Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology
ISSN: 1019-763X (Print), 2305-0284 (Online)
http://www.pakps.com

ASSESSMENT OF CITRUS GREENING DISEASE INCIDENCE AND SEVERITY IN SARGODHA, PAKISTAN: A MOLECULAR CHARACTERIZATION STUDY

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A B S T R A C T

In terms of productivity, potential for spread, and area covered, citrus is regarded as one of the major fruit crops. Citrus fruits account for around 37% of all fruit produced, with mandarins and oranges making up nearly 21% of this total. These days, citrus greening disease (CGD) is a major concern. Citrus decline is a complicated issue that causes orchard output to gradually drop until it eventually results in unproductive orchards. Citrus greening, tristeza, and gradual decline are the most prevalent illnesses that cause citrus decline. This study's goal was to determine how common citrus greening illnesses are across Sargodha's citrus-growing tehsils and kinds. Data on prevalence, incidence, and severity were collected in Sargodha's several tehsils, including Shahpur, Bhalwal, Kot-Momin, and Sargodha. Using particular primer sets A2/J5 and OI1/OI2C, the pathogen was characterized from representative citrus greening disease samples. According to the results of the Citrus Greening Disease, Bhalwal had the highest incidence rate (53.33%). Shahpur had 50% of the highest disease severity, whereas Bhalwal had 25% of the lowest disease severity. Twenty symptomatic grape fruit samples, ten fruiter samples, fifteen kinnow samples, five musambi samples, and ten sweet orange samples had their DNA isolated using the CTAB method. The pathogen DNA was recovered from a sweet orange sample that exhibits definite signs of greening and kinnow. Musambi samples were also amplified, and the amplified PCR product was sequenced to examine genetic heterogeneity in several citrus cultivars. To check for variations that would be useful for managing diseases in the future, a phylogenetic tree was generated as part of the phylogenetic analysis process.

Keywords: Citrus, Greening disease, Molecular detection, Candidatus.

INTRODUCTION

Fruit cultivation is a cornerstone of global agriculture, with citrus playing a prominent role due to its widespread cultivation and consumption (Spreen, 2010). Belonging to the Rutaceae family, citrus fruits thrive in tropical and subtropical climates across the globe, originating in northeastern India and flourishing in various soil types and temperatures (Ziegler & Wolfe, 1961). China leads global citrus production, followed by the United States, Brazil, and other nations (FAO, 2020).

Submitted: February 05, 2024
Revised: April 08, 2024
Accepted for Publication: June 03, 2024
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Pakistan stands as the 16th largest citrus producer worldwide, notably renowned for its Kinnow mandarin oranges, particularly cultivated in Punjab province. While Punjab contributes over 95% of Pakistan's citrus output, other provinces also contribute, with citrus exports generating significant foreign exchange, albeit with potential for higher yields (Rasool *et al.,* 2020). Rich in essential nutrients like vitamin C, citrus fruits offer various health benefits and serve as crucial exports for many countries, with the United States, China, Brazil, and Mediterranean nation's leading the global production landscape (Davis & Albrigo, 1994; FAO, 2020).

Numerous infections, including worms, bacteria, viruses, fungus, and *spiroplasma*, greatly lower citrus yields. Specifically, stubborn citrus disease caused by *Spiroplasma* infection results in a significant 25–32% decrease in fruit output when compared to plants that are

not infected. Orange output has significantly decreased as a result of the rising prevalence of pathogen infections. Citrus canker is caused by *Xanthomon asaxonopodis*, and greening disease is caused by Candidatus *Liberibacter*, two bacterial illnesses that reduce citrus output (Mello *et al.,* 2010).

Huanglongbing, or citrus greening disease (CGD), is a severe illness that is found all over the world. In the late 1900s, farmers in southern China began to notice the signs of citrus Huanglongbing, also known as yellow shoot disease. Similar conditions were later found in the Philippines and India, where they were known as mottle leaf disease and citrus die-back, and in Taiwan in the 1920s as likubin or drooping disease. Most experts believe that citrus species were not the main hosts of the HLB bacterium, even though it was first identified in China (Beattie *et al.,* 2005; Bove, 2006; Graca, 2011). At the 13th Conference of the International Organization of Citrus Virologists in 1995, the word "Huanglongbing" first appeared (Lin *et al.,* 1995). Greening disease was first thought to be a virus (Hoffmeyer and Oberholzer, 1948). It was later described as a mycoplasma-like organism (MLO) in 1970 (Lafleche and Bove, 1970), and Hull recognized it as a bacterium in 1972. Scholars continue to disagree on the origins of HLB; some claim it originated in citrus in India from an unidentified native Rutaceae family, and it then moved to China possibly via infected plants or budwood. According to recent conjecture, *Liberibacters* might have come from Gondwana (Beattie *et al.,* 2005; Bove, 2006).

According to surveys carried out in 1991, the incidence rates of CGD are 22% for Kinnow, 25–40% for sweet oranges, 15% for grapefruit, 10% for sweet lime, and 2% for lemons, making it a common and concerning disease in the Punjab and KPK regions (Batool *et al.,* 2007). Huanglongbing, or CGD, is a serious danger to the citrus sector and can cause significant yield losses (Graham *et al.,* 2020). It has a negative impact on yield as well as juice quality, making contaminated fruit unsellable because of its bad taste, which is linked to its high limonin and nomilin concentration. According to Shokrollah *et al.* (2011), infected fruits also have low Total Soluble Solids (TSS) and high acidity. According to study, this means that CGD plays a role in the global reduction of citrus crops.

