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Why we should care about culturing the Huanglongbing associated bacterium 'Candidatus Liberibacter asiaticus': the importance of terms and interpretations

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1 **Recently Accepted**

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3 **REVIEW**

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5 **Why we should care about culturing the Huanglongbing associated bacterium ‘*Candidatus***  
6 ***Liberibacter asiaticus*’: the importance of terms and interpretations**

7

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9

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## 31 ABSTRACT

32

33 Having bacteria grown in pure culture has been the foundation of bacteriology, by allowing a wide  
34 range of microbiological studies to determine the functionality of a specific bacterium. However,  
35 most bacteria have not been axenically cultured to date, thus hindering the understanding of their  
36 role in the context of their host or environment. Among these uncultured bacteria are the recently  
37 emergent plant pathogens '*Candidatus Liberibacter* spp.'. This group is comprised of dynamic  
38 psyllid-vectored, phloem-limited plant pathogens and endophytes that harm a wide range of  
39 economically important crops worldwide. '*Candidatus Liberibacter asiaticus*' (CLAs) is associated  
40 with Huanglongbing (HLB) in most of the main citrus-producing areas globally, a disease causing  
41 severe economic damages. Although the establishment of axenic cultures of CLAs remains a major  
42 scientific challenge, many research groups have devoted efforts to culture this bacterium to aid in  
43 elucidating its virulence mechanisms to develop effective HLB management. This has led to the  
44 development of innovative systems to culture and grow CLAs, however different authors have  
45 approached the concepts of bacterial culture and axenic culture in different manners, leading to  
46 confusion in the terminology used. In this review, we provide the scientific definitions of important  
47 terms in bacteriology, while critically reviewing the contribution of each of these important CLAs  
48 culturing studies.

49

50 Keywords: plant pathogen, unculturable, axenic culture, HLB, CLAs

51

## 52 Introduction

53

54 The establishment of pure cultures of microorganisms in laboratory conditions during the  
55 late nineteenth century has been a cornerstone of bacteriology. The ability to artificially grow  
56 bacteria enabled the development of studies to assess their physiology, taxonomy, ecology and  
57 pathology, as well as allowing studies of their morphology, virulence, antibiotic susceptibility and  
58 genome sequence, among other features (Austin 2017; Lagier et al. 2015a). However, not all  
59 bacterial species have been successfully grown in vitro. Currently, around 20,000 species have  
60 been described through culturing, while it is estimated that the total bacterial diversity ranges from  
61  $10^7$  to  $10^9$  species (Curtis et al. 2002; Parte et al. 2020). The difference between microscopic and

62 culture counts is referred to as the “great plate count anomaly” (Staley and Konopka 1985).  
63 Although microbiologists have been working to replicate the distinct natural environments of  
64 bacteria to reduce this difference between bacterial diversity and culturability, the discrepancy is  
65 still high (Lagier et al. 2015b).

66 Depending on the features of each bacterium, strategies to mimic their habitats and  
67 determine specific growth conditions to allow their culturability may include analysis of required  
68 nutrients, temperature, oxygen (aerophilic, microaerophilic and anaerobic organisms), incubation  
69 time, use of reducing agents, addition of signal compounds and co-cultivation with one or more  
70 different species, since some bacteria depend on the metabolic interactions with their community  
71 to grow (Lagier et al. 2015a; Overmann et al. 2017). In addition, the use of new technologies of  
72 the omics era, specially metagenomics, has played a pivotal role in determining unknown  
73 metabolic features of unculturable bacteria and in performing culture-independent physiological  
74 and ecological analyses of these organisms (Overmann et al. 2017). However, although some  
75 authors may argue that metagenomics has the potential to replace bacterial culture (Austin 2017;  
76 Lagier et al. 2015b), culturing still plays a key role in modern functional microbiology.

77

## 78 **The persistent case of ‘*Candidatus Liberibacter asiaticus*’ unculturability and its** 79 **implications**

80 An important group of hitherto unculturable plant pathogenic bacteria is comprised by  
81 ‘*Candidatus Liberibacter spp.*’, which are phloem-limited, fastidious Gram-negative bacteria of  
82 the  $\alpha$  subdivision of Proteobacteria (Jagoueix et al. 1994; Wang and Trivedi 2013). Species of ‘*Ca.*  
83 *Liberibacter*’ are a diverse group of plant pathogens and endophytes that cause diseases in  
84 numerous plant hosts (Merfa et al. 2019), including citrus, potato, tomato, carrot and pear (Bové  
85 2014; Nelson et al. 2012; Thompson et al. 2013). However, in this review, we will focus on the  
86 subgroup causing the citrus Huanglongbing (HLB) disease.

87 HLB is the most single devastating disease of citrus worldwide (Gottwald 2010), causing  
88 meaningful economic losses in the Americas, Asia and Africa (Bové 2014). This disease has been  
89 associated with three ‘*Ca. Liberibacter spp.*’: ‘*Ca. Liberibacter africanus*’ (CLaf), ‘*Ca.*  
90 *Liberibacter americanus*’ (CLam) and ‘*Ca. Liberibacter asiaticus*’ (CLas), which is the focus  
91 species of this review due its prevalence worldwide and greater number of published studies (Bové

92 2014). The Asian Citrus Psyllid (ACP) *Diaphorina citri* is the insect vector for both CLAs and  
93 CLam, while both CLAs and ACP are believed to be native to Asia (Bové 2006; Nelson et al. 2013).

94 Because of the difficulty in culturing these bacteria, they are given the provisional  
95 *Candidatus* status (Merfa et al. 2019). Lack of CLAs culturability impairs: (i) functional genomic  
96 analyses, which limits hypothesis testing; (ii) taxonomic identification and species name  
97 validation; (iii) fulfillment of Koch's postulates by transferring it to either insect or plant hosts;  
98 (iv) assessment of host-pathogen interactions; (v) screening of antimicrobial compounds; (vi)  
99 determination of virulence among different CLAs genotypes; (vii) strain submission to microbial  
100 collections for sharing among laboratories; and, more importantly, (viii) development of novel  
101 management approaches to control this incurable disease (Bové 2006; Merfa et al. 2019; Pinevich  
102 et al. 2018; Wang and Trivedi 2013). Understanding the strategies by which a pathogen causes  
103 disease and overcomes plant defenses may allow the development of control measures for newly  
104 emerging plant diseases (Wang and Trivedi 2013). This may be possible by interfering with key  
105 elements of the pathogen's life cycle, infection process and pathogenicity determinants. Therefore,  
106 culturing CLAs emerges as a priority, because it should enable functional studies and the  
107 development of management approaches to control HLB.

108 Although a reliable and reproducible method to culture CLAs is yet to be developed,  
109 substantial progress in culturing this bacterium has been made by using different approaches.  
110 These include mimicking the natural environments where CLAs lives, co-culture with one or more  
111 bacterial species and use of CLAs-infected plant explants, all of which will be briefly detailed  
112 below in this review (Attaran et al. 2020; Davis et al. 2008; Fujiwara et al. 2018; Ha et al. 2019;  
113 Mandadi et al. 2017; Parker et al. 2014; Sechler et al. 2009). These studies show the need of CLAs  
114 to grow in conditions close to its natural environments, and that it may obtain additional nutrients  
115 or chemical signals through a mutualistic relationship with other bacteria. However, there is great  
116 inconsistency in how these studies present the concept of an axenic bacterial culture and how this  
117 will contribute to control HLB. This is especially confusing for a lay audience, which includes  
118 citrus growers that have great interest in solving the HLB problem, as well as fund a considerable  
119 part of the ongoing research to solve this issue. Recently, we reviewed and provided insights into  
120 the requirements for CLAs culturability by mainly analyzing its genome and the chemical  
121 composition of the environments where it lives (Merfa et al. 2019). In this review, we would like  
122 to provide guidelines on how to accurately use the technical terms comprising bacterial culture. In

123 addition, we want to discuss how different culturing and non-culturing systems may be useful to  
124 study plant pathogens, particularly CLAs. We hope to help clarify and standardize these concepts  
125 for use in future publications by members of the HLB research community.

