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Citrus Huanglongbing (HLB): Diagnostic and management options

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ABSTRACT

"Huanglongbing" (HLB) or citrus greening is the most devastating diseases of citrus that affects all cultivars causing systematic death of trees worldwide. The disease is associated with the presence of the phloem-limited α-proteobacteria '*Candidatus* Liberibacter asiaticus', 'africanus', and 'americanus'. The Asian Citrus Psyllid (ACP), is the main vector that transmits the pathogen while feeding the leaves of citrus trees, affecting fruit traits including fruit shape and size, ripening, and quality of fruits and compromising plant health, eventually leading to economic loss to the citrus industry. In this review, the history of HLB, its pathosystem, and geographical distribution with a primary focus on the various diagnostic measures that are in practice are described. The HLB identification in the field is the most challenging task for the growers as the symptoms of asymmetrical, blotchy mottling patterns on leaves are often confused with nutritional deficiency. The unavailability of precise methods for identification of HLB at the initial infection is of major concern. Hence, the development of field-based detection methods could help citrus growers to take protective measures to minimize disease spread in citrus plantations. The review also highlights the existing detection and management options of HLB as well as the perspectives in this research field.

1. Introduction

Citrus greening ("huanglongbing": HLB) is one of the most severe citrus diseases which affect citrus production across the world, covering more than 50 nations in the tropics and subtropics of Asia and Africa, including India and Bangladesh [1,2]. Recently HLB has been reported in Tanzania, Nigeria, Kenya, and Ethiopia, while New Zealand, Australia, and the Mediterranean basin currently being the world's only HLB-free citrus regions [3]. HLB is caused by a phloem limited, Gram-negative α-proteobacteria '*Candidatus* Liberibacter', which significantly reduces the yield and incurs a great economic loss to citrus fruits, eventually affecting the entire tree [4]. In the 1700s, central India was the very first place wherein HLB-like symptoms were described [5]. Subsequently in the 1920's, in some south Asian countries similar infections were recorded, known locally as "likubin" (drooping disease) [6]. These infections were recognized as mottle leaf disease in Philippines [7], and citrus die-back in India [8]. Eventually the 13th conference of the International Organization of Citrus Virologists in China adopted the official name as "huanglongbing" [9]. The alternative name "greening", most likely emerged from South Africa, a place active for HLB research ever since the 1950s.

HLB affects the majority of commercially grown citrus varieties. The disease symptoms include mottling or blotchy mottle, young leaves with pale and scattered green spots, chlorosis with green veins/corky veins, root decay, thinner canopy, early flowering and yellowing of veins in different cultivars of citrus [10]. Severely infected trees were found with sparse foliation, extensive dieback of the twig, and development of yellow shoots with stunted growth. The major fruit symptoms include

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excessive and premature fruit drop, the poor color of the fruit which often remains green at the bottom, an extremely bitter taste that is not fit for juice, and reduced fruit size. The infected fruits are often lopsided, small, with a curved columella, and generally aborted seed, when present [11,12]. Since citrus juice is considered one of the important fruit drinks nationwide, representing approximately half of the fruit juice industry (USDA–ERS, 2018), significant yield loss due to HLB affected the citrus industry [13]. Despite extensive scientific investigations, there is still no available cure for HLB. The purpose of this review is to bring together a thorough understanding of HLB, with emphasis on its detection techniques, and management approaches. The review then outlines the key research gaps as well as prospective research development.

2. Pathogen, host range, and disease cycle

Different studies led to the identification of three species of '*Candidatus* Liberibacter' associated with the HLB disease which belongs to the subdivision alpha of the Proteobacteriaceae [14,15]. The species were '*Candidatus* Liberibacter africanus' (*C*Laf), '*Candidatus* Liberibacter asiaticus' (*C*Las), and '*Candidatus* Liberibacter americanus' (*C*Lam). *C*Las is the prevalent bacterium associated with Asian HLB and has been found across the world, including in the Western hemisphere. *C*Lam was initially found only in Brazil and contributed a large proportion of the total population of bacteria. *C*Laf associated with the greening in Africa is predominantly found in Saudi Arabia, South Africa, and on some islands in the Indian Ocean. The Asian Citrus Psyllid, *Diaphorina citri*, and the African citrus psyllid, *Trioza erytreae*, are the two known HLB vectors, the Asian Citrus Psyllid (ACP) being the most prevalent one [16]. Although African citrus psyllid spreads Laf in Africa, some parts of Arabia, and islands of the Indian and Atlantic Ocean, any of the three bacterial species gets transmitted by either psyllid [17]. Also, *T. erytreae* is now migrating along the north and west coasts of the Iberian Peninsula, bringing it closer to vital citrus-growing regions in Portugal and Spain [3,18–20]. The ACP transmits HLB in citrus trees when it feeds on its phloem sap. Inside ACP *C*Las multiplies in the gut and gets spread to the gonad and salivary gland by hemolymph circulation. Now, while feeding on the young flush of healthy trees, *C*Las infection is transmitted by ACP within an hour. Following inoculation, the pathogen reproduces on early flush shoots and then moves to roots, followed by uneven transmission to shoots and leaves, explaining why *C*Las concentrations are higher in roots (Fig. 1) [21]. *C*Las moves upward to reach leaves within 2 months from inoculation. The *in planta* movement of *C*Las takes place passively from source to sink via phloem sap along the sieve tube [22] wherein the development of new shoots and roots on infected citrus trees influence its vertical movement [21]. *C*Las was more likely to be detected in the root-pruned plants' roots than in the roots of non-pruned plants. In contrast, the chances of detecting *C*Las in a new flush of the top-pruned plant were lower as compared to the young flush of non-pruned plants [21]. In the context of the involvement of external appendages in the movement of *C*Las, the genome of *C*Las is found to encode flagellin-encoding genes *flaA and flgJ*. However, the expression of these genes is observed to be minimal *in planta* and highly upregulated in ACP, which suggests the movement of *C*Las through phloem sap rather than with flagella [23]. This movement of *C*Las is further confirmed by transmission electron microscope studies which also show that the structure of *C*Las, from ACP, is filamentous whereas it is in a different shape from infected citrus [23]. The movement and colonization of the pathogen in a host are found to be optimal at 25.7 °C which explains why HLB infection is more common during winter in tropical and subtropical regions [24]. *C*Las when transmitted to the host delivers different effector proteins through the Sec-dependent secretion system and type I secretion system (T1SS) [25]. Proteins of the serralysin family and Sec-dependent secretory protein 1 (SDE1) promote bacterial invasion and colonization by interacting with citrus papain proteases [26]. The effector protein SDE1 is identified to promote typical HLB symptoms of yellowing and blotchy mottled leaves by down-regulating the DDX3 expression in HLB-affected trees [27]. Additionally, the gene CLIBASIA_05315 was identified to suppress the hypersensitive response activated by the host upon infection and hence can be considered as a marker for HLB detection [28]. Although numerous research has been conducted to determine the molecular fingerprints involved in the citrus-*C*Las interaction, the identification of effectors and their targeted proteins has yet to be demonstrated at each stage of infection systematically. In this context, functional and comparative genomics, as well as transcriptomics studies will open up new directions for understanding the mechanisms engaged at each step of pathogenic invasion. However, this also urges the establishment of *in vitro* culture of the pathogen.

