Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2024 21(1):143-149. **OPEN ACCESS**

Detection of *Candidatus Liberibacter asiaticus* using molecular techniques in citrus mutants from Bangladesh

Mohammad Mahbubul Haque[1*], Md. Nazmul Hasan Mehedi[2], Jannatul Farthouse[1], Afruza Sultana[3], Ismat Ara[4]and Sushmita Dey[4]

[1]Plant Pathology Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh-2202, **Bangladesh**
[2]Horticulture Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh - 2202, **Bangladesh**
[3]Researcher, Lal Teer Seed Limited, Dhaka, **Bangladesh**
[4]Bangladesh Agricultural University, Mymensingh, **Bangladesh**

***Correspondence:** mahbub.bina@gmail.com **Received: Oct., 01, 2023, Revised: February 18, 2024, Accepted: February 21, 2024 e-Published: February 22, 2024**

Huanglongbing (HLB or citrus greening or yellow shoot disease) is a devastating disease of citrus caused by non culturable, fastidious phloem limited bacterium, *Candidatus Liberibacter asiaticus* and threatens the citrus industry in Bangladesh. The putative causal agent of the disease is transmitted through insect vector or grafting with diseased bud wood. The polymerase chain reaction (PCR) diagnosis is a more reliable and sensitive diagnostic tool for detecting greening bacterium than other conventional approaches like electron microscopy, DNA-DNA hybridization and immunofluorescence (IF) for detection of citrus greening. Results reveal that DNA extraction kit method of DNA isolation provided higher yield and better quality DNA than other methods. To confirm the reliability of PCR, the greening bacterium was also detected in graft-inoculated plants, which showed typical greening symptoms. Results show that Out of 10 samples collected from Bangladesh Institute of Nuclear Agriculture, Mymen singh, 4 (40%) belonging from 10 symptomatic trees were amplified and produced amplicons of 703 bp and 500bp from A2/J5 and LSS/LSS606 respectively in 2022 that confirmed the presence of *Candidatus Liberibacter asiaticus* in the samples. PCR suggesting sampling in March is more suitable for PCR detection of greening bacterium. The methods validated in this study will be very useful for regulatory response, effective management of infected trees, and development of a *Candidatus Liberibacter asiaticus* free nursery system.

Keywords: A2/J5, HLB, LSS/LSS606, Polymerase Chain Reaction, Citrus greening

INTRODUCTION

China was the world's largest citrus producer, with a total production of 33,888 thousand tons, cultivated in an area of 2,298 thousand hectares. Brazil ranked second, producing 19,547 thousand tons of citrus fruits in an area of 682 thousand hectares, while the USA ranked third, producing 7,069 thousand tons in an area of 616 thousand hectares. These countries together account for a significant portion of the world's citrus production and are major players in the global citrus industry (FAO, 2021). In 2020, citrus fruit production for Bangladesh was 167,104 tonnes. Citrus fruit production of Bangladesh increased from 21,632 tonnes in 1971 to 167,104 tonnes in 2020 growing at an average annual rate of 5.25% (BBS, 2021). The Sylhet region is famous for its citrus cultivation. Though Bangladesh citrus fruit, total-yield fluctuated substantially in recent years, it tended to increase through 1971-2020 period ending at 20,673 hg/ha in 2020. In Bangladesh, now a days about 400 (four hundred) hectors agriculture land are used for orange cultivation (BBS, 2021). Citrus fruits are indeed a rich source of various nutrients that are essential for maintaining good health. Apart from vitamin C, which is their most notable nutrient, citrus fruits are also abundant in other nutrients such as potassium, folate, calcium, thiamin, niacin, vitamin B6, phosphorus, magnesium, copper, riboflavin, and pantothenic acid. All these nutrients play crucial roles in various bodily functions, such as nerve and muscle function, bone health, energy production, and immune function. Therefore, including citrus fruits in our diet as part of a balanced and varied diet can be highly beneficial for our overall health. Sylhet, Moulvi bazar, Panchagarh and Hill Tracts area of Bangladesh are suitable for orange cultivation. The HLB bacteria infect citrus cultivars with characteristic symptoms of blotchy leaf mottle along with green islands on leaves and yellowing of veins that expand on leaves affixed to shoots showing the complete yellow manifestation (Baranwal et al. 2004; Wang and Trivedi, 2013). The common name “greening”