Candidatus Liberibacter, the pathogen linked to Citrus Greening Disease (CGD), has been recognized and categorized. Microorganisms that cannot be grown are referred to as "candidatus" (Murray and Schlifer, 1994). It has an about 2 µm diameter and a pleomorphic form similar to a stiff rod (Bove, 2006; Batool *et al.,* 2007). According to Subandiyah Weinert *et al.* (2004), this Gram-negative bacterium is mainly found in the phloem and cannot be grown in a lab setting. Both L. *candidatus asiaticum* and *L. candidatus* africanum, which are predominantly found in Asia and Africa, respectively, are Candidatus species belonging to the genus *Liberibacter* (Garnier *et al.,* 1984; Jagoueix *et al.,* 1996; Akhtar and Ahmad, 1999). 16s rDNA sequencing was originally used to identify CGD bacteria (Jagoueix *et al.,* 1996). *Candidatus Liberibacter asiaticus* (*CLas*), *Candidatus Liberibacter africanus* (*CLaf*), and *Candidatus Liberibacter americanus* (*CLam*) are the three strains of CGD that are known to exist based on geographic distribution, ecology, and insect vectors. Of these, *CLas* is the most common and extensively spread (Gottwald *et al.,* 2007). Considering the bacteria's obligatory parasitic nature, attempts have recently been attempted to cultivate it employing biofilm techniques (Ha, P. T et.al 2019).

One of the most important criteria for early disease detection is symptomology. Asian greening symptoms show up in warmer temperatures up to 35°C, but African greening symptoms are more common in cooler regions (20–25°C) (Halbert and Manjunath, 2004). Asymmetrical yellowing along the midribs, mottled skin mottling on leaves, and a "pen test" to differentiate symptoms from nutrient deficits are among the diagnostic indicators (Inoue *et al.,* 2020; Vashisth and Kadyampakeni, 2020). Fruit symptoms include asymmetric coloration referred to as "color inversion" and greenness at the stylar end (Batool *et al.,* 2007; Akhtar and Ahmed, 1999). Mandarins and sweet oranges are the citrus varieties most vulnerable to CGD (Knapp *et al.,* 2004). However, CGD affects all citrus varieties.

The principal vector of the disease is the Asian citrus psyllid (ACP), which feeds on phloem tissue and pierces the skin with its mouthparts to transfer the infection (Lin and Lin, 1990; da-Graca, 1991; Halbert and Manjunath, 2004). According to Lee *et al.* (2015), transmission can happen through nymphs and different developmental stages. Although there is controversy around seed transfer, most experts concur that it is not a major mechanism of transmission (Tirtawidjaja, 1981; Halbert and Manjunath, 2004). For CGD,

numerous detection techniques have been developed. Pathogen identification techniques have included the use of imaging technologies, molecular markers, polymerase chain reaction (PCR), and serological methods (Sankaran *et al.,* 2013; Knapp *et al.,* 2004; Jagoueix *et al.,* 1996; Ding *et al.,* 2020). Furthermore, the identification of diseases has showed potential for spectroscopic and canine detection approaches (Liu *et al.,* 2020; Gottwald *et al.,* 2020). A variety of management techniques are necessary to lessen the threat that greening disease (GD) poses. According to recent studies, plant immunological responses to *Candidatus Liberibacter asiaticus* (*CLas*) infection include the production of reactive oxygen species (ROS) and the deposition of callose, both of which aid in the development of symptoms (Li *et al.,* 2020). In a trial, 5-year-old *Citrus sinensis* trees infected with *CLas* received a trunk injection of streptomycin; after seven days, there was a notable decrease in the amount of hydrogen peroxide in the tree's phloem tissue, ion leakage, and *CLas* titers (Li *et al.,* 2020). Furthermore, in pathogen-infected trees, foliar treatments with Gibberellic acid (GA) at dosages of 5 mg/L and 25 mg/L reduced tissue hydrogen peroxide levels and ion leakage (Li *et al.,* 2020). Standard GD management strategies include vector control and the cultivation of resistant cultivars, even though traditional antibiotics have only modest effectiveness against the greening disease. Tetracycline, however, has demonstrated some efficacy (Cao *et al.,* 2015). Moreover, it has been shown that dietary supplements improve plant tolerance to HLB (Cao *et al.,* 2015). Citrus groves in Pakistan are suffering from GD, same like those in other parts of the world (Akhtar & Ahmed, 1999). *Citrus tristeza virus* transmission is suspected due to the presence of the citrus psylla vector in Peshawar's citrus plantations (Akhtar & Ahmed, 1999). Later studies conducted in the late 1980s verified the existence of the illness in citrus orchards in Punjab, where the greening bacteria was found in phloem sieve tubes using electron microscopy (Grimaldi &Catara, 1989). Molecular verification of GD in Pakistan was also accomplished (Saifullah *et al.,* 2015). It is difficult to quantify GD losses because the damage might range from a few impacted branches to total crop loss. According to recent surveys, GD has been linked to the reduction of citrus in both India and Pakistan, with incidence rates varying from 8 to 14 percent (Saifullah *et al.,* 2015). However, in recent years, there haven't been enough thorough surveys to track the disease's advancement.

