A Review of the ‘*Candidatus Liberibacter africanus*’ Citrus Pathosystem in Africa

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ABSTRACT

It has been nearly 100 years since citrus growers in two distinct regions in the northern provinces of South Africa noticed unusual symptoms in their citrus trees, causing significant crop losses. They had no idea that these symptoms would later become part of an almost global pandemic of a disease called greening or huanglongbing (HLB). The rapid spread of the disease indicated that it might be caused by a transmissible pathogen, but it took >50 years to identify the causative agent as ‘*Candidatus Liberibacter africanus*’. Recently, the disease appeared in more African countries, spreading by both infected planting material and *Trioza erytreae*. To date, five ‘*Ca. L. africanus*’ subspecies have been identified in various rutaceous species, with ‘*Ca. L. africanus* subsp. *clausenae*’ the only subspecies for which a biovar was detected in citrus. Efforts to detect and differentiate HLB-causing *Liberibacter* species are ongoing, and recent developments are discussed here. This review focuses on aspects of the African form of HLB, including its specific bacterial species and subspecies, its main insect vector, its geographic distribution, and current management strategies.

Keywords: bacterial pathogens

Bové (2006) described the century-old disease of citrus known as greening or huanglongbing (HLB) as “a major threat to the world citrus industry,” and 15 years later this threat is exacerbated with no indication of declining. Research by many scientists in all affected areas has shown that this is a complex disease, thus far involving three closely related *Candidatus* bacterial species, ‘*Candidatus Liberibacter asiaticus*’ (CLas), ‘*Ca. L. americanus*’ (CLam), and ‘*Ca. L. africanus*’ (CLaf); two primary insect vectors, *Trioza erytreae* (Del Guercio) for CLaf and *Diaphorina citri* (Kuwayama) for CLas and CLam; and all citrus varieties and some related genera, with environmental factors playing important roles. In this review of the African form of HLB, we revisit the historical background and symptoms of the disease and summarize the latest findings regarding the pathogen (CLaf) and its various subspecies, including their geographic distribution and associated vectors. The

progress in detection methods and the control measures applied in the South African context are also presented.

HISTORICAL BACKGROUND

In the late 1920s, citrus growers in the present-day North West Province, South Africa (near Rustenburg), noticed that some of their trees had developed a yellowing or mottling of the leaves followed by dieback; fruits on these branches were greenish, usually on one side, were misshapen, and dropped easily (Van der Merwe and Anderssen 1937). They called the disease “geel tak siekte” (Afrikaans for “yellow branch disease”). In citrus orchards in present-day Mpumalanga Province near Mbombela (Nelspruit), identical symptoms were observed, but growers there called the ailment “greening” after the fruit symptoms. Crop losses in both areas were already apparent. The name “greening” was adopted over time, possibly because of the establishment of a research institute specially for citrus and other subtropical crops in Nelspruit in 1926 (Moll 1976), and the disease received more attention from researchers there because the severity was worse in Mpumalanga in 1932 to 1936 and 1939 to 1946 than in other areas (Oberholzer et al. 1965). Initially, the symptoms were attributed to mineral toxicity or

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deficiency, although applications of zinc had no effect (Van der Merwe and Anderssen 1937). Ironically, unknown to the South African scientists and growers, a disease with identical fruit and leaf symptoms was spreading in China during the same time frame where it was called “huánglóngbīng,” which translates into English as “yellow shoot disease” (Zhao 1981), basically the same name used by Rustenburg farmers. Although an international meeting of scientists in 1993 decided to adopt “huanglongbing” as the official name of the disease (Moreno et al. 1996), “greening” is still widely used by scientists, growers, regulators, and the media, especially in Africa, and is therefore used in this review.

Field observations indicated that a transmissible agent was involved, and Oberholzer et al. (1965) demonstrated graft transmission in the field and the greenhouse, suggesting a possible viral agent. They observed high numbers of the citrus triozid *Trioza erythrae* in infected orchards, and this finding soon led to experimental proof of transmission by this species (McClellan and Oberholzer 1965a).

The last piece of the puzzle was the identification of the pathogen. In 1970 Lafèche and Bové published electron micrographic evidence for the presence of nonwalled cellular organisms, resembling mycoplasma in infected phloem sieve elements (Lafèche and Bové 1970). Similar organisms were observed in *T. erythrae* (Moll and Martin 1973). On closer examination, a thin cell wall was observed, leading Garnier and Bové (1978) to identify the organism as a true bacterium. Because all attempts to isolate the bacterium in pure culture have been unsuccessful, attention was turned to sequence analysis of its 16S rDNA. This placed the bacterium in the alpha division of the Proteobacteriaceae (Jagoueix et al. 1994). Further analyses revealed that the species in Africa and Asia were different; in Asia, the disease is associated with CLAs, whereas the African disease is associated with CLaf (Jagoueix et al. 1996). Although the symptoms in both continents are similar, the disease in Africa is confined to cooler, higher-altitude regions 900 m above sea level (masl), whereas CLAs is more heat tolerant, affecting trees in lower, warmer environments. When the same symptoms were reported from Brazil, a third species, CLam, was discovered, which shares the temperature sensitivity of CLaf (Texeira et al. 2005).

