Molecular detection of *Candidatus Liberibacter asiaticus* from citrus plants in Bangladesh

Abstract

Commercial cultivation of citrus is a vital component of the agricultural sector of a country which offering enormous nutritional and economic benefits. However, citrus plants face various disorders, including Huanglongbing (HLB), also known as citrus greening disease.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 budwood. 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, Mymensingh, 4 (40%) belonging from 10 symptomatic trees were amplified and produced amplicons of 703 bp and 500bpfrom 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.

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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.

Citrus greening or Huanglongbing (HLB) has been co-related to three uncultured "*Candidatus liberibacter* species" discovered in the phloem: "*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 (*Diaphorinacitri*) (Bove, 2006). 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 colour of the fruits. Fruits from diseased trees are usually small, lopsided with improper coloration which reduce the market value (Wang and Trivedi, 2013). HLB first became known in Southeast Asia more than 100 years ago, and it ultimately was found in Florida in the USA in 2005. Disease has rapidly spread all over the world's commercial citrus-growing regions (Kumagai et al., 2013). Symptoms of the citrus greening disease can occur throughout the entire tree and these include yellowing of leaves with a blotchy mottling pattern that is not consistent across the leaf, as well as small, upright leaves with thickened midribs and veins. As the disease progresses, leaves drop, shoots become stunted, and branches gradually die. Other signs of citrus greening include out-of-season flowering and fruiting, small and lopsided fruit with dark, undersized seeds, and excessive fruit drop. Leaf symptoms can be confusing, especially at the early stages of infection, and, thus, not always can be used for diagnosis purposes (Gottwald et al., 2007). The quantitative real-time polymerase chain reaction (qPCR) was then developed for Las, becoming the most important and widely used tool for HLB diagnosis and research (Li et al., 2006). Initially, the citrus greening disease was believed to be linked to mineral deficiency and waterlogging, as its symptoms included yellow shoots.Additionally, the disease is not seed-transmissible, as infected plants tend to have a high number of aborted seeds. The climate and type of leaves influence the acquisition of the pathogen by the vector. During winter, feeding on old leaves is observed to be ideal for pathogen acquisition, while in spring, a young flush is preferred.

Symptom-based diagnosis is not practical and the popularity of the PCR technique has grown because of its ability to quickly and sensitively detect even trace amounts of pathogen DNA in both infected plant samples and insect vectors (Sajid et al., 2022). PCR utilizes a fluorescent reporter that binds to the amplified product and emits fluorescence to indicate its presence. The intensity of the fluorescence is directly proportional to the quantity of the product that is formed. In areas where the disease is newly introduced, quarantine and eradication programs may be implemented to prevent its spread (Mei et al., 2014).Visual symptoms and biological indexing have been the historical means of diagnosis of HLB (Roistacher, 1991). Later, detection systems were developed using electron microscopy (Lafleche, D., and Bove, J. 1970), HLB-specific fluorescent substance (Schwarz, 1968), and enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (Gao et al., 1993). PCR based detection methods were developed based on sequences of the 16s ribosomal DNA and other regions of the bacterial genome (Tian et al., 1996). Sensitive detection methods for confirmation of symptoms developed include real-time quantitative PCR (qPCR) and loop-mediated isothermal amplification (Okuda et al., 2005).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.

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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). 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.

