

Brief Report

Update on ‘*Candidatus Liberibacter asiaticus*’ incidence in five districts of the Punjab province of Pakistan

Sagheer Atta^{1*}, Huawei Liu², John. S. Hartung³, Muhammad Amjad Bashir¹ and Komal Tariq⁴

¹Department of Plant Protection, Faculty of Agricultural Sciences, Ghazi University, Dera Ghazi Khan, 32200 Pakistan; ²College of Horticulture and State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Jiangsu, China; ³MPPL, ARS, BARC West, USDA, Maryland, USA; ⁴Department of Biochemistry and Molecular Biology, University of Gujrat, Pakistan.

*Correspondence to: satta@gudgk.edu.pk

Abstract

‘*Candidatus Liberibacter asiaticus*’ (CLAs) is a devastating pathogen of citrus associated with Huanglongbing (HLB, citrus greening disease). HLB is economically significant in Asia and has destroyed millions of citrus trees worldwide during the last century. Since 2007, when the first molecular evidence for the presence of CLAs in North-West Frontier Province of Pakistan (Khyber Pakhtunkhwa) were presented, there have been a limited number of studies reporting the pathogen in different citrus growing districts of the Punjab Province, one of the major citrus producing areas of the country. In this study, a small-scale survey was conducted in citrus groves exhibiting HLB-like symptoms in different districts of Punjab Province. The aim was to obtain current information on the incidence of CLAs in the area and complement the previous studies. Conventional and real time quantitative polymerase chain reaction (PCR and qPCR) assays were used for the detection of CLAs in the 94 samples tested, followed by sequencing of the PCR amplicons. Overall CLAs was detected in 43% (40/94) of the tested samples from Kinnow mandarin (n= 22), sweet orange (n= 12), grapefruit (n= 3) and Feutrell’s early mandarin (n= 3). In agreement with previous reports, this survey confirmed the presence of HLB in the Punjab Province of Pakistan and provided updated molecular evidence for the presence of CLAs and disease incidence for four citrus cultivars from five citrus growing districts of the province.

Keywords: HLB, huanglongbing, citrus greening, survey

Introduction

Huanglongbing (HLB) is one of the most economically important diseases of citrus and responsible for the destruction of millions of citrus trees in Asia, where the disease originated, and in the Americas, after its detection in the early 2000’s (Halbert and Keremane 2004; Bové 2006; Li et al. 2020; Bassanezi et al. 2020). The disease was first reported from China in 1919 and in Pakistan in 2007, when the first molecular evidence for the presence of HLB in North-West Frontier Province of Pakistan (Khyber Pakhtunkhwa) were presented (Chohan et al. 2007). However, the disease has likely been present in Pakistan for many years prior to the 2007 study as HLB-like symptoms were reported decades earlier but were attributed to damage from psyllids and/or other abiotic or biotic factors (Husain and Nath 1927; Chohan et al. 2007; Razi et al. 2014; da Graca et al. 2016). HLB in Asia is primarily associated with the Gram-negative, phloem-limited, fastidious bacterium ‘*Candidatus Liberibacter asiaticus*’ (CLAs). CLAs is transmitted by grafting and through the psyllid vector *Diaphorina citri* (Kuwayama) (Halbert and Keremane 2004; Bové 2006).

HLB has become a matter of great concern in every citrus producing area of the world as the disease affects all commercial citrus cultivars, causing decline in tree growth and the quantity and quality of fruit production (Teixeira et al. 2005; Halbert 2005, Albrecht et al. 2020). There are no effective control measures for the disease, although research has made clear the importance of a systems approach that includes among other, planting trees produced only with pathogen-free budwood, testing, identification and removal of infected trees and area wide management of the insect vector (Bové 2006; Belasque et al. 2010; Albrecht et al. 2020; Bassanezi et al. 2020; Alquézar et al. 2022).

Based on symptom expression and a small number of surveys, HLB is considered to be widespread in citrus cultivars within the growing regions of Pakistan (Catara, 1987; Catara et al. 1988; Chohan et al. 2007; Razi et al. 2014; Naqvi et al. 2017). Punjab Province is one of the most important citrus producing areas in Pakistan affected by HLB. However, only a few studies have been conducted to estimate disease incidence in different districts or citrus cultivars within the country (Razi et al. 2014; Naqvi et al. 2017; Siddique and Garnevska 2018). The most recent

HLB survey in various districts of the Punjab Province was performed in 2017 using serological and conventional polymerase chain reaction (PCR) assays (Naqvi et al. 2017). An earlier study in 2014 was focused on the seasonal variability of CLAs titer in *D. citri* and citrus trees and it was performed in a single Punjab district (Razi et al. 2014). The objective of this study was to conduct a renewed focused HLB survey in highly symptomatic orchards within five citrus producing districts of the Punjab Province using a combination of traditional PCR and quantitative real time PCR (qPCR) to complement the previous studies and update information on the incidence of CLAs in four commonly grown citrus cultivars in Punjab Province.

