



### Full Length Article

## Detection of ‘*Candidatus Liberibacter Asiaticus*’ the Causal Organism of Huanglongbing, in Mandarin Group of Citrus

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### Abstract

Huanglongbing (HLB) is a fatal disease of citrus. We report the detection of ‘*Candidatus Liberibacter asiaticus*’ the causal organism of huanglongbing, in mandarin group of citrus. Four genotypes of citrus belonging to mandarin group including: Citrus Sunki (*Citrus sunki*), Kinnow, Parson’s Special and Sun Chu Sha were studied for the detection of HLB bacterium. Taqman based real time qPCR was performed for the detection of *Candidatus Liberibacter asiaticus* in citrus host. Highest mean cycle threshold value of 25.06 was obtained for Kinnow mandarin while 24.30 for Parson’s Special, 23.95 for Citrus Sunki and 22.0 for Sun Chu Sha. These results may be helpful in the development of management plans for HLB control in Pakistan as well as round the world. © 2017 Friends Science Publishers

**Keywords:** Ct values; HLB management; Real time qPCR; Symptom

### Introduction

With respect to production, Pakistan ranks at the 13<sup>th</sup> position in the world with 2150 thousand tones production of citrus from an area of 198 thousand ha. Average yield in Pakistan is approximately 11 tons per ha that is only about 36% of per ha production in the USA (Anonymous, 2013). Citrus is one of the most important fruit crops contributing in the revenue of Pakistan. From 95.6% share of total citrus produced by Punjab, about 80% citrus includes Kinnow mandarin. Bahrain, Dubai, Indonesia, Kuwait, Malaysia, Netherlands, Oman, Qatar, Russia, Saudi Arabia, Singapore, and UK are the major market places of Pakistan’s Kinnow (Tahir, 2014). Sargodha and its neighbouring areas including Faisalabad, Toba Tek Singh, Jhang and Sahiwal are the main districts that produce good quality Kinnow (Johnson, 2006). It is suspected that HLB is one of the major reasons of low production of citrus per ha in Pakistan.

The HLB is most serious infectious disease of citrus and major threat to citrus industry (Hall *et al.*, 2012; Suszkiw, 2013). In regions, where HLB is endemic, citrus trees produce unmarketable fruit as it abscise prematurely and mostly die within 5 to 8 years (Baldwin *et al.*, 2010). In Florida, up to 2014, approximately 33% revenue of the total production of the state has been wasted due to HLB and more than 8000 persons associated with citrus industry lost their jobs (Chin *et al.*, 2014).

*Candidatus Liberibacter*, a Gram negative, non culturable and phloem limited bacterium is the causal

organism of HLB (Li *et al.*, 2009). There are three types of this bacterium: *Candidatus Liberibacter asiaticus* (*Ca. Las*), *Candidatus Liberibacter africanus* (*Ca. Laf*) (Koizumi, 1995) and *Candidatus Liberibacter americanus* (*Ca. Lam*) (Teixeira *et al.*, 2005). The complete genomes of all three bacterial species responsible for HLB have been sequenced those are: 1.23 Mbp for *Ca. L. asiaticus* (Duan *et al.*, 2009), 1.195201 Mbp for *Ca. L. americanus* (Wulff *et al.*, 2014) and 1.192232 Mbp for *Ca. L. africanus* (Lin *et al.*, 2015).

The natural vector of the pathogen is Asian citrus psyllid (ACP). There are two species of psyllid vector: *Diaphorina citri* Kuwayama (*D. citri*) and *Trioza erytreae* (*T. erytreae*) reported for citrus greening (Aubert, 1987). The *D. citri* is a natural vector of both *Ca. Las* and *Ca. Lam*, while *T. erytreae* is the vector of *Ca. Laf* (Teixeira *et al.*, 2005; Bove, 2006; Lin *et al.*, 2015).