The phloem-limited bacteria are adapted to the unique phloem environment as they lack many genes necessary for core metabolic processes; however; this property makes them unculturable in an artificial medium [30]. Davis et al. [31] reported that cultivation of *CLas* seemed to be possible with the presence of other bacteria *i.e.*, actinobacteria in the cultures. These co-cultures could survive more than 10 weeks when a fresh medium is introduced which suggests that there may be a mutually beneficial relationship between the bacterium with each other when co-cultured. Sechler et al. [32] designed a new medium named Liber A to culture all three '*Candidatus* Liberibacter'. The medium containing growth factor and citrus vein extract mediated the growth of '*Candidatus* Liberibacter' for four or five single-colony transfers after which the viability declined. Parker et al. [33] demonstrated that *C*Las' viable cultures could be maintained for several weeks when grown on grapefruit juice-containing media owing to the presence of specific elements like potassium and calcium [33]. This may facilitate the development of a culture medium for the growth of *C*Las [34]. reported the successful establishment and long-term sustainable host-free *C*Las cultures by growing the bacterium within cultured biofilms. The biofilms were grown under controlled conditions in a newly developed

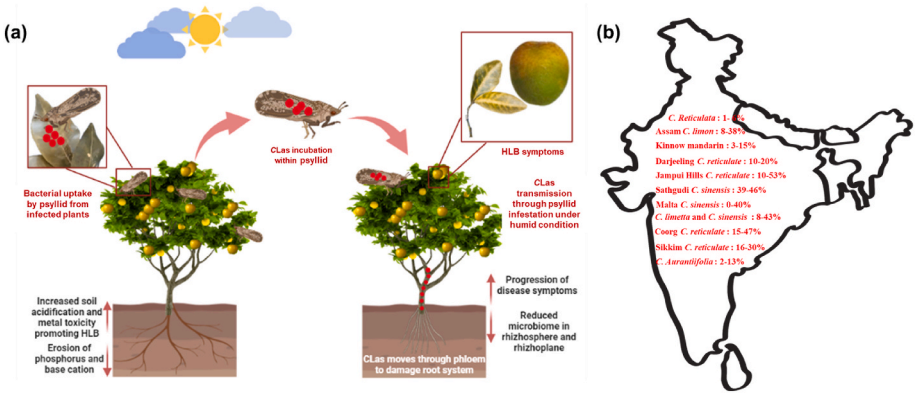

Fig. 1. Schematic illustration of citrus greening disease cycle (Created with Biorender.com) (a) and % incidence of HLB in different citrus species of India [29] (b).

growth medium and have been successfully retained for over two years undergoing sequential subculture. This host-free culture of *C*Las opens up a door to the advancement of its pure culture establishment and shall allow the researcher to analyse pathogen behaviour in response to *in vitro* management strategies [34]. However, the virulence of the established *in vitro* culture of *C*Las has not been examined indicating a gap in the understanding of the pathogenic behaviour of the established *in vitro* culture.

'*Candidatus* Liberibacter' host range includes two types of plants: those that support psyllid vectors and those that allow the bacterial pathogen to multiply. Regardless of rootstock, almost all cultivars of citrus, particularly commercial varieties are susceptible to HLB to some extent. Studies by Tsai et al. [35] stated that the best host of *D. citri* was the grapefruit when compared to *M. paniculata* (L.), *C. jambhiri* L. (Rough lemon), Jack (orange jasmine), and *C. aurantium* L. (sour orange). Based on the degree of disease severity for different strains of *C*Las, the common citrus cultivars are arranged into the following three groups: Severe: *Citrus sinensis*, *Citrus tangelo*, *Citrus reticulata*, Moderate: *Citrus paradisi*, *Citrus limon*, *Citrus aurantium*, Tolerant: *Citrus aurantiifolia*, *Citrus maxima*, *Poncirus trifoliata*. Because the majority of the findings on the reactions of citrus genotypes to HLB are based on field observations at various geographic places and times, the conclusions drawn about the resistance of different cultivars of citrus to HLB are not universal.

Studies into alternative hosts of HLB have established that *Murraya paniculata* is the favoured alternate ACP host [36]. revealed that symptoms such as leaf yellowing, dieback on branches, and defoliation, and appear in inoculated *M. paniculata* plants upon infection with either *C*Las or *C*Lam [37]. further confirmed this phenomenon and reported that in Florida *M. paniculata* was infected with *C*Las and opined *M. paniculata* as a source of infection for *C*Las as it serves as a suitable host for at least 2 months, during which it gets transmitted to sweet orange trees. However, *M. paniculata* can serve as a connecting host only if citrus is present during aparticular period. This is mainly due to the exceptionally low population of bacteria after 5 months in *M. paniculata*. Studies on potential hosts outside the Rutaceae family have shown that dodder (*Cuscuta* spp) can transmit all three citrus liberibacters to periwinkle plants inducing marked yellowing. Dodder was used to spread HLB-associated bacteria to citrus [38], in the Apocynaceae family [*Catharanthus roseus* (L.) G. Don] [39], and different solanaceous plants [40]. This indicates the wide physiological host range for *C*Las [41]. reported the non-Rutaceae plant *Pithecellobium lucidum* Benth as an opportunistic host of HLB. They showed symptomatic yellow shoots, in a severely infected citrus orchard in Fujian, China, and concluded that a low *C*Las population and a lack of psyllid propagation were present in the new host plant.

The bacteria '*Candidatus* Liberibacter' are acquired and spread throughout the psyllid's lifecycle, particularly during the nymphal stages. Eggs are placed on fresh leaves and hatch in 2–4 days. *D. citri* has five nymphal instars that take 11–15 days to mature. The psyllids feed and oviposit on leaves (flush) and young twigs, and the injection of *C*Las by ACP is frequently associated with the activities of adults feeding on the host plant's phloem sap [42]. Salivary glands and hemolymph of ACP have been observed to contain *C*Las which multiplies within its vector [43]. The entire life cycle of *C*Las takes around 15–47 days subject to temperature variation, while adults can live for several months and in a lifetime females deposits up to 800 eggs. In an orchard, diseased trees are clustered together, with secondary infections occurring 25–50 m away. Infectious adults inject saliva into flush tissues, which transmits the disease to previously uninfected trees. Also, the grafting of diseased plant materials can spread the disease [44].