126

### 127 **Historical perspective: the importance of axenic cultures for plant pathology**

128 CLAs is not the first plant pathogenic fastidious prokaryote that has been difficult to culture  
129 axenically. *Xylella fastidiosa* is a successful case study of how culturing notably accelerates  
130 studying a bacterial pathogen and aids in the development of management strategies to control the  
131 diseases it causes; even though in this case the time elapsed between disease reports and axenic  
132 culturing was nearly 100 years (Chatterjee et al. 2008; Hopkins and Purcell 2002). It is worth  
133 noting that HLB is also a century-old plant disease, however efforts into studying CLAs have been  
134 greatly delayed in comparison to *X. fastidiosa* (Bové 2006; Kruse et al. 2019).

135 *X. fastidiosa* colonizes a wide range of plant hosts and causes substantial losses in  
136 economically important crops worldwide, including grapevine, citrus and olive (Chatterjee et al.  
137 2008; Hopkins and Purcell 2002; Saponari et al. 2013). Similar to CLAs, *X. fastidiosa* is limited to  
138 the vascular system (in this case xylem vessels) of plant hosts and foregut of insect vectors, which  
139 are mainly sharpshooter leafhoppers and spittlebugs (Chatterjee et al. 2008; Hopkins and Purcell  
140 2002). The first report of Pierce's disease of grapevine in California occurred in the 1880s,  
141 although its causal agent was not known at the time (Hopkins and Purcell 2002). Culturing of *X.*  
142 *fastidiosa* only happened much later in the 1970s (Davis et al. 1978), while its genome sequence  
143 was published in 2000 (Simpson et al. 2000), being the first sequenced genome of a plant-  
144 associated bacterium. With these data at hand, research on *X. fastidiosa* quickly increased, and this  
145 bacterium was even considered one of the top 10 plant pathogenic bacteria in molecular plant  
146 pathology (Mansfield et al. 2012). Studies including functional genomics (Chen et al. 2017;  
147 Kandel et al. 2018), assessment of resistance and tolerance mechanisms to antimicrobials (Kuzina  
148 et al. 2006; Merfa et al. 2016), evaluation of colonization pattern of plant hosts and of  
149 pathogenicity and virulence mechanisms (Nascimento et al. 2016; Newman et al. 2003; Niza et al.  
150 2015), extensive genomic analyses to examine recombination among subspecies of this bacterium  
151 (Potnis et al. 2019; Vanhove et al. 2019), and inspection of innovative strategies to control *X.*  
152 *fastidiosa* (Baccari et al. 2019; Muranaka et al. 2013), among many other studies, were made  
153 possible thanks to widely available axenic cultures. They all had remarkable contributions in

154 aiding the control and avoidance of the diseases caused by this bacterium, despite the fact that a  
155 cure for plants infected by *X. fastidiosa* is still not available (EFSA Panel on Plant Health 2016).

156 Another example of a plant pathogenic fastidious bacterium being cultured is *Spiroplasma*  
157 *citri*. This organism is the causal agent of the citrus stubborn disease, which significantly reduces  
158 fruit quality and production in infected trees, and was the first phloem-limited fastidious  
159 prokaryote to be axenically cultured (Saglio et al. 1971; Shi et al. 2014). *S. citri* is a pathogenic  
160 mollicute transmitted in a circulative, persistent manner by the leafhoppers *Circulifer tenellus* (in  
161 the U.S.) and *C. haematoceps* (in the Mediterranean area) (Bové et al. 2003; Fos et al. 1986; Liu  
162 et al. 1983). As described for *X. fastidiosa*, the in vitro culturing of *S. citri*, and more recently its  
163 genome sequencing (Davis et al. 2017), have enabled significant studies on the morphology and  
164 motility of this plant pathogen, and have also elucidated the relationships among *S. citri* and its  
165 plant hosts and insect vector. In addition, the cellular and molecular features of *S. citri* have been  
166 investigated through the development of functional genomics tools for this bacterium (Bové et al.  
167 2003). Hence, these two examples of culturing fastidious prokaryotes (*X. fastidiosa* and *S. citri*)  
168 show the importance of axenic cultures in plant pathology allowing more rapid research progress  
169 geared towards controlling the diseases caused by these bacteria.

170

### 171 **Definitions of culture and axenic culture**

172 The technical definitions of “culture” and “axenic culture” are presented here to aid in  
173 reviewing the contributions of each study on CLAs culturing performed to date. These definitions  
174 were taken from well-known textbooks and a biology dictionary. Although these terms may  
175 slightly vary among authors, they share great consistency. Culture is defined as “a particular strain  
176 or kind of organism growing in a laboratory medium” (Madigan et al. 2014), more specifically “in  
177 a container of culture medium” (Tortora et al. 2019). Broadly, a culture is any “batch of cells,  
178 which can be microorganisms or of animal or plant origin, that are grown under specific conditions  
179 of nutrient levels, temperature, pH, oxygen levels, osmotic factors, light, pressure, and water  
180 content” (Martin and Hine 2008). These cultures “are prepared in the laboratory for a wide  
181 spectrum of scientific research”, and “a culture medium provides the appropriate conditions for  
182 growth” (Martin and Hine 2008).

183 On the other hand, axenic culture (synonym: pure culture) is defined as “a culture  
184 containing a single kind of microorganism” (Madigan et al. 2014), which contains “only a single

185 strain or species of microorganism” (Slonczewski and Foster 2016). Ultimately, an axenic culture  
 186 will contain “a large number of microorganisms that all descend from a single individual cell”  
 187 (Slonczewski and Foster 2016). These axenic cultures may be used to “determine the basic growth  
 188 requirements or degree of inhibition by antibiotics or other chemicals of a particular species”  
 189 (Martin and Hine 2008).

190 From these definitions it is evident that a bona fide bacterial culture only includes cells  
 191 growing through the conditions defined by the culture medium and incubation settings, and thus it  
 192 excludes any ex vivo systems that include the host cells or tissues. Moreover, an axenic culture  
 193 only considers the clonal population of a single strain (Shrestha et al. 2013). Therefore, not even  
 194 culturing of mixed strains from a same species constitute an axenic culture. Fundamentally, axenic  
 195 means culturing free of any contaminants (Pinevich et al. 2018; Shrestha et al. 2013).

196

197 **Applications of different culturing systems and non-culturing systems**

198 Because of the failure to culture CLAs axenically, researchers need to be creative and use  
 199 innovative ideas and approaches to maintain this bacterium growing in vitro – at least partially.  
 200 Thus, different culturing systems, and even non-culturing systems, may be used. However, the  
 201 range of analyses that may be performed in each system is limited. Hence, the applications and  
 202 limitations of some systems that may be useful for CLAs are presented in Table 1. The broad  
 203 definition of culture medium is defined here as “a nutrient material, either solid or liquid, used to  
 204 support the growth and reproduction of microorganisms” (Martin and Hine 2008) in “a laboratory”  
 205 (Tortora et al. 2019). Although different types of culture medium, such as defined, complex and  
 206 enriched media (Madigan et al. 2014; Tortora et al. 2019) are available, these distinctions are not  
 207 the focus of this review.

208

209 **Table 1.** Comparison among different culturing and non-culturing systems for bacterial growth.