Materials and Methods

Field survey and sample collection

Plant samples were collected showing HLB-like symptoms such as blotchy mottling and branches with small and upright leaf growth on four commonly grown cultivars of citrus in five districts within Punjab Province of Pakistan (Fig. 1; Table 1). Samples were collected from the edge of orchards along roadsides, and 4-6 leaves were collected from each tree. Leaf samples were packed in plastic bags with dry ice and shipped to United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Molecular Plant Pathology Laboratory, Beltsville, Maryland, for testing under a USDA-APHIS-PPQ Permit.

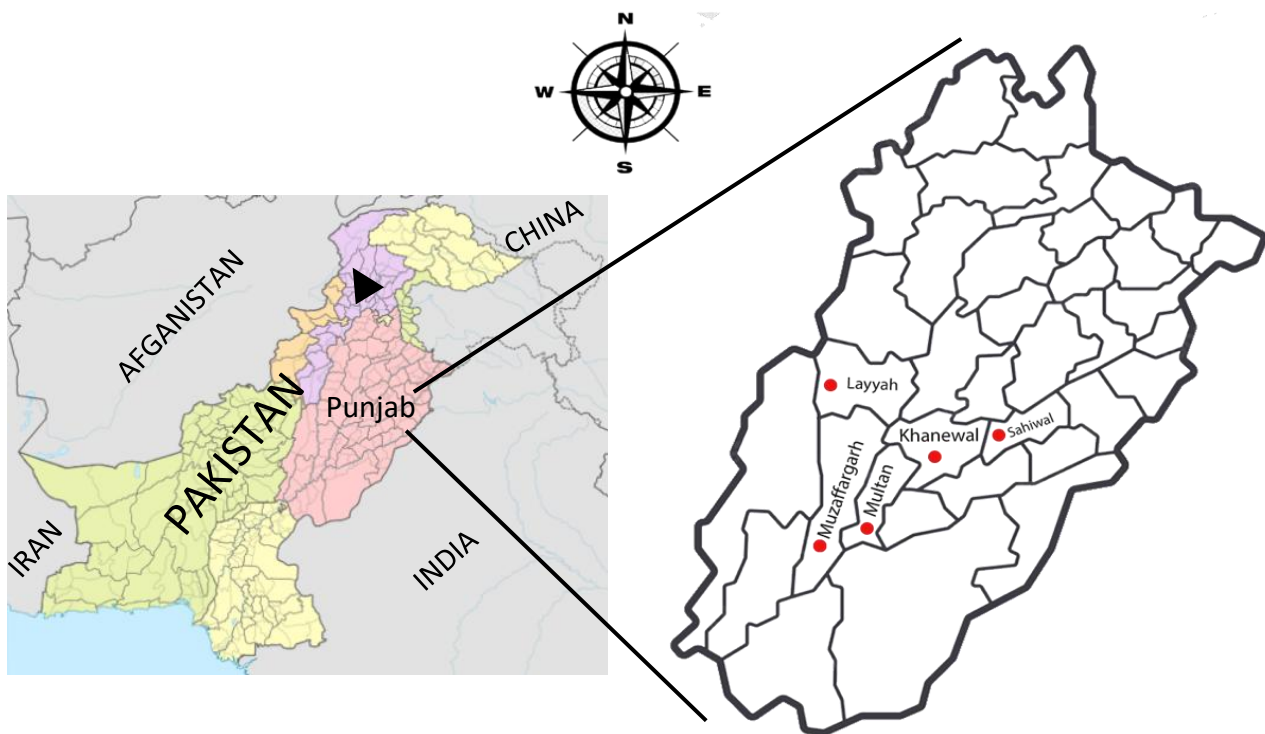


Fig. 1. Map of the country of Pakistan (left) and areas within Punjab Province (right) showing the ‘*Candidatus Liberibacter asiaticus*’ (CLAs) surveyed districts (red circles) sampled for this study. Triangle notes the North-West Frontier Province (Khyber Pakhtunkhwa) where the first molecular evidence for the presence of CLAs in Pakistan were presented in 2007 (Chohan et al. 2007).

