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Rapid colorimetric detection of *Citrus tristeza virus* combining portable sample preparation and reverse transcription-loop mediated isothermal amplification

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ABSTRACT

This article details combined portable sample preparation and colorimetric detection using loop-mediated isothermal amplification (RT-LAMP) of Citrus tristeza virus (CTV) from citrus leaves. The sample preparation employs an OmniLyse micro-homogenizer and cellulose paper disks to extract and isolate total nucleic acids in <15 min. Primer and dimethyl sulfoxide (DMSO) concentrations were optimized to minimize RT-LAMP assay reaction times and maximize the delay in the appearance of false positives due to non-specific amplification, respectively. The CTV primers were assessed against a panel of 24 CTV-positive isolates and 6 non-CTV pathogen isolates. To adapt the protocol for cold-chain-free field deployment, a lyophilized RT-LAMP reagent mix was developed and its rehydration solution was optimized to minimize false positives. In a greenhouse setting, the micro-homogenizer successfully extracted and isolated nucleic acids from CTV-positive trees, followed by the lyophilized RT-LAMP assay positively detecting the pathogen within 35 min. This study establishes the feasibility of quick and portable CTV detection without access to laboratory equipment, paving the way for larger-scale field studies, comprehensive validation of assay performance, and potential extension to other plant pathogens.

1. Introduction

Plant epidemics have long impacted crop yields and the global economy adversely, resulting in hundreds of billions of USD in crop yield losses every year [1–3]. As an example, citrus, one of the most popular cash crops in the world, has been affected by *Citrus tristeza virus* (CTV). CTV, a phloem-limited RNA virus, causes a number of devastating diseases, such as yellow dwarf and lime dieback, killing >70 million citrus plants worldwide in the past few decades [4–7]. To alleviate this situation, researchers have devised many strategies to combat CTV, including selective eradication of infected plants identified based on serological and genotypic assays, reproduction of virus-free plants, and controlling viruliferous vectors using pesticides [8,9]. However, these strategies are effective only if diseased trees can be promptly and accurately distinguished from a pool of healthy ones.

The current protocols for plant disease diagnostics rely on specimen collection and submission by growers, who need to collect multiple specimens from suspected trees, place them into clean, individually labeled containers, and mail them to centralized labs for diagnosis [10]. Any damage due to temperature or distance during transit could result in unsuccessful identification (e.g., false negatives). At the lab, a series of specimen processing steps (manual tissue homogenization and nucleic acid extraction) and the subsequent molecular (e.g., PCR/qPCR) and genotypic (e.g., NGS) assays could take anywhere from a few days to weeks, depending on the specimen amounts [11]. Despite the high-quality results provided by these commercial tools, high capital costs and expensive reagents could make routine testing unfeasible as demand increases [12–14]. Thus, an affordable method that allows in-field deployment for rapid and early detection is of great importance for effective plant disease management.

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Even field-ready serological tests such as lateral flow immunoassays (LFIAs) that are frequently used for prescreening of target plant diseases in the field (e.g., AgriStrip, Pocket Diagnostics kits, etc.) still require follow-up testing for samples tested positive with more specific techniques (e.g., qPCR, pathogen culture) in advanced labs [12,15,16]. To address this bottleneck and further benefit in-field disease management, portable and physical approaches, such as a handheld mechanical lysis device and a microneedle patch, have been adopted for quick collection of pathogen nucleic acids [17,18]. Specifically, Claremont BioSolutions' OmniLyse micro-homogenizer has been used as an effective approach to not only produce plant lysates [19] but also quickly extract total nucleic acids with the use of cellulose paper [20]. Such mechanical lysis, combined with equipment-free nucleic acid extraction (e.g., cellulose paper), has been proven sufficient for total nucleic acid extraction directly from plant lysates and is suitable for in-field use [21–24]. These nucleic acid preparation techniques are conducive to in-field nucleic acid detection tests (NAATs), especially loop-mediated isothermal amplification (LAMP) and similar isothermal tests [25,26] that operate at a constant temperature (e.g., 65 °C), have a short reaction time (30–40 min), and have a high tolerance to impurities.

Building on our previous study [20], we now present a rapid and visual detection of CTV by combining a micro-homogenizer, chromatography paper, and an RT-LAMP colorimetric assay (Fig. 1). We adapted our previously developed protocol for quick sample preparation [20], followed by RT-LAMP assays, in which the primer design, primer concentrations, and reaction times were optimized. Additionally, the role of dimethyl sulfoxide (DMSO) in minimizing false positives was investigated, and its optimal concentration was determined. To further