3. Epidemiology and geographical occurrence

*C*Las is more heat resistant and can endure a temperature range of 30–35 °C, whereas ACP prefers temperatures of 25–28 °C. *C*Laf is heat and dry weather-sensitive, found at elevations greater than 700 m and thriving at temperatures between 20 and 25 °C, whilst *C*Lam is found to be heat tolerant and grow at comparatively higher temperatures [45]. Infections of *C*Las and *C*Lam are more severe than *C*Laf leading to tree death [46]. Several fields and greenhouse studies conducted in various regions producing citruses such as California, Florida, Israel, and some other Mediterranean coasts have shown a strong correlation between soil pH and HLB occurrence [47]. The presence of bicarbonates in the irrigation water tends to increase the soil pH in these areas under the micro-sprinkler or dripper causing adverse effects on the functioning of a root, expressing yellow shoots, premature fruit, die-back, reduced fruit yield, and leaf drop in citrus affected with HLB [48]. HLB-affected plants were reported with better performance if irrigated with moderately acidic water and the soil pH was near 6.0 [49].

HLB is widely recognized as one of the major threats to citrus production and related industries around the world. According to recent data, the occurrence of HLB caused 72.2% reduction of orange production in the United States (from 7.98 to 2.22 billion tons) for processing over a period of 10 years (2007–08 to 2017–18). Also, a 20.5% reduction (2.10–1.70 billion tons) was estimated in the fresh fruit market during the same time interval. This resulted in a 3.2 times increase in the price of a box of orange from $2.89 to $9.34 in the United States [50]. In India, HLB disease is considered one of the significant causes of citrus decline. A survey on Khasi mandarin for citrus greening disease in the Northeastern States of India by indexing on *Citrus limetta* and *Citrus sinensis* showed the presence of the disease in almost all the orchards, except at Digaru, Assam [51]. Subsequent surveys conducted in 16 states of India for the period 2007 to 2012 confirmed its distribution in all surveyed states except Arunachal Pradesh [52]. *D. citri*, was similarly present in all areas except Digaru. In a preliminary survey in the northern districts of Tripura, orchards of *C. reticulata* and *C. macroptera* had 16–20% HLB-affected trees, validating HLB as a major causal factor and decline of citrus cultivation in Tripura [53]. HLB-affected Kinnow mandarin trees have shown reduced yield and fruit size in Hoshiarpur, Punjab. Additionally in Nagpur HLB has been observed in epidemic proportions in Nagpur orange and Kinnow mandarin orchards.

4. Disease severity

4.1. "Huanglongbing" and nutrient deficiencies

Since the symptomatic appearance of HLB-affected leaves includes chlorotic patterns, yellowing, and vein corking, nutrient deficits are frequently mistaken for HLB signs (Fig. 2 a-d). Zinc and boron deficiencies produce symptoms like chlorotic islands and vein corking respectively which are vital to separate from HLB symptoms for the appropriate use of fertilizer preparation. HLB symptoms on the leaf blade include chlorotic blotchy mottles, produced due to chloroplast disruption owing to the accumulation of starch in the leaf [54]. suggested a simple and crude method of differentiating nutrient deficiency from HLB by employing a test known as the "pen test" (Fig. 2 e).

4.2. Effect of HLB on phloem aberration

The *C*Las infection leads to the appearance of necrotic phloem tissue, owing to the deposition of starch and callose. Starch accumulation obstructs the passage of photoassimilates, causing symptomatic signatures like blotchy-mottle leaves and yellow shoots. In a phloem aberration study carried out with tolerant and susceptible variety phloem disruption was considerably low together with greater phloem regeneration, which contributed to HLB tolerance in Bearss lemon and LB8-9 Sugar Belle® mandarin, as compared with HLB-sensitive Valencia [55].