HLB symptoms do not appear in the host plant immediately after infestation (Chin *et al.*, 2014). HLB symptoms development under greenhouse environment from grafting may take 3 to 12 months (Lopes *et al.*, 2009). The most common symptoms of HLB are blotchy mottling, vein yellowing and vein corking. Sometimes, at initial stages of infection, the above mentioned symptoms are observed only on one part of the canopy, therefore the name of the disease in China is huang-long-bing means yellow shoot disease (Garnier and Bove, 1993). Infected leaves become small and upright, followed by leaf drop and dieback. HLB also destroys the juice quality and incorporate bitter elements in citrus juice. Fruit of small size with

loppside grows on the diseased plant. HLB affected fruit do not colour properly. Early flowering in HLB affected plants has also been observed (Albrecht and Bowman, 2008; Martinelli *et al.*, 2012).

For successful management of HLB, accurate detection of pathogen is very important but, the detection of the pathogen is very difficult, because of its low titer and uneven distribution in its citrus hosts (Bove, 2006; Li *et al.*, 2007). For HLB diagnosis, different parameters have been used including monitoring of natural vector of HLB in citrus groves, observation of HLB symptoms on citrus leaves and fruits, biochemical tests for the assessment of presence of HLB pathogen, microscopic identification of HLB pathogen and molecular detection of HLB pathogen. For the simplicity of detection by PCR, rpl/kAJL- rpoBC associated genes of HLB pathogen have been amplified. The primer set A2/J5 was designed in this method that confirms direct identification of Las and Laf (Hocquellet *et al.*, 1999). Real time PCR is highly sensitive technique that is being used frequently for HLB diagnosis all over the world. Li *et al.* (2006) developed fluorescent labelled primer sets for HLB pathogen detection in citrus and insect vector as well as internal control gene primer for plant cytochrome oxidase.

The suspected presence of HLB in Pakistan has been reported in many publications on the basis of visual symptoms (Cochran, 1976; Abbas *et al.*, 2005). Chohan *et al.* (2007) first time confirmed the presence of HLB associated bacterium in psyllid and plant host at molecular level in Peshawar province of Pakistan. It is hypothesized that the research gap was due to confusing symptoms of HLB. Most of the researchers were of the view that these symptoms are due to nutrients deficiency. Further it is suspected that due to lack of molecular knowledge about HLB diagnosis, this disease has been ignored in Pakistan.

The objectives of present study were to observe the expression of HLB symptoms and use molecular technology for HLB bacterium detection.

## Materials and Methods

### Experimental Details and Treatments

**Experimental material:** Seeds of mandarins including: *Citrus sunki*, Cleopatra, Kinnow, Kinokuni, Parson's Special, Sun Chu Sha, Nules and Scarlet Emperor were acquired from the United States Department of Agriculture (USDA) National Clonal Germplasm Repository for Citrus and Dates, Riverside, California, USA and University of Agriculture, Faisalabad, Pakistan source trees (Table 1). They were sown in controlled conditions of greenhouse of Institute of Horticultural Sciences (IHS), University of Agriculture Faisalabad (UAF) Pakistan.

**Inoculation of citrus plants:** Four genotypes of mandarins including *Citrus sunki*, Kinnow, Parson's Special and Sun Chu Sha in triplicate were inoculated by the *Candidatus*

*Liberibacter asiaticus* bacterium. The grafts and leaf midribs used for inoculation were obtained from HLB positive sweet orange (*Citrus sinensis* Osbeck.) plants that were infested by ACP in controlled conditions of growth room. Three plants for each genotype were separated as healthy controls. Each replicate of one-year-old citrus genotypes was inoculated by budding one midrib and one bud to ensure inoculation. The inoculated plants were maintained in controlled conditions of greenhouse. Application of nutrients was continued during the entire period of experiment to both treatments *i.e.* healthy control and inoculated plants to prevent expression of nutrition deficiency symptoms.