Disease detection: Detection of citrus greening disease (CGD) is critical for effective management. Initially, molecular markers are used to confirm the existence of the bacterium in citrus samples. The bacterium is characterized using 16s rDNA and detected using polymerase chain reaction (PCR) (Rasowo, 2019). PCR techniques have been used successfully to detect *Liberibacter* species in citrus trees. Furthermore, nucleic acid-based techniques such as PCR and quantitative PCR (qPCR) have helped detect and track the migration of CGD in citrus trees. Various assays have been used to detect C. *Liberibacter*, which aids in the development of management methods. Recent improvements include the creation of a rapid indexing assay, the iodine-starch test, which is a dependable and cost-effective alternative to PCR (Verma *et al.,* 2022). Furthermore, serological procedures have been refined for detecting CGD infections, with recent advances including spectroscopic and canine detection methods.

Disease management: Managing CGD presents hurdles, necessitating early detection and characterization using biochemical and molecular techniques. Integrated management approaches, such as using resistant cultivars, managing insect vectors, and administering antibiotics like tetracycline, have demonstrated some efficacy (Saini *et al.,* 2020). China has had tremendous success in eliminating CGD by removing affected trees and replacing disease-free ones. Nutritional additives have also been shown to improve plant tolerance to CGD. Insecticides, chemicals, thermotherapy, and biological control approaches are among the most cost-effective management strategies. Integrated pest management (IPM) solutions have showed potential, especially in organic settings. Effective pathogen management requires regulating the Asian citrus psyllid (ACP), the vector that spreads CGD (Bazzocchi, 2020).

Status of CGD in Pakistan: CGD also affects Pakistan's citrus groves, with surveys proving its prevalence in several places. CGD occurrence in Pakistan has resulted in significant citrus output losses (Paudyal *et al.,* 2016). Although the disease has been molecularly confirmed, there have been few rigorous surveys to evaluate its spread and impact in recent years.

MATERIALS AND METHODS

Survey and sampling: On the basis of the symptoms, a survey of infected citrus fields from several Sargodha

tehsil citrus growing locations was conducted to determine the prevalence of citrus greening. The samples were gathered from several kinds on four sides of the Sargodha tehsil. Ten samples were taken from each of the five orchards that were visited on either side. Symptomology is a primary criterion for first identification in the field. Common HLB signs in naturalistic settings include mottling and chlorosis, which are symptoms similar to zinc insufficiency. Notable were the common symptoms during the survey. Chlorosis and blotchy mottling were present in the leaf samples. There were found to be partially or totally green fruits, asymmetrical fruits, and abandoned seeds. Infected leaf samples were gathered and taken to a lab for additional processing.

Disease incidence: Ten orchards on each side were chosen in the Sargodha tehsil to track incidence, and five samples were taken from each of the four main commercially produced varietals. The percentage of plants displaying greening symptoms relative to the total number of plants evaluated indicates the presence of greening. The trees were identified as greening and their symptoms recorded when any of the following occurred: mottling, vein yellowing, lopsided fruit, aborted seeds, and bitter fruit flavor.

$$\text{Disease incidence (\%) DI} = \frac{\text{number of infected plant}}{\text{number of total plant observed}} \times 100$$

Disease severity: Over the course of two years (2020–2022), the severity of the disease was assessed using Akhtar and Ahmed's disease rating scale:

0 = No symptoms,

1 = Blotchy mottling on leaves (symptoms present on 25% of leaves),

2 = uneven fruit and color inversion; symptoms are seen in 25–50% of the canopy,

3 = Tree that has partially decreased (damage 51-75%), and

4 = Tree with severe deterioration (>75%)

Molecular characterization: Total DNA extraction: Leaf samples with symptomatic CGD infection were collected in order to extract DNA. Selected were commercially grown cultivars in the Sargodha region. Ten specimens from various varaites were gathered for the in vitro experiment. With minor adjustments, Murray & Thompson's 1980 protocol was used to extract the whole DNA from citrus plants (Naz *et al.,* 2013). After weighing the sample, crush 0.5 mg of fresh leaf midrib tissues. After that, 800 µl of extraction buffer, 1.4 M NaCl, and 2 percent (wv-1) 2-mercaptoethanol were added, and the mixture was incubated for 30 minutes at 65 °C. The samples were centrifuged for ten minutes at 12,000 rpm, and the supernatant was then moved to new Eppendorf tubes. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the recovered supernatant. Subsequently, the DNA pellet was resuspended in 100 ums of TE buffer after being cleaned in 70% ethanol. The temperature of the DNA extracts was maintained at -20°C. After adding RNAase-A and dissolving it in DEPC-treated water, it was incubated for 30 minutes at 37°C before being suspended in PCR.

Molecular detection: PCR: Methods based on molecules that are utilized to identify CGD. The 16s rDNA and β operon from the processed citrus samples were amplified using two sets of primers: the universal primer of *CLas* (A2TATAAAGGTTGACCTTTCGAGTTT is forward and J5ACAAAAGCAGAA ATAGCACGAACAA) (primer described Hocquellet *et al.,* 1999) is reversed, and the OI1CGCGTATGCAATACGAGCGGCAPCR forward and OI2C GCCTCGCGACTTCGCAACCCAT reverse) (primer described Jagoueix *et al.,* 1996).