Over time, African greening has been reported in several other central, eastern, and southern African countries and most recently in Nigeria (Ajene et al. 2019, 2020d) and Angola (Fourie et al. 2021). A serious development has been the identification of CLAs in Ethiopia (Saponari et al. 2010) and Kenya (Ajene et al. 2020b) and the presence of *D. citri* in Kenya (Rwomushana et al. 2017), Tanzania (Ajene et al. 2020b), and Nigeria (Oke et al. 2020).

GREENING SYMPTOMS ON CITRUS

CLaf infection can cause a range of symptoms on citrus trees that are influenced by several factors, including the citrus type or cultivar, the season, the age of the tree when it became infected, and environmental factors. Together all these factors influence the degree to which a plant or the fruit is affected. Plants can display any combination of the greening-associated symptoms described here but not necessarily the full complement.

A tree infected early in its life can display symptoms in all parts simultaneously, but if infection occurs later, especially under low vector pressure, symptoms will appear in only some sectors of the tree and spread throughout the tree in the coming seasons.

Leaf symptoms are usually the first to become apparent. Initially, leaves of a normal size develop a yellow color along the veins and asymmetric mottled blotch (Fig. 1A). McClellan and Schwarz (1970) suggested “blotchy-mottle” as a more appropriate name, but this was not adopted. When numerous leaves are symptomatic, the entire branch has an overall yellow appearance (Fig. 1B), hence the initial name “yellow branch” disease. Leaf symptoms are more pronounced

in winter (Schwarz 1968), probably because of the temperature sensitivity of CLaf. Later symptom development appears as smaller leaves, with the same yellow-green patterns, and can be more upright, sometimes referred to as the “rabbit-ear” symptom. In chronically infected trees the tree density decreases (sparsely foliated), and extensive twig dieback can occur (Fig. 1C) (Oberholzer et al. 1965). Infected trees decline over seasons and become unproductive when continuous and premature fruit drop occurs. This loss of vigor can be attributed in part to poor root development (Manicom and Van Vuuren 1990). Tree death, as has been reported for the Asian form (Zhao 1981), has not been reported for African greening.

Fruit from infected trees can exhibit several symptoms. Fruit size and shape can be affected, with diseased trees bearing more small fruits, which are often lopsided with a curved columella (Fig. 1D) and can contain aborted seeds. The vascular bundles in the fruit albedo can also develop a brown discoloration (Fig. 1D). Fruit tends not to color up fully, with the stylar end remaining green, resulting in the color inversion symptom (Fig. 1E). The fruit flavor is also affected, and fruit has a bitter, salty flavor (McClellan and Schwarz 1970). If pressure is applied to the peel, a grayish-white color develops on the rind surface (Oberholzer et al. 1965).

Generally, greening-affected sweet oranges (*Citrus sinensis*) display the color inversion and lopsided fruit symptom with mild to severe leaf symptoms, whereas mandarin (*Citrus reticulata*) types present a severe leaf symptom with small fruit and color inversion. Lemons (*Citrus limon*) do not present leaf symptoms clearly distinguishable from nutrient deficiencies or a clear fruit symptom.

‘CANDIDATUS LIBERIBACTER AFRICANUS’

Apart from divergent natural insect vectors, a primary characteristic differentiating CLaf from CLAs is its comparative heat sensitivity, with optimal symptom expression occurring under cool conditions from 22 to 24°C. Symptoms were observed to diminish when infected trees were exposed to temperatures >27°C for extended periods (Bové et al. 1974). This heat-sensitive characteristic is also correlated with the natural vector of CLaf, *T. erythrae*, which survives only cooler, humid climates mostly found at higher altitudes (Catling 1969).

The search for the origin of CLaf led to the identification of other liberibacters in various rutaceous hosts with nucleic acid sequence identities closest to CLaf. Importantly, CLaf was not identified in any of these host species (Roberts et al. 2015; Roberts and Pietersen 2017). However, ‘*Ca. L. africanus* subsp. *clausenae*’ (CLafCl), originally identified from *Clausena anisata* (horsewood) in South Africa (Roberts et al. 2015), was later identified in citrus in East Africa (Rasowo et al. 2019; Roberts et al. 2017). An erroneous identification of CLAs in East Africa (Shimwela et al. 2016) was found to be a biovar of CLafCl, designated ‘CLafCl bv. Citrus’ and was associated with typical HLB symptoms observed on citrus in Tanzania, Uganda, and Kenya. In a few instances, CLafCl was found to be coinfections with CLaf, indicating that these liberibacters are adapted to similar environmental conditions (Roberts et al. 2017). Both *T. erythrae* and *D. citri*, collected in Tanzania, tested positive for the presence of CLafCl, indicating a probable overlap of vectors between CLaf, CLafCl, and CLAs (Rasowo et al. 2019; Roberts et al. 2017).

