editing tool after ZFN (Zinc-finger nuclease) and TALEN (transcription activator like effector nuclease) (Gaj *et al*., 2013) [19]. Genome editing tools were successfully explored in enhancing resistance in crop. TALEN explored three Mildewresistance Locus (MLO) alleles in bread wheat to confer resistance to powdery mildew (Yanpeng *et al*., 2014) [47]. CRISPR was used to edit LOB1, a susceptible gene of citrus canker, at coding region and promoter region to confer citrus canker resistance, respectively (Aihong *et al*., 2017; Hongge *et al*., 2017) [1, 25]. CRISPR has one advantage over all other technologies: the modified plant may not contain foreign DNA, so the edited plant can be released to market as commercial production for human consumption (Waltz E., 2016) [43]. CRISPR could not achieve the goal of conferring citrus HLB resistance at present as HLB susceptible gene from citrus was not identified yet. However, CRISPR was a powerful tool with great potential in breeding HLB resistant citrus after susceptible gene discovery. Hence, future study needs to be focused on identification of pathogen effectors and elucidation on the virulence mechanisms. Interestingly, heat treatment evidenced in reducing HLB symptoms and perhaps it was due to the activation of PTI at higher temperature (Cheng *et al*., 2013) [10]. Genes which function to enhance PTI for curtailing HLB proliferation and could be explored in CRISPR-CAS. Moreover, multi-disciplinary approach on HLB might return ray of hope in future. Additionally, ethnic integration from scientists, growers, and governments against HLB will be the best clue for battle.

A stable antimicrobial peptide identified for effective treatment on citrus Huanglongbing

A stable peptide was recognized and identified by comparative proteomic analysis between HLB-susceptible citrus cultivars and HLB-tolerant citrus hybrids and relatives. This novel peptide was stable and effective in antimicrobial activity. The peptide is known as stable antimicrobial peptides (SAMPs) which was novel class of peptide identified (Huang *et al*., 2021). This SAMP isolated from *Microcitrus australiasica* could potentially kill *Liberibacter crescens* (Lcr), a culturable *Liberibacter* strain. The SAMP also effective on reducing infection from CLas in plants. The author showed effective recover from HLB-diseased plant under controlled greenhouse trials. The SAMP treated plant enhanced induced innate immunity to further prevent the infections. This SAMP is heat stable unlike antibiotics, thus field application through spraying will be ray of hope in the future. From the controlled experiment under controlled environment, SAMP spray was taken up by citrus leaves, stayed stable inside the plants for at least a week, and moved systemically through the vascular system where CLas is located. Definitely, this finding will give a potential ray of hope in near future.

Conclusion

HLB was recognized as the biggest and unresolved threat to citrus industry. Many efforts were completed to decipher the mechanisms on CLas mediated disease symptoms. But the mechanism is still in elusive zone for the disease development. The fundamental query on disease symptoms development over time was not addressed till now. No specific phloem cell specific study was carried out to understand how phloem response to CLas infection temporally in early time point. Field observations also indicated that some ACP hosts were more tolerant to CLas than others. The resistant plants produce a wide spectrum of defense compounds such as terpenoids, alkaloids, nonprotein amino acids, benzoxazinoids, cyanogenic glucosides, and phenolic compounds. The phloem contains many cells that were implicated in the synthesis, distribution, and release of large number of defense compounds such as terpenes, alkaloids, ketones, and glucosinolates. Although many studies had been conducted on HLB disease, the mechanisms underpinning citrus tolerance to CLas had not been determined yet. Exploring this mystery is essential for development of economically important CLas tolerant varieties. Some citrus varieties were tolerant to CLas were also tolerant to *Citrus Tristeza Virus* (CTV). Although the mechanism of *P. trifoliata* resistance to CTV had not been revealed yet, it seemed that it was due to the failure of the CTV to spread within its host despite its ability to replicate as several antibacterial compounds have been identified in the fruit and seed of *P. trifoliata*. Therefore, it was suggested that the phloem sap of CLas-tolerant varieties contain some antibacterial compounds. A number of issues were still pending which could be explored in future research study. Like, no susceptible gene for HLB was identified. Hence, CRISPR-CAS will be a promising tool to control HLB in future if identification of susceptible genes will be identified.

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