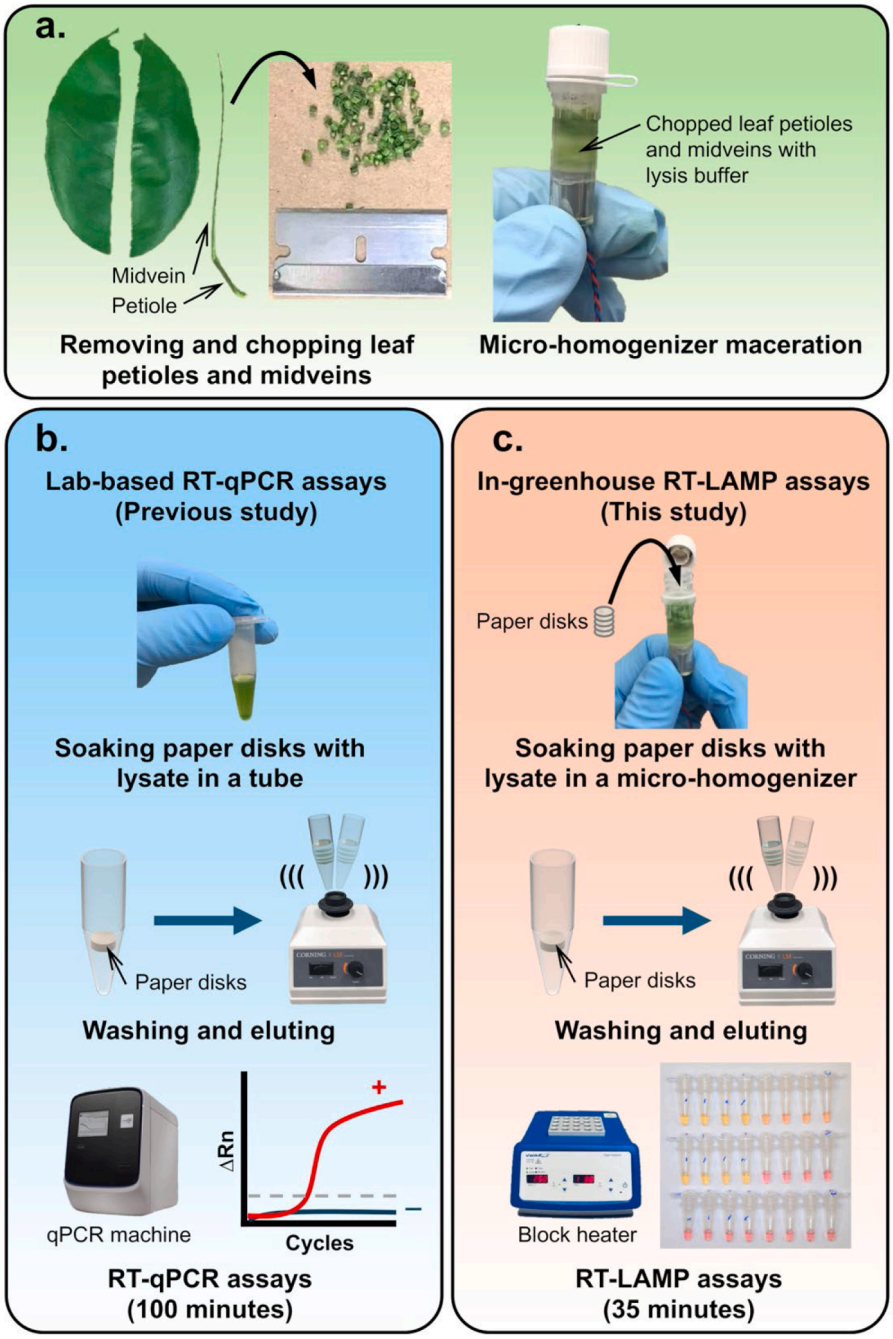

Fig. 1. Stepwise workflow starting from (a) sample collection and processing to (b) lab-based nucleic acid extraction and RT-qPCR [20] and (c) in-field nucleic acid extraction and detection utilizing colorimetric RT-LAMP (present study).

enable in-field plant disease diagnosis via elimination of the need for a cold chain, lyophilization of the RT-LAMP reaction mix was attempted and compared side by side with freshly prepared controls. Finally, colorimetric RT-LAMP assays for CTV detection with the lyophilized RT-LAMP reaction mix were successfully performed in a greenhouse setting.

2. Materials and methods

2.1. Materials

Guanidine isothiocyanate (GITC), polyvinylpyrrolidone (PVP), dimethyl sulfoxide (DMSO), D-(+)-trehalose dihydrate, guanidine hydrochloride (GuHCl) and Whatman Grade 1 CHR cellulose chromatography paper (Cat. No. 3001–861) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate trihydrate (pH 5.0), sodium chloride, ethylenediaminetetraacetic acid (EDTA), Tris-EDTA 1X solution (TE, pH 8.0), Tris-borate-EDTA (TBE) solution and MagMAX-96 Viral RNA isolation kit (Cat. No. AM1836) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 1 M Tris–HCl solution (pH 7.5) (Cat. No. 15567–027) was purchased from Invitrogen (Waltham, MA, USA). AgPath-ID One-Step RT-PCR kit was purchased from Life Technologies (Carlsbad, CA, USA). WarmStart Colorimetric LAMP kit (Cat. No. M1800S/L), gel loading dye (10X), and 100 bp DNA ladder were purchased from New England BioLabs (Ipswich, MA, USA). Green Fluorescent Dye (Item ID 30078–1) was purchased from Lucigen (Middleton, WI, USA). Primers and probes for RT-qPCR and RT-LAMP assays were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Nuclease-free water was used to prepare all buffers and Master Mix throughout the study. OmniLyse X-mini Cell & Tissue Lysis Device (Item# 01.532.48), also referred to as OmniLyse micro-homogenizer, was purchased from Claremont BioSolutions (Upland, CA, USA).

2.2. Preparation of buffers

The lysis buffer was prepared with 4 M GITC, 0.2 M sodium acetate trihydrate, 2 mM EDTA, and 2.5 % PVP, with the final pH adjusted to 5.0 using 1 N HCl solution [20]. The wash buffer was prepared by diluting a 1 M Tris–HCl solution to 10 mM for washing paper disks after soaking in the crude lysate. The elution buffer used for the benchtop extraction protocol was 1X TE solution, with an additional 0.1 M sodium chloride added for eluting nucleic acids from paper disks [20].

2.3. Plant materials and sample collection

The RT-LAMP primers were evaluated using 24 CTV-positive citrus accessions representing different cultivars and infection backgrounds (Table S4) and 6 non-CTV-infected citrus isolates infected with other viruses, viroids, and bacterial pathogens (Table S5). Healthy sour orange (*Citrus aurantium* L.) was used throughout the study as a negative control to evaluate the developed protocols. These sources were all provided by the Citrus Clonal Protection Program (CCPP) disease bank at the University of California, Riverside. All tested leaves were evenly collected on the east, west, south, and north sides of the tree at eye level to ensure consistency of sampling. Before processing, the leaves were gently wiped with wet paper towels to remove any dust.

2.4. Benchtop protocol for sample preparation

2.4.1. Preparation of crude lysates

Phloem-rich petioles and midveins removed from freshly picked healthy and diseased leaves were ground using a mortar and pestle with a gradual addition of liquid nitrogen until finely pulverized. The tissues were then aliquoted to 250 mg each in 2 mL centrifuge tubes, lysed by mixing with 750 μL of the lysis buffer and incubating for 15 min at 4 °C. The mixture was centrifuged at 14,000 × *g* at 4 °C for 25 min (Allegra X-22R centrifuge, Beckman Coulter, Hercules, CA, USA), after which 150 μL of the supernatant was collected for subsequent nucleic acid extraction.

2.4.2. Nucleic acid extraction using benchtop equipment

Nucleic acids in crude lysates were isolated using MagMAX Express-96 Magnetic Particle Processor (Thermo Fisher Scientific) with a MagMAX-96 Viral RNA isolation kit. Each reaction contained 150 μL of supernatant (crude lysate), 22 μL of RNA binding bead mix (10 μL lysis/binding enhancer + 10 μL RNA binding beads + 2 μL carrier RNA), 139 μL of lysis/binding solution as recommended by the manufacturer and extra 139 μL of 100 % isopropanol as previously reported by Dang et al. [11]. The resulting nucleic acids were eluted in 100 μL of 1X TE solution and were quickly assessed using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) to confirm the quality before being aliquoted and stored at −80 °C.

2.5. Micro-homogenizer-based protocol for sample preparation

2.5.1. Preparation of crude lysates

As introduced and optimized in our previous work [20], OmniLyse X-mini Cell & Tissue Lysis Devices were used to macerate 100 mg of chopped petioles and midveins from freshly picked healthy and diseased leaves, along with 300 mg of ceramic microbeads and 300 μL of lysis buffer for 5-min lysis at 11 V. The resulting crude lysate (250 μL) was drawn out using a filtered syringe tip and collected in a centrifuge tube for subsequent nucleic acid extraction.

2.5.2. Nucleic acids extraction using paper disks

Five paper disks (d = 6.35 mm), made of chromatography paper, were subject to a 5-min soaking in 250 μL of crude lysate for rapid nucleic acid capture. Disks were then transferred to a clean centrifuge tube containing 1 mL of 10 mM Tris–HCl solution for a 1-min wash with mild vortexing. These washed disks were suitable for either immediate elution in 100 μL of modified 1X TE solution for molecular assays or storage at room temperature for later use after being fully air dried.

2.6. RT-qPCR assays

Primers and probes were adapted from previous reports, with their quality and homology validated [27]. Cytochrome oxidase (COX) genes in plant mitochondrial genomes are routinely used as a reliable internal control to confirm nucleic acid integrity. The sequences and the corresponding nucleotide positions of these primers and probes are listed in Table 1. All RT-qPCR assays were performed using an Applied Biosystems QuantStudio™ 12 K Flex Real-Time PCR System (Thermo Fisher Scientific), as previously described [20].