4.3. Nutrient depletion resulting in metabolic imbalances

Functional analysis of the *C*Las genome depicts that *C*Las can digest

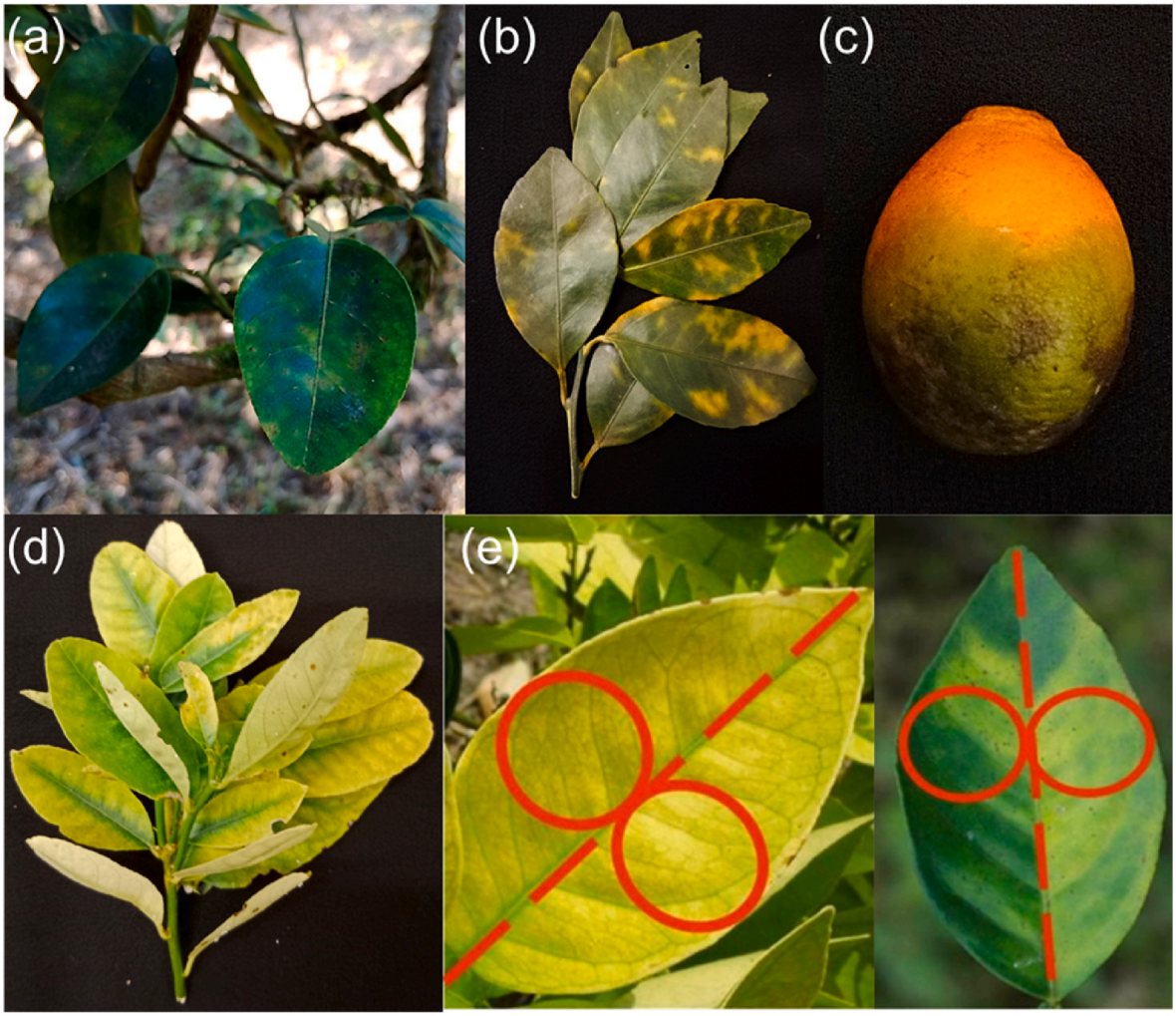

Fig. 2. HLB disease symptoms in leaf (a, b), in fruit (c), Zn deficiency symptoms in citrus leaf (d) and analysis of pen test [54] in citrus leaf (e).

sugars like glucose, fructose, and xylulose but not galactose, mannose, cellulose, or rhamnose [56]. noticed that there is a significant glucose accumulation as opposed to fructose, suggesting that *C*Las might favourably utilize fructose thereby indicating that reduced fructose concentration post-infection is coupled with an increased glucose accumulation in the infected host tissues. According to Wang et al. [57]; glucose buildup favors the suppression of photosynthesis-related enzymes, which contributes to the advancement of HLB symptoms. *C*Las expresses only one sugar transmitter for glucose/galactose [58], and it is still unknown how fructose is imported by *C*Las from its host, thus this idea needs to be validated further. The de novo genome analysis of *C*Las provided a basis for the amino acid biosynthetic pathways which have shown that they can produce glycine, serine, lysine, cysteine, glutamate, threonine, aspartate, and arginine but are incapable of producing phenylalanine, histidine, thiamine, tyrosine, tryptophan, isoleucine, asparagine, alanine, methionine, leucine, valine, and proline. These insufficiencies in the biosynthesis of amino acids might be defied by the bacterium by importing amino acids exogenously [57]. As a result, the genome of *C*Las encodes a collection of generic L-amino acid permease proteins capable of transporting into the cell a wide range of amino acids. In addition, the genome encodes a gene for branched-chain proton-glutamate transporter that can import both aspartate and glutamate. Also, *C*Las has a thiamine ABC transporter which is absent in *L. crescens*, the only culturable *Liberibacter* species, possibly to counteract the inability to manufacture thiamine [59]. Li et al. [60] investigated all genes related to ABC transporter-protein in the *C*Las genome and discovered 14 numbers of ABC transporter systems as well as 7 non-transporting ABC proteins. Sequence analysis and functional annotation have shown that the bacterium uses these ABC transporters to import metabolites (amino acids and phosphates) and enzyme co-factors (choline, manganese, thiamine, iron, and zinc); resist organic solvents, lipid-like drugs, and heavy metals; secrete virulence factors and maintain the outer membrane composition. These transporter proteins play an important function in supplying nutrients to *C*Las and causing a metabolic imbalance in citrus. It has been reported that the *C*Las genome encodes one gene for the zinc transport system (*znuABC*) [58] which was further explained by Vahling-Armstrong et al. [61] to be responsible for the uptake of zinc thereby contributing to zinc deficiency in HLB-infected trees [59]. *C*Las genome has also been shown to encode an ATP/ADP translocase enzyme, which, like its ATP synthase, is used to obtain energy directly from its host [62].

4.4. *Effect of HLB on plant hormone balance*

The pathogen infection in plants is known to be affected by Phytohormones. In a study by Rosales and Burns, [63] in Valencia *C. sinensis* (L.) Osbeck trees, it was shown that symptomatic and asymptomatic fruits harvested 7 and 12 months post full bloom generate considerably lower concentrations of ethylene than healthy fruits. The analysis of flavedo from the middle section, stem end, or stylar end of fruit showed that the abscisic acid (ABA) and indole-3-acetic acid (IAA) were higher in symptomatic fruits than in fruits harvested from asymptomatic and

healthy trees. The results suggest that a lower concentration of IAA in the stem end of asymptomatic fruits could lead to accelerated abscission, despite lower production of ethylene in the whole fruit [63]. Tang and Vashisth, [64] compared the expression analysis of genes in 'Hamlin' sweet orange which is HLB-susceptible and HLB-tolerant 'Minneola' tangelo, 'LB8-9' mandarin, and 'Dancy' tangerine. They revealed that HLB increases mature fruit abscission in susceptible 'Hamlin' sweet orange than 'LB8-9' mandarin due to the upregulation and downregulation of differentially expressed genes (DEGs) between the cultivars. 368 DEGs were found wherein 310 were in the abscission zone of Hamlin orange and 58 in mandarin fruit 'LB8-9'. DEGs associated with cell wall loosening were found to be upregulated in Hamlin orange thereby indicating the progression of abscission induced in the abscission zone of the citrus. DEGs linked with the metabolism and signal transduction pathways of hormones *viz.* auxin, cytokinin, ethylene, abscisic acid, and gibberellic acid, suggest that HLB possibly brings about changes in inherent hormone balance that induce fruit abscission.

4.5. Effect of HLB on biochemical properties of citrus fruit

Research into the impact of greening on citrus peel oils seems to indicate that this disease harms essential oil production. Xu et al. [65] carried out studies on volatiles secreted from cold-pressed orange oils with and without HLB symptoms from Valencia and Hamlin, Florida. The study revealed that oils derived from fruit with HLB symptoms had reduced concentrations of decanal, linalool, geranial, neral, carvone, 2-decenal, and dodecanal, and higher citronellal concentrations when compared to asymptomatic fruit. These findings may be attributed to the disruption in the biochemical pathways forming orange oils which may have caused these volatiles to alter concentration based on the severity of HLB infection. Furthermore, in an extensive study conducted in HLB-affected sweet orange trees in Florida, it was reported that "Valencia" orange trees with HLB symptoms had lower concentrations of conventional peel oil components, including octanal, valencene, decanal, and were abundant in oxidative/dehydrogenated terpenes, such as limonene oxide and carvone [66]. This further substantiates the notion that HLB disrupts certain biochemical pathways responsible for the formation of essential oils.