DNA extraction and isolation

DNA was extracted from phloem rich midrib tissues of the citrus leaves. Midrib tissues were aseptically excised and ground using liquid nitrogen with a mortar and pestle. A 100 mg subsample of the resulting homogenized tissue from each sample was used for DNA isolation following Naqvi et al. (2017). The extracted DNA was stored at -20°C until used for PCR testing.

Conventional PCR testing and sequencing

Conventional PCR assays were carried out in $25\ \mu\text{l}$ of reaction mixture containing $1 \times$ PCR buffer, $2\ \mu\text{M}$ of each

primer, $2.0\ \mu\text{M}$ MgCl_2 , $0.3\ \mu\text{M}$ of each dNTP, 0.4 units *Taq* DNA polymerase (Fermentas, Waltham, MA, USA) and 100 ng genomic DNA as template. Specific CLAs primers from 16S rDNA intergenic region (Forward primer OI1 5'-GCGCGTATGCAAGAGCGGCA-3' and reverse primer OI2c 5'-GCCTCGGACTTCGCAACCCAT-3' (Jagoueix et al. 1994) were used for the amplification in a thermal cycler (MyCycler, Biorad Inc., Foster, CA, USA). The reaction conditions for OI1 and OI2 primers were: one cycle at 94°C for 3 min, 34 cycles of 94°C for 30 sec, 64°C for 30 sec and 72°C for 1 min, followed by one cycle of extension at 72°C for 10 min.

PCR products were analyzed by gel electrophoresis using 1.5% agarose gels in TBE buffer and stained with Gel Red (Biotium, Fremont, CA, USA). The agarose gels were visualized using a FluorChem Q (Alpha Innotech, Batern, Germany) gel documentation system to evaluate if the samples were positive based on the expected 1,160 bp product. Expected size amplified products were eluted from gels using Pure Link gel elution kit (Invitrogen, Waltham, MA, USA) prior to DNA sequencing.

The eluted PCR products were sent for sequencing (Genewiz, Rockville, MD, USA) and were sequenced using the automated fluorescent DNA sequencing Sanger method. All successful sequences were subjected to an NCBI BLAST search using the default parameters for sequence identity.

qPCR testing

Samples tested positive with the primers OI1/OI2c were subsequently tested with qPCR. The qPCR assays were carried out as described by Li et al. (2006). All

reactions were performed in triplicate and each run contained one known negative and one known positive control obtained from the greenhouse. The tests were performed and the results were analyzed using the Smart CyclerII machine and software (IDT, Coralville, IA).

Results

CLas incidence in Punjab Province, Pakistan

A total of 94 samples exhibiting HLB-like symptoms were collected from five citrus growing districts in the Punjab Province of Pakistan, and testing revealed a CLas incidence ranging from 36% to 46% (Table 1). Kinnow mandarin (*Citrus reticulata* Blanco) was infected at the rate of 52%, followed by sweet orange (*C. sinensis* (L.) Osbeck) at the rate of 33%, grapefruit (*C. paradisi* Macfad.) and Feutrell's Early mandarin (*C. reticulata* Blanco) at the rate of 38%, each. The overall CLas incidence was 43% (Table 1).

Table 1. Distribution and incidence of 'Candidatus Liberibacter asiaticus' (CLas) in four citrus cultivars expressing Huanglongbing-like symptoms in five districts within Punjab Province, Pakistan.

Surveyed Districts And CLas Incidence	Citrus Cultivar ¹ and CLas Incidence ²			
	Kinnow Mandarin	Sweet Orange	Grapefruit	Feutrell's Early Mandarin
Sahiwal 8/22 (36%)	5/10 (50%)	2/8 (25%)	NT ³	1/4 (25%)
Multan 11/24 (46%)	6/10 (60%)	3/10 (30%)	NT	2/4 (50%)
Khanewal 8/18 (44%)	5/8 (63%)	2/6 (33%)	1/4 (25%)	NT
Layyah 9/20 (45%)	4/8 (50%)	3/8 (38%)	2/4 (50%)	NT
Muzaffar Garh 4/10 (40%)	2/6 (33%)	2/4 (50%)	NT	NT
Total 40/94 (43%)	22/42 (52%)	12/36 (33%)	3/8 (38%)	3/8 (38%)

¹Kinnow mandarin and Feutrell's Early mandarin (*Citrus reticulata* Blanco), grapefruit (*C. paradisi* Macfad.) and sweet orange (*C. sinensis* (L.) Osbeck).