2.7. RT-LAMP assays

Two primer sets (one for CTV and one for COX), with six specific primers (F3/B3, FIP/BIP, and LB/LF) in each, were designed using PrimerExplorer v5 (Eiken Chemical, Japan) and subject to BLAST analysis to confirm their specificities. The CTV primers were designed based on the conserved region determined through proper sequence alignment (Table S1). A similar approach was used for designing COX primers (Table S2). The sequences of RT-LAMP primer sets are detailed in Table 2, while the overall comparison of nucleotide positions between RT-qPCR and RT-LAMP primers is available in Tables S1 and S2. The protocols for preparing the RT-LAMP reaction mixture were also modified to adapt to various needs, which are detailed below. All the assays were conducted isothermally at 65 °C either in a CFX Connect Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) or a portable dry block heater to fit specific purposes. To evaluate the performance of the lyophilized RT-LAMP formulation relative to the conventional liquid RT-LAMP assay, both formats described in Sections

Table 1
CTV and COX primers and probes for RT-qPCR assays.

Gene	Primers/probes*	Sequence 5′ – 3′	Nucleotide position**	Amplicon size
CTV	Forward 1	TGTGTGCGGATTTCTTGACTG	524–544	133 bp
	Forward 2	TGTGTGCAGATTTCTTGACCG	524–544	
	Reverse	TTCCCAAGCTGCCTGACATT	656–637	
	Probe-NED	AAGCGAGGGGCTGAT	607–621	
COX	Forward	AATCTGACCTTCTTTCCCATGC	32–53	163 bp
	Reverse	AAGTGATTGTTACGACCACGAAGA	194–171	
	Probe-FAM	ATCCAGATGCTTACGCTGG	96–114	

* Primer and probe sequences were adapted from Osman et al. [27].
** Nucleotide position is based on GenBank accessions: CTV-AJ518842, COX-CX297817.

Table 2
CTV and COX primers for RT-LAMP assays.

Gene	Primers/probes	Sequence 5′ – 3′	Nucleotide position*
CTV	FIP (F1c + F2)	CGGAATCCCTGCATCTAGCG-CTTTTGTAGACAGAACCGCA	507–488; 447–466
	BIP (B1c + B2)	GCTGGGTATCATTATTTGTGTGC-AGCTTGTATGTACACAGCA	508–530; 588–570
	F3	CTAACGATGCTCTTTACTTAGC	425–446
	B3	CCCCTCGCTTTTTCAACAA	616–598
	LF	GACGTCCGCCATAACTCAA	487–469
	LB	GCTGGCTTGACTGATTTAGAATG	547–569
COX	FIP (F1c + F2)	CGGATATATAAGAGCCAAAACTGCT-GCATTCCAGATTATCCAGATG	152–128; 84–104
	BIP (B1c + B2)	GGATTTGTTGTTTCTTCGTGGTCG-CAACAGCCCAAGGACTTG	159–182; 242–225
	F3	TTAGGGCTTTCGGGTATG	59–76
	B3	CCAGTGTGGTTGAATTCTGT	263–244
	LF	GGCATTCCATCCAGCGTAAG	124–105
	LB	GCAGTGGAAATAACAAAAGATGTGC	198–222

* Nucleotide position is based on GenBank accessions: CTV-AJ518842, COX-CX297817.

2.7.2 and 2.7.3 were systematically compared using the same sets of CTV-positive and CTV-negative citrus samples.

2.7.1. Preparation of RT-LAMP reaction mixture

The original protocol suggested by the manufacturer [28] was modified below. Each reaction contained 12.5 μL of 2X Master Mix, 2.5 μL of 10X Primer Mix (final concentration: 1.6 μM of FIP/BIP, 0.2 μM of F3/B3 and 0.75 μM LF/LB), 1.25 μL of Green Fluorescent Dye, 6.75 μL of nuclease-free water, and 2 μL of RNA template (or nuclease-free water for non-target control). Green Fluorescent Dye was added for visualization via CFX Connect Real-Time PCR Detection System. This mixture was used for the determination of primer concentrations and the assessment of the CTV primers' pathogen selectivity.

2.7.2. Preparation of DMSO-based RT-LAMP reaction mixture

The reaction mixture as described in Section 2.7.1 was modified by adding some amount of DMSO (4 %, 6 %, and 8 % v/v). For the 6 % DMSO reactions, each reaction contained 12.5 μL of 2X Master Mix, 2.5 μL of 10X Primer Mix (final concentration: 1.6 μM of FIP/BIP, 0.2 μM of F3/B3 and 0.75 μM LF/LB), 1.5 μL of DMSO, 1.25 μL of Green Fluorescent Dye, 5.25 μL of nuclease-free water, and 2 μL of RNA template (or nuclease-free water for non-target control). Green Fluorescent Dye was added for visualization via CFX Connect Real-Time PCR Detection System. The 6 % DMSO solution was used for the survey of CTV isolates, determination of the lowest detectable concentration and rehydration of the lyophilized reaction mix.

2.7.3. Preparation of lyophilized RT-LAMP reaction mixture

The lyophilization protocol previously developed by Song et al. [29] was adopted in the study. Each reaction contained 12.5 μL of 2X Master Mix, 2.5 μL of 10X Primer Mix (final concentration: 1.6 μM of FIP/BIP, 0.2 μM of F3/B3 and 0.75 μM LF/LB), 5.75 μL of D-(+)-trehalose dihydrate solution (4.296 g/10 mL), 1.25 μL of Green Fluorescent Dye, 1 μL of GuHCl solution (0.955 g/10 mL) and 2 μL of RNA template (or nuclease-free water for non-target control). The reaction mixtures in 1.5 mL Eppendorf tubes were placed at −80 °C until fully frozen before being freeze-dried for 18–20 h in a FreeZone Triad Cascade Benchtop Freeze Dryer (Labconco, Kansas City, MO). The resulting freeze-dried powders were then stored either at −80 °C or in a sealed Ziploc with desiccant at room temperature for later use. Lyophilized RT-LAMP reagents were prepared as described above and rehydrated immediately prior to use with either nuclease-free water or a 6 % DMSO solution. For method comparison, lyophilized reactions were run in parallel with the conventional RT-LAMP assay (Section 2.7.1) on the same CTV-positive, CTV-negative, and non-template control samples. Both assays were incubated under identical conditions, and results within the reaction window were used to compare the false negative rates between the lyophilized format and the conventional assay.

2.8. Gel electrophoresis

Amplicons from RT-qPCR and RT-LAMP were visualized by agarose gel electrophoresis (2 % w/v) in 0.5X TBE solution. Amplified samples were mixed with gel loading dye (10X) and loaded into the wells. A 100 bp DNA ladder was used as a size marker (M). The gel was run for 30 min at 100 V using a PowerPac Basic Electrophoresis Power Supply (BIO-RAD). The resultant gel was immersed in ethidium bromide solution (0.06 % v/v) with gentle shaking for 10 min for DNA post-staining and was imaged using a ChemiDoc Imaging System (BIO-RAD).