4.6. Effect of HLB on water uptake

Scheduling of irrigation is a significant element in HLB-affected trees since HLB-affected citrus trees have been observed to lose up to 80% of their root mass based on the severity of the disease, potentially affecting the uptake of water and nutrient. HLB-affected trees experience premature fruit drop due to slight water stress, the canopy size is reduced and there is a significant reduction in the number and their size. Furthermore, the growth of leaves, shoots, and roots is slowed. A study carried out in a Florida citrus grove (2011–2015), reported consumption of ~22%–25% more water by healthy trees than by HLB-affected trees, which is probably due to better leaf area and root density [67]. Since diseased trees produced lower leaf area index and canopy density, they took up minimal water and so fewer nutrients from the soil. However daily irrigation of affected trees significantly improves their water uptake as compared with infrequent irrigation [68,69].

5. Disease detection

Different disease detection techniques have emerged such as biological indexing, microscopic and spectroscopic imaging techniques, biochemical characterization, hybridization assays, and most predominantly amplification techniques (Table 1). In a recent study, laser-induced breakdown spectroscopy (LIBS) has been displayed as a favourable alternative in identifying *C*Las-infected citrus plants. Without sample pretreatment, LIBS spectra and pictures were acquired by focusing a laser on both infected and healthy fruit surfaces. The

Table 1
Different diagnostic techniques for HLB disease.

Detection system	Properties		References
Symptomology	Tree symptoms	Yellow shoots, twig dieback, defoliation, fruit drop, blotchy mottle leaves	[83]
	Leaf symptoms	Asymmetric blotchy leaf mottle (a characteristic symptom of HLB)	[83]
	Fruit symptoms	Fruit color inversion (orange color peduncular end and green stylar end) Reduced fruit size with bitter taste with low soluble acids.	[84]
	Root symptoms	30–50% loss of trees' fibrous roots. Roots are affected long before the appearance of disease symptoms in leaves	[85]
Biological indexing		Use of indicator plants Most easy and cheapest	[86]
Microscopic and Biochemical Techniques	Microscopy	A reliable technique for the initial diagnosis of the presence of *C*Las. Visualizing salivary sheaths at feeding sites using epifluorescence and confocal microscopy	[22,87]
	Biochemical techniques	Uses a fluorescent chromatograph to detect gentiosyl-glucoside from diseased tissues from fruits and bark. This method was non-specific and a definitive diagnosis was not possible when used alone	[88]
	Volatile organic compounds (VOCs)	Chemical and fluorescence image analysis of VOCs and secondary metabolites released from diseased trees, can be used for disease diagnosis	[80]
Serological and DNA hybridization Assay	DNA Hybridization	Probe hybridization with In-2.6 for *C*Las and AS-1.7 for *C*Laf.	[89,90]
	Fluorescence in-situ hybridization (FISH)	A *C*Las-specific probe is used to identify the presence of *C*Las bacterial cells. Uses LSS primer for 16S rRNA gene of *C*Las as a probe with fluorescein-conjugated anti-probe antibody	[84]
Amplification techniques	Traditional PCR	Qualitative detection can be used to detect a low concentration of pathogens.	[22,91,92]
	Real-time PCR	Quantitative detection, sensitive, specific, and reproducible results.	[1]
	Ultrasensitive detection system	Two-step PCR using the specific-species-specific TaqMan outer and inner primer.	[71]
	Loop-mediated isothermal amplification (LAMP)	Used for detection of amplification with dye instead of gel-electrophoresis. Also associated with other techniques such as RPA, LFA	[72,81,93]

(*continued on next page*)

Table 1 (*continued*)

Detection system	Properties		References
	DNA microarray	Transcriptional profiles are compared between healthy and diseased plants.	[94]
	Direct sensitive PCR	Bacterial cells are extracted from the mid rib of the infected plant at feeding sites for highly sensitive direct PCR.	[22,84]
	Droplet Digital PCR (ddPCR)	Sensitive and precise detection of low-concentration DNA in the individual reaction. Target DNA positive amplification with droplet shows fluorescence and vice versa	[87,95]
	Recombinase Polymerase Amplification (RPA)	Amplifies a specific DNA target. DNA purifications, costly equipment, or highly trained technicians are not required Advancement has been done to complete the assay within 15 min	[72,96]
Spectroscopic/ Imagi-ng techniques	Visible, Infrared, and thermal imaging techniques	Based on the difference in the average reflectance between infected and healthy plant at infrared wavelengths 560 and 710 nm.	[97,98]
	Polarized Imaging Technique	Uses time-lapse images of citrus leaves to follow the hyper-accumulation of starch in leaves by highlighting the regions in citrus leaf samples with an increase in average grey value as an indication of starch accumulation in the pre-symptomatic stage.	[99]

