A new method for early detection of latent infection by ‘Candidatus Liberibacter asiaticus’ in citrus trees [version 1; peer review: 1 approved, 1 approved with reservations]

Kazuki Fujiwara1, Kenta Tomimura2, Toru Iwanami3, Takashi Fujikawa4

1Kyushu Okinawa Agricultural Research Center, National Agriculture and Food Research Organization (NARO), Koshi, Kumamoto, 861-1192, Japan
2Department of Citrus Research, Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization (NARO), Shimizu, Shizuoka, 424-0292, Japan
3Department of Agriculture, Faculty of Agriculture, Tokyo University of Agriculture, Atsugi, Kanagawa, 243-0034, Japan
4Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki, 305-8605, Japan

Abstract

Background: ‘Candidatus Liberibacter asiaticus’ (CLas) is a major causal agent of citrus greening disease. The disease primarily involves an asymptomatic, often latent infection of CLas. However, there is no effective technique to distinguish latent-infected trees from healthy ones. This study describes the development of a new detection method for latent CLas infection using cuttings.

Methods: Root tissues regenerated from cuttings using symptomatic and asymptomatic citrus trees were prepared for real-time a polymerase chain reaction (PCR) test which was used to investigate latent CLas. When some of the regenerated roots were negative for CLas in the first real-time PCR assay, a subsequent cultivation in soils was performed using the CLas-negative cuttings. CLas development during cultivation was evaluated by a second real-time PCR assay using soil-grown roots from seedlings.

Results: Previously, CLas had not been detected from leaves of the latent-infected trees in our greenhouse by real-time PCR. In this study, however, CLas was detected at a moderate frequency from the root tissues of cuttings derived from the latent-infected trees, by the same PCR test. For cuttings with regenerated roots that tested negative for CLas by real-time PCR, CLas was frequently detected from roots grown in nursery soil with autoclaving, after cultivation for a month or more.

Conclusions: Latent infection with CLas was detectable by real-time PCR using root tissues regenerated by cuttings and roots grown in nursery soil with autoclaving. These results suggest that the new method of investigation would provide great opportunities for early detection of CLas in asymptomatic citrus trees from field surveys, and would accelerate the eradication practice of citrus greening.
Keywords
Citrus greening disease, cuttings, latent infection, real-time PCR

This article is included in the Agriculture, Food and Nutrition gateway.

Corresponding author: Takashi Fujikawa (ftakashi@affrc.go.jp)

Author roles: Fujiwara K: Funding Acquisition, Investigation, Writing – Original Draft Preparation; Tomimura K: Investigation, Writing – Review & Editing; Iwanami T: Writing – Review & Editing; Fujikawa T: Investigation, Writing – Review & Editing

Competing interests: The authors declare a competing interest in the form of a Japan patent application (2016-45274). Kazuki Fujiwara and Takashi Fujikawa are directly involved in applying for the patent, and the application was filed under the name of NARO. The patent application comprises methodologies of cuttings, detection of CLas in cuttings with root-sprouts, and subsequent cultivation of cuttings.

Grant information: This work was supported in part by grants from the Project of the NARO Bio-oriented Technology Research Advancement Institution (Research program on development of innovative technology). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2021 Fujiwara K et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Fujiwara K, Tomimura K, Iwanami T and Fujikawa T. A new method for early detection of latent infection by 'Candidatus Liberibacter asiaticus' in citrus trees [version 1; peer review: 1 approved, 1 approved with reservations]
F1000Research 2021, 10:250 https://doi.org/10.12688/f1000research.51546.1
First published: 29 Mar 2021, 10:250 https://doi.org/10.12688/f1000research.51546.1
**Introduction**

Citrus greening disease (huanglongbing; HLB) is one of the most devastating diseases for the citrus industry worldwide and is caused by the phloem-limited phytopathogenic bacteria *Candidatus Liberibacter* spp. (Bové, 2006; Jagoueix et al., 1994). In particular, *Candidatus Liberibacter asiaticus* (CLas) has been an important causal agent in many countries including parts of Asia (Bové, 2006). In Japan, the occurrence of citrus greening disease has been limited to the south of the islands including the Okinawa prefecture and Oshima Islands, with Tokunoshima Island being the upper limit (Moji Plant Protection Station, 2012). Disease management has been carried out to prevent a further spread of the disease to citrus production areas on the main islands. A combination of PCR techniques for CLas detection and the disposal of infected citrus trees is the most practical approach to control the disease as there is currently no cure. On Kikaijima Island, located among the Amami Islands, a program for eradication of citrus greening disease was conducted in 2005 and had accomplished the eradication of the disease by 2012, resulting in an official declaration of eradication on Kikaijima Island (Moji Plant Protection Station, 2012). Currently, the eradication program is in progress on the larger Island of Tokunoshima, which is located to the south of Kikaijima Island.

Citrus greening disease is not often distinguishable from physiological disorders. Early symptoms of citrus greening disease appear in the leaves and include yellowish, blotchy chlorosis and mottling of leaves (Bové, 2006). On the other hand, these symptoms resemble other disorders including virus infections and nutrient deficiencies (Tian et al., 2014). Also, the disease primarily involves latent infection of CLas. In this asymptomatic condition, CLas is often undetectable with PCR techniques because the pathogen has infected the asymptomatic tree at a very low density (Gottwald et al., 2007). Asymptomatic trees that are overgrown in the fields are an important source of infection through vector-mediated transmission by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (Pelz-Stelinski et al., 2010). Because current disease management only allows us to identify citrus greening disease when CLas is detected by PCR and real-time PCR, the sensitivity and robustness of PCR detection is indispensable (Arrendondo Valdés et al., 2016; Folimonova & Achor, 2010; Fujikawa & Iwanami, 2012). Recent studies have made significant progress in understanding the primary localization of CLas in roots before systemic distribution (Louzada et al., 2016; Park et al., 2018). CLas density in roots is relatively higher than that in leaves (Johnson et al., 2014). This suggests that root samples would be an alternative target for early detection of CLas. On the other hand, one drawback to using crude roots for detecting CLas would be the difficulties in processing DNA-based techniques. In contrast to leaf samples, root samples are not easily available in field surveys because of the hardened tissues in mature citrus trees, and it may be a struggle to dig up root tissues for further assays. In this study, we developed a new approach for early detection of CLas using root samples recovered from cuttings of CLas-infected trees. This approach allowed us to detect CLas from asymptomatic citrus trees that had previously tested negative when using leaves for conventional PCR and real-time PCR.

**Methods**

**CLas-infected and asymptomatic tree preparation**

One-year-old seedlings of rough lemon (*Citrus jambhiri* Lush.) were prepared in 12 cm-diameter × 24 cm-deep pots. Graft-inoculation of 12 seedlings was carried out with a small amount of bark tissue of CLas-infected flat lemon trees (*Citrus depressa* Hayata), which has been maintained in a quarantined greenhouse under a special permission (26Y1214) issued by the Ministry of Agriculture, Forestry and Fisheries (Fujikawa et al., 2013). CLas infection on seven rough lemon trees was confirmed by both the development of foliar symptoms and the presence of CLas by real-time PCR using HLBas/HLBr/HLBp primer–probe set (Li et al., 2006). Five rough lemon trees did not develop any foliar symptoms, and CLas was undetectable from leaves over two years in a monthly survey with both the conventional PCR (Fujikawa et al., 2012) and real-time PCR using HLBas/HLBr/HLBp primer–probe set (Li et al., 2006). These inoculated but asymptomatic rough lemon trees (five pots in all) were used as asymptomatic trees for this study.