System *		Description	Applications	Limitations	Koch’s postulates	References
Culturing systems	Solid medium	Culture medium containing agar, or other inert solidifying agent, at a concentration of 1.0 to 2.0%	Useful for isolating bacteria in pure culture and determining the colony characteristics of the isolate. Also used in the following assays: - Screening of antimicrobials - Bacterial motility	Does not allow renewal of nutrients over time, thus access to nutrients may be limited	Yes	(Anjum 2015; Balouiri et al. 2016; Bonnet et al. 2020; Kandel et al. 2017; Madigan et al. 2014; Naranjo et al. 2020)



			- Counting of colony forming units (CFU)			
	<b>Semisolid medium</b>	Cultured medium prepared with agar, or other inert solidifying agent, at a concentration of 0.1 to 0.4%	Enables culturing microaerophilic bacteria. Also useful for determining bacterial motility, specifically swarming and swimming motilities, and chemotaxis		Yes	(Hashsham 2007)
	<b>Liquid medium</b>	Also called culture broth. Contains all required nutrients for growth of the desired bacteria dissolved in water, however without the presence of any solidifying agent	Used to propagate large number of cells and for specific assays including: <ul style="list-style-type: none"> <li>- Biofilm formation</li> <li>- Growth curve <sup>x</sup></li> <li>- Time lapse assays, including antimicrobials screening <sup>x</sup></li> </ul>	- Not suitable for isolating bacteria from a mixed sample <ul style="list-style-type: none"> <li>- Does not allow identification of the morphological characteristics of bacterial species</li> </ul>	Yes/No <sup>y</sup>	(Anjum 2015; Balouiri et al. 2016; Bonnet et al. 2020; Kandel et al. 2017; Naranjo et al. 2019; Naranjo et al. 2020)
	<b>Co-culture</b>	Two or more distinct bacterial species or strains are cultured together with some level of contact among them	- Allows studying metabolic interactions among co-cultured bacteria <ul style="list-style-type: none"> <li>- May be used to culture fastidious and unculturable bacteria that rely on other organisms to grow</li> <li>- Different organisms may be axenically cultured by physical separation through a membrane that allows only metabolic interactions</li> </ul>	- Physical separation among organisms can only be performed at a small scale and with a limited number of members <ul style="list-style-type: none"> <li>- Although possible, axenic cultures are not easily achieved</li> </ul>	Yes/No <sup>z</sup>	(Goers et al. 2014; Hashsham 2007; Merfa et al. 2019; Tanaka and Benno 2015)
	<b>Liquid medium in flow conditions</b>	Bacterial cells are cultured in flow systems or microfluidic chamber (MC) mimicking the plant vasculature, in which fresh culture medium broth is continuously supplied through a current flow	MC allows real-time observations in a microscope. MCs and flow systems are suited for the following assays: <ul style="list-style-type: none"> <li>- Bacterial motility</li> <li>- Biofilm formation</li> <li>- Measurement of adhesion force to a surface</li> <li>- Screening of antimicrobials</li> </ul>	Not suitable for isolating bacteria. Cells must be previously cultured axenically for downstream analysis using this system	Not applicable**	(De La Fuente et al. 2007; Meng et al. 2005; Naranjo et al. 2019; Naranjo et al. 2020)
<b>Non-culturing systems</b>	<b>Intracellular culture</b>	Intracellular bacteria are grown within host cells, which are cultured in vitro	- Ability to grow bacteria that would otherwise be unculturable <ul style="list-style-type: none"> <li>- Allows assessing the interaction of the target bacterium with its host</li> </ul>	- Do not allow a bona fide axenic culturing of bacteria <ul style="list-style-type: none"> <li>- Number of assays that can be performed is limited</li> </ul>	No	(Lagier et al. 2015a)
	<b>Detached leaves</b>	Leaves are detached from a desired plant host, surface-sterilized, inoculated with the respective bacterial pathogen to	Allows screening the pathogenicity/virulence of different strains/species of the pathogen; and screening resistance/susceptibility of		No	(Francis et al. 2010; Randhawa and Civerolo 1985)

		be studied and incubated in 0.5% water-agar plates	different genotypes of the plant host(s)			
	<b>Leaf discs</b>	Infected leaves of plant host are detached, surface-sterilized and leaf discs of 5 mm dia. are taken. Leaf discs are pooled and inoculated into test media to assess bacterial growth in planta over time by quantitative PCR (qPCR)	Allows evaluating unculturable bacteria growth in planta over different physicochemical and nutritional conditions		No	(Attaran et al. 2020)
	<b>Hairy root</b>	Hairy root formation is induced in infected roots of plant host by <i>Rhizobium rhizogenes</i> . Infected hairy roots are then inoculated into test media to assess bacterial growth in planta over time by quantitative PCR (qPCR)			No	(Mandadi et al. 2017)

210 \* The differentiation between culturing and non-culturing systems was made based on the culture definition presented  
 211 here. Thus, only systems in which cells are consistently grown in vitro were considered a culturing system; while  
 212 systems that depend on other living organisms that do not rely directly on a culture medium were considered as non-  
 213 culturing. Take note that non-culturing does not mean no growth, but that microorganisms rely on ex vivo tissues to  
 214 survive and multiply.

215 \*\* Studies in flow conditions using MCs are usually not aimed for subsequential analysis in planta.

216 <sup>x</sup> Assays performed by measuring turbidity of culture over time.

217 <sup>y</sup> Bacteria grown in liquid media may be used for fulfillment of Koch's postulates only if they have been previously  
 218 isolated in pure culture, usually by plating in solid media.

219 <sup>z</sup> Co-culture system may be used for Koch's postulates when cells are physically separated by a membrane, allowing  
 220 only for exchange of metabolites. Therefore, each member of the co-culture is considered axenic.

221

222 Among the systems described in Table 1, culturing in solid medium is the most desired for  
 223 CLAs due to ease of work and ability to isolate cells in axenic cultures (Bonnet et al. 2020). The  
 224 conditions established in this system could then be applied in liquid medium to increase the range  
 225 of assays to be performed. However, because of the recalcitrant nature of CLAs to culturing, other  
 226 systems may also be explored (Table 1). It has been suggested that CLAs may have a  
 227 microaerophilic respiration (Wang and Trivedi 2013), which would require a semi solid medium  
 228 and/or incubation in controlled oxygen conditions to grow. Moving further, transient co-cultures  
 229 of CLAs with other bacteria show the potential of the co-culture system to (co-)culture CLAs in  
 230 vitro (Davis et al. 2008; Fujiwara et al. 2018; Ha et al. 2019; Parker et al. 2014; Sechler et al.

231 2009). Moreover, the use of microfluidic chambers mimicking the plant phloem system may allow  
232 optimizing the culturing conditions for this phloem-limited pathogen (Jagoueix et al. 1994;  
233 Naranjo et al. 2020). Finally, the use of the non-culturing systems is based on the intracellular  
234 nature of CLAs, which is obligatory in planta but transitory in ACPs (Ghanim et al. 2017; Merfa et  
235 al. 2019). Although these systems do not constitute an actual culture, since they are ex vivo  
236 systems, they are valuable resources to grow CLAs and study this bacterium.

237 We would like to note that there are other culturing systems being used to grow hitherto  
238 unculturable bacteria from different environmental sources. However, since these systems have  
239 not been tested so far with CLAs, they are not the focus of this review. These systems include, but  
240 are not limited to: (i) growing marine bacteria in microtiter plates using extinction culturing with  
241 in situ concentrations of substrate, coupled to sensitive detection methods of microbes to assess  
242 growth and determine microbial diversity (Connon and Giovannoni 2002); (ii) establishing pure  
243 cultures of marine bacteria by encapsulating cells in gel microdroplets, which allows parallel  
244 microbial culturing in low nutrient flux conditions (Zengler et al. 2002); (iii) growing previously  
245 uncultured microorganisms by encapsulating them in polysulfone-coated agar spheres and  
246 incubating in simulated or natural environments (Ben-Dov et al. 2009); and (iv) culturing bacteria  
247 using a device, called I-tip, which allows cells and natural chemical compounds to diffuse into it  
248 and promote bacterial growth (Jung et al. 2014). For a more thorough review of different culturing  
249 systems, we suggest referring to other reviews published elsewhere (Lagier et al. 2015a; Lewis et  
250 al. 2020; Overmann et al. 2017).