2.9. Statistical analysis

All data analysis and graphing were performed using GraphPad Prism (version 10.4.2; GraphPad Software, San Diego, CA, USA). Unless otherwise specified, $N = 3$ indicates technical replicates, i.e., three parallel reactions prepared from the same nucleic-acid extract within a single experiment. Biological replicates (distinct trees or isolates) are described in the corresponding sections. For comparative analyses of amplification performance (e.g., threshold time, Tt) under different reaction conditions, data are presented as mean ± standard deviation (SD). Where applicable, differences between two groups were assessed using an unpaired two-tailed Student's *t*-test, and differences among multiple conditions were evaluated using one-way ANOVA and Tukey's multiple-comparisons (unpaired) test. As specified in the figure captions, a p-value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Optimization of CTV RT-LAMP assay

3.1.1. Determination of primer concentrations for CTV RT-LAMP assays

CTV isolate T529 (accession no KC841789.1) was selected as the target for determining the primer concentrations that would result in the minimum CTV RT-LAMP assay time. All primer sets were evaluated

using the conventional RT-LAMP assays described in Section 2.7.1, which were carried out at 65 °C with varying concentrations of inner primers (1.2 μM, 1.6 μM, and 2 μM), loop primers (0.5 μM, 0.75 μM, and 1 μM) and a fixed outer primer concentration (0.2 μM) in different combinations [30]. As presented in Table 3, all combinations showed nearly equivalent threshold times, ranging from 9.6 to 10.4 min. Notably, the combination with 1.6 μM of inner primers, 0.2 μM of outer primers, and 0.75 μM of loop primers (bolded in Table 3) performed almost one minute shorter in threshold time (09′33″) than the longest one (10′21″). Thus, this primer combination was chosen and used throughout the study for the preparation of both CTV and COX primer mixtures.

3.1.2. Minimization of false positives for CTV RT-LAMP assays

DMSO is frequently used in PCR and LAMP assays to inhibit the formation of secondary structures in DNA templates and primers, as reported by Shao et al. [31]. It reduces the melting temperature of DNA complexes, including primer dimers, facilitating their dissociation and minimizing unwanted self-complementarity [32,33]. This suppresses the amplification of non-specific strands and results in fewer and delayed false positives. Thus, RT-LAMP reaction mixtures with varying concentrations of DMSO (0 %, 4 %, 6 %, and 8 % (v/v)) added were tested with CTV positive controls (PC, $N = 3$), negative controls (NC, $N = 8$), and non-target controls (NTC, $N = 8$) and incubated at 65 °C for 80 min. The greater number of negative controls was chosen to account for the random nature of non-specific amplification, allowing a better estimate of an appropriate reaction cut-off time.

As depicted in Fig. 2, the addition of DMSO significantly delayed the threshold times of all controls. To maximize the detection window for RT-LAMP assays, the time differences between the average threshold times of PCs and the earliest false positive in each NC+NTC were compared across different DMSO concentrations. The RT-LAMP assays with 6 % DMSO had the broadest detection window, significantly delaying the occurrence of false positives compared to the assay with 0 % and 4 % DMSO (Fig. 2). Increasing the DMSO concentration to 8 % did not statistically enhance the suppression of false positives compared to the 6 %, while it extended the threshold time of true positives and shortened the detection window. This suggests diminishing returns with higher DMSO concentrations. Therefore, 6 % DMSO was selected as the optimal protocol for preparing RT-LAMP mixtures. As the first false positive with 6 % DMSO did not occur until slightly after 40 min, that was chosen as the cut-off threshold for subsequent experiments. Without DMSO, the cut-off threshold would be 30 min.

3.1.3. Evaluating detectable concentrations for CTV and COX RT-LAMP assays

To evaluate the detectable concentrations of the RT-LAMP primers for CTV and COX, total nucleic acids of a CTV-positive source (isolate T529) were selected as a positive control and a series of six 10-fold dilutions were prepared for RT-qPCR assays. The results of the RT-qPCR assays for CTV and COX were calibrated with previously generated standard curves for the same primer sets as this study [27]. From this calibration, the concentrations of the solutions were able to be determined and are presented in Fig. 3a and 3c. The curves generated from these calibrated points had good linearity ($R^2 > 0.99$ for both genes), indicating a high amplification efficiency. RT-LAMP assays were then conducted with the same set of samples. It was observed that the 5th and 6th dilutions were not detected in the CTV RT-LAMP assay, suggesting that the lowest detectable quantity of CTV is on the order of 10^3 copies per reaction. The calculation process is detailed in Table S3. The threshold times of the RT-LAMP assays demonstrated high linearity over at least five orders of magnitude ($R^2 = 0.97$ for CTV and $R^2 = 0.99$ for COX). Although the lowest detected quantity of RNA from the RT-LAMP assay was approximately 1–2 orders of magnitude higher than that of

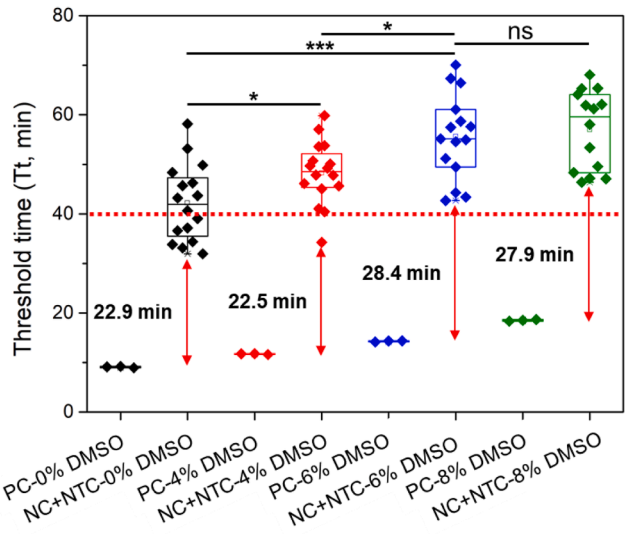

Fig. 2. Comparison of threshold times of RT-LAMP assays with 0 %, 4 %, 6 %, and 8 % DMSO. PC denotes CTV-positive control, NC is negative control, and NTC is non-target control. Increasing DMSO concentration delayed the threshold times for both positive and negative controls due to inhibition of template–primer binding and polymerase activity. The time difference between the average threshold time for PC and the earliest false positive for NC+NTC defines the "detection window." One and two true negatives were observed in the 6 % and 8 % DMSO groups, respectively, where no signal occurred within the 80-minute reaction. A 40-minute reaction time (dashed line) was set as the laboratory's cutoff, within which the fewest false positives occurred. $N = 3$ for PC, $N = 16$ for NC and NTC combined. * and *** denote $p \leq 0.05$ and $p \leq 0.001$, respectively; ns (not significant) indicates $p > 0.05$.

Table 3
Optimization of primer concentrations for RT-LAMP assay for CTV.