C. anisata is found throughout tropical Africa, from Guinea and Sierra Leone and eastward to Ethiopia and Sudan and southward. They occur naturally in the Eastern and Western Cape Provinces of South Africa but not in arid regions of these provinces. Despite the widespread distribution of both *C. anisata* and CLafCl in southern Africa, CLafCl has not been detected in citrus in southern Africa (R. Roberts, unpublished data). A host jump to citrus might

therefore have required specific adaptations not commonly occurring in the natural populations of CLafCl and the vector.

The first complete genome sequence of CLaf (CP004021) was reported in 2015 to be 1,192,232 nucleotides in length, with 1,089 predicted genes (Lin et al. 2015). The genome was assembled from genomic DNA isolated from CLaf-infected *T. erythrae*. In 2020 a

second nearly complete genome was assembled from DNA extracted from infected plant material (Fourie et al. 2021). These two reference sequences are 99.7% identical on the nucleotide level and provide an extensive resource to improve detection protocols. Genomic resources allow gene copy number analyses, useful in tailoring detection assays toward high copy number regions to

FIGURE 1

Greening symptoms observed on citrus leaves and fruit. **A**, Leaves displaying typical blotchy mottle (*Citrus reticulata* cultivar Tango). **B**, Greening-affected branch appears yellow from a distance (*Citrus reticulata* cultivar Tango). **C**, Sectoral nature of '*Candidatus Liberibacter africanus*' infection and twig dieback (*Citrus sinensis* cultivar Valencia late). **D**, Longitudinal section of a lopsided fruit, with curved columella and brown discoloration of vascular bundles clearly visible (*Citrus sinensis* cultivar late navel). **E**, Color inversion symptom on fruit with the stylar end remaining green.

increase sensitivity. Conventionally, the three-copy 16S rRNA gene is targeted to detect *Candidatus Liberibacter* species associated with HLB (Bertolini et al. 2014; Jagoueix et al. 1996; Li et al. 2006; Orce et al. 2015). An alternative to the 16S rRNA is the ribonucleotide reductase β -subunit gene, which showed an increased detection sensitivity for CLas (Zheng et al. 2016). Genomic analyses revealed that CLas has five copies of the ribonucleotide reductase β -subunit gene; however, CLaf only has one copy (Zheng et al. 2016). This could result in a decreased sensitivity for CLaf if this gene is targeted for universal liberibacter detection.

Two prophage segments were identified in the first CLaf genome (Lin et al. 2015), and it remains to be determined whether these integrated prophage-like sequences could be used to investigate CLaf genomic diversity or targeted for the development of universal or liberibacter-specific markers. Population-specific prophage sequences were identified in CLas from Pakistan (Cui et al. 2021), and comparative analyses of the available CLas genomes revealed that all of the genomes contained at least one prophage-like sequence out of the 33 identified prophage-like elements (Dominguez-Mirazo et al. 2019).

The availability of the CLaf genome also allows the use of technology such as microsatellite markers to study the genetic composition of CLaf. Research using microsatellite markers to study genetic variation of CLaf populations in South Africa identified two major genetic groups, based on distinct geographies, while alluding to the existence of potential third and fourth genetic groups (Roberts et al. 2021).

Bové (2006) proposed that each of the then-known liberibacters affecting citrus emerged independently as a citrus pathogen from where they were first described (i.e., Africa, Asia, and South America). Various studies have found evidence of founder haplotypes for CLas originating from India, from where it spread to China, having evolved along with commercial citrus species (Beattie et al. 2008; Islam et al. 2012; Teixeira et al. 2008). However, support for an African origin of CLaf lies within the geographic limitations of this liberibacter, because it has not yet been detected outside Africa. The existence of the various CLaf subspecies further supports an African origin for CLaf (Roberts et al. 2017).

CLAF GEOGRAPHIC DISTRIBUTION

Historically, HLB distribution in Africa was limited to cooler, humid climates, generally occurring at altitudes 600 masl, or cooler microclimates at lower altitudes and was also correlated to the prevalence of *T. erythrae*, indicating the probability of CLaf as the causative agent (Aubert et al. 1988; Bové 2014; Oberholzer et al. 1965; Schwarz 1967). Initial surveys conducted in East and West Africa and in countries south of the Sahara identified “greening disease” in Ethiopia, Rwanda, Burundi, Cameroon, Malawi, and Eswatini (formerly Swaziland) (Aubert et al. 1988; Catling and Atkinson 1974). The first confirmation of CLaf in Africa was in South Africa, Zimbabwe, and the Mascarene islands of Madagascar, Réunion, and Mauritius (Bové 2014; Garnier and Bové 1996).

Surveys in South Africa indicated the presence of greening in climatically conducive citrus producing regions in Mpumalanga, Limpopo, North-West, Gauteng, and KwaZulu-Natal provinces (Pietersen et al. 2010; Schwarz 1967). Wider spread of the disease to areas of absence or sporadic prevalence of the vector was linked to the sale of infected nursery trees from areas of high disease incidence (Schwarz 1967). This is relevant to regions of the Western Cape and Eastern Cape provinces, and further spread was noted in the Western Cape Province over time (Garnier et al. 2000a; Pietersen et al. 2010). The Northern Cape Province has remained disease free because the environment is not conducive to vector and pathogen spread, with high temperatures and low relative humidity.