soluble solids content and pH value of the juice were used as acidity and sugar indicators, while the level of Ca, Zn, and K in the peel and pulp were investigated. Statistical analysis revealed a significant difference between the healthy and diseased fruit samples [70]. Nucleic acid-based detection technologies are routinely used strategies for *CLas* detection [71,72,73]. A study has been reported on the advancement of Cas12a-based DNA endonuclease-targeted CRISPR trans reporter (DETECTR) assay for precise and sensitive detection of *CLas* from diseased samples. The DETECTR assay targeting the five-copy nrdB gene, couples Cas12a transcleavage associated fluorescent reporter oligonuceotide with isothermal amplification which was capable of specific detection of *CLas* presence in citrus leaves at the attomolar level [74]. These methods omit the conventional amplification step and thereby hold promise in facilitating rapid and sensitive in-field detection of HLB. In the context of rapid and field-deployable detection of HLB, a handheld portable RAMAN system coupled with chemometric analysis was used to detect HLB infection. The diagnostic was based on unique chemical spectra for infected leaves and the accuracy of identification was 100% for grapefruits and 94% for oranges [75]. In recent times mass spectroscopic imaging technique has been employed in the detection of HLB in leaf samples based on the increased concentration of metabolites such as quinic acid, sucrose, nobiletin, and phenylalanine. In the study, metabolites abieta-8,11,13-trien-18-oic acid, and 4-acetyl-1-methylcyclohexane showed a higher distribution in diseased leaves. Also, mass spectroscopy has been coupled with desorption electrospray ionization for rapid and specific detection of HLB biomarkers in sweet oranges [76]. Recently, a methodology has been developed using unmanned aerial vehicles (UAVs) with RGB cameras to quantify the health of citrus trees. Disease detection was carried out using true color (red/-green/blue) visible-near infrared and thermal infrared reflectance imaging-based calculation of triangular greenness index (TGI) wherein changes in spectral reflectance were correlated to the overall health of the tree [77]. Concerning HLB detection based on biomarker, a study has reported a subtle and selective label-free biosensor combining single-walled carbon nanotubes (SWNTs) field-effect transistor (FET)/chemiresistor with selective antibodies for detection of Sec-delivered effector 1 (SDE1), a secreted protein biomarker in HLB. The study reported that the chemiresistive biosensor could detect SDE1 biomarkers in tissue extract of citrus greening samples up to 5 nM concentration [78]. Although most of the studies on HLB detection uses citrus leaf and fruit for the presence of *C*Las, a study has been reported on fibrous root tissue of citrus as alternate source material for the evaluation of HLB infection. Here, quantitative PCR carried out with primer-probe set TXCChlb has been established based on 16S rDNA of the pathogen *C*Las. In the study, disease detection before the emergence of symptoms has been carried out using fibrous root tissue to facilitate early detection and prevent the spread of disease [79]. It has been noted that the decline of fibrous roots occurs in citrus trees affected with *C*Las owing to the evenly distributed presence of the pathogen within the roots. As such diagnosis of HLB using root samples mediated the early detection of *C*Las in HLB-affected trees [1]. In a different study reflectance and fluorescence images with photosynthesis and secondary metabolites were implied for the rapid detection of HLB disease. Here, a handheld device has been developed that captures the images to infer physiological and structural information. For high throughput detection of HLB, the study was carried out in combination with deep learning technologies [80]. In the context of rapid detection of HLB, the loop-mediated isothermal amplification (LAMP) method has been proven to be a reliable, highly sensitive, and specific detection method. In a study, the LAMP method was found to be 10-fold higher in sensitivity towards detection of *C*Las at 62 °C of amplification temperature as compared to conventional PCR [81]. In continuation, the recombinase polymerase-based LAMP method has been combined with lateral flow assay (HLB-RPA-LFA) for the detection of *C*Las. The developed HLB-RPA-LFA diagnostic tool was able to identify the presence of *C*Las at an isothermal temperature of 38 °C for 20–30 min in *Citrus reticulata*, *Citrus sinensis* and *Citrus aurantiifolia* [72]. In a recent study 10 canines have been trained and used for the detection of *C*Las with 0.9905 accuracies, 0.9961 specificities, and 0.8579 sensitivity. The identification was possible before the visual detection of *C*Las within two weeks post-infection [82]. Targeted early detection strategies have been developed by visualizing salivary sheaths of ACP at feeding sites using Coomassie brilliant blue stain followed by PCR assays for the detection of *C*Las in surrounding areas of feeding sites [22]. As can be seen, the most widely used reliable approach for the detection of HLB involves laboratory-based PCR methods, which are both expensive and time-consuming. Similarly, the numerous advanced technologies addressed in recent studies necessitate laboratory-based equipment. It is also worth noting that all of these detection devices can detect HLB at a late stage, when the infection has progressed to an alarming level of severity and has spread throughout the orchard. As such, there is a need to develop an early detection tool for HLB diagnosis that is both rapid and cost-effective.

6. Prevention and control

6.1. Cultural method

One of the efficient ways to control HLB is to restrict its spread and that of its insect vectors by intercropping citrus with a non-host crop like

guava. To decrease the disease's impact removal of infected branches, trees, and the use of liberibacter-free planting material are encouraged along with aggressive control of the vector psyllid. In China, HLB was successfully controlled by promoting the use of healthy saplings, systematic planting and early detection and exclusion of diseased plants from infected orchards [100]. Vuuren et al. (2000) noted that isolates of the Citrus Tristeza virus could also play a role in protecting trees from HLB infection. In the search for the use of repellent plants as a cultural control method, intercropping has since been a means to fulfil this end. In the Mekong Delta of Vietnam, growers have discovered that citrus intercropped with guava (*Psidium guajava*) survived several years longer. Citrus/guava interplanting has shown significant initial reductions in disease rates when compared to citrus monocultures, according to research conducted in Indonesia. This was thought to be due to volatiles released by the guava, which repelled citrus psyllids [101]. Yan et al. [102] showed that *Gynura bicolor* and *Capsicum annuum* could potentially be used as interplants in citrus orchards. The use of the tree species, *Camptotheca acuminate*, the shrubs, *Mimosa bimucronata* and *Lantana camara* as a landscape barrier which may provide a more environmentally sound and cost-effective alternative to manage HLB provided further research and investigation are carried out to ascertain the findings.

6.2. Chemical control

In earlier times the use of tetracycline antibiotics mediated the control of HLB disease to a great extent when applied by trunk injection [103]. However, because tetracycline is bacteriostatic rather than bactericidal, it must be injected repeatedly, resulting in phytotoxicity [104]. As such the resultant phytotoxicity and the involved labor cost led to a drop in its usage in recent years. Contrary to this Hussain et al. [105], studied the effect of different antibiotic combinations (Rifampicin + Ampicillin sodium, Ampicillin sodium + Cefalexin, Cefalexin + Rifampicin, Cefalexin + Ampicillin sodium + Rifampicin) in 10-year-old Kinnow mandarin orchard with HLB infection located at Multan, Pakistan. The antibiotics were applied in February before flowering, in April during the fruit set, and in June at the fruit growth stage. An increase in fruit weight and yield by five times was observed with the application of antibiotics, along with an improvement in the ripening index, juice content, total sugars, total soluble solids, and phenolic and vitamin C content. It was further reported that antimicrobials such as benzbromarone and tolfenamic acid were effective against *C*Las in commercial orchards of *Citrus paradise* and *Citrus sinensis*. These treatments induced the expression of genes linked with metabolism and growth, without negatively affecting the indigenous microbiota of citrus and did not compromise the tree viability [106]. Therefore, the results indicate a link between treatment-related reductions in *C*Las and the expansion of numerous key taxa linked with citrus health [106]. Ghosh et al. [72] evaluated the antibacterial efficacy of two antimicrobial agents, 2S albumin (a plant-based protein) and Nano-Zinc Oxide along with their combinations against *C*Las in HLB-affected *Citrus sinensis* plants. Accordingly, they demonstrated that trunk injections of the combination of 2S albumin-Nano-ZnO formulation might potentially be established as a novel anti-*C*Las treatment to lower the *C*Las population and hence mitigate the severity of HLB in citrus.