Inner primers (FIP/BIP) (μM)	Outer primers (F3/B3) (μM)	Loop primers (LF/LB) (μM)	Threshold time* (min's")			Mean Time (min's")	SD
			R1	R2	R3		
1.2	**0.2**	0.5	09′51″	09′41″	09′35″	09′43″	0′08″
1.6			09′38″	09′37″	09′42″	09′39″	0′02″
2			10′04″	10′15″	10′16″	10′12″	0′07″
1.2		**0.75**	09′39″	09′34″	09′43″	09′39″	0′05″
1.6			**09′36″**	**09′36″**	**09′27″**	**09′33″**	**0′05″**
2			10′28″	10′20″	09′57″	10′15″	0′16″
1.2		1	09′46″	09′46″	09′38″	09′43″	0′05″
1.6			10′01″	09′51″	09′57″	09′56″	0′05″
2			10′18″	10′29″	10′17″	10′21″	0′07″

* In real-time RT-LAMP assays, the threshold time (Tt) was defined as the time from the start of incubation until the fluorescence signal exceeded a threshold above baseline background. This Tt is analogous to the quantification cycle (Cq) in qPCR and reflects the time required for the accumulating amplicons to generate a detectable increase in fluorescence.

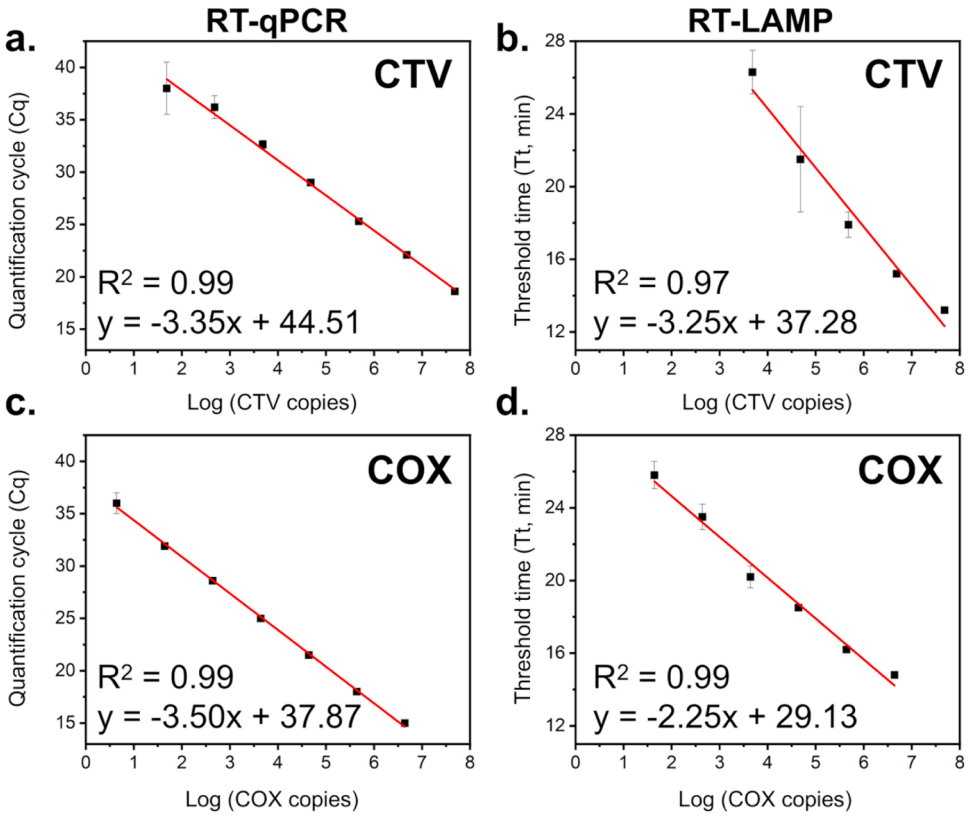

Fig. 3. Amplification comparison of RT-qPCR and RT-LAMP assays using 10-fold serially diluted total nucleic acids of a CTV-positive source (T529). Cq values and threshold times (Tt) derived from (a) CTV RT-qPCR, (b) CTV RT-LAMP, (c) COX RT-qPCR, and (d) COX RT-LAMP assays. ($N = 3$).

RT-qPCR, target RNA with $10^5 - 10^6$ copies per reaction can be detected within a short period of time (16–17 min). Additionally, the gel patterns of both the RT-qPCR and RT-LAMP assays indicate correct amplification, as does the color change of the RT-LAMP tubes (Fig. S1).

3.1.4. Assessment of the CTV RT-LAMP assay with various CTV isolates and non-CTV pathogens

The optimized RT-LAMP primer concentrations were tested using 24 CTV-positive isolates from CCPP greenhouses at the University of California, Riverside (Table S4). Total nucleic acids were extracted

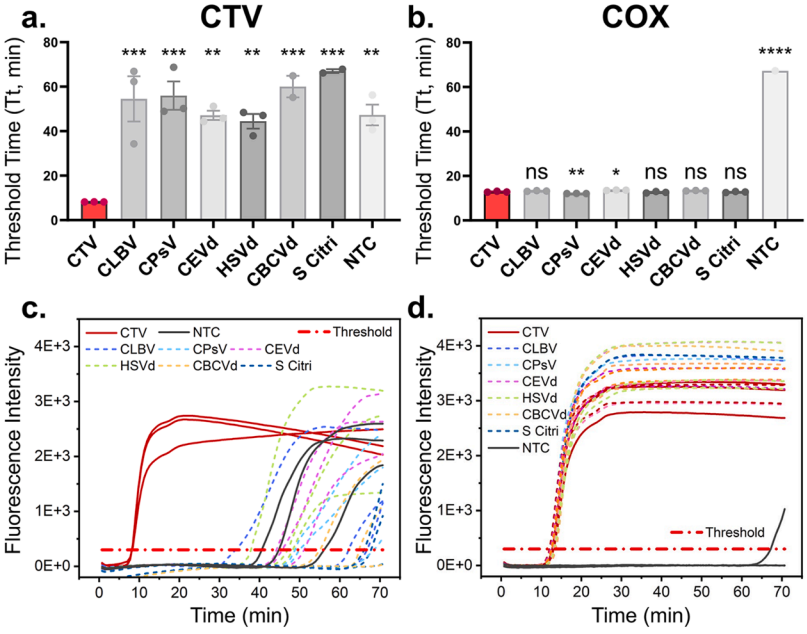

Fig. 4. The selectivity assessment of CTV and COX RT-LAMP primer performance. (a) CTV- and (b) COX-targeting RT-LAMP primers against CTV and non-target citrus pathogens ($N = 3$, 0 % DMSO added). Bar graphs summarize Tt (mean ± SD, $N = 3$) for each group, assessing the selectivity performance of (c) CTV and (d) COX primer sets. *, **, and *** denote $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively; ns (not significant) indicates $p > 0.05$.

following the standard protocol (Section 2.4), and both RT-qPCR and RT-LAMP were performed in triplicate on the same samples. Fourteen out of the 24 isolates were correctly identified as positive by RT-LAMP, with 100 % accuracy, 7 isolates had partially positive identification (17 %−33 %), and 3 isolates were negative (Table S4). The ten isolates that resulted in partial or no identification with the CTV RT-LAMP primers were not included in the pool of 100 CTV isolates used to design the primers (Table S1). Although the CTV primer set is not perfect match for all known CTV variants, it was successful in detecting the isolates chosen for assay optimization (T529) and its greenhouse implementation (T520)

Primer selectivity was examined using a panel of non-CTV citrus pathogens. Six pathogens were chosen: two viruses (Citrus leaf blotch virus and Citrus psorosis virus), three viroids (Citrus exocortis viroid, Hop stunt viroid, and Citrus bark cracking viroid), and one bacterium (*Spiroplasma* citri). The isolate information is detailed in Table S5. The CTV RT-LAMP assays remained negative for all non-CTV targets for longer than 30 min, the cut-off time for non-DMSO reactions (Fig. 4a,c). All isolates showed the expected consistent positive detection for COX (Fig. 4b,d). This confirms that our CTV primer set is highly selective for CTV.