**Cutting preparation**

Cuttings were prepared from the upper part of young green shoots and branches, which were taken from seven symptomatic and five asymptomatic rough lemon trees. Cuttings of approximately 4 cm in length containing one leaf with no disease symptoms were selected and treated with 1% “Menedael” Fe (II) aq. fertilizer (Menedael Co., Ltd., Osaka, Japan; Cat. No. 242461) and then dipped in “Rooton”, a synthetic auxin α-naphthyl acetamide, (Sumitomo chemical garden products inc., Tokyo, Japan; JAN code 4975292090625) for promotion of rooting. The cuttings that had been treated were planted in a box that contained autoclaved “Kanuma” pumice and kept at approximately 32°C for a month under natural light/dark conditions, covered with a plastic film. Care was taken to keep the Kanuma pumice gently wet but not saturated to ensure optimum rooting. Roots that regenerated a month after cutting were used for CLas detection in the real-time PCR (Li et al., 2006).

**Cultivation test**

For detecting CLas in cuttings with root-sprouts that tested negative in the first PCR test, subsequent cultivation of the cuttings in various soils was carried out in a quarantined greenhouse and monitored for two months. Five cuttings were individually transplanted into the following soils: two commercial soils which were “Kenbyo” nursery soil (Yaenougei Co., Ltd., Nagasaki, Japan) and “Sanyo composted bark” soil (Sanyo Chip Co., Ltd., Yamaguchi, Japan); one natural soil from an area where citrus greening disease has occurred, namely “Kunigami Maaji” clay soil collected from Kunigami-son village (around N26°44′44.8″, E128°10′42.0″), Okinawa Prefecture; and two natural soils from areas where citrus greening disease has not occurred, which were Acrisols collected from Isen-cho.
DNA preparation, PCR and real-time PCR

Leaf samples were cleaned and wiped with a kimwipe paper (Kimberly Clark Corp., Irving, TX) impregnated with 70% ethanol. Midribs were excised and chopped into 2–3 mm pieces, providing approximately 0.05 (±0.01 SD) g of the midribs for DNA extraction. For the root samples, a single root was vortexed vigorously to remove soils. Approximately 4 cm of the root segment was cut out from the bottom of the root and then wiped with a kimwipe impregnated with 70% ethanol.

All sample materials were individually ground with a mortar and pestle, and then subjected to DNA extraction using the commercial kit DNeasy Plant Mini Kit (QIAGEN, Valencia, CA; Cat. No. 69106) according to the manufacturer’s instructions. DNA was eluted in 200 μL of a given AE buffer. CLas detection in cuttings was performed by TaqMan probe real-time PCR in a QuantStudio 5 real-time PCR system (Thermo Fisher Scientific Inc., Waltham, MA) using the HLBas (5’T-CGACGCGGT ATGCCAATACG-3’) / HLBr (5’-GCCTATCCCGTAGAAA AGGTAG-3’) / HLBrp (6-FAM-AGACGGTGATGTAACGC G-BHQ-1) primer–probe set (Li et al., 2006). Premix Ex Taq (Probe qPCR) (Takara Bio Inc., Shiga, Japan) was used according to the manufacturer’s protocol. The PCR reaction volume of 20 μL contained 0.4 μL of each primer, 10 μL of Premix Ex Taq, 0.8 μL of HLBrp, 0.4 μL of ROX Reference Dye II, 2 μL DNA template, and 6 μL of sterile distilled water. The real-time PCR consisted of pre-denaturation at 95°C for 30 s, and 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. CLas concentration was calculated based on the relationship between quantity and molarity of CLas 16S rDNA (as outlined by Degen et al., 2006), and the standard curve analysis was determined using the amount of template DNA adjusted to the DNA content in 10⁶ cells/μL and a 10-fold dilution series. The CLas density in leaves was calculated from the total amount of DNA eluted from leaves according to the representative standard curve (y = −1.538x + 42.278, R² = 0.9999). The results of the CLas development were analyzed using Tukey-Kramer multiple comparison test in the R statistical software (v. 3.5.0).

Results

Detection of CLas recovered from latent infection using cuttings

Most of the cuttings from asymptomatic trees survived and were well rooted (Figure 1a–c), but the regeneration of cuttings using symptomatic trees was evidently influenced by CLas infection. A total of 224 clones regenerated from 240 prepared cuttings using asymptomatic trees whereas a total of 24 clones were recovered from 45 prepared cuttings using symptomatic trees. One root sample was collected from each cutting and these were used for the primary PCR test. CLas was detected in a total of 115 cuttings in the real-time PCR (Fujiwara, 2021a), which corresponds to a 51.3% detection rate (Table 1), while none of the cuttings showed foliar symptoms of citrus greening. In addition to this test, nine leaf samples from cuttings showing CLas negative in roots were randomly selected and subjected to real-time PCR, the results of which showed no detection of CLas within the leaf samples. In regeneration of cuttings using symptomatic trees, CLas was detected in all root samples, which corresponds to a 100% detection rate (Table 1), as was detected in the symptomatic leaves.

Effect of soil types on CLas recovery from latent infection

A subsequent cultivation test was performed (Fujiwara, 2021b) to assess the CLas recovery from latent infection using the cuttings which showed no CLas detection in the primary PCR test (Supplementary Figure 1, Extended data (Fujiwara, 2020)). To examine suitable soil types for CLas development...
Table 1. Detection of *Candidatus* Liberibacter asiaticus (CLas) from roots recovered from cuttings.

| Root samples* | CLas detection |
|---------------|----------------|
| Root samples  | CLas detection |
|               |                |
| Asymptomatic  | 224            |
|               | 115 (51.3%)    |
|               | 109 (48.7%)    |
| Symptomatic   | 24             |
|               | 24 (100%)      |
|               | 0              |

* Roots re-generated a month after cutting were used to detect CLas by the real-time polymerase chain reaction. Raw data is provided on Figshare ([Fujiwara, 2021a](#)).

In cultivation, we tested five different types of soils including two commercial soils (“Kenbyo” nursery soil and “Sanyo composted bark” soil) and three natural soils (Acrisols, Andosols, and “Kunigami Maaji” soil). Overall, a cultivation of the cuttings in prepared soils successfully provided CLas detection by real-time PCR from asymptomatic trees (Figure 2). In the nursery soil, CLas was detectable from both leaves and roots a month after transplanting ($P < 0.01$). The density of CLas in seedlings grown in the “Kenbyo” nursery soil with autoclaving ($3.4 \times 10^5$ cells/leaf and $2.1 \times 10^5$ cells/root) was relatively higher than that in the same soil without autoclaving ($3.8 \times 10^5$ cells/leaf and $4.9 \times 10^5$ cells/root), and the detection was stable throughout the cultivation for two months (Figure 2; Supplementary Figure 2, Extended data ([Fujiwara, 2020](#))). In contrast, although CLas was likely to be detected in both leaves and roots grown in a composted soil ($1.7 \times 10^5$ cells/leaf and $4.5 \times 10^5$ cells/root with autoclaving), the CLas density detected in cultivations in the composted soil without autoclaving fluctuated highly (Figure 2; Supplementary Figure 3, Extended data ([Fujiwara, 2020](#))). Seedlings grown in composted soil without autoclaving showed CLas development a month after cultivation, no CLas was detected in the subsequent cultivation. In the natural soils, which comprised Acrisols, Andosols, and “Kunigami Maaji” soils, it was also the case that CLas detection fluctuated in both the leaves and roots similar to that in the composted bark soil without autoclaving (Figure 2; Supplementary Figure 4–6, Extended data ([Fujiwara, 2020](#))). Autoclaving of the Andosols and “Kunigami Maaji” soil had little effect on the propagation of CLas in the cuttings, showing $2.3 \times 10^5$ cells/leaf and $1.1 \times 10^4$ cells/root in Andosols without autoclaving; $5.2 \times 10^5$ cells/leaf and $9.5 \times 10^5$ cells/root in a Kunigami Maaji soil without autoclaving. In Acrisols, CLas development was relatively varied compared with other natural soils and appeared to be affected by the soil conditions with other natural soils and appeared to be affected by the soil conditions with other natural soils and autoclaving (Figure 2; Supplementary Figure 4, Extended data ([Fujiwara, 2020](#))). In short, the soil conditions of a nursery soil treated with autoclaving was suitable for early detections of CLas recovered from latent infection using cuttings.

### Discussion

This study showed that while it remains difficult to identify CLas from latent infection in host trees, latently infected CLas can be detectable by real-time PCR using roots regenerated from cuttings and grown in autoclaved nursery soil. The results indicated that detection from cuttings with or without cultivation in autoclaved nursery soil can be practically applicable to detect CLas from latently infected trees. In field tests, it...
might be possible to detect CLas directly from the root samples of latently infected trees, but in some seasons and locations digging on site and finding new, fine, roots would be extremely difficult. Instead, the CLas detection from cuttings reported here is suitable in any surrounding as long as twigs are available.

Soil chemicals and microorganisms evidently affect the occurrence and distribution of CLas in citrus (Blaustein et al., 2017) as is the case with other soil-borne diseases (Philippot et al., 2013). CLas is likely to accumulate in roots prior to systemic distribution in citrus trees (Johnson et al., 2014) and requires CLas-associated microbiota in the host plant for its own survival (Fujiwara et al., 2018). This suggests that disturbance of CLas-associated microbiota by soil microorganisms may affect CLas development in citrus trees. A subset of CLas-associated microbiota in roots was structured by CLas infection, resulting in distinct microbial and metabolological profiles in roots (Padhi et al., 2019; Zhang et al., 2017). Thus, soil and rhizosphere management may be critical for disease control management. On the other hand, there is a concern that improved root integrity slows CLas growth and delays disease development, making it difficult to detect CLas in roots and other plant tissues. Our study has shown that CLas recovery from latent infection varied with soil type and conditions. While we still need to clarify the interplay between soil functions and CLas behaviors, further accumulation of knowledge on the relationship between root zone and the occurrence of citrus greening diseases is expected to enable more effective monitoring and detection of CLas in roots (Blaustein et al., 2018).

A citrus greening disease eradication program in Japan has been promoted by both the early detection of CLas from leaf samples using PCR methods and the removal of the infected trees. Detection of CLas from latent infection was once carried out by a validation method using grafting over six months (Figure 3a). The disease management has been successful in increasing citrus greening disease-free areas in the Okinawa prefecture and Amami Islands. In addition to the conventional survey, a new approach developed in this study could be provided as part of an eradication program for finding latent infection of CLas and is expected to contribute to time-and cost-efficient validation of CLas infection detection. In practice, the process of a new approach consists of two sequential steps (Figure 3b; Supplementary Figure 7a-b, Extended data (Fujiwara, 2020)). As foliar samples from an asymptomatic tree test negative in the primary PCR test, cuttings should be prepared from the asymptomatic tree. Roots regenerated from the cuttings are then used for CLas detection in the second PCR test (Figure 3b; Supplementary Figure 7a, Extended data (Fujiwara, 2020)). If CLas is detected from any of the cuttings, the asymptomatic tree would be removed from the field. Next, a subsequent cultivation test is conducted as the third step if all cuttings from the asymptomatic tree test negative in the second PCR test (Figure 3b; Supplementary Figure 7b, Extended data (Fujiwara, 2020)). Root samples are collected within a few months after cultivation, and the results would allow us to decide whether or not to eliminate the asymptomatic tree. When CLas infection is not validated throughout the process, further monitoring or a re-start of the survey would be followed if necessary.

The techniques described in this paper are only applicable for citrus cultivars that root readily by cutting. These cultivars include lemon, trifoliate orange, murcot, Cleopatra mandarin, as well as the flat lemon that is common in the citrus greening-affected subtropical islands of Japan. For satsuma mandarin, oranges, and others that root poorly by cutting, propagation by air-layering would be needed. Exploration on media considering plant hormone formula and temperature conditions for promoting rooting from these cultivars is in progress in our laboratory.

**Conclusion**

The latent infection of CLas is detectable with the new detection method using cuttings. Our study revealed that the autoclaved nursery soils allowed for early detection of CLas. The new method using cuttings is time efficient in CLas
surveying, in contrast to the conventional method which uses grafting. It is expected that the new method may provide opportunities for identification and validation of other ‘Candidatus Liberibacter’ spp. under quarantine conditions, as well as providing opportunities in monitoring surveys of citrus greening disease.

**Data availability**

**Underlying data**

Figshare: Raw data for “Detection of CLas from roots recovered from cuttings” shown in Table 1. https://doi.org/10.6084/m9.figshare.13985021.v1 (Fujiiwara, 2021a).

This project contains the following underlying data:
- Raw Data 1 (raw, unaveraged real-time PCR data).

Figshare: Raw data for “A subsequent cultivation in different soil types using CLas-negative cuttings” shown in Supplementary Figures 2–6. https://doi.org/10.6084/m9.figshare.13985198.v1 (Fujiiwara, 2021b).

This project contains the following underlying data:
- Raw Data 2 (raw, unaveraged real-time PCR data).

**Extended data**

Figshare: Supplementary Figures 1–7. https://doi.org/10.6084/m9.figshare.13466676.v1 (Fujiiwara, 2020).

This project contains the following extended data:
- Figure 1. Schematic representation of the experimental design.
- Figure 2. A subsequent cultivation test for CLas detection in “Kenbyo” nursery soil using seedlings with no CLas detection in a primary PCR test.
- Figure 3. A subsequent cultivation test for CLas detection in “Sanyo composted bark” soil using seedlings with no CLas detection in a primary PCR test.
- Figure 4. A subsequent cultivation test for CLas detection in Acrisols using seedlings with no CLas detection in a primary PCR test.
- Figure 5. A subsequent cultivation test for CLas detection in Andosols using seedlings with no CLas detection in a primary PCR test.
- Figure 6. A subsequent cultivation test for CLas detection in “Kunigami Maaji” soil using seedlings with no CLas detection in a primary PCR test.
- Figure 7. A new approach for validation of CLas infection in asymptomatic trees.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

**Ethics**

No special permissions or quarantines were required for this study.

**Acknowledgements**

We thank Yuko Shimizu from Okinawa Prefectural Plant Protection Center. We also thank Katsuhiko Miyaji, Yoshihiro Ogawa, and Tomohiro Fukumoto from Kagoshima Prefectural Institute for Agricultural Development for supporting the soil collection. We are also grateful to Mariko Taguchi, Akane Sasaki, Hiroe Hatomi, and Yuko Fujii from NARO for their support throughout the experiments.

**References**

Arredondo Valdés R, Ortiz JC, Deheza MB, et al.: *A review of techniques for detecting Huanglongbing (greening) in citrus*. Can J Microbiol. 2016; 62(10): 803–811. PubMed Abstract | Publisher Full Text

Blaustein RA, Lorca GL, Meyer LJ, et al.: *Defining the core citrus leaf- and root-associated microbiota: Factors associated with community structure and implications for managing huanglongbing (citrus greening) disease*. Appl Environ Microbiol. 2017; 83(11): e0210–17. PubMed Abstract | Publisher Full Text | Free Full Text

Blaustein RA, Lorca GL, Teplicki M: *Challenges for Managing Candidatus Liberibacter spp. (Huanglongbing Disease Pathogen): Current Control Measures and Future Directions*. Phytopathology. 2018; 108(4): 424–435. PubMed Abstract | Publisher Full Text

Bövé JM: *Huanglongbing: a destructive, newly emerging, century-old disease of citrus*. J Plant Pathol. 2006; 88(1): 7–37. Reference Source

Degen HJ, Deufel A, Eisel D, et al.: *PCR Application Manual*. 3rd Ed. (Roche Diagnostics GmbH, Mannheim). 2006. Reference Source