is instigated from the symptoms on mandarin fruits with an uneven ripening of fruits, which appearing half orange and half green color of the fruits. Fruits from diseased trees are usually small, lopsided with improper coloration which reduces the market value (Wang and Trivedi, 2013).Huanglongbing (HLB) is one of emerging diseases of citrus in Bangladesh. The symptoms of the disease include blotchy chlorosis and/or mottling of leaves, stunted growth and finally death of the plants. HLB is caused by three species of α-Proteobacteria "*Candidatus Liberibacter asiaticus*"(CLas) (Jagoueix et al. 1994), "Ca. L. africanus" (CLaf) (Planet et al. 1995) and "Ca. L. americanus" (CLam) (Teixeira et al. 2005) . 'Ca. Liberibacter spp. is transmitted by grafting as well as citrus Psyllid (Diaphorina citri) (Bove, 2006). A number of molecular markers have been used worldwide to detect Candidatus Liberibacter spp. (Gottwald, 2010) and to analyze their population diversity (Puttamuk et al. 2014; Zhang et al. 2016). The aim of this study was to use the polymerase chain reaction method to identify the presence of *Candidatus Liberibacter asiaticus* in citrus plants at a molecular level.

MATERIALS AND METHODS

For the diagnosis of HLB disease, Germplasm of Horticulture division, BINA, Mymensingh were surveyed in 2022. During survey of orchards special emphasis was given on the presence of *Diaphorina citri* adults or nymphs and typical symptoms of HLB especially blotchy mottling and vein yellowing. Total ten symptomatic trees (including control) were selected for HLB diagnosis (Table 1).

Table 1: List of citrus mutants

Sample id	Mutant Line	Sample id	Mutant Line
1	BARI Malta-1	6	Thai Malta 30Gy
2	Malta (Washington) 40Gy	7	Malta India 20Gy
3	Malta(Washington) 50Gy	8	Malta Malaysia 40Gy
4	Washington Naval mother	9	Malta EMS 0.5% 3h
5	BARI Malta 40Gy	10	Thai Malta Control

Three samples were collected from each tree. In the month of March 2022, for each replicate, 4 mature leaves from a tree with blotchy mottling and vein yellowing symptoms as well as from healthy controls were collected all around the canopy (Figure 1). Leaves collected from HLB suspected sweet orange trees and healthy/negative controls were kept in zip lock bags. Bags were labeled and placed in box with ice. Samples were transported to the laboratory as soon as possible. Samples were then kept at 4°C and used for DNA extraction next day.

Figure 1: Blotchy mottles symptoms in sweet orange (Malta) leaves

Molecular studies

Molecular studies for the diagnosis of HLB were conducted at Molecular lab, Plant Pathology division, BINA, Mymensingh, Bangladesh.

DNA extraction

Leaf samples were washed with sterile distilled water and 70% ethanol and dried on blotting paper to remove excess water. Leaf midribs were ripped off and chopped with sterilized scissors. Approximately 60 mg of leaf midribs were placed into Eppendorf tube and ground by micro-pestle adding liquid nitrogen. The Las genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following manufacturer's instructions. The pellet was re-suspended in 25*µ*l volume of DNA rehydration solution. The rehydrated genomic DNA was incubated at 4°C for overnight and stored at -20°C to serve as the template for PCR amplification.

DNA quantification

Genomic DNA extracted from suspected to be HLB positive as well as healthy sweet orange leaf midrib and petiole was quantified by gel electrophoresis technique. Agarose gel (1.5 %) prepared in 1X TBE buffer (Tris base, boric acid, EDTA) of 40ml stained with 0.6g

ethidium bromide was used for DNA quantification. Gel was visualized in the gel documentation system (BioRad) by using software quantity one.

Polymerase Chain Reactions (PCR) for HLB Detection

Conventional PCR performed after DNA extraction and quantification. 16S rDNA primer LSS/LSS606 (Jagoueix et al. 1996) and ribosomal protein gene of the rplKAJL-rpoBC operon (β operon) primer A2/J5 (Hocquellet et al. 1999) were used for the detection of HLB bacterium in suspected positive samples and healthy controls (Table 1). A total volume of 25 µL was used in the PCR reaction mix. Thin walled, flat capped, 0.2 mL, nuclease free, individual PCR tubes were used for PCR reaction mix. Amplification was carried out in Applied Biosynthesis thermo cycler with the following thermal profile for LSS/LSS606: one cycle for initial denaturation at 96°C for 9 minutes; followed by 35 cycles at 96°C for 30 seconds, 55°C for 30sec and 72°C for 1 minute; one cycle for final extension at 72°C for 7 minute and thermal profile for A2/J5: one cycle for initial denaturation at 94°C for 3 minutes; followed by 35 cycles at 94°C for 1min, 58°C for 1min and 72°C for 1 minute; one cycle for final extension at 72°C for 10 minute.

Analysis of PCR product

The PCR products were analyzed by gel electrophoresis using a 1.5% agarose in 1X TBE buffer (Tris base, boric acid and 0.5 M EDTA [pH 8.0]) containing ethidium bromide (0.6g). Gel was visualized and analyzed using the Alpha Imager HP System (Protein Simple, San Jose, CA, USA). A 100 bp DNA ladder set (Invitrogen, Carlsbad, CA, USA) was included to determine fragment size.

Sequencing

The partial sequencing was done through outsourcing in the designated laboratory of Invent Technologies Limited, Bangladesh. PCR products were sequenced directly in both orientations from primers A2/J5 according to the standard protocols for the ABI PRISM 3500 (version 3.7) automated DNA sequence (Perkin Elmer) with ABI PRISM Ready Reaction Dye Termination Cycle Sequencing Kit. The quality of nucleic acid sequences was evaluated using Chromas (Version 2.6) software to avoid the use of low quality bases. Amplified DNA sequences were compared with other *Candidatus Liberibacter spp*. sequence database available in the GenBank using Basic Local Alignment Search Tool (BLAST) algorithm to identify closely related sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA (version 5.22).

RESULTS

In total, 10 citrus leaf samples (4 mature leaves per sample) collected from 10 trees of sweet orange were tested for the presence of *Candidatus Liberibacter asiaticus*. Highly intact genomic DNA with smear in few samples obtained was used for the amplification. When conventional PCR was performed by using 16S rDNA primer pair LSS/LSS606 and rplKAJL - rpoBC operon (β operon) primers A2/J5, out of 4 DNA samples suspected to be HLB positive, 4 (40%) were amplified belonging from three symptomatic trees (Table 2) and produced amplicons of ≈1160bp as observed by Jagoueix et al. (1996) and ≈703 bp as reported by Hocquellet et al. (1999) that confirmed the presence of *Candidatus Liberibacter asiaticus* in the samples (Figure 2 and 3).

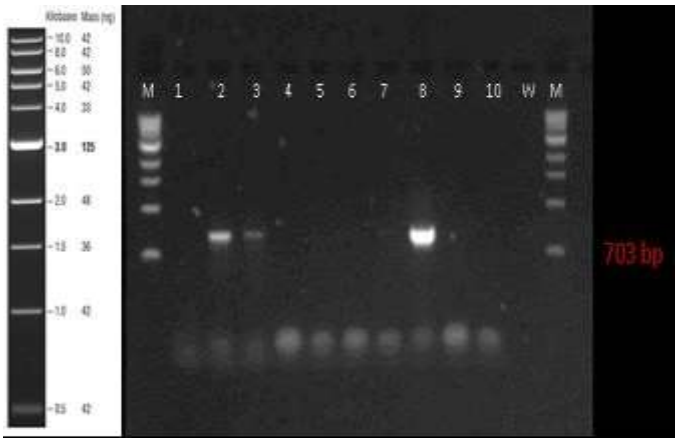

Figure 2: PCR amplification of a 703 bp from the suspected samples confirmed the presence of *Candidatus Liberibacter Asiaticus* using A2/J5

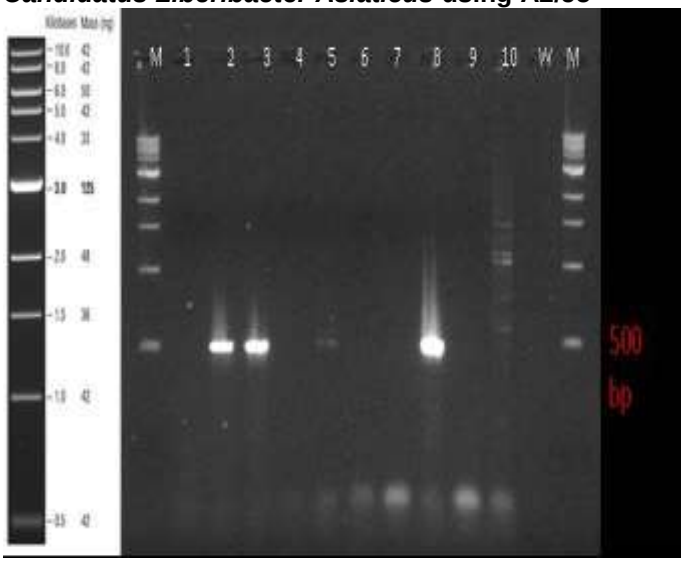

Figure 3: PCR based detection of *Ca. Liberibacter* asiaticus using Las606/Lss

Table 2: Primers used in conventional PCR studies to amplify genomic regions of *Candidatus Liberibacter asiaticus*

Primer	Sequences	Target DNA	Orientation	Regions of amplification	Comments
LSS	ACC CAA CAT CTA GGT AAA AAC C	Las	Forward	16s ribosomal RNA	Primer described by Jagoueix et al. 1996
LSs606	GGA GAG GTG AGT GGA ATT CCG	Las	Reverse	16s ribosomal RNA	Primer described by J agoueix et al. 1996
A2	TAT AAA GGT TGA CCT TTC GAG TTT	Las	Forward	rplKAJL-rpoBC (operon)	Primer described by Hocquellet et al.1999
J5	ACA AAA GCA GAA ATA GCA CGA ACA A	Las	Reverse	rplKAJL-ropBC (operon)	Primer described by Hocquellet et al.1999

Table 3: Closest relatives of the *Candidatus Liberibacter asiaticus* isolates based on A2/J5 primer

Isolates	Closest relatives	Accession no.	Alignment	Homology
BDMalta_Washington_50gy	*Candidatus Liberibacter asiaticus* isolate Gondar_GNDGJ ribosomal protein	MK542517.1	636/636	100
	Candidatus Liberibacter asiaticus isolate Yaracuy ribosomal protein	MG418842.1	636/636	100
	Candidatus Liberibacter asiaticus isolate MA3 50S ribosomal subunit protein	KM889668.1	636/636	100
BDmalta_Malaysia_40gy	*Candidatus Liberibacter asiaticus* clone CHN13-1 50S ribosomal subunit protein	KC133068.1	637/637	100
	Candidatus Liberibacter asiaticus clone CHN8-1 50S ribosomal subunit protein	KC133066.1	637/637	100
	Candidatus Liberibacter asiaticus ribosomal protein gene, partial cds	FJ177536.1	638/640	99
BDmalta_Malaysia_40gy	*Candidatus Liberibacter asiaticus isolate SSL* clone HLB-LIME 50s ribosomal subunit protein	KP210472.1	651/651	100
	Candidatus Liberibacter asiaticus strain CHE-Unshu 50S ribosomal subunit protein	KC477384.1	651/651	100
	Candidatus Liberibacter asiaticus isolate MA3 50S ribosomal subunit protein	KM889668.1	651/651	100

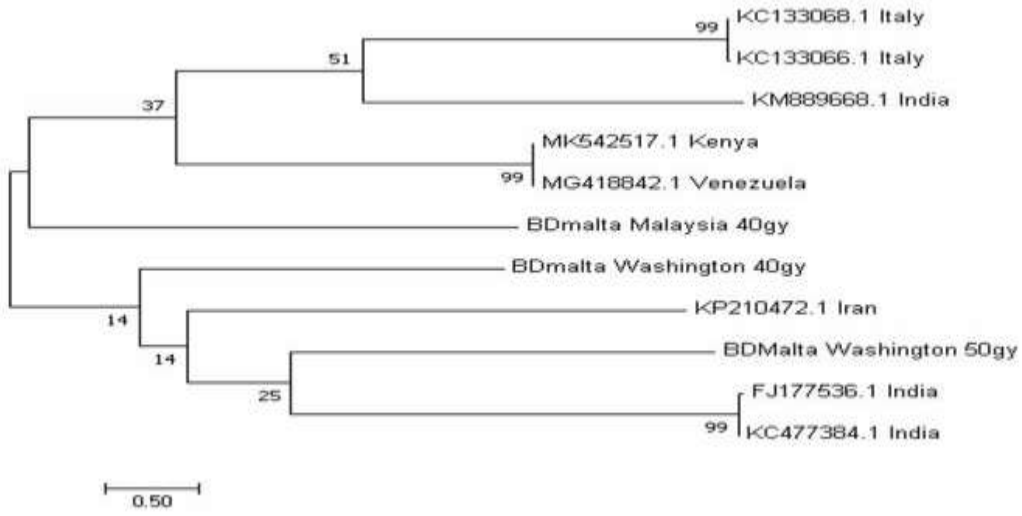

Figure 4: Phylogenetic relationships of the *Candidatus liberibacter asiaticus* isolates using MEGA7 software

Sequence analysis

One representative PCR product after amplification with primers A2/J5 of the ribosomal protein genes of the *rpl*KAJL-*rpo*BC operon were sequenced (Table 3). BLAST homology showed 99-100% sequence identity with the corresponding nucleotide sequence of ribosomal protein gene of *Candidatus Liberibacter asiaticus* strains found in India, China, Vietnam, Iran and Kenya Phylogenetic tree constructed from translated protein showed close relationship with Indian, Iranian isolates. The origin of *Candidatus Liberibacter asiaticus* in Bangladesh is uncertain because of the perfect homology between the sequence of the Bangladeshi strain and strains from India. We suspect that it was introduced by infected materials from the South Asian regions. Results from PCR amplification and aligning ribosomal gene sequences with other identified strains of different countries gave strong evidence that the symptomatic mandarin orange leaf samples collected from the surveyed areas were infected with *Candidatus Liberibacter asiaticus* and not due to micronutrient deficiencies or disorder. It revealed the mystery of citrus declining in Bangladesh which was associated with bacterial pathogen. These results confirmed the occurrence of citrus greening (HLB) on *Citrus reticulata* in Bangladesh.

DISCUSSION

Firstly, the initial detection of the disease is an important aspect to have an overview of the infection mode. A proper understanding of HLB symptoms is necessary for the preliminary step for disease survey and investigation. HLB-like symptoms, viz., leaf mottling, yellowing of leaf, yellow shoot, Zn-deficiency-like symptoms, vein clearing, and twig dieback, which resembled the symptoms (Gopal et al. 2010; Das et al. 2014; Kaipeng et al. 2017) were encountered in different citrus-growing sites. Routine monitoring of HLB-like symptoms is needed to manage the disease transmission. However, there is a lack of precision in detecting HLB based on symptomatology due to the similarity of symptoms to nutrient deficiency, for example (Gopal et al. 2010; Li et al. 2009), that associated with long latency period requires diagnosis of the causal agent to confirm the infection. Also, there might be confusion between other plant disorder symptoms and HLB infection which can lead to misidentification. In this work, some of the symptomatic leaf samples did not give positive PCR results which showed that certain symptomatic leaves resemble HLB symptoms without the bacterial infection. In such a situation, PCR detection of "*Ca. Liberibacter* spp." is one of the top-notch choices for HLB diagnosis compared with other techniques such as symptomatology.

Huanglongbing was present in northeastern and north-western India in the 1800s and early 1900s (Husain and Nath, 1927; Gottwald et al. 2007). Husain and Nath (1927) described severe damage caused by populations of *Diaphorina citri* at Sargodha from 1915 to 1920. Detailed assessments of the incidence and distribution patterns of HLB are important for management decisions and control strategies for reducing pathogen transmission. The present study was an important step towards the management of HLB in Bangladesh. We used primer pairs LSS/LSS606 and A2/J5 for the detection of *Ca. L. asiaticus* in sweet orange succari leaf samples. After amplification by conventional PCR, discrete bands of ≈500bp and ≈703bp were obtained in 70% samples from LSS/LSS606 and A2/J5 respectively as described by Jagoueix et al. (1996) and Hocquellet et al. (1999). Efforts are being made to control HLB all around the world but, complete control has not yet discovered. One of the main reasons may be the non cultureable nature of the bacterium (Davis et al. 2008; Sechler et al. 2009; Parker et al. 2014). Huanglongbing management may be difficult in older orchards due to severe damage caused by *Ca. L. asiaticus* and its insect host *D. citri.* However, the development of infrastructure for molecular studies proved helpful for HLB management experiments by early diagnosis of disease in the bud wood and nursery plants raised in the greenhouse used as healthy control were confirmed disease free.

We have reported an assessment of the greening bacterium *"Ca. Liberibacter asiaticus*" based on traditional symptomology and then confirmed by PCR techniques and phylogeny analysis. The detection of the disease is one of the most important aspects of managing the disease. As morphological symptoms are not sufficient in disease detection, PCR is precise in the detection even during the latest phase of infection. Early detection and disease diagnostics systems can be adopted in these regions by the regular use of the PCR or other advanced diagnostic tools. However, proper attention needs to be drawn to take up measures against this dreadful disease which is a threat towards all the citrus cultivars in this biodiversity-rich region. Otherwise, this might lead to a critical challenge for rejuvenating citrus cultivation.

CONCLUSIONS

Candidatus Liberibacter asiaticus is the bacterial species responsible for causing HLB disease in Bangladesh, as confirmed by the amplification of a 703bp and 500bp amplicon using primer pairs A2/J5 and LSS/LSS606, respectively.

Supplementary materials

The supplementary material / supporting for this article can be found online and downloaded at: https://www.isisn.org/article/

Author contributions

MMH: investigation, conceptualization, methodology, supervision and writing original draft JF: investigation, formal analysis, performed the statistical analysis and writing original draft, SD: edited the manuscript, SB: edited the manuscript, MNHM: edited the manuscript, IA: edited manuscript; AS: edited the manuscript. All authors carefully read and authorized the final manuscript.

Funding statement
This study was conducted by the financial support of Bangladesh Institute of Nuclear Agriculture (BINA).

Institutional Review Board Statement
The study was approved by the Bioethical Committee of the …….

Informed Consent Statement
Not applicable.

Data Availability Statement
All of the data is included in the article/Supplementary Material.

Acknowledgments
The authors would like to acknowledge BINA for the availability of fund.

Conflict of interest
The authors declared that present study was performed in absence of any conflict of interest.

REFERENCES

Anonymous. 2019. FAO statistical year book. Food and Agriculture Organization of the United Nations, pp: 169.

Baranwal, V.K., Mazumder, S., Singh, J., Suryanarayana, V., Ghosh, D.K. and Ahlawat, Y.S. 2004. PCR detection of *Candidatus Liberibacter asiaticus*, the agent of Huanglongbing or greening disease in citrus. *Indian Phytopathol.* 57:164–168.

BBS, 2021. Statistical Yearbook of Bangladesh. Bangladesh Bureau of statistics, Ministry of planning, Dhaka, Bangladesh.

Bove, J.M. 2006. Huanglongbing: a destructive, newly emerging, century old disease of citrus. *J. Plant Pathol.* 88: 7-37.

Das, A.K., Nerkar, S., Bawage, S., Kumar, A. 2014. Current distribution of Huanglongbing (citrus greening disease) in India as diagnosed by real-time PCR. *Journal of Phytopathology* 162:402–406.

Davis, M.J., Mondal, S.N., Chen, H., Rogers, M.E. and Brlansky, R.H. 2008. Co-cultivation of '*Candidatus Liberibacter asiaticus'* with actinobacteria from citrus with huanglongbing. *Plant Dis.* 92:1547-1550.

Gopal, K., Gopi, V., Kalyani, L., Sreelatha, M. ad Sreenivasulu, B. 2010. Symptom-based diagnosis of Huanglongbing (citrus greening) disease by PCR in sweet orange (*Citrus sinensis* osbeck) and acid lime (*Citrus aurantifolia* swingle). *Archives of Phytopathology and Plant Protection* 43:863–870.

Gottwald, T.R., Bergamin-Filho, A., Bassanezi, R.B., Amorim, L., Irey, M., Zhao, X. and Aubert, B. 2007. Concepts in Huanglongbing Epidemiology. Pages 1-10 in:Proc. of the Intl. Workshop for the Prevention of Citrus Greening Disease in Severely Infected Areas. Intl. Res. Div., Agric. Forestry Fisheries Res. Counc. Secretariat, Ministry of Agric., Forestry and Fisheries, Tokyo, Japan.

Gottwald, T.R., Dixon, W., Parnell, S. and Riley, T. 2010. Huanglongbing: The dragon arrives in the USA. Proc. Huanglongbing Greening Int. Workshop. Ribeirão Preto, São Paulo, Brazil, 16-20 July 2010.

Hocquellet, A., Toorawa, P., Bove, J.M. and Garnier, M. 1999. Detection and identification of two *Candidatus liberobacter* species associated citrus huanglongbing by PCR amplification of ribosomal protein genes of the beta operon. *Mol. Cell. Probes.*13: 373-379.

Hussain, M.A. and Nath, D. 1927. The citrus psylla (*Diaphorina citri Kuwayama*) [Psyllidae: Homoptera]. Memories of the Department of Agriculture in India, *Entomological Series.* 10:1-27.

Jagoueix, S., Bove, J.M. and Garnier, M. 1994. The phloemlimited bacterium of greening is a member of the alpha subdivision of the proteobacteria. *Int. J. Syst. Bacteriol.* 44: 379-386.

Jagoueix, S., Bove, J.M. and Garnier, M. 1996. PCR detection of two *candidatus Liberibacter* species

associated with greening disease of citrus. *Mol. Cell. Probes.*10: 43-50.

Kaipeng, D.L., Karmakar, K., Ghosh, S., Rahaman, L., Nath, N. and Sharma, B.K. 2017. Huanglongbing: causal disease for the decline of *Citrus reticulata* and *Citrus macroptera* production in Tripura, *Northeast India. Journal of Plant Pathology* 99:273–278.

Li, W., Levy, L. and Hartung, J.S. 2009. Quantitative distribution of "*Candidatus Liberibacter asiaticus*" in citrus plants with citrus Huanglongbing. *Phytopathology* 99:139–144.

Parker, J.K., Wisotsky, S.R., Johnson, E.G., Hijaz, F.M., Killiny, N., Hilf, M.E. and De La Fuente, L. 2014. Viability of 'Candidatus Liberibacter asiaticus' prolonged by addition of citrus juice to culture medium. *Phytopathology* 104:15-26.

Planet, P., Jagoueix, S., Bové, J.M. and Gar-nier, M. 1995. Detection and characterization of the African citrus greening liberobacter byamplification, cloning, and sequencing of therplKAJL-rpoBC operon. *Curr. Microbiol.* 30:137-141.

Puttamuk, T., Zhou, L., Thaveechai, N., Zhang, S. and Armstrong, C.M. 2014. Genetic diversity of *Candidatus Liberibacter asiaticus* based on two hypervariable effector genes in Thailand. *PLoS ONE* 9(12):e112968.

Sechler, A., Schuenzel, E.L., Cooke, P., Donnua, S., Thaveechai, N., Postnikova, E., Stone, A.L., Schneider, W.L., Damsteegt, V.D. and Schaad, N.W. 2009. Cultivation of *'Candidatus Liberibacter asiaticus', 'Ca. L. africanus',* and *'Ca. L. americanus'* associated with huanglongbing. *Phytopathology* 99:480-486.

Texeira, D.C., Saillard, C., Eveillard, S., Danet, J.L., da Costa, P.I., Ayres, A.J. and Bové, J.M., 2005. '*Candidatus Liberibacter americanus'* associated with citrus huanglong-bing (greening disease) in São Paulo State, Brazil. *Int. J. Syst. Evol. Microbiol.* 55:1857-1862.

Wang, N. and Trivedi, P. 2013. Citrus huanglongbing: A newly relevant disease presents unprecedented challenges. *Phytopathology* 103:652–665.

Zhang, Z., Bao, M., Wu, F., Chen, J. and Deng, X. 2016. Predominance of single prophase carrying a CRISPR/cas system in *"Candidatus Liberibacter asiaticus"* Strains in Southern China. PLoS ONE 11(1):e0146422.