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

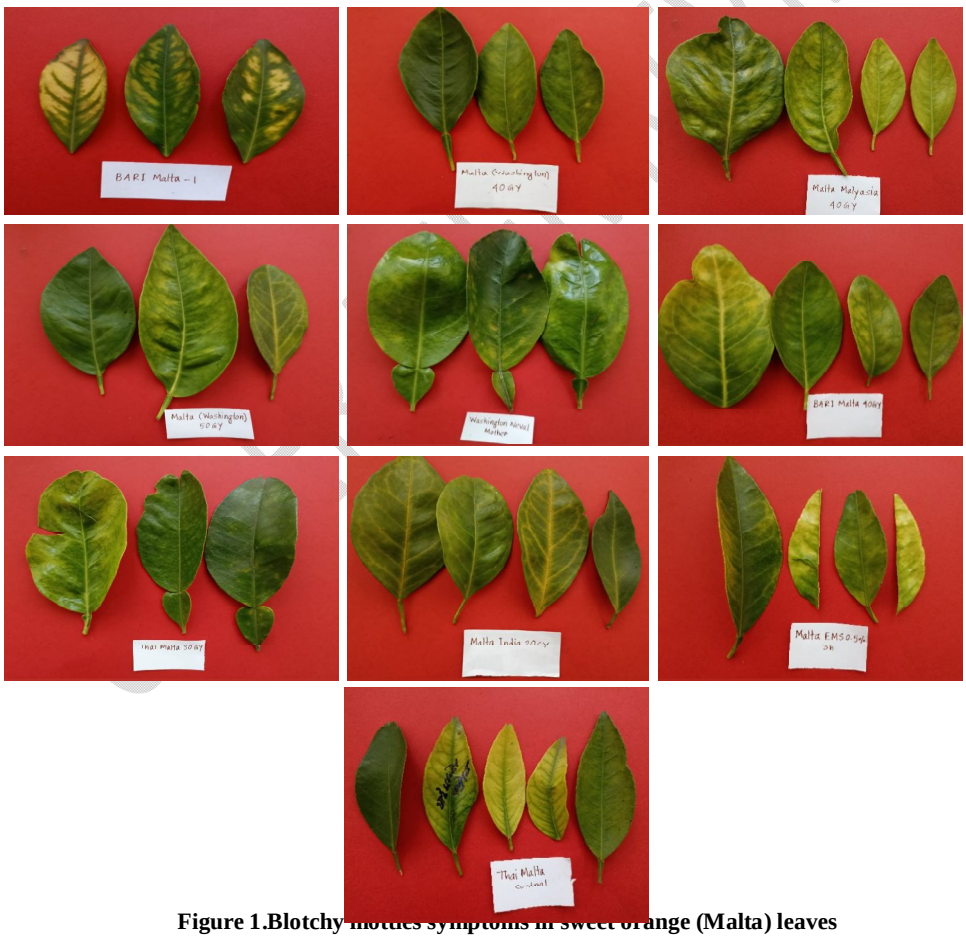

Figure 1.Blotchy mottles symptoms in sweet orange (Malta) leaves

Molecular studies

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

DNA extraction

Leaf samples were washed with sterile distilled water and 70% ethanol and dried onblotting 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 tubeand ground by micro-pestle adding liquid nitrogen. The Las genomic DNA was extractedusing Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) followingmanufacturer's instructions. The pellet was re-suspended in 25μl volume of DNArehydration 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 thermocycler 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 TBEbuffer (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 (ProteinSimple, 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 ofInvent Technologies Limited, Bangladesh. PCR products were sequenced directly in both orientations from primers A2/J5 according to the standard protocols for the ABIPRISM 3500 (version 3.7) automated DNA sequencer (Perkin Elmer) with ABI PRISM Ready Reaction Dye Termination Cycle Sequencing Kit.The quality of nucleic acidsequences was evaluated using Chromas (Version 2.6) software to avoid the use of lowqualitybases. 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 (Table2) 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 (Figure2 and 3).

Table2. 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 Jagoueix *et al.*, 1996
A2	TAT AAA GGT TGA CCT TTC GAG TTT	Las	Forward	rpIKAJL-rpoBC (operon)	Primer described by Hocquellet *et al.*,1999
J5	ACA AAA GCA GAA ATA GCA CGA ACA A	Las	Reverse	rpIKAJL-ropBC (operon)	Primer described by Hocquellet *et al.*,1999

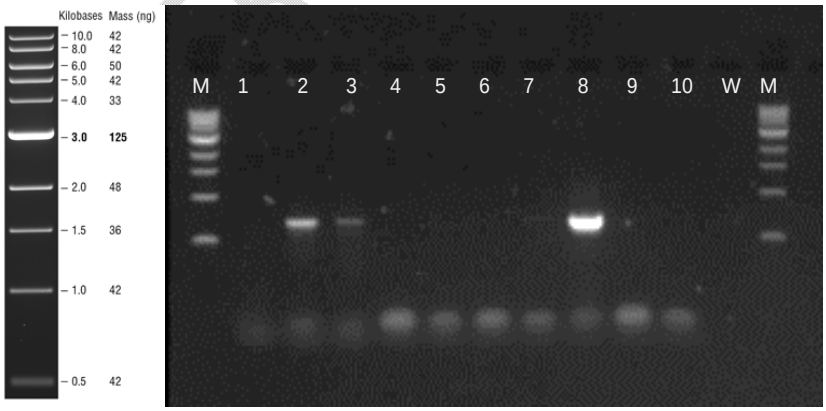

Figure 2. PCR amplification of a 703 bp from the suspected samples confirmed the presence of *CandidatusLiberibacter Asiaticus* using A2/J5

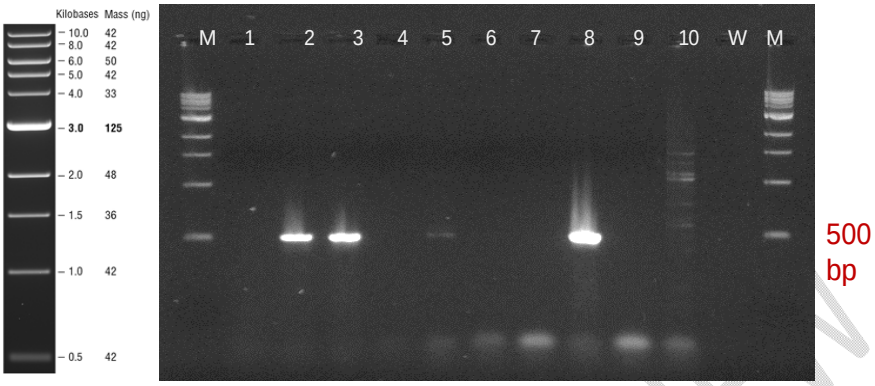

Figure 3. PCR based detection of *Ca. Liberibacter*asiaticus using Las606/Lss

Sequence analysis

One representative PCR product after amplification with primers A2/J5 of the ribosomalprotein genes of the *rpl*KAJL-*rpo*BC operon were sequenced (Table3). BLAST homology showed 99-100% sequence identity with the correspondingnucleotide sequence of ribosomal protein gene of *Candidatus Liberibacter asiaticus* strains found in India, China, Vietnam, Iran and KenyaPhylogenetic tree constructed from translated protein showed close relationship withIndian, Iranian isolates.The origin of *Candidatus Liberibacter asiaticus* in Bangladesh is uncertain because of the perfect homologybetween the sequence of the Bangladeshi strain and strains from India. We suspect that itwas introduced by infected materials from the South Asian regions.Results from PCR amplification and aligningribosomal gene sequences with otheridentified strains of different countries gave strong evidence that the symptomaticmandarin orange leaf samples collected from the surveyed areas were infected with *Candidatus Liberibacter asiaticus* and not due to micronutrient deficiencies or disorder. Itrevealed the mystery of citrus declining in Bangladesh which was associated with bacterialpathogen. These results confirmed the occurrence of citrus greening (HLB) on *Citrus reticulata* in Bangladesh.

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	FJ177536.1	638/640	99

	protein gene, partial cds			
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

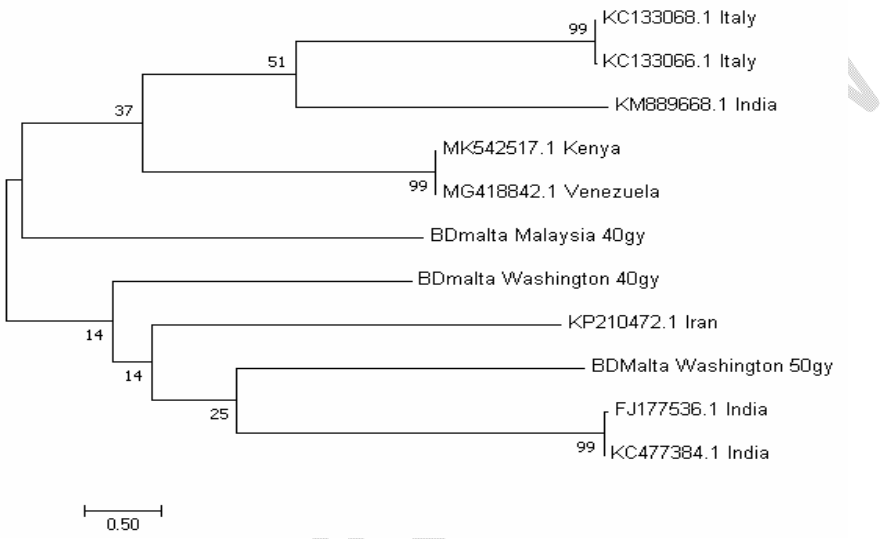

Figure 4. Phylogenetic relationships of the *Candidatus liberibacter asiaticus* isolates using MEGA7 software

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.

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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 *Diaphorinacitri* 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.*

Nauman et al. (2021), claim that although HLB disease exhibits characteristic symptoms such as mosaic or mottling patterns on leaves, stunted plant growth, deformed shape, premature fruit drop, and yellowing of reticulate venation, the detection of the bacteria and downstream studies requires high-quality DNA, which is challenging to extract from citrus leaves due to the plant's various species, age groups, thick waxy cuticles, and high production of compounds like phenolics and polysaccharides. Shafiq et al. (2018), took a study molecularly characterize the mtco1 gene in ACP, and evaluate the incidence of CLas in different citrus cultivars in Pakistan. Zafarullah et al. (2016), have conducted a study to identify and characterize the HLB pathogen in commercially grown citrus varieties, specifically Kinnow and sweet oranges. The prevalence of *Candidatus liberibacter asiaticus* exhibited seasonal variation, with a decrease during the spring season characterized by conditions of temperature and humidity that are considered unfavorable for the bacterium. CLas is known to produce symptoms under relative humidity below 40% and high-temperature conditions, with temperatures up to 35°C.

Several management strategies have been used to manage this disease. The effective decrease in this disease is dependent upon the effective administration of micronutrients, which serve a crucial function in enhancing the resilience of citrus plants to the disease. The characteristic indicators of citrus greening include the presence of discolored leaves on citrus plants that were affected by the disease. The fruits that were infected exhibited stunted growth and were unable to achieve the characteristic orange hue of mature fruit. Subsequently, a combination of Zinc sulfate and Manganese sulfate was administered to specifically chosen diseased plants at varying concentrations. Then, various vegetative, physiological in nature, and biochemical characteristics of citrus fruit that were suffering from citrus greening, including fruit diameter, juice percentages, total soluble solids, weight, total solids insoluble in water, titratable acidity ascorbic acids were statistically analyzed. Zhang et al. (2011), stated no established cure for citrus HLB has been around for over a century. To control HLB spread, a propagation test system was used to screen therapeutic compounds on both infected periwinkle and citrus plants with CLas. Li et al. (2019), took a study to determine the lowest dosage of oxytetracycline (OTC) required to effectively control citrus HLB disease using a new method developed to evaluate the effect of OTC treatment on CLas titers in infected plants. Munir et al. (2022), conducted a study to establish a valuable method of managing HLB by modifying the citrus endophytic bacteria with the use of an indigenous endophyte, thereby ensuring sustainability. Umair et al., (2023), conducted a study to investigate phylogenic silver nanoparticles' (AgNPs') potential to successfully recover the condition of citrus plants afflicted with HLB disease in a biocompatible manner.However, the development of infrastructure for molecular studies

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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 latent 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.

Conclusion

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. HLB is a severe threat to the citrus industry, as it affects all commercial varieties and regions. The disease is caused by Las, which is transmitted by psyllids. The psyllid transmission of HLB is a complex and dynamic process that involves multiple interactions among biotic and abiotic factors. Understanding these interactions can help to design better strategies to reduce the psyllid population and prevent Las transmission. Opportunities for future improvements in the management of HLB disease may involve the development of resistant citrus cultivars to mitigate susceptibility to the disease, as well as the study of biological control strategies, such as the development of beneficial microorganisms or predators, to specifically target the bacteria responsible for the disease or its vectors.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.

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