3.2. *In-greenhouse RT-LAMP assays*

3.2.1. *Evaluation of lyophilized RT-LAMP reagents*

To further adapt the current RT-LAMP protocol for in-field application, it is critically important to streamline the procedures by having all the required reagents, except for the viral template, mixed in a tube in advance. Furthermore, reagents that can be stored and shipped under less stringent conditions, such as at room temperature, are even more ideal, particularly for diagnostic purposes in resource-limited regions. Given these requirements, lyophilization of the RT-LAMP reagents was considered an appealing solution. It has been reported that trehalose, a disaccharide, provides cryo- and lyo-protection for proteins (e.g., polymerase) during the lyophilization process and enhances stability during long-term storage [34]. Further, the addition of guanidine hydrochloride in the mixture helps expedite the appearance of positive signals [35]. Therefore, a previously reported protocol by Song et al. [29] was adapted for the preparation of lyophilized RT-LAMP reagents. To evaluate the performance of the lyophilized reaction mix after one-week storage, the resulting lyophilized mixtures were stored at −80 °C and room temperature separately. Serving as controls for comparison, the original DMSO-based mixture and fresh trehalose-based control were also included to evaluate the threshold time of PC and the false positive rate within a 40-min window. The images of the lyophilized reaction mix before and after one-week storage, and the comparison between fresh controls and rehydrated reaction mix are all detailed in Fig. S2.

As depicted in Fig. 5a, trehalose-based assays for CTV generally resulted in earlier thresholds for PC and no significant difference in thresholds for NC+NTC compared to DMSO-based assays, although a false positive in lyophilized reaction mix (−80 °C) and in fresh trehalose control were found within the 40-min window. Fewer false positives, as annotated in brackets (true-negative rate), were also found in these trehalose-based assays, indicating the combination of trehalose and guanidine hydrochloride is a promising alternative to DMSO without a negative impact on PC's threshold time. In RT-LAMP assays of COX (Fig. 5b), a false positive in the lyophilized reaction mix (room temp) was found very close to its PC's threshold, while the rest showed no significant difference between each other. Similarly, true negatives were also observed in most of these reactions. Notably, regardless of storage conditions, trehalose-based assays with a lyophilized reaction mix showed no significant differences from the fresh control for CTV and COX assays. Therefore, room temperature storage was selected for our next study. Although trehalose-based assays overall demonstrated better outcomes with fewer false positives and earlier thresholds for PC, random false positives within the 40-min window (as indicated in Fig. 5a, b) could still be an uncertainty for in-field application, leading to unreliable detection. Overall, these data demonstrate that the lyophilized RT-LAMP reagents maintained performance comparable to freshly prepared mixes for up to one week under the tested storage conditions; longer-term stability was not evaluated in this study. However, the study by Song et al. [29] reported that the lyophilized RT-LAMP reagents remained viable after 30 days of storage at 4 °C and ten days at room temperature. Furthermore, a new commercially available product (LyoPrime WarmStart LAMP, New England Biolabs) has a shelf-life of 24 months at 25 °C.

To eliminate false positives within the reaction window, a 6 % DMSO solution was used to rehydrate the lyophilized reaction mix stored at room temperature, and was compared with the fresh trehalose-based control and the H_2O-rehydrated reaction mix. As presented in RT-LAMP CTV assays (Fig. 6a), the lyophilized mix rehydrated with 6 % DMSO solution delayed false positives for 3.5 min, whereas the first false positives in DMSO-rehydrated assays and the fresh control almost occurred at the same time. Notably, in COX assays (Fig. 6b), no false

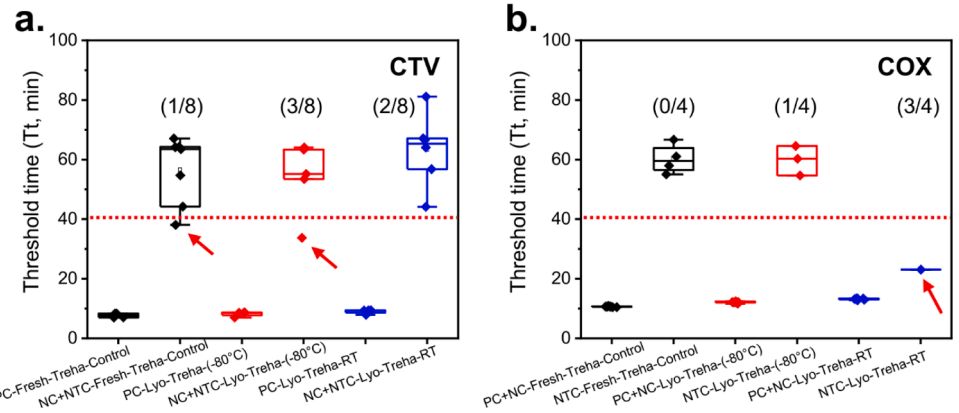

Fig. 5. Comparison of RT-LAMP threshold times for true and false positives for three trehalose-based formulations: fresh trehalose control; water rehydration of lyophilized mix stored at –80 °C; and water rehydration of lyophilized mix stored at room temperature (RT). The red dashed line marks the 40-minute cutoff. The following naming conventions are used: "Control type" – "Reagent preparation condition" – "Reagent composition" – "Reagent storage condition (only for lyophilized samples)." Control type: positive control (PC), negative control (NC), or non-target control (NTC). Reagent preparation: freshly prepared (Fresh) or lyophilized (Lyo). Reagent composition: Trehalose (Treha). Reagent storage: stored at –80 °C or room temperature (RT). For example, PC+NC-Lyo-Treha-(−80 °C) indicates a combination of both positive and negative controls, lyophilized with trehalose, and stored at –80 °C before rehydration. The fractions in parentheses indicate the number of undetected cases over the tested samples. For the CTV assays, $N = 4$ for PC, $N = 8$ for NC and NTC combined. For the COX assays, $N = 8$ for PC and NC combined, $N = 4$ for NTC.