251

## 252 **CLAs culturing studies**

253 With all the technical definitions and culturing systems detailed above, following we will  
254 assess the contribution of each CLAs culturing study to reach the ultimate long-sought goal of  
255 obtaining an axenic culture of CLAs. These studies will be detailed here separated by culturing and  
256 non-culturing systems.

257

258 **Culturing systems.** To our knowledge, the first report on CLAs culturing was published in  
259 2008. In that study, CLAs was co-cultured with an accidental skin commensal contaminant  
260 Actinobacteria commonly inhabiting citrus and ACPs, *Propionibacterium acnes*. The CLAs/*P.*  
261 *acnes* co-culture was able to survive multiple passages. However, attempts to purify CLAs in

262 axenic cultures were non successful. The authors concluded that the relationship among CLAs and  
263 *P. acnes* was mutually beneficial, in which CLAs would likely obtain nutrients and/or chemical  
264 signals, thus not allowing its axenic growth (Davis et al. 2008). Shortly after, Sechler and  
265 collaborators claimed in 2009 to successfully culture all three ‘*Ca. Liberibacter* spp.’ suspected as  
266 causal agents of HLB in axenic conditions (CLAs, CLam and CLaf). A culture medium mainly  
267 composed of citrus vein extract, and named Liber A, was able to maintain bacterial growth for four  
268 to five passages before viability started to decline. In addition, two isolates of CLAs and one of  
269 CLam cultured in this system displayed pathogenicity on citrus plants and were isolated from  
270 noninoculated tissues of inoculated plants. The authors thus declared a partial fulfillment of Koch’s  
271 postulates (Sechler et al. 2009). However, results of this study are controversial, since no other  
272 research lab could reproduce these cultures and no follow-up studies have even been published by  
273 the authors. A few years later, Parker and collaborators (2014) used a similar approach of  
274 mimicking the natural environment of CLAs to culture this bacterium. They used culture media  
275 containing commercial grapefruit juice and were able to maintain viable CLAs co-cultures in vitro  
276 with other microflora from grapefruit seeds (source of CLAs inoculum of that study) for several  
277 months in biofilm. The growth pattern of CLAs resembled cryptic growth over time, with  
278 oscillations in the population numbers. This indicates that the persisting population of CLAs could  
279 partially grow using the content of dead cells (nutrients and/or signaling components) as growth  
280 stimulators, in an ongoing cycle of growth followed by death and release of nutrients. Thus, since  
281 there was no continuous nor axenic CLAs growth, no bona fide culturing of this bacterium was  
282 claimed (Parker et al. 2014). For some years, these three reports were the main studies to contribute  
283 for achieving the goal of culturing CLAs. However, new studies and strategies have been reported  
284 on recent years.

285         In 2018, co-culturing of the Japanese CLAs strain Ishi-1, which bears no phages in its  
286 genome, was reported in association with phloem-associated microbiota (Fujiwara et al. 2018).  
287 The population of CLAs was followed over time by quantifying DNA through qPCR, however with  
288 no direct quantification of viability. Moreover, distinctive colonies of CLAs were not present in  
289 agar plates, but few cells were found under microscopic investigations. The presence of the phloem  
290 microflora was deemed as essential for CLAs growth, since suppression of certain bacterial families  
291 by antibiotics decreased CLAs survival. Furthermore, CLAs was resistant to oxytetracycline and  
292 multiple other antibiotics (Fujiwara et al. 2018), contradicting previous reports (Zhang et al. 2014).

293 Therefore, reproducibility of these findings by other research groups is still needed, as we have  
294 already noted elsewhere (Merfa et al. 2019). Another report of CLAs being grown in co-culture  
295 with citrus-associated microflora has been published recently in 2019 (Ha et al. 2019). By  
296 developing a novel culture medium based on BM7 medium, which is used to culture *Liberibacter*  
297 *crescens*, the only culturable species of the *Liberibacter* genus (Fagen et al. 2014a; Leonard et al.  
298 2012), a long-term co-culture of CLAs was established, with many successful sub-cultures, using  
299 a membrane biofilm reactor system. The authors argue that the long-term growth of CLAs in their  
300 system is an advantage to the other studies on CLAs culturing. However, even though that is an  
301 important contribution of their study, the number of CLAs cells assessed by qPCR as genome  
302 equivalents only reached the order of  $10^3$  cells per mL of culture (Ha et al. 2019). This constitutes  
303 only a minor fraction of the entire biofilm population and is likely a bottleneck to study CLAs using  
304 this system. By comparison, Parker and collaborators (2014) obtained CLAs titers in their culturing  
305 system in the order of  $10^5$  to  $10^6$  cells per mL of culture. In addition, Ha and collaborators (2019)  
306 erroneously employ the term “axenic” to describe their cultures. As defined in this review, axenic  
307 means culturing a single clonal population of an isolate or strain. Therefore, the culture described  
308 by them is not axenic. Finally, a host-free co-culture of CLAs with ACP-associated microbiota has  
309 been established recently using the same culture medium developed by Ha and collaborators  
310 (2019). In this study, mixed cultures of CLAs were treated with different antibiotics, which were  
311 previously shown to not affect CLAs in ex vivo assays (Zhang et al. 2014), to alter their  
312 composition (Molki et al. 2020). Authors were able to show that the presence of bacteria from the  
313 *Pseudomonadaceae* family has a positive correlation with CLAs growth, while an abundance of  
314 *Bacillus aureus* decreased the CLAs population to below the detection limit. The study thus  
315 suggests that enriching *Bacillaceae* within CLAs-infected trees could possibly be a biocontrol  
316 strategy for HLB, which is currently being addressed by them (Molki et al. 2020).

317 Unfortunately, no follow-up studies have been published to date using any of these  
318 culturing systems described here. This shows their practical limitation to study the cellular and  
319 molecular features of CLAs. However, they remain as seminal contributions to the effort of  
320 axenically culturing this organism. These studies have shown that CLAs may grow in vitro outside  
321 either its plant or insect hosts, and more remarkably, that CLAs usually required the host’s  
322 microbiota to grow, since most studies reported co-cultures of this bacterium. This is an usual  
323 particular feature of intracellular pathogens with reduced genomes, since they rely on the

324 ecological services provided by the host and associated microbiota to grow, while scavenging for  
325 nutrients and energy (Merfa et al. 2019).