It was in East Africa where unique citrus liberibacter diversity was discovered. Ribosomal DNA sequence data of Kenyan isolates from various ecological zones, all at elevations >1,200 masl,

indicated a variant liberibacter with sequence identity closest to CLaf (Magomere et al. 2009). The identity of this variant was clarified 8 years later. In 2015, a report of CLas and CLaf detection, in high-elevation regions in Uganda, sparked an urgent effort to clarify the presence of citrus liberibacters in the region (Kalyebi et al. 2016). A survey to corroborate the report was conducted in Uganda, Kenya, and Tanzania and included the regions of Budaka and Tororo of Uganda where CLas was reportedly found. Results of this later investigation indicated a likely nontarget amplification via a real-time PCR assay, resulting in a misidentification of CLafCI as CLas (Roberts et al. 2017). This was nonetheless the first report of a subspecies of CLaf to be identified in citrus, in the same ecological niches as that of CLaf, which was detected not only in Uganda but also in Kenya, Tanzania, and Ethiopia (Ajene et al. 2020c; Rasowo et al. 2019; Roberts et al. 2017). Three studies indicated the predominance of CLafCI in the western and central regions of Kenya before the introduction of CLas, which was detected only in the coastal regions of Lungalunga, Matuga, and Muhaka in Kenya (Ajene et al. 2020c; Rasowo et al. 2019; Roberts et al. 2017), thus resolving the variant liberibacter species detected by Magomere et al. (2009). CLafCI was likewise identified as the predominant citrus liberibacter in Uganda (Ajene et al. 2020c). In contrast to Kenya and Uganda, in Ethiopia CLas was identified as the predominant citrus liberibacter and was identified at altitudes between 161 and 2,116 masl (Ajene et al. 2020a). Although vector transmission of CLas at high elevations is unlikely because the vector was not observed in a majority of the positive sites, human-mediated transmission plays a significant role in the distribution of the liberibacter in the region, as seen in Ethiopia, and cannot be overlooked. The liberibacter population structure in East Africa is expected to change with the presence of both CLas and *D. citri* in the region (Ajene et al. 2020c; Rwomushana et al. 2017; Saponari et al. 2010), as was observed in Brazil with the displacement of CLam by CLas as the dominant liberibacter (Lopes et al. 2009). Furthermore, the detection of CLas in field populations of *T. erythrae* in the Ethiopian highlands (Ajene et al. 2019) adds to the potential for increased spatial distribution of the liberibacter species because both vector species (*T. erythrae* and *D. citri*) have been experimentally shown to transmit both liberibacter species (Lallemand et al. 1986; Massonie et al. 1976). A survey in Nigeria confirmed the presence of CLaf in the central region where the natural vector *T. erythrae* is absent (Ajene et al. 2020d). Considering the limited scope of the study, it is too early to speculate on the overall distribution of liberibacters in Nigeria, although *D. citri* was subsequently reported from the southwestern region (Oke et al. 2020).

VECTOR

T. erythrae (superfamily Psylloidea), also known as the African citrus psyllid, is the primary insect vector for CLaf (Fig. 2A). Psyllids (Hemiptera: Psylloidea) are sap-feeding insects that often complete their life cycle on only a few host plant species (Burckhardt et al. 2014). The family Trioizidae is well represented in Africa, and the species *T. erythrae* is widespread in sub-Saharan Africa, Madagascar, Réunion, Mauritius, St. Helena, Madeira, Canary Islands, Yemen, Saudi Arabia, Portugal, and northwestern Spain (Cocuzza et al. 2017). In South Africa it occurs in most citrus production areas except for areas best suited to grapefruit production, which are too hot. Hollis (1984) provided a detailed description on the Afrotropical Trioizidae that emphasized the close link between trioizid species and their host plants. His opinion was that *T. erythrae* completed its life cycle only on plants belonging to Rutaceae. He also considered *T. erythrae* to be one of a group of 10 similar species that were often hard to distinguish morphologically but had different host plants (Hollis 1984). One of the species in this group is *T. catlingi* Hollis, which completes its life cycle on

the creeper *Stephania abyssinica* (Quart.-Dill. & A. Rich.) Walp. var. *abyssinica* but is not found on citrus. However, DNA barcoding could not distinguish it from *T. erythrae* (Khamis et al. 2017). Furthermore, analysis of the complete mitochondrial genome and an in-depth analysis of the *cytochrome c oxidase I* (COI) gene region indicated that not all sequences identified as *T. erythrae* belong to the same species and that some degree of specificity with different plant hosts is likely to exist (Ajene et al. 2020e). Correctly identifying these species from samples collected on sticky yellow traps is therefore challenging. Another similar species to *T. erythrae* is *T. litseae* Borge, which has occasionally been found to complete its life cycle in citrus on Mauritius, Rodrigues, and Réunion islands, although its primary host plant is *Litsea glutinosa* (L.) (Aubert

1987). Samways (1987) captured an unknown species that was morphologically similar to *T. litseae* on sticky yellow traps near Komatipoort, Mpumalanga, South Africa, a region that is considered climatically unsuitable for *T. erythrae*.