Gel electrophoresis: Using a 0.8% agarose gel, the amplified product was found. Gel was made ready.0.8 mg of powdered agarose dissolved in 100 milliliters of TBE buffer (0.5M) is 0.8% weight by volume. Bake the mixture until it becomes translucent, then let it cool. Once the temperature dropped to a point where it could be touched, 12µl of ethidium bromide was added. Transfer into the gel caster and let it solidify. Either a 5µl PCR result isolated in a noteworthy serial or a 1 kb DNA ladder loaded on either side. Gel was detected over UV light tranaluminator band development after the desired amount of time at the appropriate voltage was applied.

Genetic variability: The PCR amplified product was employed in the DNA sequencing process. DNA sequencing is used to determine whether genetic variation in pathogens from various citrus cultivars is useful for managing citrus greening disease. Building the phylogenetic analysis allowed for the study of genetic variability. The genetic variation among the citrus greening bacteria isolated from several kinds was shown by the phylogenetic tree.

RESULTS AND DISCUSSION

The common symptoms were identified during the survey. Figure 1 A depicts blotchy mottling and chlorosis in the leaf samples. Figure 1D and Figure 1E depict whole or partially green fruits, respectively, and asymmetrical fruits. In CGD-infected trees, we found asymmetrical fruits, vertical development, and mottled skin with mild discoloration during our investigation. These findings are completely comparable with the observations made by McCollum and Baldwin (2017). We also saw yellowing of the leaves. As the disease worsens, the branches finally perish, the leaves fall, and the shoots become stunted.44 Asian and Latin American nations have reported cases of CGD (Ajene *et al.,* 2019). In 2016, Zafarullah *et al.* discovered that kinnow and sweet orange had a 42 percent CGD incidence. Previous studies have shown that the disease incidence in the Sargodha district ranged from 8 to 11 percent in all of the tehsils. The provinces of Punjab and KPK in Pakistan are where CGD was first reported. The most common symptoms were blotchy mottling and vein yellowing of the leaf veins; in areas where the illness had spread widely, dieback may also be noted; during the survey, vein corking symptoms were hardly noticed, and symptoms of asymmetrical fruit were also hardly noticeable. The most noticeable and common signs of greening that were seen, as well as table 1 above, indicate that lopsidedness and vein yellowing were the most severe symptoms in various Sargodha tehsils during the survey. Employing a pen test to address this issue, vein yellowing frequently corresponds with symptoms of zinc deficiency. Throughout the year, a varied number of samples were collected and confirmed to be positive for CGD. According to Razi *et al.* (2014), the months with the largest number of cases were February, April, and May. Consequently, early detection of CGD in the field depends heavily on symptomology.

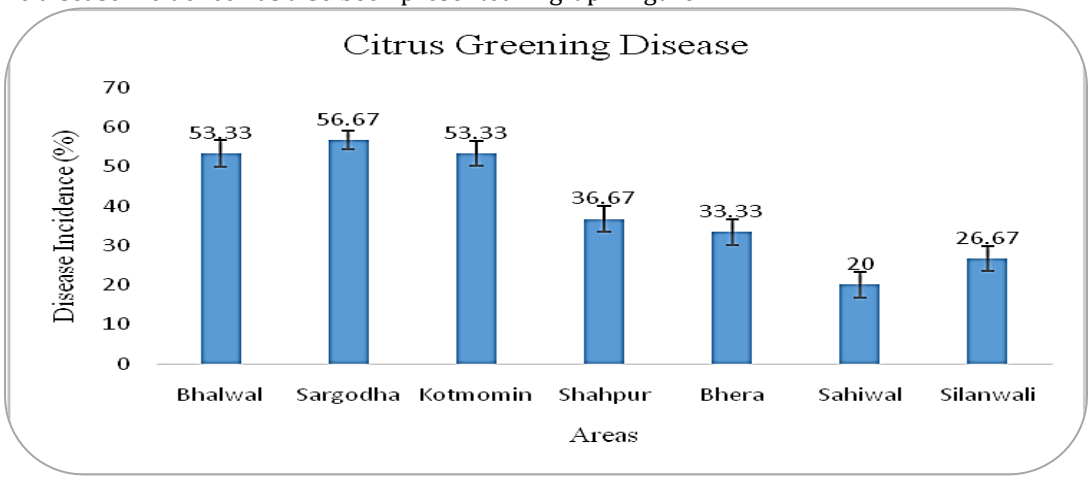
Table 1. Citrus greening signs on various types seen during the survey in several tehsils of Sargodha. Scale 0: no symptoms at all Two strong symptoms and one minor symptom.

Locations	Varieties	Vein Yellowing	Mottling	Color Inversion	Lopsided	Vein Corking
Sargodha	Musambi	2	1	2	2	0
	Sweet Lemon	2	1	0	2	0
	Kinnow	2	2	1	2	0
	Fruiter Early	2	1	1	1	0
	Grape Fruit	2	2	2	1	1
Shahpur	Musambi	2	1	0	2	1
	Sweet Lemon	1	2	0	1	1
	Kinnow	2	1	1	2	0
	Fruiter Early	1	1	2	1	0
	Grape Fruit	2	1	1	2	1
Tehsil Bhalwal	Musambi	1	1	2	1	0
	Sweet Lemon	2	2	1	1	0
	Kinnow	1	2	1	2	0
	Fruiter Early	2	1	2	2	1
	Grape Fruit	1	2	1	1	0
Kotmomin	Musambi	2	1	2	2	1
	Sweet Lemon	1	1	1	2	1
	Kinnow	2	1	2	2	0
	Fruiter Early	1	0	1	1	0
	Grape Fruit	1	0	1	1	0

Figures 1A, 1D, and 1F in the narrative illustrate the various symptoms observed in the several tehsils of Sargodha. The Sargodha tehsil itself indicates that the two types most vulnerable to severe symptoms are kinnow and musambi, which exhibit vein yellowing, lopsidedness, and color inversion. Similar patterns were also noted in Tehsil Kotmomin; however, in Tehsil Bhalwal, early and sweet lemons were the most vulnerable to the citrus greening disease. All kinds in Shahpur Tehsil had severe symptoms, and this tehsil also had the greatest level of illness severity.

Figure 1 (a) vein yellowing; (b) leaves with mixing of yellow and green color; (c) leaves with zinc defiency; (d) fruit showing color inversion; (e) vector of CGD citrus psylla (*Diaphorinacitri*) (f)lopsided symptoms were noted during survey.

Severity: The disease incidence has also been presented in graph Figure 1.1.



Graph 1.1. In Citrus Greening Disease result showed that Bhalwal had the highest percentage of incidence which was 53.33% and Silanwali had the lowest incidence 26.67%.

The disease severity has also been presented in graph Figure.1.2: Severity of Citrus Greening: Result presented in table (Table 4.10) showing ANOVA for disease severity of citrus greening at different location in Sargodha. Disease severity was significantly different at different locations at (F=74.5, P<0.001). Disease severity was maximum in Shahpur (56%) followed by KotMomin (38%) and Sargodha (34%). Lowest disease severity was recorded in Bhalwal (27%) (Figure 4.8).

Analysis of variance for percent disease severity of citrus greening at different locations in Sargodha

Source of Variation	Degree of Freedom	Sum of Squares	Means Squares	F-value	P-value
Ion	-	2293.75	764.583	27.2	-
Error	16	450	28.125	-	-
Total	19	2743.75	-	-	-

P<0.001 shows significance

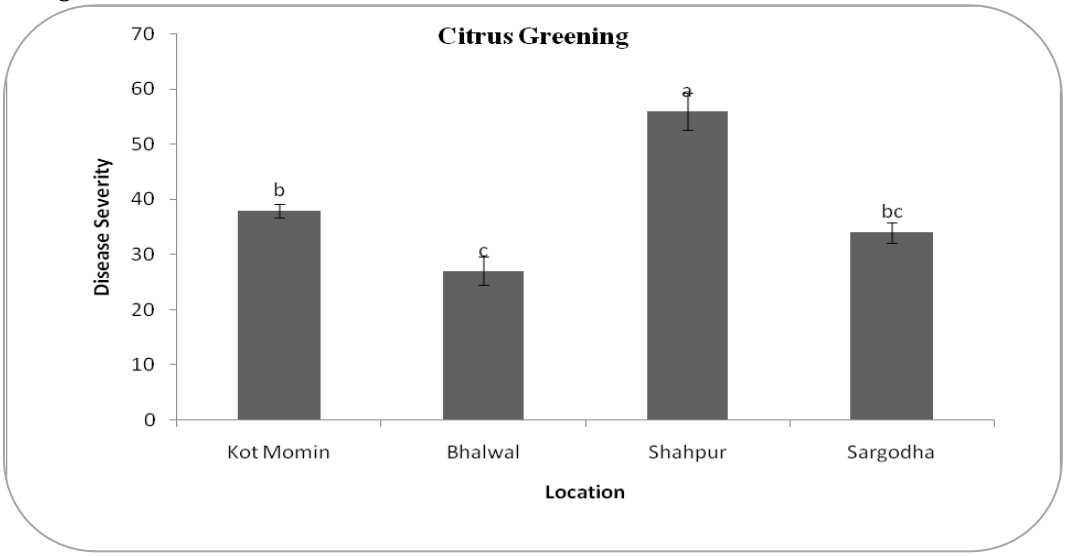


Figure 1.2. Disease severity (%) of Citrus greening disease at different locations. The highest disease severity was recorded in shahpur and the lowest disease severity was noted in Bhalwal. Means sharing similar letters are not significantly different at *P<0.05.*

Molecular Characterization of Citrus Greening: The economic significance of citrus growing in Pakistan stems from its ability to withstand difficult weather conditions over an extended period. However, maintaining productivity requires both ideal crop nutrition and efficient disease management techniques. In Sargodha, Pakistan, we conducted a study to molecularly define Huanglongbing (HLB), commonly referred to as citrus greening, in different citrus cultivars.

Several citrus-growing districts were sampled, with a primary focus on the tehsils of Sargodha. Citrus greening disease was confirmed using molecular markers A2, J5, OI1, and OI2C after four tehsils were chosen for sampling. After amplifying DNA samples using Polymerase Chain Reaction (PCR), the samples were sequenced. In order to gain important management insights for citrus greening disease, this sequencing was done in order to evaluate genetic differences within the isolated pathogens. To clarify the genetic heterogeneity among bacterial strains isolated from several citrus cultivars, phylogenetic analysis was performed. The 16S rDNA-based PCR detection technique, which was created in 1996, is extensively used in nations where citrus greening disease is endemic. *Candidatus Liberibacter* species, which are very common in Asia and Africa, have been identified thanks to this technique, which is well-known for its sensitivity and specificity (T. Fujikawa *et al.,* 2012). Determining the presence of the HLB pathogen in host plants was essential for managing diseases and reviving Pakistan's citrus industry. Our goal was to determine the molecular existence of the causal agent of citrus greening disease, which we hypothesized to be *Candidatus Liberibacter asiaticus.* The PCR method was used to identify positive citrus plants, making it easier to locate HLB-positive plants for possible antibiotic and thermotherapy treatment. Additionally, developing management plans for HLB requires examining the genetic variety of PCR-amplified products.

Table 2. Specific primers and their sequence for the identification of *CLas*

Primer	Sequence (5' to 3')	Putative Gene	Product size	Temp	Reference
A2 Forward	TAT AAA GGT TGA CCT TTC GAG TTT	rplKAJL-rpoBC(β operon)	669-703	55°C	Hocquellet *et al.* (1999)
J5 Reverse	ACA AAA GCA GAA ATA GCA CGA ACA A	rplKAJL-rpoBC(βoperon)	669-703	55°C	Hocquellet *et al.* (1999)
OI1 Forward	(5'GCGCGTATGCAAGAGCG GCA-3')	16S rDNA	1160	58°C	Sandrine Jagoueix *et al.* (1966)
OI2c Reverse	(5'GCCTCGCGACTTCGCAACC CAT- 3')	16S rDNA	1160	58°C	Sandrine Jagoueix *et al.* (1966)

Figure 2. CGD infected tree: (A)CGD infected sweet lemon; B, pathogen affected kinnow leaves showing vein yellowing and (C) is HLB affected tree and (D) blotchy mottle symptoms

DNA was extracted from symptomatic grape fruit samples (20), fruiter samples (10), kinnow samples (15), and musambi samples (5), using the CTAB technique. The procedure is described in full above. Using primer OI1/OI2C, which is specific for asiaticus and africanus species, the sample is first amplified in sweet orange, which exhibits clear symptoms of greening in fig (1A, B,2B). PCR is then run on the gradient shown below (fig. 3), and our primer is annealed at 56°C after confirming the asiaticus strain with primers set of OI1/OI2C that specific africanus strain. Run the same sample on PCR with specific primer of asiaticus A2/J5 to confirm asiaticus strain, and result is positive, yielding an asiaticus strain product. Considering that "*CLas*" cannot be cultured in vitro. It is quite challenging to investigate the ecology, epidemiology, and management of this pathogen (Kokane *et al.,* 2020). PCR detected the greening pathogen in leaf samples from different citrus types (given in the top figure). Citrus variety DNA was amplified using primer pairs, yielding PCR products of the proper sizes, 1160 bp and 703 bp, respectively. Previous works of literature have They were able to confirm the PCR product size by utilizing their various primer combinations and reporting comparable band sizes. The goal of the primer employed in this work was to amplify the operon regions and 16s rDNA of the bacteria. When *CLas* was amplified, a 703 bp band was obtained, whereas *CLaf* yielded a 669 bp band. Using A2/J5 primers, a particular amplification of 703 bp was seen in all infected HLB-CM samples. They also found that in healthy and control plants, there was no amplification. Molecular pathogen detection, which includes CGD, has shown to be a dependable method for identifying pathogens at low concentrations even prior to the manifestation of physical signs.

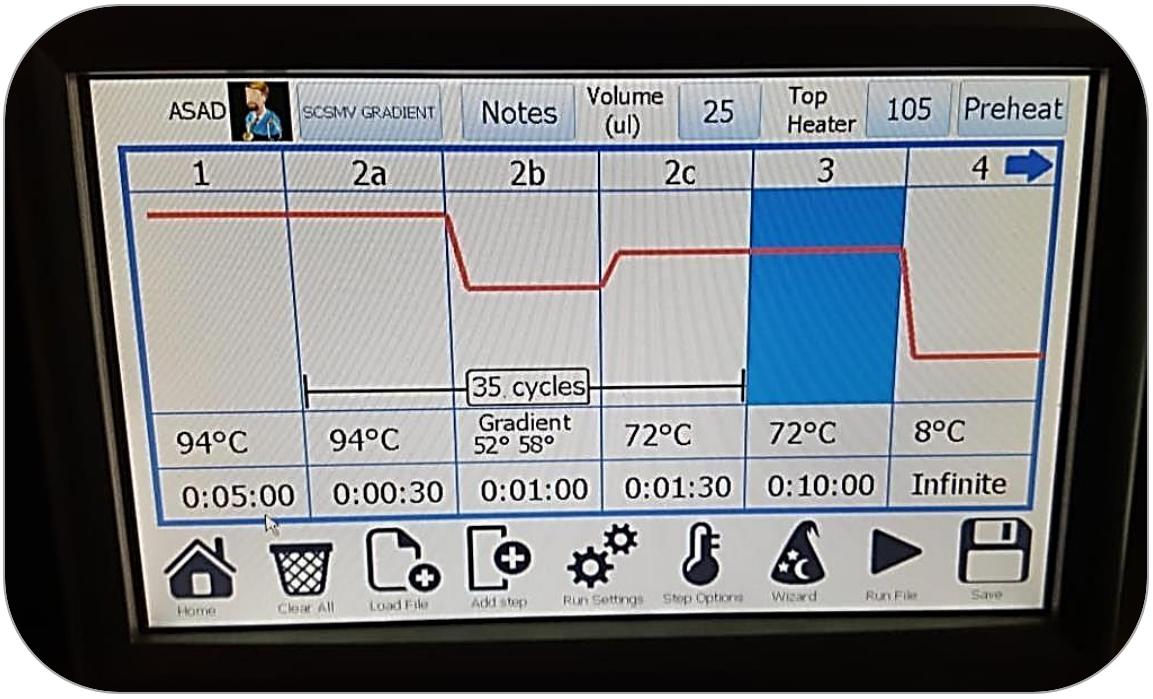


Figure 3. PCR run on the gradient base different temperature ranges (52 to 58°C) for primer annealing.

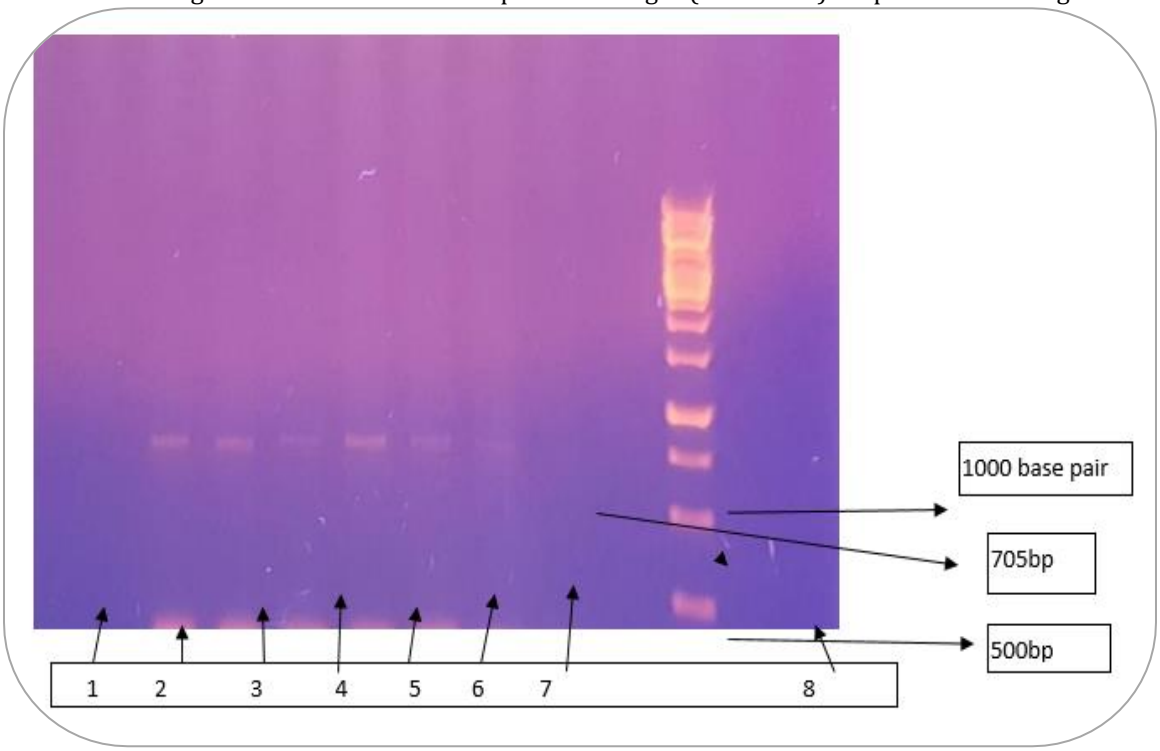


Figure 3. Visualization of bands of primer A2/J5 at 705bp. (A) Lane 8= 1kb DNA Ladder, Lane 7: Negative Control (dH2O), Lanes 6 = DNA samples from healthy trees (asymptomatic); Lanes 1,2 = DNA samples from CGD infected (Symptomatic plants) (Kinnow and Mosambi, respectively). Lanes 4 = DNA samples from CGD infected (Symptomatic plants grape fruit). Lane 3,5 = having very low quantity of pathogen DNA.

Visualization of bands of primer OI1/OI2C at 1160bL: Sequencing of the causal agent of HLB: PCR amplified products were send to USA for sequenced and analyzed to further examine the genetic diversity of *Candidatus Liberibacter asiaticus* on the 16S rDNA gene.

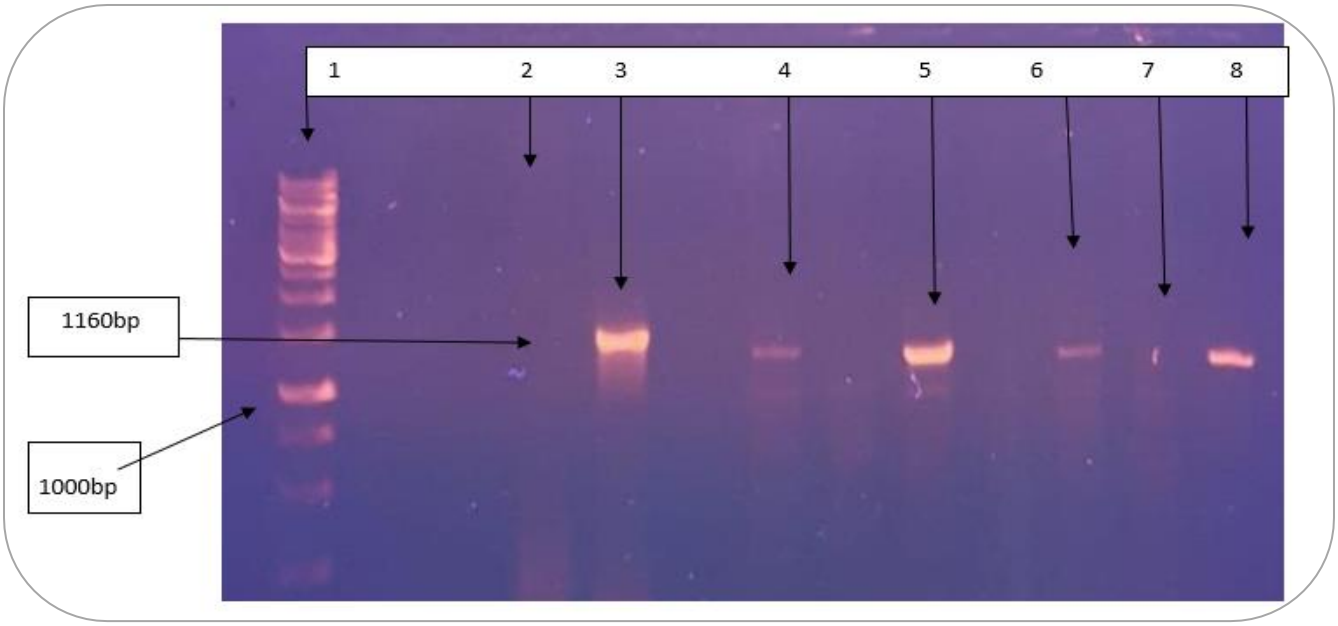


Figure B. Lane 1 = 1kb DNA Ladder, Lane 2: Negative Control (dH2O), Lanes 7 = DNA samples from healthy trees (asymptomatic); Lanes 3,5= DNA samples from CGD infected (Symptomatic plants) (Kinnow and musambi, respectively). Lane 4, 6= CGD infected (symptomatic plants.

CONCLUSION

The study concludes that there is a serious threat to citrus crops globally, including in Pakistan, due to the several diseases, most notably *Candidatus Liberibacter asiaticus*, which are responsible for citrus production losses. As an essential fruit crop with significant nutritional and therapeutic significance, citrus is frequently threatened by pathogens such viruses, nematodes, prokaryotes, and fungus. Huanglongbing (HLB), which is spread by the Asian citrus psyllid *Diaphorina citri* and is brought on by the Gram-negative alpha *proteobacterium Candidatus Liberibacter asiaticus*, is a specific cause for concern. HLB symptoms include pale yellow leaves with blotchy mottling and vein yellowing, asymmetrical, bitter-tasting fruits with black seeds that haven't fully developed, and yellowing and thinning of the canopy.

The purpose of the study was to evaluate genetic variability among various citrus types impacted by citrus greening. Samples were collected based on symptomology and visits to orchards as part of a survey carried out in the Sargodha tehsil. The CTAB method was used to extract DNA, and disease incidence and severity were noted. Sequencing after PCR analysis identified genetic diversity in positive samples. This study emphasizes how crucial it is to comprehend genetic variety in order to fight citrus illnesses, especially in light of disease control techniques and sustainable citrus production. However, breakthroughs, the genetic variability and disease vectors related with citrus greening in Pakistan remain unknown, highlighting a crucial research requirement for future studies. This work attempts to close this gap by doing molecular characterization to better understand the occurrence and severity of citrus greening disease in Pakistan. The new emerging evidence supporting the idea and need to more study on host responses may play a role in shaping HLB development, management employing host defense mechanisms should no longer be ignored. To the proper management of disease need to work in future on host-pathogen interactions, population genetics and vectoring of the causal agent are discussed. Some recent studies show that the pathogen have small antimicrobial peptides (SAMPs) were isolated which can suppress the growth of HLB causing bacteria and promote host immunity in citrus and in Pakistan there are still no work on it. So, it is need to work on the isolation of (SAMPs) from *Candidatus Leberbacter asiaticus* species and promote host immunity by using (SAMPs). Need a complete genome study of citrus species to identify the resistant gene and citrus breeding to support long-term control of this devastating disease. There is no cure for this disease, but have a solution to do research and study on citrus cultivars, to find resistant gene, meaning their genes could be used to create immunity in other citrus. Work on the genetic variation and unique horticultural

features in different species of citrus in Pakistan and to develop resistant cultivars, and the first step towards that is identifying these important resistant genes in different citrus cultivars that should have an attractive character against pathogen resistant and will used for domestication. Sequencing the genomes of plants and to find the genetic variability will give us a new platform for genetic improvements and better management of their production into the future.

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