² Number of CLas positive samples/tested samples (ratio outside parenthesis) and percent positivity rate (% in parenthesis).

³ NT: Not tested.

Conventional PCR detection and sequence analysis of genomic region of CLas isolates.

The CLas detection in the 40 positive samples showed that primers OI1/OI2c amplified the 16s rDNA region with the expected product size of 1,160 bp. The nucleotide similarity of the PCR products using NCBI BLAST, revealed a query coverage of 99-100% with E values of 0, with multiple CLas sequences deposited within GenBank. The identified sequences shared high homology (98-99%) with the Pakistani accession LN835770.1 (isolate

Kinnow), as well as several international sequences from Malaysia (EU224393.1), USA, Florida (EU130552.1, EU130554.1, CP001677.5, DQ471900.1, FJ236554.1, FJ263703.1, FJ263700.1), Mexico (JQ867409.1, JQ867421.1, JQ867407.1), China (CP010804.1, CP004005.1), Japan (AP014595.1, AB555706.1), and India (KC551941.1). This widespread sequence identity highlights the global dissemination of CLas from its center of origin in Southeast Asia to relatively newer production regions in the Americas.

qPCR detection of CLAs.

The 40 samples tested positive with conventional PCR were also tested with qPCR for confirmation. The Cq values of the positive samples were between 20 and 28 for most samples while a few samples yielded higher Cq values, indicating lower CLAs titers in some of the trees tested (Supplementary Table 1).

Discussion

Citrus is an important crop in Pakistan and suffers from many biotic and abiotic problems (Khan 1992; Arif et al. 2005; Siddique and Garnevska 2018). Punjab Province is one of the most important citrus producing areas in Pakistan affected by HLB. However, there are no effective long term management strategies to control the disease and only a few studies have been conducted to estimate the disease incidence between different districts or citrus cultivars within Pakistan (Razi et al. 2014; Naqvi et al. 2017). The latest HLB survey in six districts of the Punjab Province was performed in 2017 using serological and conventional PCR assays and reported disease incidences ranging from 10% in sweet orange to 51.7% in Kinnow mandarin. Incidences in grapefruit (26.2%) and Feutrell's Early mandarin (22.2%) exhibited intermediate levels of disease (Naqvi et al. 2017). A 2014 study focused on seasonal variability of CLAs titer in *D. citri* and citrus trees reported an overall disease rate of 25% for four citrus cultivars within Pakistan (Kinnow mandarin, Feutrell's Early mandarin, Mosambi sweet orange, and grapefruit) (Razi et al. 2014). Although the number of sampled trees was not so high for all genotypes assessed, the results of the present study indicate an increase in the incidence of CLAs in the Punjab Province with an overall disease rate of over 40%. Kinnow mandarin CLAs infection rate was consistently over 50% and the infection rates of grapefruit, Feutrell's Early mandarin, and sweet orange were elevated above 30% (Table 1). The observed increase in CLAs incidence could be a result of the natural progression of the infection or because of the focused sampling in this study of symptomatic trees. This indicates that a more detailed survey to include testing of other pathogens that can induce HLB-like symptoms in citrus, such as phytoplasmas reported previously from Pakistan, need to take place (Noorizadeh et al. 2022; Fahmeed et al. 2009).

Molecular detection of CLAs with a combination of methods such as conventional PCR for pathogen identification (i.e., possibility of sequencing) and qPCR (i.e., sensitivity) provided complementary information important to understand the distribution of HLB within Pakistan (Hung et al. 1999; Li et al. 2007; Kokane et al. 2020; Teixeira et al. 2008). Such molecular assays can also be utilized for the detection of additional virus and virus-like pathogens that could be associated with citrus decline in Pakistan (Khan 1992; Arif et al. 2005; Dang et al. 2022). Utilizing such molecular tools in citrus surveys in Pakistan can provide a more complete picture of the citrus disease

status for HLB and various other diseases within this citrus growing region of the middle-east.

Knowing the disease rate for HLB, or any other citrus disease, is one of the most important pieces of information for the successful implementation of a management program. Management of HLB in Pakistan is challenging not only because of the absence of a robust survey program but also due to the absence of certified nurseries and lack of regulations regarding the use of disease-free planting materials (Shamoon et al. 2020). This requires the implementation of a comprehensive HLB management program, which should include measures such as the use of pathogen-tested citrus propagative materials, large-scale survey programs utilizing appropriate detection methods, areawide coordinated vector management, and extension and education programs for citrus nurseries and growers (Bassanezi et al. 2020; Alquézar et al. 2022).

HLB has been suspected to be present in Punjab Province of Pakistan as early as 1917-18. However, the information on disease incidence still remains limited (Hussain and Nath 1927; Naqvi et al. 2017; Razi et al. 2014; Catara et al. 1988). Despite the limited sampling in this study, it appears that HLB continues to spread within the Punjab Province of Pakistan and highlights the need for a more comprehensive citrus health survey and HLB management program for the province and the country.

Acknowledgments

We give special thanks to Drs. Greg Douhan and Georgios Vidalakis, University of California, Riverside, for their significant contributions in improving the manuscript. We thank Dr. John S. Hartung and his team at USDA, ARS, BARC, MPPL, Beltsville, Maryland for providing financial and technical assistance. We thank the Higher Education Commission of Pakistan Funded NRPU Project No. 17001 "Identification and Monitoring of Important Citrus Diseases and Establishment of Viral-Free Propagation Scheme.

References

- Albrecht C, Hicks A, Hornbaker V, Khalid S, Kumagai L, Morgan D, and Okasaki K. 2020. Action Plan for Asian Citrus Psyllid and Huanglongbing (Citrus Greening) in California. *Journal of Citrus Pathology*, 7(1). <http://dx.doi.org/10.5070/C471049054>
- Alquézar B, Carmona L, Bennici S, Miranda MP, Bassanezi RB, Peña L. 2022. Cultural Management of Huanglongbing: Current Status and Ongoing Research. *Phytopathology* 112:11-25. 10.1094/phyto-08-21-0358-ia.
- Arif MA, Ahmed M, Ibrahim and S. Hassan. 2005. Occurrence and distribution of virus and virus-like diseases of citrus in north-west frontier province of Pakistan. *Pak. J. Bot.* 37:407-421.

- Bassanezi RB, Lopes SA, de Miranda MP, Wulff NA, Volpe HXL, and Ayres AJ. 2020. Overview of citrus huanglongbing spread and management strategies in Brazil. *Trop. plant pathol.* 45, 251–264.
- Belasque J, Bassanezi RB, Yamamoto PT, Ayres AJ, Tachibana A, Violante AR, Tank A, Di Giorgi F, Tersi FE, Menezes GM, Dragone J, Jank RH, and Bové JM. 2010. Lessons from Huanglongbing management in SÃO PAULO State, Brazil. *Journal of Plant Pathology*, 92(2), 285–302.
- Bove JM. 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *J Plant Pathol* 88:7–37.
- Catara A. 1987. Compendium of citrus diseases and disorders in Pakistan. Project for Research and Development in cultivation of Fruits, Vegetables and Olives. Pak. Agric. Res. Council, Islamabad, Pakistan.
- Catara A, Azzaro A, Mughal SM and Khan DA. 1988. Virus, viroids and prokaryotic diseases of citrus in Pakistan. In: Proceedings 6th Int. Citrus Cong. Mar.6-11. pp.957-962.
- Chohan SN, Qamar R, Sadiq I. 2007. Molecular evidence for the presence of huanglongbing in Pakistan. *Australasian Plant Disease Notes* 2, 37–38. <https://doi.org/10.1071/DN07019>
- da Graça JV, Douhan GW, Halbert SE, Keremane ML, Lee RF, Vidalakis G, Zhao H. 2016. Huanglongbing: an overview of a complex pathosystem ravaging the world's citrus. *J Integr Plant Biol* 58:373–387.
- Dang T, Bodaghi S, Osman F, Wang J, Rucker T, Tan S-H, Huang A, Pagliaccia D, Comstock S, Lavagi-Craddock I, Gadhavre KR, Quijia-Lamina P, Mitra A, Ramirez B, Uribe G, Syed A, Hammado S, Mimou I, Campos R, Abdunour S, Voeltz M, Bae J, Dang E, Nguyen B, Chen X, Siddiqui N, Hsieh YT, Abu-Hajar S, Kress J, Weber K and Vidalakis G. 2022. A comparative analysis of RNA isolation methods optimized for high-throughput detection of viral pathogens in California's regulatory and disease management program for citrus propagative materials. *Front. Agron.* 4:911627.
- Fahmeed F, Rosete YA, Pérez KA, Boa E. and Lucas J. 2009. First Report of '*Candidatus* Phytoplasma asteris' (Group 16SrI) Infecting Fruits and Vegetables in Islamabad, Pakistan. *Journal of Phytopathology*, 157: 639-641.
- Halbert S. 2005. The discovery of huanglongbing in Florida. In: Proceedings of 2nd international citrus canker and huanglongbing research workshop, Orlando, H-3.
- Halbert SE, Keremane ML. 2004. Asian citrus psyllids (Sternorrhyn- cha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. *Fla Entomol* 87:330–353.
- Hung TH, Wu ML and Hong SJ. 1999. Development of a rapid method for the diagnosis of citrus greening disease using the polymerase chain reaction. *Journal of Phytopathology*, 147:599-604.
- Husain MA, Nath D .1927. The citrus psylla (*Diaphorina citri*, Kuw) [psyllidae: Homoptera]. *Mem Dept Agric India, Entomol Ser* 10: 5–27.
- Jagoueix S, Bove JM, and 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 Jul;44(3):379-86. doi: 10.1099/00207713-44-3-379. PMID: 7520729
- Khan IA. 1992. Virus and virus like diseases of citrus. In: Proceedings 1st Int. Sem. Citricul. Pakistan 2-5 Dec". Univ. Agric. Faisalabad. pp. 343-352
- Kokane SB, Bhose S, Kokan EA, Gubyad M, Ghosh DK. 2020. Molecular detection, identification, and sequence analysis of '*Candidatus Liberibacter asiaticus*' associated with Huanglongbing disease of citrus in North India. *3 Biotech.* 10:341.
- Li S, Wu F, Duan Y, Singerman A, Guan Z. Citrus greening: Management strategies and their economic impact. *HortScience.* 2020 May 1;55(5):604-12.
- Li W, Hartung JS, Levy L. 2006. Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus Huanglongbing. *J Microbiol Methods* 66: 104–115. PMID: 16414133
- Li W, Hartung JS, Levy L. 2007. Evaluation of DNA amplification methods for improved detection of "*Candidatus Liberibacter* species" associated with citrus huanglongbing. *Plant Dis.* 91:51.
- Naqvi SAH, Umar U, Atta S, Liu H, Rehman AU, Khan AA. 2017. Serological and molecular based detection of graft transmissible pathogens associated with citrus from non-core areas of Pakistan. *Pakistan J Agric Sci* 54(4):793–799.
- Noorizadeh S, Golmohammadi M, Bagheri A, Bertaccini A. 2022. Citrus industry: Phytoplasma-associated diseases and related challenges for Asia, America and Africa. *Crop Protection* 151:105822. <https://doi.org/10.1016/j.cropro.2021.105822>.
- Razi MF, Keremane ML, Ramadugu C, Roose M, Khan IA, Lee RF. 2014. Detection of citrus huanglongbing-associated '*Candidatus Liberibacter asiaticus*' in citrus and *Diaphorina citri* in Pakistan, seasonal variability, and implications for disease management. *Phytopathology* 104:257–268.
- Shamoon S, Ali M, and Yasin A .2020. Citrus cluster feasibility and transformation study. In Ali Mubarik,. (ed.) (2020). Cluster Development Based Agriculture Transformation Plan Vision-2025. Project No. 131(434)PC/AGR/CDBAT-120/2018. Unpublished Report, Planning Commission of Pakistan, Islamabad, Pakistan and Centre for Agriculture and Biosciences International (CABI), Rawalpindi, Pakistan.
- Siddique MI, Garnevska E. 2018. Citrus value chain (s): A survey of Pakistan citrus industry. *Agric. Val. Chain* 37, 37–56. doi: 10.5772/intechopen.70161



- Teixeira DC, Saillard C, Eveillard S, Danet JL, Ayres AJ, Bove J. 2005. '*Candidatus Liberibacter americanus*', associated with citrus huanglongbing (greening disease) in Sao Paulo State, Brazil. *Int J Syst Evol Microbiol* 55:1857–1862.
- Teixeira DC, Saillard C, Couture C, Martins EC, Wulff NA, Jagoueix S, Yamamoto PT, Ayres, AJ, Bové JM. 2008. Distribution and quantification of *Candidatus Liberibacter americanus*, agent of huanglongbing disease of citrus in São Paulo state, Brasil, in leaves of an affected sweet orange tree as determined by PCR. *Molecular and Cellular Probes*, v.22, p.139-150.