**DNA extraction and quantification:** After one year of inoculation, leaf samples with HLB symptoms were excised and their DNA was extracted to detect the HLB pathogen. DNA was extracted from ~ 0.5 g midribs and petioles of leaf of inoculated and healthy plants by the CTAB (2% CTAB, 100 mM Tris HCl, 1.4 mM NaCl, 20 mM EDTA) method modified from protocol 3 of Ruangwong and Akarapisan (2006). The DNA was quantified by spectrophotometric method using 1 µL genomic DNA from the total with spectrophotometer NanoDrop ND 2000™ (Thermo Scientific) at the Institute for Integrative Genome Biology (IIGB), University of California Riverside, USA. The concentration of DNA was obtained by absorbance at 260 nm. The ratio of nucleic acids to proteins in the sample was evaluated by the ratio of the absorbance at 260 and 280 nm (A<sub>260</sub>/A<sub>280</sub>) (Sambrook and Russel, 2001).

**Real-time PCR for *Candidatus Liberibacter asiaticus* detection:** In the DNA samples of *Citrus sunki*, Kinnow, Parson's Special and Sun Chu Sha, TaqMan based multiplex quantitative real time PCR was conducted for the detection of Las sequence. Sweet orange cultivar Succari DNA was used as positive control. Quantitative PCR was conducted using 16S rDNA based primer-probe set HLBasfpr, specific to Las (5'→3' sequences: forward GTCGAGCGGTATGCAATAC, reverse TGCCTTATCCCGTAGAAAAAGGTAG and probe AGACGGGTGAGTAACGCG). A primer-probe set based on plant cytochrome oxidase (COX) gene was used as a positive internal control to assess the quality of the DNA extracts (5'→3' sequences: forward GTATGCCACGTTCGATTCCAGA, reverse GCCAAAAGTCTAAGGGCATTTC and probe ATCCAGATGCTTACGCTGG) as described by Li *et al.* (2006). Cycling conditions of PCR consisted of an initial denaturation step at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 10 sec and annealing at 58°C for 20 sec.

### Statistical Analysis

iQ5 Optical System software version 2.1 was used for data analysis conditions including baseline and threshold.

## Results

Out of eight genotypes evaluated in the current study only four including *Citrus sunki*, Kinnow, Parson's Special and Sun Chu Sha survived (Table 1). To see the success and response of inoculation, observation of HLB symptom expression was started after one month of inoculation. Despite nutrients application, the leaf colour of inoculated plants was found to be light green as compared to dark green for the healthy control plants. All genotypes of mandarin expressed HLB symptoms in leaves including: blotchy mottle, vein yellowing, vein corking and leaf yellowing. Parson's Special and Sun Chu Sha expressed more symptoms as compared to *Citrus sunki* and Kinnow (Table 2).

**Real-time PCR for *Candidatus Liberibacter asiaticus* detection:** Each PCR reaction was run in triplicate for each genotype for the detection of HLB bacterium. The measurement was made after each amplification cycle, and this is the reason why this method is called real time PCR. The DNA samples were considered positive for HLB bacterium with cycle threshold (Ct) values up to or less than 36.9 as described by Hoffman *et al.* (2013). The DNA samples with Ct values above 36.9 or no amplification (NA) were considered negative for the presence of HLB pathogen. No amplification was observed in healthy controls of all of four mandarins. All of the inoculated varieties of citrus were found infected upon testing for HLB pathogen with a high rate of infection (Table 3). Correlation coefficient value of -0.60018 was obtained

for the correlation between Ct values and HLB symptoms using Pearson's correlation formula.

## Discussion

In present study, from eight sown genotypes of mandarin, 50% were survived and tested for the presence of *Candidatus Liberibacter asiaticus*. Vein corking symptom of HLB was observed in *Citrus sunki*, while blotchy mottle symptom was observed in Kinnow mandarin. Parson's Special and Sun Chu Sha expressed vein corking, vein yellowing, blotchy mottling and leaf yellowing symptoms of HLB. Hussain and Nath (1927) also described the symptoms of HLB that lookalike symptoms reported today. Due to vascular blockage, starch accumulation occurs in HLB symptomatic leaf tissue as described by Etxeberria *et al.* (2009). Upregulation of starch synthesizing genes incorporating ADP- glucose pyrophosphorylase, granule bound starch synthase and starch debranching enzyme result in starch accumulation in HLB infected leaves (Kim *et al.*, 2009). Starch accumulation is the major cause of symptom expression in HLB affected citrus plants. The HLB was found to be present in north-eastern and north-western India in the 1800s and early 1900s. A severe damage caused by populations of *D. citri* at Sargodha and Lyallpur from 1915 to 1920 has been reported in detail indicating the presence of *D. citri* in Pakistan (Indo-Pak subcontinent) for 100 years (Hussain and Nath, 1927; Gottwald *et al.*, 2007). Diseased trees are yet producing fruit in Pakistan, but in low amount.

**Table 1:** Mandarin cultivars used for the detection of *Candidatus Liberibacter asiaticus*

Sr. No.	Group	Cultivar	Binomial	Accession No.
1	Mandarin	Citrus Sunki	<i>Citrus sunki</i>	PI 539678
2	Mandarin	Cleopatra	<i>Citrus reshni</i>	PI539492
3	Mandarin	Kinnow	<i>Citrus reticulata</i> Blanco	Pak
4	Mandarin	Kinokuni	<i>Citrus kinokuni</i>	PI 539270
5	Mandarin	Parson's Special	<i>Citrus reticulata</i>	PI539497
6	Mandarin	Sun Chu Sha	<i>Citrus reticulata</i>	PI 539544
7	Mandarin	Nules	<i>Citrus clementina</i> hort.ex Tanaka	Pak
8	Mandarin	Scarlet Emperor	<i>Citrus reticulata</i>	PI 539505

**Table 2:** Expression of huanglongbing symptoms in mandarin cultivars

Sr. No.	Group	Cultivar	Blotchy mottle	Vein yellowing	Vein corking	Leaf yellowing
1	Mandarin	Citrus Sunki	-	-	✓	-
2	Mandarin	Kinnow	✓	-	-	-
3	Mandarin	Parson's Special	✓	✓	✓	✓
4	Mandarin	Sun Chu Sha	✓	✓	✓	✓

**Table 3:** Mean cycle threshold values for detection of Las in mandarins and positive control sweet orange

Sr.No.	Group	Cultivar	Fluor
			FAM
			Ct Mean
1	Mandarin	Citrus Sunki	23.95
2		Kinnow	25.06
3		Parson's Special	24.30
4		Sun Chu Sha	22
5	Sweet orange	Succari	20.0

Many factors could be involved in the survival of HLB infected trees like, hot summers in Pakistan or resistance development in citrus host against Las.

To detect the Las in HLB infected 4 genotypes of citrus belonging to mandarin group and succari sweet orange, we performed taqman based qPCR targeting 16S rRNA gene of Las according to Li *et al.* (2006). Bacterial titre based on cycle threshold (Ct) values was found significantly higher in positive control succari with mean Ct value of 20. Among 4 genotypes of mandarin, Sun Chu Sha was found to have lowest mean Ct value of 22 indicating highest titre of HLB bacterium. Kinnow mandarin produced lowest signals for HLB pathogen detection in real time qPCR with highest mean Ct value of 25.06. The negative value for the correlation coefficient indicates that expression of symptoms does not depend upon ct values for that represent the bacterial titre. Efforts are in progress to control HLB all over the world but it is rapidly spreading in new areas.

In conclusion, all studied genotypes of citrus belonging to mandarin group were found susceptible to HLB pathogen with less susceptibility for Kinnow mandarin. Further studies are needed to test commercially grown citrus varieties in Pakistan for Las detection to control this disease.

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