326

327 **Non-culturing systems.** The first report of a non-culturing system to grow CLas cells is a  
328 patent application in the U.S. published in 2017 (Mandadi et al. 2017). In that application, the  
329 authors describe the induction of hairy roots by *Rhizobium rhizogenes* in plants already infected  
330 by CLAs. These roots can then be propagated and inoculated into test media to evaluate CLas  
331 growth in planta over time in different conditions by DNA quantification through qPCR. In 2020,  
332 Zuñiga and collaborators used this system to validate their predictions about nutrient requirements  
333 of CLAs, which were obtained through a genome-scale metabolic model of this bacterium. Among  
334 the metabolic model predictions and validation using the hairy root system, the authors conclude  
335 that CLas requires essential compounds from their hosts to survive, including aromatic amino  
336 acids, vitamins, saccharides and fatty acids (Zuñiga et al. 2020). However, both of these studies  
337 (the patent and the manuscript) call the hairy root system a culture of CLAs, even though the  
338 manuscript clearly states that this is an ex vivo system (Mandadi et al. 2017; Zuñiga et al. 2020).  
339 Based on the definitions presented in this review, we propose that a better definition would be  
340 calling hairy roots an ex vivo system to grow CLas cells in planta, similar to what has been done  
341 previously (Yang et al. 2018), but enabling higher throughput assessments. In addition, neither of  
342 these publications show the titer that CLas has reached in hairy roots. Zuñiga and collaborators  
343 (2020) present their results as relative growth rate of CLAs. However, the lack of data concerning  
344 uniformity of infection of root cells and actual number of CLas cells, as assessed by qPCR,  
345 precludes the analysis of how feasible this system is to grow this bacterium. Nevertheless, a recent  
346 published study has shown the applicability of the hairy root system to establish a relatively fast  
347 high throughput screening method of antimicrobials against '*Ca. Liberibacter spp.*'. By using this  
348 system, authors were able to determine a range of antimicrobial peptides and chemicals that inhibit  
349 CLAs, and thus have the potential to be used as therapies to control HLB (Irigoyen et al. 2020).

350 Finally, another ex vivo assay to grow CLas was described in 2020 using leaf discs from  
351 infected citrus plants (Attaran et al. 2020). In this system, leaf discs are inoculated into test media  
352 to assess CLas growth in planta over time in different physicochemical and nutritional conditions  
353 by DNA quantification through qPCR. As the main findings, the authors observed CLas growth in  
354 the presence of glucose only when grown in microaerophilic conditions (10% O<sub>2</sub>), while the

355 presence of the antibiotic amikacin further increased CLas growth (Attaran et al. 2020). The  
356 authors suggest that glucose may be either used directly by CLas or after glucose oxidation by the  
357 leaf tissue, through ATP uptake from the plant host by the bacterium. Additionally, the authors  
358 argue that, although CLas lacks the enzyme glucose 6-phosphate isomerase (PGI) of the glycolytic  
359 pathway, it could reroute its metabolism to generate glyceraldehyde-3-phosphate through the  
360 pentose phosphate pathway (PPP), which would then allow production of pyruvate from glucose  
361 (Attaran et al. 2020). However, in addition to the probable absence of the enzyme transaldolase of  
362 the PPP (Fagen et al. 2014b), another study has noted that, in combination to an incomplete  
363 glycolytic pathway, CLas also lacks a methylglyoxal detoxification system to eliminate this  
364 cytotoxic byproduct of glycolysis (Jain et al. 2017). Thus, it is more likely that CLas uses the  
365 metabolic products of glucose metabolism performed by the host, possibly by directly importing  
366 ATP from its hosts through its ATP/ADP translocase (Jain et al. 2017; Vahling et al. 2010). In  
367 addition, the authors provide the results of CLas growth in leaf discs only after three days of  
368 incubation (Attaran et al. 2020). It would be useful to know how the bacterium behaves during a  
369 longer time of growth and why this fastidious prokaryote presented optimal growth in such a short  
370 time. To conclude, similarly to the hairy root system (Mandadi et al. 2017; Zuñiga et al. 2020), the  
371 authors also call the leaf disc system a culture of CLas (Attaran et al. 2020), which we again  
372 propose that it would be more suitable to classify it as an ex vivo system to grow CLas cells in  
373 planta.

374         These non-culturing systems described here have the potential to be powerful tools to  
375 assess the CLas response to different conditions in planta. Although they may be similar to keeping  
376 CLas-infected plants in greenhouse conditions to perform a range of assays (Yang et al. 2018),  
377 they possibly represent a faster screening method to assess this bacterium. Additionally, they are  
378 focused on analyzing the bacterium itself, and not the plant host. However, although they are well-  
379 suited to evaluate CLas in planta, the analysis of compounds required for CLas growth is masked,  
380 since it is not possible to determine whether CLas may use the provided nutrients directly or after  
381 they are metabolized by the plant host and/or associated microbiota. To sum up, we reinforce that  
382 these systems do not fit the culture definition presented here, since growth of CLas occurs in planta  
383 using ex vivo tissues and may not directly rely on the culture medium to multiply and survive.

384

### 385 **Final considerations**

386           Although an axenic culture of CLAs has not been established to date, researchers have been  
387 using different methods to study the features of this bacterium and its interactions with its insect  
388 and plant hosts. Some of these strategies include the employment of genomic tools, performance  
389 of in vivo assays with plant hosts and ACP, and use of surrogate bacteria, including *Sinorhizobium*  
390 *meliloti* and *Agrobacterium tumefaciens*, which are also bacteria of the Rhizobiaceae family  
391 (Andrade and Wang 2019; Vahling-Armstrong et al. 2012), and *L. crescens*, that has the highest  
392 genome synteny with CLAs (Jain et al. 2019). A few of the studies made possible by using these  
393 methods include: (i) antimicrobial screening of CLAs in planta (Yang et al. 2018; Zhang et al.  
394 2014); (ii) assessment of the colonization patterns of CLAs in plant and insect hosts (Achor et al.  
395 2020; Ghanim et al. 2017); (iii) determination of the energetic requirements of CLAs (Jain et al.  
396 2017); (iv) performance of indirect functional genomics using surrogates (Andrade and Wang  
397 2019; Vahling-Armstrong et al. 2012); and (v) analysis of pathogenicity factors (Clark et al. 2018;  
398 Jain et al. 2018).

399           However, lack of axenic culture still precludes many studies to be performed with CLAs  
400 itself, and many hypotheses cannot be tested directly with this bacterium. Thus, the key question  
401 that we had in this review was: are researchers culturing CLAs already? Considering the literal  
402 definition of culture presented here, the answer is yes, but only as co-cultures. Some studies have  
403 shown growth of CLAs in vitro with only the aid of the nutrients present in the culture medium and  
404 of the incubation conditions, which means without the presence of any host, either plant or insect.  
405 However, another question arises: are these CLAs culturing systems entirely suitable for answering  
406 standing questions on CLAs biology? This time, the answer is no. An axenic culture of CLAs has  
407 yet to be available and current culturing systems do not allow continuous growth of this bacterium  
408 (Davis et al. 2008; Parker et al. 2014). In addition, subsequent transfers of CLAs cannot either be  
409 performed, or its titer only reaches a small proportion of the entire microbial population (Ha et al.  
410 2019). Hence, with so many different strategies to culture CLAs being reported and different  
411 concepts being presented, we hope that this review may help authors to standardize the  
412 terminology used in their publications on CLAs culturing. We strive to avoid misunderstandings  
413 of the audience and/or disseminate erroneous concepts about CLAs cultures and axenic culturing.  
414 Ideally, an axenic culture would be able to grow to high titers in both solid and liquid medium, be  
415 pathogenic to citrus plants, amenable to store as glycerol stocks at -80°C and survive indefinite  
416 sub-cultures.



417

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423

424 **References**

- 425 Achor D, Welker S, Ben-Mahmoud S, Wang C, Folimonova SY, Dutt M, Gowda S, Levy A. 2020.  
426 Dynamics of *Candidatus Liberibacter asiaticus* movement and sieve-pore plugging in  
427 citrus sink cells. *Plant Physiol.* 182(2):882-891.
- 428 Andrade M, Wang N. 2019. The Tad pilus apparatus of '*Candidatus Liberibacter asiaticus*' and its  
429 regulation by VisNR. *Mol Plant Microbe Interact.* 32(9):1175-1187.
- 430 Anjum MN. 2015. Screening methods for the detection of antimicrobial resistance genes present  
431 in bacterial isolates and the microbiota. *Future Microbiol.* 10(317-320).
- 432 Attaran E, Berim A, Killiny N, Beyenal H, Gang DR, Omsland A. 2020. Controlled replication of  
433 '*Candidatus Liberibacter asiaticus*' DNA in citrus leaf discs. *Microb Biotechnol.* 13(3):747-  
434 759.
- 435 Austin B. 2017. The value of cultures to modern microbiology. *Antonie Van Leeuwenhoek.*  
436 110(10):1247-1256.
- 437 Baccari C, Antonova E, Lindow S. 2019. Biological control of Pierce's disease of grape by an  
438 endophytic bacterium. *Phytopathology.* 109(2):248-256.
- 439 Balouiri M, Sadiki M, Ibsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity:  
440 A review. *J Pharm Anal.* 6(2):71-79.
- 441 Ben-Dov E, Kramarsky-Winter E, Kushmaro A. 2009. An in situ method for cultivating  
442 microorganisms using a double encapsulation technique. *FEMS Microbiol Ecol.*  
443 68(3):363-371.
- 444 Bonnet M, Lagier JC, Raoult D, Khelaifia S. 2020. Bacterial culture through selective and non-  
445 selective conditions: The evolution of culture media in clinical microbiology. *New*  
446 *Microbes New Infect.* 34:100622.
- 447 Bové JM. 2006. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus.  
448 *Journal of Plant Pathology.* 88:7-37.
- 449 Bové JM. 2014. Huanglongbing or yellow shoot, a disease of Gondwanan origin: Will it destroy  
450 citrus worldwide? *Phytoparasitica.* 42(5):579-583.
- 451 Bové JM, Renaudin J, Saillard C, Foissac X, Garnier M. 2003. *Spiroplasma citri*, a plant  
452 pathogenic mollicute: Relationships with its two hosts, the plant and the leafhopper vector.  
453 *Annu Rev Phytopathol.* 41:483-500.
- 454 Chatterjee S, Almeida RPP, Lindow S. 2008. Living in two worlds: The plant and insect lifestyles  
455 of *Xylella fastidiosa*. *Annu Rev Phytopathol.* 46:243-271.
- 456 Chen H, Kandel PP, Cruz LF, Cobine PA, De La Fuente L. 2017. The major outer membrane  
457 protein MopB is required for twitching movement and affects biofilm formation and  
458 virulence in two *Xylella fastidiosa* strains. *Mol Plant Microbe Interact.* 30(11):896-905.

- 459 Clark K, Franco JY, Schwizer S, Pang Z, Hawara E, Liebrand TWH, Pagliaccia D, Zeng L, Gurung  
460 FB, Wang P et al. 2018. An effector from the Huanglongbing-associated pathogen targets  
461 citrus proteases. *Nat Commun.* 9(1):1718.
- 462 Connon SA, Giovannoni SJ. 2002. High-throughput methods for culturing microorganisms in  
463 very-low-nutrient media yield diverse new marine isolates. *Appl Environ Microbiol.*  
464 68(8):3878-3885.
- 465 Curtis TP, Sloan WT, Scannell JW. 2002. Estimating prokaryotic diversity and its limits. *Proc Natl*  
466 *Acad Sci U S A.* 99(16):10494-10499.
- 467 Davis MJ, Mondal SN, Chen H, Rogers ME, Brlansky RH. 2008. Co-cultivation of ‘*Candidatus*  
468 *Liberibacter asiaticus*’ with Actinobacteria from citrus with Huanglongbing. *Plant Dis.*  
469 92:1547-1550.
- 470 Davis MJ, Purcell AH, Thomson SV. 1978. Pierce’s disease of grapevines: Isolation of the causal  
471 bacterium. *Science.* 199:75-77.
- 472 Davis RE, Shao J, Zhao Y, Gasparich GE, Gaynor BJ, Donofrio N. 2017. Complete genome  
473 sequence of *Spiroplasma citri* strain R8-A2(t), causal agent of stubborn disease in *Citrus*  
474 species. *Genome Announc.* 5(16).
- 475 De La Fuente L, Montanes E, Meng Y, Li Y, Burr TJ, Hoch HC, Wu M. 2007. Assessing adhesion  
476 forces of type I and type IV pili of *Xylella fastidiosa* bacteria by use of a microfluidic flow  
477 chamber. *Appl Environ Microbiol.* 73(8):2690-2696.
- 478 Fagen JR, Leonard MT, Coyle JF, McCullough CM, Davis-Richardson AG, Davis MJ, Triplett  
479 EW. 2014a. *Liberibacter crescens* gen. nov., sp. nov., the first cultured member of the  
480 genus *Liberibacter*. *Int J Syst Evol Microbiol.* 64:2461-2466.
- 481 Fagen JR, Leonard MT, McCullough CM, Edirisinghe JN, Henry CS, Davis MJ, Triplett EW.  
482 2014b. Comparative genomics of cultured and uncultured strains suggests genes essential  
483 for free-living growth of *Liberibacter*. *PLoS One.* 9(1):e84469.
- 484 Fos A, Bové JM, Lallemand J, Saillard C, Vignault JC, Ali Y, Brun P, Vogel R. 1986. The  
485 leafhopper *Neotalitrus haematoceps* is a vector of *Spiroplasma citri* in the Mediterranean  
486 area. *Ann Inst Pasteur Microbiol.* 137A:97-107.
- 487 Francis MI, Peña A, Graham JH. 2010. Detached leaf inoculation of germplasm for rapid screening  
488 of resistance to citrus canker and citrus bacterial spot. *European Journal of Plant Pathology.*  
489 127(4):571-578.
- 490 Fujiwara K, Iwanami T, Fujikawa T. 2018. Alterations of *Candidatus Liberibacter asiaticus*-  
491 associated microbiota decrease survival of *Ca. L. asiaticus* in in vitro assays. *Front*  
492 *Microbiol.* 9:3089.
- 493 Ghanim M, Achor D, Ghosh S, Kotsedalov S, Lebedev G, Levy A. 2017. ‘*Candidatus*  
494 *Liberibacter asiaticus*’ accumulates inside endoplasmic reticulum associated vacuoles in  
495 the gut cells of *Diaphorina citri*. *Sci Rep.* 7(1):16945.
- 496 Goers L, Freemont P, Polizzi KM. 2014. Co-culture systems and technologies: Taking synthetic  
497 biology to the next level. *J R Soc Interface.* 11(96):20140065.
- 498 Gottwald TR. 2010. Current epidemiological understanding of citrus Huanglongbing. *Annu Rev*  
499 *Phytopathol.* 48:119-139.
- 500 Ha PT, He R, Killiny N, Brown JK, Omsland A, Gang DR, Beyenal H. 2019. Host-free biofilm  
501 culture of “*Candidatus Liberibacter asiaticus*,” the bacterium associated with  
502 Huanglongbing. *Biofilm.* 1:100005.

- 503 Hashsham S. 2007. Culture techniques. In: Reddy C, Beveridge T, Breznak J, Marzluf G, Schmidt  
504 T, Snyder L, editors. Methods for general and molecular microbiology. Third edition ed.  
505 Washington: ASM Press. p. 270-285.
- 506 EFSA Panel on Plant Health. 2016. Treatment solutions to cure *Xylella fastidiosa* diseased plants.  
507 EFSA Journal. 14(4):e04456.
- 508 Hopkins DL, Purcell AH. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and  
509 other emergent diseases. Plant Dis. 86:1056-1066.
- 510 Irigoyen S, Ramasamy M, Pant S, Niraula P, Bedre R, Gurung M, Rossi D, Laughlin C, Gorman  
511 Z, Achor D et al. 2020. Plant hairy roots enable high throughput identification of  
512 antimicrobials against *Candidatus Liberibacter* spp. Nat Commun. 11(1):5802.
- 513 Jagoueix S, Bové JM, Garnier M. 1994. The phloem-limited bacterium of greening disease of  
514 citrus is a member of the alpha subdivision of the Proteobacteria. Int J Syst Bacteriol.  
515 44:379-386.
- 516 Jain M, Cai L, Fleites LA, Munoz-Bodnar A, Davis MJ, Gabriel DW. 2019. *Liberibacter crescens*  
517 is a cultured surrogate for functional genomics of uncultured pathogenic '*Candidatus*  
518 *Liberibacter*' spp. and is naturally competent for transformation. Phytopathology.  
519 109(10):1811-1819.
- 520 Jain M, Munoz-Bodnar A, Gabriel DW. 2017. Concomitant loss of the glyoxalase system and  
521 glycolysis makes the uncultured pathogen "*Candidatus Liberibacter asiaticus*" an energy  
522 scavenger. Appl Environ Microbiol. 83:e01670-01617.
- 523 Jain M, Munoz-Bodnar A, Zhang S, Gabriel DW. 2018. A secreted '*Candidatus Liberibacter*  
524 *asiaticus*' peroxiredoxin simultaneously suppresses both localized and systemic innate  
525 immune responses in planta. Mol Plant Microbe Interact. 31(12):1312-1322.
- 526 Jung D, Seo EY, Epstein SS, Joung Y, Han J, Parfenova VV, Belykh OI, Gladkikh AS, Ahn TS.  
527 2014. Application of a new cultivation technology, I-tip, for studying microbial diversity  
528 in freshwater sponges of Lake Baikal, Russia. FEMS Microbiol Ecol. 90(2):417-423.
- 529 Kandel PP, Almeida RPP, Cobine PA, De La Fuente L. 2017. Natural competence rates are  
530 variable among *Xylella fastidiosa* strains and homologous recombination occurs in vitro  
531 between subspecies *fastidiosa* and *multiplex*. Mol Plant Microbe Interact. 30(7):589-600.
- 532 Kandel PP, Chen H, De La Fuente L. 2018. A short protocol for gene knockout and  
533 complementation in *Xylella fastidiosa* shows that one of the type IV pilin paralogs  
534 (PD1926) is needed for twitching while another (PD1924) affects pilus number and  
535 location. Appl Environ Microbiol. 84:e01167-01118.
- 536 Kruse A, Fleites LA, Heck M. 2019. Lessons from one fastidious bacterium to another: What can  
537 we learn about *Liberibacter* species from *Xylella fastidiosa*. Insects. 10(9).
- 538 Kuzina LV, Miller TA, Cooksey DA. 2006. In vitro activities of antibiotics and antimicrobial  
539 peptides against the plant pathogenic bacterium *Xylella fastidiosa*. Lett Appl Microbiol.  
540 42(5):514-520.
- 541 Lagier JC, Edouard S, Pagnier I, Mediannikov O, Drancourt M, Raoult D. 2015a. Current and past  
542 strategies for bacterial culture in clinical microbiology. Clin Microbiol Rev. 28(1):208-  
543 236.
- 544 Lagier JC, Hugon P, Khelaifia S, Fournier PE, La Scola B, Raoult D. 2015b. The rebirth of culture  
545 in microbiology through the example of culturomics to study human gut microbiota. Clin  
546 Microbiol Rev. 28(1):237-264.
- 547 Leonard MT, Fagen JR, Davis-Richardson AG, Davis MJ, Triplett EW. 2012. Complete genome  
548 sequence of *Liberibacter crescens* BT-1. Stand Genomic Sci. 7(2):271-283.

- 549 Lewis WH, Tahon G, Geesink P, Sousa DZ, Ettema TJG. 2020. Innovations to culturing the  
550 uncultured microbial majority. *Nat Rev Microbiol*.
- 551 Liu HY, Gumpf DJ, Oldfield GN, Calavan EC. 1983. Transmission of *Spiroplasma citri* by  
552 *Circulifer tenellus*. *Phytopathology*. 73:582-585.
- 553 Madigan MT, Martinko JM, Bender KS, Buckley DH, Stahl DA. 2014. Brock biology of  
554 microorganisms. Churchman K, editor. New York: Pearson.
- 555 Mandadi KK, Irigoyen SC, Mirkov TE. 2017. Methods, compositions, and systems for culturing  
556 and characterizing fastidious plant microbes. *US Pat Appl*. 15:1-70.
- 557 Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier V, Beer  
558 SV, Machado MA et al. 2012. Top 10 plant pathogenic bacteria in molecular plant  
559 pathology. *Mol Plant Pathol*. 13(6):614-629.
- 560 Martin E, Hine R. 2008. A dictionary of biology. Oxford: Oxford University Press.
- 561 Meng Y, Li Y, Galvani CD, Hao G, Turner JN, Burr TJ, Hoch HC. 2005. Upstream migration of  
562 *Xylella fastidiosa* via pilus-driven twitching motility. *J Bacteriol*. 187(16):5560-5567.
- 563 Merfa MV, Niza B, Takita MA, De Souza AA. 2016. The MqsRA toxin-antitoxin system from  
564 *Xylella fastidiosa* plays a key role in bacterial fitness, pathogenicity, and persister cell  
565 formation. *Front Microbiol*. 7:904.
- 566 Merfa MV, Pérez-López E, Naranjo E, Jain M, Gabriel DW, De La Fuente L. 2019. Progress and  
567 obstacles in culturing '*Candidatus Liberibacter asiaticus*', the bacterium associated with  
568 Huanglongbing. *Phytopathology*. 109(7):1092-1101.
- 569 Molki B, Call DR, Ha PT, Omsland A, Gang DR, Lindemann SR, Killiny N, Beyenal H. 2020.  
570 Growth of '*Candidatus Liberibacter asiaticus*' in a host-free microbial culture is associated  
571 with microbial community composition. *Enzyme Microb Technol*. 142:109691.
- 572 Muranaka LS, Giorgiano TE, Takita MA, Forim MR, Silva LFC, Coletta-Filho HD, Machado MA,  
573 de Souza AA. 2013. N-acetylcysteine in agriculture, a novel use for an old molecule: Focus  
574 on controlling the plant-pathogen *Xylella fastidiosa*. *PLoS One*. 8(8):e72937.
- 575 Naranjo E, Merfa MV, Ferreira V, Jain M, Davis MJ, Bahar O, Gabriel DW, De La Fuente L.  
576 2019. *Liberibacter crescens* biofilm formation in vitro: Establishment of a model system  
577 for pathogenic '*Candidatus Liberibacter spp.*'. *Sci Rep*. 9(1):5150.
- 578 Naranjo E, Merfa MV, Santra S, Ozcan A, Johnson E, Cobine PA, De La Fuente L. 2020. Zinkicide  
579 is a ZnO-based nanoformulation with bactericidal activity against *Liberibacter crescens* in  
580 batch cultures and in microfluidic chambers simulating plant vascular systems. *Appl  
581 Environ Microbiol*. 86:e00788-00720.
- 582 Nascimento R, Gouran H, Chakraborty S, Gillespie HW, Almeida-Souza HO, Tu A, Rao BJ,  
583 Feldstein PA, Bruening G, Goulart LR et al. 2016. The type II secreted lipase/esterase LesA  
584 is a key virulence factor required for *Xylella fastidiosa* pathogenesis in grapevines. *Sci Rep*.  
585 6:18598.
- 586 Nelson WR, Munyaneza JE, McCue KF, Bové JM. 2013. The Pangaeian origin of '*Candidatus  
587 Liberibacter*' species. *Journal of Plant Pathology*. 95:455-461.
- 588 Nelson WR, Sengoda VG, Alfaro-Fernandez AO, Font MI, Crosslin JM, Munyaneza JE. 2012. A  
589 new haplotype of "*Candidatus Liberibacter solanacearum*" identified in the Mediterranean  
590 region. *European Journal of Plant Pathology*. 135(4):633-639.
- 591 Newman KL, Almeida RPP, Purcell AH, Lindow SE. 2003. Use of a green fluorescent strain for  
592 analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. *Appl Environ Microbiol*.  
593 69(12):7319-7327.

- 594 Niza B, Coletta-Filho HD, Merfa MV, Takita MA, de Souza AA. 2015. Differential colonization  
595 patterns of *Xylella fastidiosa* infecting citrus genotypes. *Plant Pathology*. 64(6):1259-1269.
- 596 Overmann J, Abt B, Sikorski J. 2017. Present and future of culturing bacteria. *Annu Rev*  
597 *Microbiol*. 71:711-730.
- 598 Parker JK, Wisotsky SR, Johnson EG, Hijaz FM, Killiny N, Hilf ME, De La Fuente L. 2014.  
599 Viability of ‘*Candidatus Liberibacter asiaticus*’ prolonged by addition of citrus juice to  
600 culture medium. *Phytopathology*. 104:15-26.
- 601 Parte AC, Sardà Carbasse J, Meier-Kolthoff JP, Reimer LC, Göker M. 2020. List of prokaryotic  
602 names with standing in nomenclature (LPSN) moves to the DSMZ. *Int J Syst Evol*  
603 *Microbiol*.
- 604 Pinevich AV, Andronov EE, Pershina EV, Pinevich AA, Dmitrieva HY. 2018. Testing culture  
605 purity in prokaryotes: Criteria and challenges. *Antonie Van Leeuwenhoek*. 111(9):1509-  
606 1521.
- 607 Potnis N, Kandel PP, Merfa MV, Retchless AC, Parker JK, Stenger DC, Almeida RPP, Bergsma-  
608 Vlami M, Westenberg M, Cobine PA et al. 2019. Patterns of inter- and intrasubspecific  
609 homologous recombination inform eco-evolutionary dynamics of *Xylella fastidiosa*. *ISME*  
610 *J*. 13(9):2319-2333.
- 611 Randhawa PS, Civerolo EL. 1985. A detached-leaf bioassay for *Xanthomonas campestris* pv.  
612 *pruni*. *Phytopathology*. 75:1060-1063.
- 613 Saglio P, Laflèche D, Bonissol C, Bové JM. 1971. Isolement et culture in vitro des mycoplasmes  
614 associés au stubborn des agrumes et leur observation au microscope électronique. *C R*  
615 *Acad Sci Paris*. 272:1387-1390.
- 616 Saponari M, Boscia D, Nigro F, Martelli GP. 2013. Identification of DNA sequences related to  
617 *Xylella fastidiosa* in oleander, almond and olive trees exhibiting leaf scorch symptoms in  
618 Apulia (southern Italy). *J Plant Pathol*. 95:668.
- 619 Sechler A, Schuenzel EL, Cooke P, Donnua S, Thaveechai N, Postnikova E, Stone AL, Schneider  
620 WL, Damsteegt VD, Schaad NW. 2009. Cultivation of ‘*Candidatus Liberibacter asiaticus*’,  
621 ‘*Ca. L. africanus*’, and ‘*Ca. L. americanus*’ associated with Huanglongbing.  
622 *Phytopathology*. 99:480-486.
- 623 Shi J, Pagliaccia D, Morgan R, Qiao Y, Pan S, Vidalakis G, Ma W. 2014. Novel diagnosis for  
624 citrus stubborn disease by detection of a *Spiroplasma citri*-secreted protein.  
625 *Phytopathology*. 104:188-195.
- 626 Shrestha PM, Nevin KP, Shrestha M, Lovley DR. 2013. When is a microbial culture "pure"?  
627 Persistent cryptic contaminant escapes detection even with deep genome sequencing.  
628 *mBio*. 4(2):e00591-00512.
- 629 Simpson AJG, Reinach FC, Arruda P, Abreu FA, Acencio M, Alvarenga R, Alves LMC, Araya  
630 JE, Baia GS, Baptista CS et al. 2000. The genome sequence of the plant pathogen *Xylella*  
631 *fastidiosa*. *Nature*. 406(6792):151-157.
- 632 Slonczewski JL, Foster JW. 2016. *Microbiology: An evolving science*. Twitchell B, editor. New  
633 York: W. W. Norton & Company.
- 634 Staley JT, Konopka A. 1985. Measurement of in situ activities of nonphotosynthetic  
635 microorganisms in aquatic and terrestrial habitats. *Annu Rev Microbiol*. 39:321-346.
- 636 Tanaka Y, Benno Y. 2015. Application of a single-colony coculture technique to the isolation of  
637 hitherto unculturable gut bacteria. *Microbiol Immunol*. 59(2):63-70.

- 638 Thompson S, Fletcher JD, Ziebell H, Beard S, Panda P, Jorgensen N, Fowler SV, Liefting LW,  
639 Berry N, Pitman AR. 2013. First report of '*Candidatus Liberibacter europaeus*' associated  
640 with psyllid infested scotch broom. *New Dis Rep.* 27:6.
- 641 Tortora GJ, Funke BR, Case CL. 2019. *Microbiology: An introduction.* Beauparlant S, editor.  
642 Boston: Pearson.
- 643 Vahling CM, Duan Y, Lin H. 2010. Characterization of an ATP translocase identified in the  
644 destructive plant pathogen "*Candidatus Liberibacter asiaticus*". *J Bacteriol.* 192(3):834-  
645 840.
- 646 Vahling-Armstrong CM, Zhou H, Benyon L, Morgan JK, Duan Y. 2012. Two plant bacteria, *S.*  
647 *meliloti* and *Ca. Liberibacter asiaticus*, share functional znuABC homologues that encode  
648 for a high affinity zinc uptake system. *PLoS One.* 7(5):e37340.
- 649 Vanhove M, Retchless AC, Sicard A, Rieux A, Coletta-Filho HD, De La Fuente L, Stenger DC,  
650 Almeida RPP. 2019. Genomic diversity and recombination among *Xylella fastidiosa*  
651 subspecies. *Appl Environ Microbiol.* 85:e02972-02918.
- 652 Wang N, Trivedi P. 2013. Citrus Huanglongbing: A newly relevant disease presents unprecedented  
653 challenges. *Phytopathology.* 103:652-665.
- 654 Yang C, Zhong Y, Powell CA, Doud MS, Duan Y, Huang Y, Zhang M. 2018. Antimicrobial  
655 compounds effective against *Candidatus Liberibacter asiaticus* discovered via graft-based  
656 assay in citrus. *Sci Rep.* 8(1):17288.
- 657 Zengler K, Toledo G, Rappe M, Elkins J, Mathur EJ, Short JM, Keller M. 2002. Cultivating the  
658 uncultured. *Proc Natl Acad Sci U S A.* 99(24):15681-15686.
- 659 Zhang M, Guo Y, Powell CA, Doud MS, Yang C, Duan Y. 2014. Effective antibiotics against  
660 '*Candidatus Liberibacter asiaticus*' in HLB-affected citrus plants identified via the graft-  
661 based evaluation. *PLoS One.* 9(11):e111032.
- 662 Zuñiga C, Peacock B, Liang B, McCollum G, Irigoyen SC, Tec-Campos D, Marotz C, Weng NC,  
663 Zepeda A, Vidalakis G et al. 2020. Linking metabolic phenotypes to pathogenic traits  
664 among "*Candidatus Liberibacter asiaticus*" and its hosts. *NPJ Syst Biol Appl.* 6(1):24.  
665