Folimonova SY, Achor DS: *Early events of citrus greening (huanglongbing) disease development at the ultrastructural level*. Phytopathology. 2010; 100(9): 949–958. PubMed Abstract | Publisher Full Text

Fujikawa T, Iwanami T: *Sensitive and robust detection of citrus greening (huanglongbing) bacterium “Candidatus Liberibacter asiaticus” by DNA amplification with new 16S rDNA-specific primers*. Mol Cell Probes. 2012; 26(5): 194–197. PubMed Abstract | Publisher Full Text | Free Full Text

Fujikawa T, Miyata SI, Iwanami T: *Convenient Detection of the Citrus Greening (Huanglongbing) Bacterium ‘Candidatus Liberibacter asiaticus’ by Direct PCR from the Midrib Extract*. PloS One. 2013; 8(2): e57011. PubMed Abstract | Publisher Full Text | Free Full Text

Fujiiwara K: Raw data for “Detection of CLas from roots recovered from cuttings” shown in Table 1. figshare. Dataset. 2021a. http://www.doi.org/10.6084/m9.figshare.13985021.v1

Fujiiwara K: Raw data for “A subsequent cultivation in different soil types using CLas-negative cuttings” shown in Supplementary Figures 2-6. figshare. Dataset. 2021b. http://www.doi.org/10.6084/m9.figshare.13985198.v1
Fujiiwara K: Supplementary Figures 1-7, figshare. Figure, 2020. http://www.doi.org/10.6084/m9.figshare.13466676.v1
Fujiiwara K, Iwanami T, Fujikawa T: Alterations of Candidatus Liberibacter asiaticus-Associated Microbiota Decrease Survival of Ca. L. asiaticus in in vitro Assays. Front Microbiol, 2018; 9: 3089. PubMed Abstract | Publisher Full Text | Free Full Text
Gottwald TR, da Graça JV, Bassanezi RB: Citrus Huanglongbing: The pathogen and its impact. Plant Health Prog. 2007; 8(1). Publisher Full Text
Jagoueix S, Bove JM, Garnier M: The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the Proteobacteria. Int J Syst Bacteriol. 1994; 44(3): 379-386. PubMed Abstract | Publisher Full Text
Johnson EG, Wu J, Bright DB, et al.: Association of 'Candidatus Liberibacter asiaticus' root infection, but not phloem plugging with root loss on huanglongbing-affected trees prior to appearance of foliar symptoms. Plant Pathology. 2014; 63(2): 250–298. PubMed Abstract | Publisher Full Text
Li W, Hartung JS, Levy L: Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associated with citrus huanglongbing. J Microbiol Methods. 2006; 66(1): 104-115. PubMed Abstract | Publisher Full Text
Louzada ES, Vazquez OE, Braswell WE, et al.: Distribution of 'Candidatus Liberibacter asiaticus' Above and Below Ground in Texas Citrus. Phytopathology. 2016; 106(7): 702-709. PubMed Abstract | Publisher Full Text
Mogi Plant protection station: Emergency action and eradication on citrus greening disease in Kikai Island. Plant Protection. (In Japanese). 2012; 66(6): 348–349. Reference Source
Padhi EMT, Maharaj N, Lin SY, et al.: Metabolome and Microbiome Signatures in the Roots of Citrus Affected by Huanglongbing. Phytopathology. 2019; 109(12): 2022-2032. PubMed Abstract | Publisher Full Text
Park JW, Louzada ES, Evan Braswell W, et al.: A new diagnostic real-time PCR method for huanglongbing detection in citrus root tissue. J Gen Plant Pathol. 2018; 84(5): 359-367. PubMed Abstract | Publisher Full Text
Philippot L, Raaijmakers JM, Lemanceau P, et al.: Going back to the roots: the microbial ecology of the rhizosphere. Nat Rev Microbiol. 2013; 11(11): 789-799. PubMed Abstract | Publisher Full Text
Tian S, Lu L, Labavitch JM, et al.: Spatial imaging of Zn and other elements in Huanglongbing-affected grapefruit by synchrotron-based micro X-ray fluorescence investigation. J Exp Bot. 2014; 65(4): 953–964. PubMed Abstract | Publisher Full Text | Free Full Text
Zhang Y, Xu J, Riera N, et al.: Huanglongbing impairs the rhizosphere-to-rhizoplane enrichment process of the citrus root-associated microbiome. Microbiome. 2017; 5(1): 97. PubMed Abstract | Publisher Full Text | Free Full Text
Open Peer Review

Current Peer Review Status: ✔️ 🤔

Version 1

Reviewer Report 29 April 2021

https://doi.org/10.5256/f1000research.54728.r82414

© 2021 Kunta M. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Madhurababu Kunta

Texas A&M University-Kingsville Citrus Center, Weslaco, TX, USA

This study contributes to detection of CLas from asymptomatic trees using root samples which is highly useful for the stakeholders to take appropriate HLB management strategies and to prevent the spread of the disease. This is a valuable scientific report that provides early and pre-symptomatic Candidatus L asiaticus (CLas) detection in citrus using root samples. However, the authors of the manuscript used HLBaspr 16 S rDNA-based qPCR test to detect CLas, causal organism for citrus HLB, from the citrus roots. I recommend that the authors test for CLas all the samples using another primer set targeting a region other than 16S rDNA (for example, RNR) to confirm the results. I would be glad to review the manuscript if the authors can conduct the tests and submit a revised manuscript based on my recommendations. This recommendation is based on the previous scientific reports.

Kunta et al. (2014) tested both leaf and root samples and concluded that HLBaspr primers are not suitable for CLas detection in root samples due to false positives. The reason to include my paper is that it was the first study published in 2014 that determined that HLBaspr based qPCR to amplify 16S rDNA region is not suitable to detect CLas from citrus root samples due to non-specific amplification from non-target organisms which leads to false positive results. However, HLBaspr works excellent for Clas detection in the leaf samples.

Another study, Shin et al. (2018), reported that Bradyrhizobium sp. closely related CLas within Rhizobiales contributed to the false positives in the roots when HLBaspr primers targeting 16rDNA are used. This paper was published in 2018, which describes that rhizobiales such as Bradyrhizobium (Bradyrhizobium 16S rDNA sequences are highly similar to CLas) could interfere and lead to unreliable CLas detection in roots. This study also recommends that primers other than HLBaspr should be used to avoid potential cross reactions with members of Rhizobiales for accurate CLas detection.

References
1. Kunta M, Viloria Z, del Rio H, Louzada E: Diverse DNA extraction methods and PCR primers for detection of Huanglongbing-associated bacteria from roots of ‘Valencia’ sweet orange on sour
orange rootstock. *Scientia Horticulturae*. 2014; **178**: 23-30 Publisher Full Text

2. Shin K, van Bruggen A: Bradyrhizobium isolated from huanglongbing (HLB) affected citrus trees reacts positively with primers for Candidatus Liberibacter asiaticus. *European Journal of Plant Pathology*. 2018; **151** (2): 291-306 Publisher Full Text

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
No

**If applicable, is the statistical analysis and its interpretation appropriate?**
Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**
No

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Citrus Pathology, Citrus Pathogen Detection, Horticulture

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 21 April 2021

https://doi.org/10.5256/f1000research.54728.r82413

© 2021 Subandiyah S. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Siti Subandiyah
Department of Plant Protection, Universitas Gadjah Mada, Yogyakarta, Indonesia

The manuscript is well written with good English throughout the whole paper. However, a little improvement is needed in the Methods (e.g. quantification of the inoculum source) or when some methodological sentences may need to be moved from the Results to Methods.
The Discussion may be improved on the explanation of the soil characteristic differences among the soil types used in the experiment. The cultivar(s) recommended for root stocks when air layering propagation may be needed.

I have provided some further notes here.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant Pathology, some on the similar area studying of huanglongbing

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com