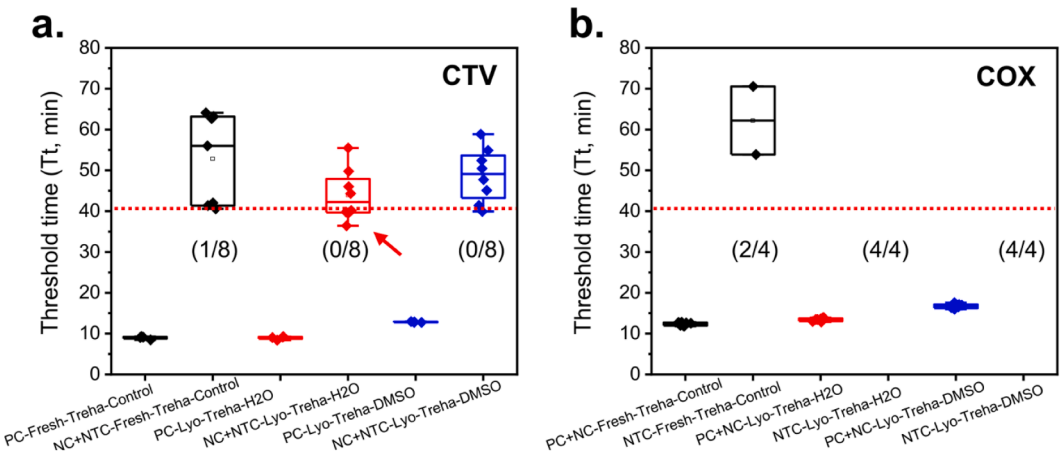

Fig. 6. Comparison of RT-LAMP threshold times for true and false positives across three trehalose-based protocols. CTV (a) and COX (b) assays compare: fresh trehalose control; rehydration with water of lyophilized mix stored at room temperature; and rehydration of lyophilized mix at room temperature with 6 % DMSO. The red dashed line indicates the 40-minute cutoff. In CTV assays (a), rehydration with 6 % DMSO delayed the first false positives by ~3.5 min compared to water controls. In COX assays (b), both rehydration methods prevented false positives, unlike fresh controls. These results support 6 % DMSO rehydration of room-temperature-stored lyophilized mixes ("modified trehalose-based protocol") as the best option for minimizing false positives. The following naming conventions are used: "Control type" – "Reagent preparation condition" – "Reagent composition". Control type: positive control (PC), negative control (NC), or non-target control (NTC). Reagent preparation condition: freshly prepared (Fresh) or lyophilized (Lyo). Reagent composition: Trehalose-dimethyl sulfoxide (Treha-DMSO) or Trehalose-DI water (Treha-H_2O). For example, NC+NTC-Lyo-Treha-DMSO refers to a combination of negative and non-target controls, lyophilized with trehalose, and stored at room temperature before rehydration with 6 % DMSO. The fractions in the parentheses indicate the number of undetected cases over the tested samples. For the CTV assays, $N = 4$ for PC, $N = 8$ for NC and NTC combined. For the COX assays, $N = 8$ for PC and NC combined, $N = 4$ for NTC.

positives were found in either rehydrated assay, while 2 out of 4 were still found in the fresh control. This set of tests suggested that the addition of DMSO effectively suppressed false positives from happening early. However, even with the DMSO, a false positive occurred right before the 40-minute cut-off and so to further reduce the possibility of a false positive, the reaction window was reduced to 35 min for subsequent experiments using the lyophilized reaction mix.

Overall, RT-LAMP assays with H_2O-rehydrated lyophilized reaction mix showed no difference in PC threshold times, regardless of storage conditions. Rehydration using a 6 % DMSO solution further delayed the false-positive threshold, ensuring reliable detection within the designated reaction time. Given the reported outcomes, a lyophilized reaction mix (room temperature) rehydrated with 6 % DMSO solution (referred to as modified trehalose-based protocol) was then selected for in-greenhouse CTV RT-LAMP assays in the next section.

3.2.2. *Implementation of in-greenhouse colorimetric RT-LAMP assays*

Our next attempt was to conduct on-site CTV RT-LAMP assays in a greenhouse using our previously reported micro-homogenizer and cellulose paper protocols for tissue lysis and nucleic acid extraction. Leaves were directly collected from a CTV-positive plant (isolate T520) and a CTV-negative sweet orange for onsite sample preparation. The lyophilized reaction mix for CTV and COX detection was rehydrated on the spot and temporarily placed on ice before use. Total nucleic acids were eluted from soaked paper disks after washing and were also placed on ice for later use. RT-LAMP assays were conducted by inserting the tube strips into a heat block for 35 min. The tube strips were then placed on ice immediately after incubation to halt the reaction.

As depicted in Fig. 7a, the CTV-positive, CTV-negative, and water control were ordered sequentially from top to bottom for both CTV and COX RT-LAMP assays ($N = 4$). The color changes were distinguishable for all tests after a 35-min incubation at 65 °C, allowing quick visual detection. However, the CTV assays produced a less distinct color transition than the COX assays in the CTV-positive samples, likely due to the low initial copies of CTV in the sample. In addition, differences in the fluorescence between positive and negative reactions (Fig. 7b) were

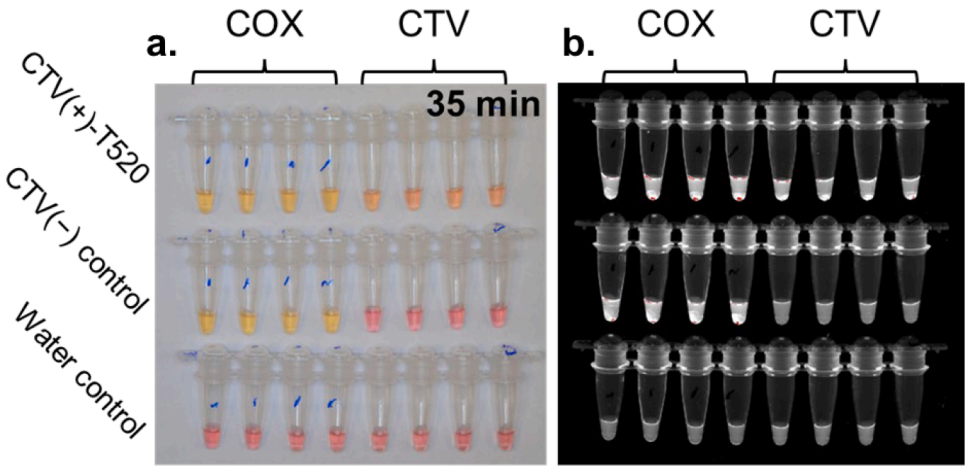

Fig. 7. The evaluation of in-greenhouse RT-LAMP assays for CTV and COX detection. Colorimetric assays (a) and fluorescence readout (b) after a 35-min incubation at 65 °C.

consistent, although the change was not as obvious as seen in the colorimetric assays. This study demonstrates the feasibility of a rapid, lyophilized RT-LAMP assay for the direct detection of CTV from citrus leaf samples in greenhouse conditions. The combined protocol, including efficient sample preparation and isothermal amplification, achieved reliable CTV detection within 35 min, without false positives. This approach offers a promising, field-adaptable diagnostic tool for real-time monitoring of CTV in citrus cultivation.

4. Conclusion

In this study, we developed and evaluated an integrated protocol that combines rapid sample preparation with colorimetric RT-LAMP for the detection of CTV from citrus leaves. Total nucleic acids were prepared using a micro-homogenizer and cellulose paper, followed by RT-LAMP assays optimized for quick signal generation and reduced non-specific amplification. The CTV RT-LAMP assay reliably produced a fluorescent signal in just 15 min of amplification with false negatives being delayed with the addition of 6 % DMSO. Furthermore, a lyophilized colorimetric RT-LAMP reaction mix enabled on-site, in-greenhouse endpoint detection within 35 min, without false-positive or false-negative responses, demonstrating the feasibility of near-source testing. Based on current reagent and consumable use, the per-test consumable cost is estimated to be on the order of $10-$15, with the majority of the cost being the micro-homogenizer (Table S6). This per-test cost can be further reduced through the development and use of a re-usable tissue homogenizer [18]. Our future studies will extend this portable sample preparation and sensing framework to the detection of other plant pathogens using existing LAMP primers and performing large-scale validations. Additionally, we will improve the utility of this platform by streamlining the extraction and detection processes, prolonging the shelf-life of the lyophilized reagents, and reducing the overall testing cost.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Chia-Wei Liu: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Sohrab Bodaghi:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition. **Manjunath L. Keremane:** Writing – review & editing, Methodology. **Brent Kalish:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Conceptualization. **Georgios Vidalakis:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Hideaki Tsutsui:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Hideaki Tsutsui serves on the editorial board of SLAS Technology. He had no involvement in the peer review of this article. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.slast.2026.100398.

References

[1] He S, Krainer KMC. Pandemics of people and plants: which is the greater threat to food security? Mol Plant 2020;13(7):933–4.
[2] Singh BK, Delgado-Baquerizo M, Egidi E, Guirado E, Leach JE, Liu H, Trivedi P. Climate change impacts on plant pathogens, food security and paths forward. Nat Rev Microbiol 2023;21(10):640–56.
[3] Tatineni S, Hein GL. Plant viruses of agricultural importance: current and future perspectives of virus disease management strategies. Phytopathology® 2023;113(2):117–41.
[4] Atta S, Cao M, Umar UUD, Zhou Y, Yang F, Zhou C. Biological indexing and genetic analysis of Citrus tristeza virus in Pakistan. J Gen Plant Pathol 2017;83:382–9.
[5] Hajeri S, Killiny N, El-Mohtar C, Dawson WO, Gowda S. Citrus tristeza virus-based RNAi in citrus plants induces gene silencing in Diaphorina citri, a phloem-sap sucking insect vector of citrus greening disease (Huanglongbing). J Biotechnol 2014;176:42–9.
[6] Karasev A, Boyko V, Gowda S, Nikolaeva O, Hilf M, Koonin E, Niblett C, Cline K, Gumpf D, Lee R. Complete sequence of the citrus tristeza virus RNA genome. Virology 1995;208(2):511–20.
[7] Ayllón M, López C, Navas-Castillo J, Garnsey S, Guerri J, Flores R, Moreno P. Polymorphism of the 5′ terminal region of Citrus tristeza virus (CTV) RNA: incidence of three sequence types in isolates of different origin and pathogenicity. Arch Virol 2001;146:27–40.
[8] Walker WB, Allen ML. Expression and RNA interference of salivary polygalacturonase genes in the tarnished plant bug, Lygus lineolaris. J Insect Sci 2010;10(1):173.
[9] Wang J, Bozan O, Kwon SJ, Dang T, Rucker T, Yokomi RK, Lee RF, Folimonova SY, Krueger RR, Bash J. Past and future of a century old Citrus tristeza virus collection: a California citrus germplasm tale. Front Microbiol 2013;4:366.
[10] California Department of Food & Agriculture. Sample preparation and submission to the plant pest diagnostics branch plant pathology laboratory. July 19, https://www.cdfa.ca.gov/plant/ppd/PDF/Submission_guidelines_Plant_Pathology.pdf; 2025. July 19.
[11] Dang T, Osman F, Wang J, Rucker T, Bodaghi S, Tan SH, Pagliaccia D, Lavagi-Craddock I, Vidalakis G. High-throughput RNA extraction from citrus tissues for the detection of viroids. Methods Protoc 2022:57–64.
[12] Miller SA, Beed FD, Harmon CL. Plant disease diagnostic capabilities and networks. Annu Rev Phytopathol 2009;47:15–38.
[13] QIAGEN. RNeasy plant mini Kit for RNA extraction. August 28, https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-plant-mini-kit; 2024. August 28.
[14] QIAGEN. DNeasy plant kits. August 28, https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-plant-kits; 2024. August 28.
[15] Singh J, Cobb-Smith D, Higgins E, Khan A. Comparative evaluation of lateral flow immunoassays, LAMP, and quantitative PCR for diagnosis of fire blight in apple orchards. J Plant Pathol 2021;103:131–42.
[16] Kanapiya A, Amanbayeva U, Tulegenova Z, Abash A, Zhangazin S, Dyussembayev K, Mukiyanova G. Recent advances and challenges in plant viral diagnostics. Front Plant Sci 2024;15:1451790.
[17] Paul R, Saville AC, Hansel JC, Ye Y, Ball C, Williams A, Chang X, Chen G, Gu Z, Ristaino JB. Extraction of plant DNA by microneedle patch for rapid detection of plant diseases. ACS nano 2019;13(6):6540–9.
[18] Liu CW, Kalish B, Bodaghi S, Vidalakis G, Tsutsui H. A 3D-printed handheld device for quick citrus tissue lysis and nucleic acid extraction. Adv Sample Prep 2025:100199.

[19] Haveman NJ, Schuerger AC, Yu PL, Brown M, Doebler R, Paul AL, Ferl RJ. Advancing the automation of plant nucleic acid extraction for rapid diagnosis of plant diseases in space. Front Plant Sci 2023;14:1194753.
[20] Liu CW, Bodaghi S, Vidalakis G, Tsutsui H. Quick plant sample preparation methods using a micro-homogenizer for the detection of multiple citrus pathogens. Chemosensors 2024;12(6):105.
[21] Liu CW, Tsutsui H. Sample–to-answer sensing technologies for nucleic acid preparation and detection in the field. SLAS Technol 2023;28(5):302–23. https://doi.org/10.1016/j.slast.2023.06.002.
[22] Zou Y, Mason MG, Wang Y, Wee E, Turni C, Blackall PJ, Trau M, Botella JR. Nucleic acid purification from plants, animals and microbes in under 30 s. PLoS Biol 2017;15(11):e2003916.
[23] Mathews DM, Bodaghi S, Heick JA, Dodds JA. Detection of avocado sunblotch and other viroids using RNA filter paper capture and RT-PCR. Viroids: Methods Protoc 2022:219–33.
[24] Li P, Li M, Yue D, Chen H. Solid-phase extraction methods for nucleic acid separation. A review. J Sep Sci 2022;45(1):172–84.
[25] Moehling TJ, Choi G, Dugan LC, Salit M, Meagher RJ. LAMP diagnostics at the point-of-care: emerging trends and perspectives for the developer community. Expert Rev Mol Diagn 2021;21(1):43–61.
[26] Notomi T, Mori Y, Tomita N, Kanda H. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. J microbiol 2015;53(1):1–5.
[27] Osman F, Hodzic E, Kwon SJ, Wang J, Vidalakis G. Development and validation of a multiplex reverse transcription quantitative PCR (RT-qPCR) assay for the rapid detection of Citrus tristeza virus, Citrus psorosis virus, and Citrus leaf blotch virus. J Virol Methods 2015;220:64–75. https://doi.org/10.1016/j.jviromet.2015.04.013.
[28] New England Biolabs. WarmStart colorimetric LAMP 2X master mix typical LAMP protocol (M1800), https://www.neb.com/en-us/protocols/2016/08/15/warmstart-colorimetric-lamp-2x-master-mix-typical-lamp-protocol-m1800. August 2, 2024.
[29] Song X, Coulter FJ, Yang M, Smith JL, