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Quantitative Real-Time PCR reveals latent infections of *Candidatus Liberibacter* asiaticus in Persian lime (*Citrus latifolia* Tan.) from different sources

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Abstract

Persian lime (*Citrus latifolia* Tan.) is a crop of high economic and social relevance in Mexico due to its nutritional properties, associated health benefits, and particularly the foreign exchange generated through exportation. In recent years, the state of Veracruz has become the leading production region for this species at the national level. However, this crop is susceptible to several vascular diseases that cause significant economic losses in the citrus industry. Among these, Huanglongbing (HLB) is considered the most destructive disease, exerting the greatest impact on Persian lime production. This study involved the collection of Persian lime leaf tissues from different sources: field-grown plants, certified nursery plants, and plants previously sanitized through micrografting and thermotherapy processes. Genomic DNA was extracted from the collected samples. DNA integrity was verified by agarose gel electrophoresis and quantified using a NanoDrop One-C spectrophotometer. Quantitative Real-Time Polymerase PCR (qPCR) was performed using a QuantStudio One thermocycler and SYBR Green Master Mix as the fluorescent detection system. Samples were considered HLB-positive when the cycle threshold (Ct) value was below 35 during the quantification of *Candidatus Liberibacter* asiaticus. The results support the early, precise, and efficient detection of *C*Las by qPCR in Persian lime plants, thereby contributing to improved diagnostic and phytosanitary management strategies for this crop.

Keywords: *Citrus latifolia*; HLB; Molecular diagnostics; qPCR detection

1. Introduction

Huanglongbing (HLB), also known as "citrus greening," is a devastating disease caused by the Gram-negative bacterium *Candidatus Liberibacter* asiaticus (*C*Las), which colonizes the phloem of host plants and induces systemic infection in citrus species [1]. HLB is transmitted by the Asian Citrus Psyllid (*Diaphorina citri* Kuwayama) and can also spread through the grafting of infected buds [2]. The disease severely affects the physiological performance and reduces productivity in both young and mature plants across species and hybrids within the *Citrus* genus [3-5].

Symptoms of HLB are diverse and include leaf mottling, starch accumulation in leaves, stunted vegetative growth, zinc deficiency, fruit deformation, and reduced juice quality, among others [6]. Once trees are infected, no effective treatment is currently available, and symptoms may take several months to become visible, thereby complicating disease detection and management [7]. In Mexico, the emergence of HLB has raised significant concern among citrus producers and

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phytosanitary authorities due to the lack of effective control strategies. Current management relies on an integrated approach that includes the timely detection and removal of infected trees, suppression of vector populations, and the use of disease-free planting material propagated in certified nurseries, with the aim of mitigating the detrimental effects of the disease [8].

Therefore, the timely and accurate detection and quantification of *Candidatus Liberibacter* asiaticus *(C*Las*)* in Persian lime is critical for limit its spread; however, conventional diagnosis methods are often insufficiently sensitive or time-consuming. In this context, traditional diagnostic methods, such as visual inspection or serological assays, are largely ineffective during the early stages of infection. As a result, a substantial number of *C*Las-infected trees remain undetected, facilitating the dissemination of the pathogen and its vector within the plantations.

Recent advances in molecular diagnosis, particularly Quantitative Real-Time PCR (qPCR), have emerged as the most sensible and reliable tools for the early detection of HLB in citrus. qPCR enables the precise detection of *Candidatus Liberibacter* asiaticus (*C*Las) prior to the onset of visible symptoms, thereby facilitating proactive disease management and more effective phytosanitary interventions [9].

Therefore, the aim of this study was:

To develop a reliable qPCR-based molecular diagnostic approach for the detection and quantification of *Candidatus Liberibacter* asiaticus (*C*Las) in leaf samples of Persian lime (*Citrus latifolia* Tan.) obtained from different sources (field-grown plants [FG], certified nursery plants [CN], and sanitized plants [SP]), with the objective of facilitating early disease detections and improving phytosanitary management strategies.

2. Materials and Methods

2.1. Plant material

For the experimental design, three groups of Persian lime (*Citrus latifolia Tan.*) plants were randomly selected: 1) nine one-year-old plants grafted onto Citrumelo Swingle rootstocks [FG]; 2) nine twelve-month-old plants grafted onto *Citrus volkameriana* rootstocks, obtained from a nursery certified under the NOM-079-FITO-2002 standard [CN]; and 3) nine twelve-month-old plants grafted onto *Citrus volkameriana* rootstocks that had previously undergone a sanitation process through micrografting and thermotherapy [SP], following the protocol developed by our research group [10-11]. Leaf tissue samples were collected from all plants and transported to the laboratory in sterile polyethylene bags, where molecular diagnostic analyses were performed using qPCR.

2.2. DNA extraction in Persian lime leaves

Genomic ADN was extracted from 200 mg of macerated leaf tissue using the Plant DNA Purification Kit (Norgen BIOTEK Corp., Thorold, ON, Canada), according to the manufacturer's instructions. DNA concentration and purity were measured using a NanoDrop One-C® spectrophotometer (Thermo Scientific NanoDrop Technologies, LLC, Wilmington, DE, USA), and DNA integrity was evaluated by 1% agarose gel electrophoresis at 80 V for 45 min.

2.3. *C*Las detection by qPCR

qPCR assays were performed using a QuantStudio™ 1 Real-Time PCR System thermocycler (Applied Biosystems, Foster City, CA, USA) and PowerTrack™ SYBR Green Master Mix for fluorescence detection. For *C*Las detection, the primers HLB-4G (5′AGTCGAGCGCGTATGCGAAT-3′) and HLBr (5′-GCGTTATCCCGTAGAAAAAGGTAG-3′) reported by Bao et al [12], were used. All reactions were carried out in triplicate in a final reaction volume of 10 μL, including *C*Las-positive DNA and nuclease-free water as positive and negative controls, respectively. Cycle threshold (Ct) values were used to determine HLB infection status. Plants were considered PCR-positive for *C*Las when the CT value was below 35, whereas plants were considered negative when Ct values were greater than 35.

3. Results

3.1. qPCR assay optimization and standard curve validation for *C*Las detection

As previously described, DNA extraction for all samples was performed using pooled leaf tissue consisting of three Persian lime leaves collected from each randomly selected plant. The results showed that the DNA Purification Kit was effective in yielding high-quality DNA at adequate concentrations. Consistently, the 260/280 and 260/230 absorbance ratios were within the expected purity ranges. DNA integrity was further assessed by 1% agarose gels electrophoresis, which revealed intact and well-preserved genomic DNA across all foliar samples analyzed.

Figure 1 shows the standard curve generated by quantitative PCR (qPCR), illustrating the amplification of *C*Las-positive foliar DNA sample exhibiting HLB (Huanglongbing) symptoms across a range of concentrations. The oligonucleotide primer pair specific for *C*Las quantification produced a clear proportional dose–response relationship. To generate a robust standard curve, five data points spanning several orders of magnitude were obtained using a 1:5 serial dilution series. In Figure 1, the Ct values are plotted on the y-axis, while the corresponding DNA quantities are represented on the x-axis. The resulting standard curve exhibited an amplification efficiency of 98 % and a coefficient of determination R^2 of 0.99, indicating high linearity and assay reliability. These results validate the suitability of the selected marker gene for *C*Las quantification by qPCR, consistent with the findings previously reported by Bao et al. [12].

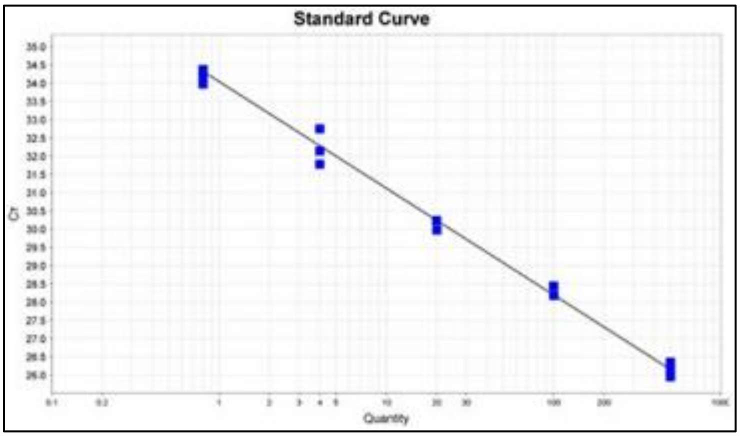

Figure 1 Standard curve generated by qPCR for the detection and quantification of *Candidatus Liberibacter* asiaticus (*C*Las). The primer pair reported by Bao et al. [12] were used for *C*Las amplification. A 1:5 serial dilution series was prepared from foliar DNA samples obtained from trees exhibiting HLB symptoms

3.2. Specific amplification and melting curve analysis for *C*Las detection in Persian lime from different plant sources

The marker gene reported by Bao et al. [12] proved effective for the detection of *C*Las in Persian lime leaf samples obtained from different sources. As shown in Figure 2, a single peak was observed in the melting curve analysis, indicating high specificity of the amplified product. This result confirms the reliability of the quantification data, as no non-specific amplification products, including primer-dimers, were detected.

qPCR analysis enabled clear discrimination of *C*Las infection among Persian lime plants from different sources (Figure 2). Distinct amplification profiles were observed depending on plant origin. Field-grown plants exhibited typical exponential amplification curves that crossed the threshold line at relatively low Ct values, indicating high bacterial loads. Melting curve analysis revealed a single, sharp peak, confirming specific amplification of the target gene without evidence of non-specific products or primer-dimer formation. Certified nursery plants showed more variable amplification behavior. Some samples displayed delayed amplification with higher Ct values, suggesting lower bacterial titers, while others showed irregular or late amplification signals. Nonetheless, melting curve analysis consistently produced a defined peak corresponding to the expected melting temperature, confirming assay specificity. In contrast, sanitized plants (pathogen-free) exhibited no detectable amplification or only late, non-specific signals that did not cross the established threshold. Their melting curves lacked the characteristic peak observed in positive samples, supporting the absence of detectable *C*Las DNA.

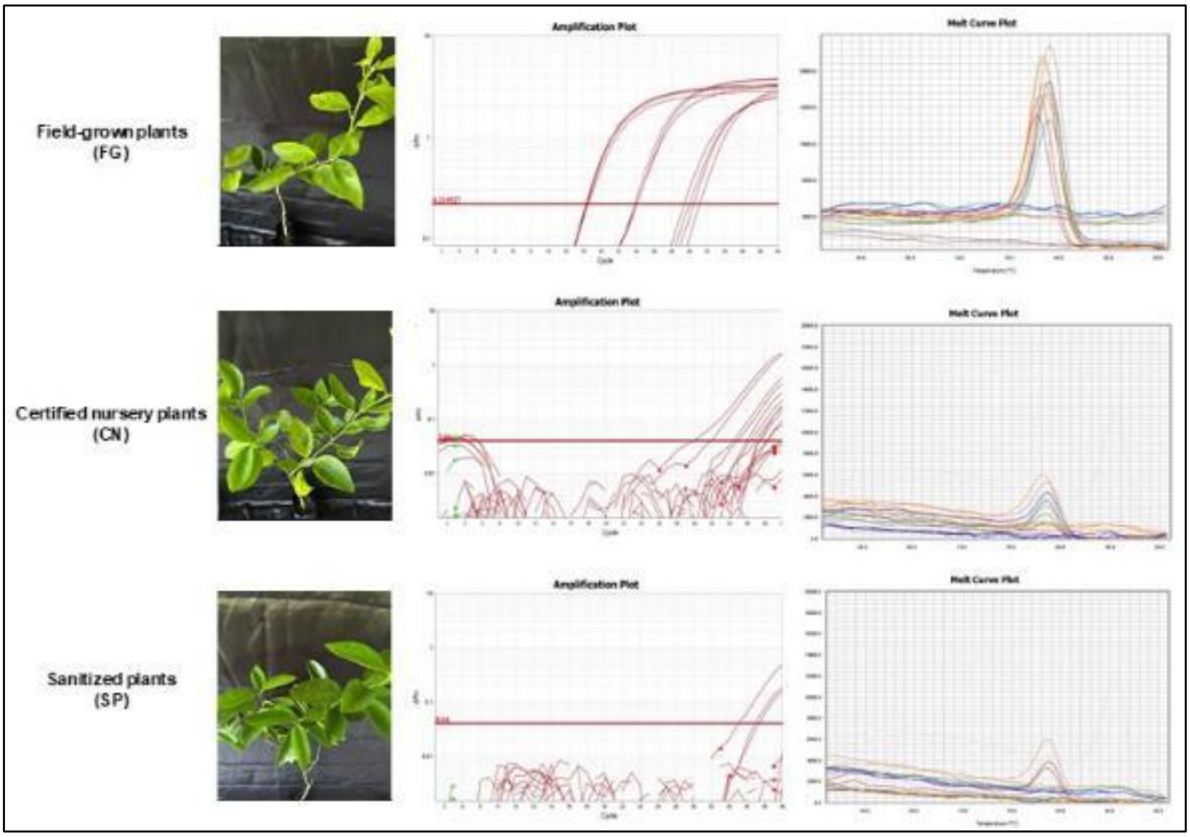

Figure 2 Amplification plots and melting curve analysis obtained by qPCR for the detection and quantification of *C*Las in Persian lime leaf samples from different sources, including field-grown plants, certified nursery plants, and sanitized plants (pathogen-free). Each amplification curve corresponds to an individual sample, with three technical replicates performed per sample

3.3. qPCR detection and quantification of *CLas* in Persian lime from different plant sources

RT-qPCR analysis revealed clear differences in *C*Las detection among Persian lime plants from different origins (Table 1). All field-grown plants (9/9) tested positive for *C*Las, with Ct values ranging from 17.72 ± 0.44 to 29.01 ± 0.78. Most samples exhibited Ct values below 22, indicating high bacterial loads. Only one sample (FG-9) showed a comparatively higher Ct value (29.01 ± 0.78), suggesting a lower infection level.

Respect to certified nursery plants, *C*Las detection was more variable. Three out of nine samples (CN-1, CN-7, and CN-8) tested positive, with Ct values ranging from 32.65 ± 0.72 to 34.55 ± 0.62. The remaining six samples showed either not detectable amplification (N.D.) or Ct values above the established threshold (>35), and were therefore classified as negative. Notably, positive certified plants exhibited higher Ct values compared to field-grown plants, indicating lower bacterial titers. Contrary, sanitized plants showed the lowest frequency of *C*Las detection. Only one sample (SP-2) tested positive, with a Ct value of 34.21 ± 1.22, whereas the remaining eight samples showed no detectable amplification (N.D.) or Ct values above 35 and were classified as negative. These results suggest that the sanitation process substantially reduced detectable *C*Las levels in Persian lime plants. Positive controls consistently amplified with Ct values ranging from 25.02 to 25.36, confirming assay performance and reproducibility. No amplification was observed in negative controls, demonstrating the absence of contamination and high assay specificity (Table 1). Overall, the qPCR assay effectively discriminated between heavily infected field-grown plants, partially infected certified nursery plants, and predominantly *C*Las-free sanitized plants, confirming its sensitivity and reliability for HLB diagnosis in Persian lime.

Table 1 Detection and quantification of *C*Las by qPCR in Persian lime leaf tissue

Origin	Code	Ct value	Diagnosis
Field-grown plants	FG-1	18.01 ± 0.56	Positive
	FG-2	17.72 ± 0.44	Positive
	FG-3	18.85 ± 0.85	Positive
	FG-4	20.01 ± 1.11	Positive
	FG-5	21.23 ± 0.75	Positive
	FG-6	19.65 ± 0.98	Positive
	FG-7	18.45 ± 0.65	Positive
	FG-8	22.06 ± 1.45	Positive
	FG-9	29.01 ± 0.78	Positive
Certified nursery plants	CN-1	34.55 ± 0.62	Positive
	CN-2	N.D.	Negative
	CN-3	N.D.	Negative
	CN-4	36.58 ± 1.12	Negative
	CN-5	N.D.	Negative
	CN-6	N.D.	Negative
	CN-7	32.65 ± 0.72	Positive
	CN-8	33.95 ± 1.03	Positive
	CN-9	N.D.	Negative
Sanitized plants	SP-1	N.D.	Negative
	SP-2	34.21± 1.22	Positive
	SP-3	N.D.	Negative
	SP-4	N.D.	Negative
	SP-5	36.67 ± 0.88	Negative
	SP-6	N.D.	Negative
	SP-7	N.D.	Negative
	SP-8	N.D.	Negative
	SP-9	N.D.	Negative
Control +	PC-1	25.19	Positive
	PC-2	25.02	Positive
	PC-3	25.36	Positive
Control -	NC-1	N.D.	Negative
	NC-2	N.D.	Negative
	NC-3	N.D.	Negative

4. Discussion

Accurate and sensitive detection of *Candidatus Liberibacter asiaticus* (*C*Las) remains essential for effective management of Huanglongbing (HLB) and the implementation of reliable certification programs. In this study, the DNA extraction kit consistently yielded high-quality DNA suitable for qPCR analysis, thereby minimizing the effects of PCR inhibitors commonly present in citrus tissues. High DNA quality is a critical prerequisite for sensitive *C*Las detection, as previously reported in diagnostic studies [9].

The optimized qPCR assay showed high analytical performance, with an amplification efficiency of 98% and R^2 value of 0.99, meeting widely accepted standards for quantitative assays. These parameters indicate strong linearity across dilution ranges and confirm the suitability of the selected marker gene for *C*Las quantification, consistent with previous reports [12]. The presence of a single melting peak in all positive samples confirmed assay specificity and excluded non-specific amplification, strengthening the reliability of SYBR Green-based detection.

Clear differences in bacterial titers were observed among plants from different sources. All field-grown plants tested positive, with predominantly low Ct values (<22), indicating high bacterial loads typical of systemic infections in symptomatic trees. Such elevated titers are consistent with advanced phloem colonization and disease progression described in established HLB pathosystems [2, 13]. The observed variability in Ct values likely reflects the uneven distribution of *C*Las within host tissues, a recognized limitation in HLB diagnostics. In contrast, certified nursery plants showed limited detection, with only three samples testing positive and Ct values >32, suggesting low bacterial titers. These results may reflect early or latent infections, emphasizing the importance of highly sensitive molecular diagnostics in nursery certification programs. Low-titer infections near the detection threshold may constitute a significant phytosanitary risk if not accurately identified.

Recent advances in on-site rapid detection tools and integrated sanitation protocols further highlight the importance of continuous monitoring. Field-deployable recombinase polymerase amplification (RPA) assays and highly sensitive qPCR methods have been validated to provide timely and accurate detection of *C*Las in both plant tissues and insect vectors, facilitating timely interventions post-sanitization [14]. Moreover, broader HLB management frameworks emphasize that sanitation strategies should be combined with vector control, deployment of pathogen-free planting material, and continuous molecular surveillance to suppress pathogen resurgence and delay reinfection in newly established trees [15]. Our sanitized plants showed minimal *C*Las detection, with only one Ct positive sample. These results indicate that the sanitation procedure markedly reduced bacterial titers, demonstrating its effectiveness in lowering pathogen load under the conditions evaluated. Nevertheless, complete eradication cannot be definitively assumed, as *Candidatus Liberibacter* asiaticus is known to persist at low and uneven titers within host tissues [9]. The detection of a residual low-level infection, evidenced by high Ct values, suggests that surviving bacterial populations may remain below the threshold of consistent detection while retaining the potential for systemic recolonization [16]. This finding underscores the biological complexity of *C*Las–host interactions and highlights the limitations of single-time-point diagnostics. Therefore, sustained post-sanitation molecular monitoring using sensitive qPCR assays is essential to verify long-term pathogen suppression and to prevent inadvertent propagation of asymptomatic but infected plant material.

The assay exhibited high reproducibility, as evidenced by the consistent amplification of positive controls and the absence of detectable signal in negative controls. Overall, the validated qPCR protocol reliably discriminated among heavily infected field-grown plants, low-titer nursery plants, and predominantly *C*Las-free sanitized plants. These results confirm its utility as a robust and sensitive diagnostic tool for HLB surveillance, nursery certification programs, and epidemiological assessments in Persian lime production systems.

5. Conclusion

Ct values ranging from 18 to 20 were obtained for field-grown samples, indicating high titers of *Candidatus Liberibacter* asiaticus (*C*Las), consistent with their origin from symptomatic field plants. In contrast, previously sanitized plants tested negative, supporting the effectiveness of the implemented sanitation protocol. Notably, *C*Las was detected in three nursery-certified plants, suggesting the presence of latent or undetected infections in materials presumed to be pathogen-free. These findings demonstrate that the validated qPCR protocol constitutes a reliable and sensitive tool for the early detection of HLB in Persian lime. Moreover, they underscore the importance of its systematic application in surveillance, certification, and phytosanitary management programs in citrus-producing regions. The consistent implementation of this diagnostic approach could strengthen field-level decision-making and contribute to mitigating the economic impact associated with this disease.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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