Broad-spectrum insecticides have been shown to lower ACP numbers, particularly during tree dormancy while targeting overwintering adult populations [107]. In Florida, the use of systemic insecticides neonicotinoid, thiamethoxam, imidacloprid, and clothianidin, in addition to cyantraniliprole, are permitted to be used to aid in citrus production barring some restrictions on their use on young trees [108, 109]. Although the fact that the use of insecticides has an impact on citrus fruit yield, their intensive use has led to the development of resistance in ACP populations which may lead to the emergence of secondary pest outbreaks [110]. With regard to this phenomenon [111], reported field evidence of moderate to high levels of resistance by psyllid populations against thiamethoxam. A recent study recommended that frequent application of neem products, soft and organic foliar insecticides like horticultural spray oil, kaolin clay, neem oil, and insecticidal soap every 7–14 days when psyllids are observed can help in reducing the spread of the disease [112]. As such, economic thresholds for the application of insecticides are of utmost importance in Integrated Pest Management practice to diminish the possibility of the development of resistance and secondary pest outbreak.

6.3. Biological control

Research into biological control saw the successful use of two psyllid parasitoids in Reunion Island *viz*., *Tamarixia radiata* and *Diaphorencyrtus aligarhensis* [113]. This has led to a reduction of Asian psyllids populations on the island. The use of these parasitoids along with the disease-free saplings and the elimination and destruction of Liberibacter-infected trees has significantly reduced huanglongbing on the island. In a new study, a noble class of heat-stable antimicrobial peptides (SAMPs) has been derived from *Microcitrus australiasica* which has been found to suppress *C*Las growth in HLB-positive trees and engage host immunity against future infections from *C*Las under controlled conditions in the green house. It was found that the α-helix-2 domain of SAMPs was most effective in killing α-proteobacteria via prompt leakage in cytosol resulting in cell lysis. When administered as a foliar spray, SAMPs are taken up by citrus trees, stay within, and then move progressively towards the *C*Las' location [114]. Strain EB92-1 of *Xylella fastidiosa* was able to colonize citrus and reduce the severity of disease in HLB-infected trees over three years [115]. A study has shown the potential use of gibberellin, uric acid, and rutin in citrus trees to upregulate H_2O_2-scavenging enzymes thereby reducing H_2O_2 concentrations and cell death when infected with *C*Las [116].

6.4. Thermotherapy

Citrus seedlings are treated to continuous heat (40 °C–42 °C) for a minimum of 48 h in a thermotherapy treatment before plantation, which greatly reduces or eliminates Las in HLB-infected nursery plants [117]. According to Ehsani et al. [118]; thermal treatment could effectively reduce the disease symptoms of citrus trees groves. Thermotherapy, however, had a key flaw as high-temperature treatment is known to effectively reduce the pathogen load above ground level, leaving the temperature in the roots as such due to the soil's buffering capacity. Such roots then acted as a source for canopy re-infection [119] which has been the main reason why the efficacy of thermotherapy in lowering *C*Las populations in roots needs to be improved before it can be employed as a treatment in integrated HLB control. Although, under controlled greenhouse conditions, *C*Las titers were significantly reduced with 45 and 48 °C treatments in seedlings affected with HLB four weeks after treatment [120], studies have indicated that in-field thermotherapy is not an efficient method for the control of HLB during commercial production of citrus. After thermotherapy, a molecular and biochemical examination of citrus trees revealed that robust growth is an artefact of defoliation that is mistaken for disease healing [121].

6.5. Balanced nutrition application

HLB symptoms are minimized by foliar micronutrient treatments, particularly Mg, Zn, and Mn [122]. However, the efficacy of these treatments has been questioned due to mixed results and significant discrepancies in administration rates, and unavailability of the standard rate of applications of these macro- and micronutrients. Shen et al. [123] reported that an improved nutrient program was found to reduce the *C*Las population while also having a positive impact on leaf size, fruit quality, and yield which showed that foliar nutrient application promotes productivity and improves the overall health of Citrus. Morgan et al. [124]; studied a 5-year-long foliar application program consisting

of Zn, B, and Mn on Valencia trees, 5–7-year-old, grafted on single rootstock to determine the outcome of enhanced leaf nutrient status and yield volume. The nutrients, Zn, B, and Mn were applied in March, May, and September at three rates, three times per year following flushes. It was found that Mn and Zn application at higher rates caused the greatest increase of those nutrients in foliar parts. However, their concentration decreased right after application, as noted by the fact that there was no difference found compared to Mn and Zn concentration between the treated trees and controls before the subsequent application. Interestingly, an increased application of Mn and Zn showed a marked increase in canopy volume. This suggests that as compared to healthy trees, the HLB-affected trees have smaller and weaker root systems thus warrant frequent and small doses of fertilizer. Fertigation and controlled-release of fertilizer can be alternative strategies to applying conventional dry granular fertilizer many times. Similarly, micronutrients should be applied at slightly higher doses than the standard rate. Before making any adjustments to a fertilizer program, it is normative to perform leaf and soil nutrient analysis to ensure a successful nutrient management regime [54].

6.6. Genetic manipulation in citrus for resistance

Over expression of pathogenesis-related gene 1 (*NPR1*) and antimicrobial proteins such as plant defensins and thionins, or antimicrobial peptides like cecropin B are some of the main approaches in genetic manipulation for resistance to HLB [125,126]. The US Environmental Protection Agency has approved the development and field testing of HLB-resistant transgenic citrus varieties that expresses spinach defensins developed by Erik Mirkov (Texas A&M University). However, constraints regarding acceptance by the consumer of transgenic varieties have prevented the commercialization of the product. To develop resistant crops, targeted genome-editing technologies based on transcription activator-like effector nucleases, zinc finger nucleases, or CRISPR/Cas9–single guide RNA (sgRNA) could be investigated. *CsLOB1*, a disease susceptible gene that favours bacterial canker disease in Duncan grapefruit has been manipulated to develop disease-resistant plants using Cas9-sgRNA [127–129]. *CsLOB1* belongs to the plant transcription factor gene family Lateral Organ Boundaries Domain (LBD) involves in plant growth regulation. Mutation of *CsLOB1* mediated the development of disease-resistant plant with no adverse effect on their phenotype. Similar research in the above techniques could potentially lead to breakthroughs in citrus greening control. Gene *NPR1* acts as a key regulator of the transcription factors controlling signal transduction pathway leading to induced PR gene expression and systemic acquired resistance (SAR). It has been reported that the induced expression of *Arabidopsis thaliana NPR1*, the broad-spectrum disease-resistance gene in transgenic citrus lines led to effective resistance against HLB [125]. found that some transgenic Valencia and Hamlin orange exhibiting over-expression of the *AtNPR1* gene under a constitutive CaMV35S promoter and *AtSUC2* phloem-specific promoter resulted in reduced severity of disease, and also a few lines were found to be disease-free. According to Robertson et al. [129]; high levels of *AtNPR1* expression in citrus can give HLB tolerance under high disease pressure in the greenhouse, which is also in line with findings by Wang et al. [130]; that showed the HLB-tolerant genotypes had higher expression levels of four *AtNPR1*-like genes as compared to the HLB-susceptible genotype after HLB infection. Recently, the *NPR1* gene from HLB-tolerant *Citrus paradisi* Macf. CiNPR4, was introduced into *Citrus sinensis* Obseck and the transgenic variety was found with a significant reduction in *C*Las titer levels. The tolerance was likely to be mediated by the salicylic acid and jasmonic acid signalling pathways [131]. All of these results suggest that defence-signalling pathway mediated by the *AtNPR1*-like gene may play a role in HLB tolerance [132]. reported the induced expression of attacin A (*attA*) in sweet orange Hamlin and Pera for HLB resistance. The attacins are secreted in the hemolymph of Trichoplusia ni (Hübner) (Lepidoptera: Noctuidae), upon infection with bacteria. It binds with lipopolysaccharides of Gram-negative bacterial membrane and alters membrane structure and permeability. The *attA*-modified citrus plants dramatically reduced the symptoms of HLB as compared to the non-transgenic cultivar of these plants. Agrobacterium-mediated introduction of a modified antimicrobial protein thionin in citrus, demonstrated increased resistance to HLB and citrus canker [133]. In *Citrus sinensis* Osbeck phloem specific expression of a gene for cationic lytic peptide cecropin B with *GRP1.8*, the phloem-specific promoter from the French bean, minimizing the effect of HLB [134]. This antimicrobial peptide, Cecropin B, has been isolated from the Chinese tasar moth *Antheraea pernyi*. Antimicrobial peptide AMP sarcotoxin IA (*stx IA*) gene isolated from the flesh fly *Sarcophaga peregrine* was introduced into Pera sweet orange plants and the transgenic citrus line STX-11 was found to be less susceptible against *C*Las while maintaining fruit and juice quality [135]. It has been reported that polyploid citrus species show specific physiological and anatomical characteristics that are associated with increased tolerance to biotic and abiotic stress [136]. In a recent study, the traits of the triploid Persian lime *Citrus latifolia*, one of the least susceptible citrus species were compared to diploid Mexican lime *Citrus aurantiifolia* when infected with HLB. The leaf petiole analyzed using scanning electron microscopy (SEM) was found with callose deposition and increased starch contents with enhanced rate of photosynthesis, transpiration, and stomatal conductance in Persian lime as compared to Mexican lime [137].

Since *Candidatus* Liberibacter spp. proliferates throughout phloem tissue, the use of antimicrobials as foliar sprays is the most convenient approach for managing HLB. However, these antimicrobials need to enter the vascular system of infected plants to have an inhibitory effect on the pathogen. This calls for caution in the use of antimicrobial dosages, as greater levels can have a phytotoxic effect on the host. Furthermore, because the pathogen is known to translocate to the roots, the antimicrobials utilized must also be delivered to the root system to prevent HLB infection. Also, the use of antibiotics may adversely impact the soil microbiota which is critical for the managing of healthy citrus trees. As discussed, HLB is often carried out after the symptomatic detection of infection such as blotchy mottle leaves, scattered green spots, and chlorosis. However, the latent period between the inoculation of the pathogen and the onset of symptoms is the most crucial time to manage HLB infestation in the orchard. In this context, the development of a field-applicable point-of-care diagnostic kit shall undeniably facilitate the rapid and precise detection of the pathogen in this latent phase. Also, the best efficacy of prescribed antimicrobials can be achieved if the presence of pathogens can be recognized during this latent phase. Additionally, the inherent issues with the use of antimicrobials can be addressed by detecting the disease at an early stage, when there is less pathogen inoculum, and managing it before it becomes endemic. The different control strategies adopted against HLB are summarized in Fig. 3.

7. Conclusion

Although it has been more than a century since the disease was first described, there has been a substantial gap in the early detection of HLB infection to curtail it and stop it from spreading. The lacuna in field-based disease detection systems augmented the spread of the disease. In this context, the development of rapid and field-based diagnostic tools would ease disease detection at the initial level of infection and the identification of disease-free saplings to mitigate HLB disease. Moreover, since the availability of *C*Las in roots before symptomatic visualization of HLB infection has been confirmed, protecting plants in the root system with the appropriate antagonist would undeniably be a crucial management approach for the devastating disease.

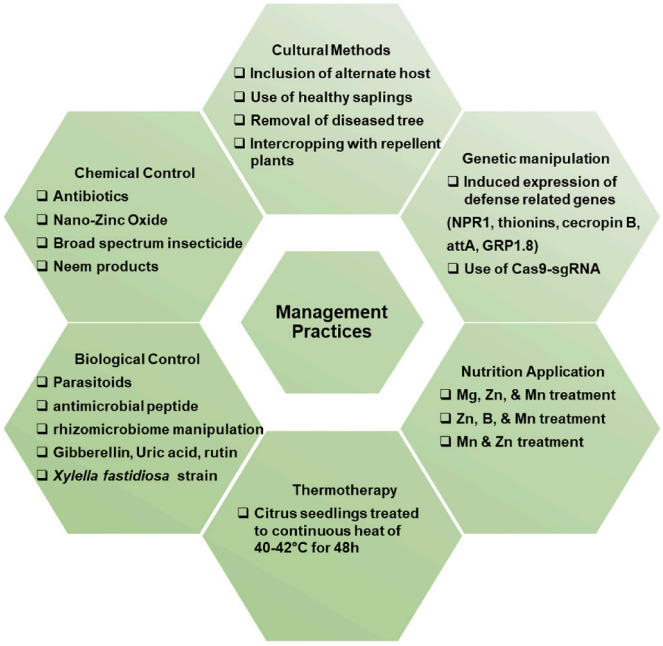

Fig. 3. Schematic representation of control strategies of Huanglongbing disease.

Authors' contributions

DT, conceptualized, designed the framework, wrote and proof read the manuscript. CC, designed the framework, wrote the manuscript. SS and PU, contributed in literature survey and compilation. PD, SBS, SH, LS, LLK, and DS, proof read the manuscript and provided critical feedback that helped shape the manuscript. All authors read and approved the final version of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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