T. erythrae completes its life cycle on all citrus types and cultivars and on a few indigenous Rutaceae plants, in addition to *Murraya* species that are sometimes grown in residential areas. Population fluctuations are closely associated with the presence of new foliage growth because the orange eggs are oviposited on soft, expanding leaves. The nymphs develop in open galls or “pockmarks” on the abaxial side of the leaf that form bumps on the adaxial side (Fig. 2B). These are permanent features that can be used to demonstrate that *T. erythrae* occurs in the locality when neither new growth nor live

FIGURE 2

Different life stages of *Trioza erythrae*. **A**, Adult with distinctive wing pattern and eggs. **B**, Nymph. Feeding nymphs leave pockmarks on leaves. **C**, Young flush with nymphs in newly formed pockmarks. **D**, Older vacated pockmarks on greening-affected branch.

insects are present. There are five nymphal instars, and although the last instar may move a short distance before molting to adults, the adult is the main dispersal life stage. The adults are therefore the vectors responsible for spreading CLaf from infected citrus trees to uninfected trees or orchards, and border rows of citrus are most likely to be infected first. Sticky yellow traps (Benhadi-Marín et al. 2021; Samways 1987) are a convenient method of monitoring adult *T. erytrae*, and specimens can be carefully removed and tested for the presence of liberibacter species via molecular techniques.

T. erytrae has been found more abundantly in highland areas that are generally cool, in contrast to coastal regions (Ajene et al. 2020c; Catling 1969; Shimwela et al. 2016). Protracted high temperatures, together with relative humidity <25%, are detrimental to the psyllids' development and induce mortality, specifically in eggs and first instar nymphs (Catling 1969; Van den Berg et al. 1990).

Surveys in East Africa indicated the presence of *T. erytrae* in citrus producing regions. The vector was seen in highland areas where the cooler temperatures favored its development, as observed in Ethiopia, Kenya, and Uganda (Ajene et al. 2020c). In Ethiopia, *T. erytrae* was found in the highlands of the Amhara region at elevations >1,800 masl. Likewise, in Uganda *T. erytrae* was found in the conducive areas in the central and western regions and was not detected in the eastern region of the country, where the climatic conditions are not favorable for the development of the triozyd (Ajene et al. 2020c). Similarly, *T. erytrae* is widely distributed in the western and central regions of Kenya, where the elevation and climatic conditions are conducive, but the triozyd was not found in the hotter, coastal regions at elevations from 20 to 1,100 masl (Aidoo 2019). Suitable areas where *T. erytrae* is most likely to establish can be predicted by ecological niche modeling. This information can be used to tailor monitoring efforts in a region in the unlikely event of an incursion (Espinosa-Zaragoza et al. 2021).

D. citri (Hemiptera: Psyllidae), also known as the Asian citrus psyllid, is the primary vector of CLas and CLam and was detected in mainland Africa (Tanzania) for the first time in 2015 (Shimwela et al. 2016). Subsequently, the psyllid was detected in coastal and western Kenya (Rwomushana et al. 2017), the North Wollo region in Ethiopia (Ajene et al. 2020a), and southwestern Nigeria (Oke et al. 2020). The ideal temperature range for the population development of *D. citri* is 25 to 31°C (Liu and Tsai 2000; Milosavljević et al. 2020), although *D. citri* has been reported to withstand temperatures >35°C (Milosavljević et al. 2020). The psyllid has been reported to have a native altitudinal range (<800 masl) (Jenkins et al. 2015). However, the psyllid was detected at altitudes of ≤2,112 masl in Ethiopia (Ajene et al. 2020a).

NON-CITRUS-INFECTING CLAF SUBSPECIES

Studies into the potential of indigenous rutaceous trees serving as reservoir hosts of CLaf have been a research focus in South Africa. In the early 1990s CLaf graft transmission studies were conducted on *C. anisata* (Van den Berg et al. 1991), and CLaf, probably a subspecies of CLaf, was detected in *Vepris lanceolata* with the use of a Southern blot assay in 1996 (Korsten et al. 1996). This possibility was explored further in the Western Cape Province after an outbreak of greening that was thought to be due to the presence of an indigenous reservoir host for this pathogen. Garnier et al. (2000b) subsequently identified a liberibacter species from the indigenous rutaceous species, *Calodendrum capense* (Cape chestnut), growing next to a citrus orchard near Stellenbosch, Western Cape. This liberibacter is similar, albeit distinct from CLaf, and was thus afforded subspecies status, now known as '*Ca. L. africanus* subsp. *capensis*'. CLafC was shown to be widely distributed in *C. capense* trees across South Africa (Phahladira et al. 2012). However, this liberibacter does not appear to infect citrus trees. In the study by Phahladira et al. (2012), 272 citrus trees tested negative for CLafC despite being within 8 m of a lane of 43 CLafC-positive *C. capense* trees for >15

years, with evidence of *T. erytrae* being present in the citrus orchard. Furthermore, in a study using primers from Hocquellet et al. (1999) that can detect both CLaf and CLafC, no CLafC was found in citrus trees collected throughout South Africa, which had tested positive by PCR and sequencing for CLaf (Pietersen et al. 2010). A number of these samples were within short distances of *C. capense* trees. The lack of transmission to citrus may be because *C. capense* is not a preferred host of *T. erytrae* (Moran 1968). The vector of CLafC is still unknown.

Studies to find potential reservoir hosts for CLaf were conducted among known natural hosts of *T. erytrae* (Roberts et al. 2015; Roberts and Pietersen 2017). Host plants were collected throughout the natural distribution of these trees in South Africa and included *C. anisata*, *V. lanceolata*, *Zanthoxylum capense*, *Teclea* spp., and *Oricia* spp. CLaf sensu stricto was not identified from any of these samples, but liberibacters with high sequence identity with CLaf unique to each of these hosts were detected. This finding was based on the amplicon sequences from three genes (16S, *rplJ*, and *omp*) (Roberts et al. 2015; Roberts and Pietersen 2017). These liberibacters are now known as CLafCl, '*Ca. L. africanus* subsp. *teclea*', '*Ca. L. africanus* subsp. *vepridis*', and '*Ca. L. africanus* subsp. *zanthoxyli*', named for the hosts from which they were identified (Roberts et al. 2015; Roberts and Pietersen 2017). Mottling symptoms were observed for some of the trees collected; however, no clear correlation could be made between the occurrence of CLaf subspecies from their indigenous hosts and symptom expression, with the majority of trees testing positive for subspecies seemingly being healthy. CLafCl was identified from *C. anisata* trees, distributed throughout the South African range of this tree species. '*Ca. L. africanus* subsp. *vepridis*' was detected in *V. lanceolata* trees distributed along the east coast of South Africa (Western Cape, Eastern Cape, and KwaZulu-Natal), '*Ca. L. africanus* subsp. *zanthoxyli*' was detected in *Z. capense* also along the east coast, and '*Ca. L. africanus* subsp. *teclea*' was detected in a single *T. gerrardii* specimen collected in southern KwaZulu-Natal (Roberts et al. 2015; Roberts and Pietersen 2017). No liberibacters were obtained from any of the *Oricia* spp. collected in southern KwaZulu-Natal (Roberts and Pietersen 2017). Liberibacters were also not found among fynbos members of the Rutaceae (R. Roberts and G. Pietersen, unpublished results). The distribution of the CLaf subspecies on native hosts outside South Africa is unknown, with the exception of CLafCl, which occurs in Ethiopia, Uganda, and Kenya (Ajene et al. 2020c).

The phylogenetic relationship between the CLaf subspecies in relation to citrus-infecting liberibacters is shown in Figure 3.

FIGURE 3

Maximum likelihood phylogeny constructed with representative sequences of '*Candidatus Liberibacter africanus*' (CLaf) subspecies associated with rutaceous species in South Africa using available *omp* gene sequences. The phylogeny was inferred via the general time reversible model with gamma correction to account for site variations. Bootstrap support values based on 1,000 replicates are indicated at branches. CLaf sequences are included for reference and '*Ca. L. asiaticus*' and '*Ca. L. americanus*' sequences as outgroups.

CLAF DETECTION

The earliest disease verification was achieved by bark inoculation to healthy seedlings and subsequent monitoring for symptom development (McClean and Oberholzer 1965b). A laboratory assay was additionally used that detected fluorescence obtained with a chemical treatment of plant extracts spotted to chromatographic paper (Schwarz 1965), and this approach was further simplified by observing fluorescence in the albedo of cut fruit (Schwarz 1967). CLaf was the first citrus liberibacter to be observed with electron microscopy (Lafleche and Bové 1970; Moll and Martin 1973). With the association of a causative organism, serologically based assays using monoclonal antibodies were developed that could differentiate CLaf and CLas (Garnier et al. 1987). The latest application of a serology-based detection assay was the development of a direct tissue blot immuno assay for the detection of CLas and CLaf from different geographic origins (Ding et al. 2020).

Nucleic acid-based diagnostics were originally based on the 16S rRNA gene, which was widely used for sequence-based bacterial phylogeny and taxonomy. Initial classification of citrus liberibacters as Alphaproteobacteria was based on 16S rRNA sequence data for isolates of African and Asian origin, showing the first sequence diversity between CLaf and CLas (Jagoueix et al. 1994). Development of PCR diagnostics for CLaf and CLas logically developed concurrently thereafter, targeting the 16S rRNA for conventional (Jagoueix et al. 1996) and real-time PCR (Bertolini et al. 2014; Li et al. 2006; Orce et al. 2015). Details of the various assays are listed in Table 1. The Li et al. (2006) species-specific assays became widely used, partly because of the use of a common probe for the species-specific assays for CLaf, CLam, and CLas detection but also as multiple copies of the gene were detected, allowing increased sensitivity (Kim and Wang 2009). Two genus-specific protocols were developed with the probe of Li et al. (2006). Bertolini et al. (2014) designed new primers to be used with the probe, whereas a new universal forward primer, LibUF, was used with the probe and HLBr reverse primer (Roberts et al. 2015).

Use of the 16S rRNA gene to provide resolution at the species level for citrus liberibacters was questioned when CLas was reported in Kenya, with the Li et al. (2006) assays used as the only validation (Kalyebi et al. 2016). A subsequent survey to verify this report found that CLafCl, a subspecies of CLaf, was misidentified as CLas with the 16S rRNA assays (Roberts et al. 2017). This finding suggests that alternative regions should be used for species differentiation.

The other genome domain that was used for CLaf inclusive diagnostic development was the β -operon for ribosomal protein genes. Species-specific hybridization probes for Southern and dot blots were derived from genes *rpIKAJL-rpoBC* of the β -operon for CLas and CLaf (Hocquellet et al. 1997; Planet et al. 1995). Thereafter, conventional PCR targeting the *RplA* and *RplJ* genes of the β -operon followed that allowed amplification of both CLaf and CLas but with slight differences in amplicon size for differentiation (Hocquellet et al. 1999). Real-time genus- and species-specific assays for CLaf, CLam, and CLas were designed targeting the *rpoB* and *RplJ/RplK* genes, respectively, but with lower sensitivity compared with 16S rRNA assay of Li et al. (2006) (Ananthakrishnan et al. 2013). A loop-mediated isothermal amplification assay, targeting the same ribosomal protein gene region but including the *nusG* gene, was developed for CLas detection (Okuda et al. 2005), but this assay cross-reacted with CLaf (Li et al. 2007).

The outer membrane protein *Omp*-gene diversity of citrus liberibacters was investigated and showed significant phylogenetic differentiation between citrus liberibacter species and subspecies (Bastianel et al. 2005; Roberts et al. 2017). Similarly, the β -operon for ribosomal protein genes showed significant differentiation for citrus liberibacters (Roberts et al. 2017).

Accurate and sensitive detection assays for liberibacter species from insects and plant material are the cornerstone of HLB monitoring and management strategies and require continuous development and validation.

GREENING DISEASE MANAGEMENT

Eradication of citrus greening, where the vector is endemic, is not possible, and disease management is reliant on integrated prevention and control strategies. Greening disease control was pioneered in South Africa (Buitendag and Von Broembsen 1993), and many of these strategies were later adopted and expanded in Brazil to contain the ravages of HLB (Bassanezi et al. 2020).

Preventive measures include quarantine actions such as restricted movement of plants from infected regions to those with pathogen and vector absence and preemptive scouting to detect disease outbreaks and vector incursion to enable early control interventions. The supply of certified disease-free nursery trees to growers is vital for reducing inoculum pressure, in parallel with the removal of abandoned, unproductive, and infected trees. With CLaf, unlike with CLas, it is possible to control disease spread within the tree through early removal of symptomatic branches of sectoral infections. This

TABLE 1
PCR-based assays that detect '*Candidatus Liberibacter africanus*' according to the genome target regions, PCR assay type, and target specificity

Gene target region	PCR assay type	Genus (G) and species (S) specific ^a	Primer designations	Reference
16S rRNA	Conventional	S (CLaf)	OAI/OI2c	Jagoueix et al. 1996
	TaqMan real-time	S (CLaf)	HLBaf/HLBp/HLBr	Li et al. 2006
	TaqMan real-time	G (CLaf/CLas/CLam)	CaLsspF/HLBp/CaLsspR	Bertolini et al. 2014
	TaqMan real-time	G (CLaf/CLas/CLam)	LibUF/HLBp/HLBr	Roberts et al. 2015
	SYBR Green real-time	G (CLaf/CLas)	f-p3G/r-p3G	Orce et al. 2015
Ribosomal protein genes <i>RplA</i> and <i>RplJ</i> of the β -operon	Conventional	G (CLaf and CLas)	A2/J5	Hocquellet et al. 1999
Ribosomal protein gene <i>RpoB</i> of the β -operon	TaqMan and SYBR Green real-time	G (CLaf /CLas/CLam)	<i>rpoB</i>	Ananthakrishnan et al. 2013
Ribosomal protein gene <i>RplJ/RplK</i> of the β -operon	TaqMan and SYBR Green real-time	S (CLaf)	<i>RplJ/RplK</i>	

^a CLaf, '*Ca. L. africanus*'; CLam, '*Ca. L. americanus*'; CLas, '*Ca. L. asiaticus*'.

indicates that CLaf does not translocate in the plant at the same rate as has been shown for CLas (Pandey et al. 2021), and disease progression in mature trees is slow (Moll and van Vuuren 1977).

There are currently no commercial therapeutic treatments available that target the bacterium. Antibiotics were tested as curative treatments for CLaf by application through trunk injections (Moll and van Vuuren 1977). However, antibiotic treatments were not sustainable because application was labor intensive and expensive. Additionally, disease remission was only temporary, but the most adverse finding was that high antibiotic residues were detected in the fruit of treated trees (Buitendag and Von Broembsen 1993). Disease control is therefore dependent on reducing vector activity. The use of systemic insecticides with long residual action, applied before the spring flush, followed by shorter-acting insecticides, remains the primary means to effectively control the vector. This method has brought about a distinct reduction in the incidence of greening-infected trees in commercial plantings in greening-affected areas in South Africa.

Biological control of *T. erytrae* is another supportive intervention and is effective in the absence of hyperparasitoids. The parasitic wasp *Tamarixia dryi* (= *Tetrastichus dryi*) significantly reduced populations of *T. erytrae* on the Indian Ocean island of Réunion, leaving a greatly limited population of the vector (Aubert and Quilici 1984).

The Mpumalanga Lowveld, which ranges from 600 to 850 masl, was severely affected by greening, rendering parts of the region unsuitable for citrus production. The Lowveld has a subtropical climate with mild winters and warm to hot, humid summers, conditions mostly favorable to the vector and greening (Schwarz and Green 1972). However, the disease has been successfully managed in this region for >40 years, which is testament to a focused, integrated management strategy with an area-wide approach. Growers in the region agreed to refrain from planting lemons, which have numerous flush cycles throughout the year. Because chemical treatments are applied before flush, unsynchronized flushing could compromise area-wide vector management. A synchronized spring flush is maintained through irrigation and fertilization interventions. Fertilization before winter is avoided, and limited irrigation is applied in winter to prevent out-of-season, winter flushing. Young, nonbearing trees ≤5 years old need protracted protection with multiple systemic insecticide treatments throughout the year. In established orchards all symptomatic branches are removed annually and treated to prevent regrowth. The success of this region has been the result of continuous cooperation between various stakeholders, and grower participation has been maintained by industry-driven information sessions and advisory consultants (J. Warrington, *personal communication*).

Attempts to breed citrus cultivars resistant to greening were made in South Africa by De Lange et al. (1985), who produced hybrids of sweet orange and Tahiti lime. However, the hybrid lines were not resistant to greening. Van Vuuren and Manicom (2009) investigated whether embryos rescued from symptomless segments of fruit chimeras of diseased fruits would yield resistant or tolerant plants. Plants produced from these embryos eventually became infected when exposed to infective psyllids (Van Vuuren and Manicom 2009; S. P. Van Vuuren, *unpublished data*).

Resistance to CLas was first reported in two Australian citrus genera, *Microcitrus* and *Eremocitrus*, providing an ideal source of possible resistance (Ramadugu et al. 2016). Through conventional breeding technology (hybridization), the resistance from *E. glauca* was bred into several citrus species, producing lime-like hybrids with HLB resistance (Ramadugu et al. 2019). The development and screening of antimicrobial peptides (AMPs) active against CLas is being pursued (Stover et al. 2013). These could be long-term solutions to the various forms of this disease; however, the latter approach might require the use of transgenic trees that may be resisted by consumers. Recently, a new class of AMPs, stable

AMPs, were discovered in *Microcitrus australasica*. These AMPs were found to be active against liberibacter species and hold great promise as a general HLB therapeutic (Huang et al. 2021). The advances in genome editing technology allow for the creation of cisgenic plants expressing liberibacter active molecules naturally occurring in citrus, mitigating consumer concerns. Editing the host genome to be resistant to the liberibacter might soon be a reality.

CONCLUSION

The disease is a major challenge for which no simple remedy has proven totally effective for disease control. There are several reasons, including initial obstacles to identify the causal organism, which still defies isolation in pure culture, together with the complex interaction between hosts, pathogen, vector, and the environment. Although much progress has been made, the disease still causes loss to citrus producers in many countries in Africa. The introduction of CLas and the Asian citrus psyllid to the continent presents a serious additional challenge, which will affect citrus in areas unsuitable for CLaf and *T. erytrae*. Additionally, CLas may outcompete CLaf, as has occurred in Brazil with CLam.

CLaf and its vector *T. erytrae* are confined largely to cooler areas with higher relative humidity and can be managed through the use of systemic plant protection products to control the vector and pruning in winter to reduce inoculum. However, ACP and CLas will negatively affect hotter and drier citrus production regions, so their combined effect will have a greater impact on future citrus production in Africa.

The African form of greening differs from the Asian, not only in the temperature sensitivities of CLaf and the vector but also with the discovery of numerous subspecies of CLaf and the ongoing mystery of the origin of CLaf. Continuing investigations should reveal how citrus first became infected, probably somewhere in South Africa when native vegetation was cleared for citrus cultivation, and *T. erytrae* transmitted a bacterium from a native tree to the monoculture of vigorous new citrus trees.

The latest advances in molecular technology open up more possibilities for the development of control measures to complement existing efforts. The first area that showed significant progress was the development of more sensitive monitoring and detection assays that allow earlier detection, fast tracking interventions to curb secondary spread of the disease. The availability of the liberibacter genomes opens up new possibilities for innovative control strategies.

The detailed molecular characterization of the CLaf pathosystem lags significantly behind that of CLas, and future research efforts should characterize the pathogen–insect vector–plant host interactions to address these knowledge gaps.

The control of the African form of HLB caused by CLaf will certainly benefit from the increase in international HLB research on the more severe Asian form caused by CLas. The complexity of this disease will require multitiered management strategies drawn from the global research effort to minimize its impact.

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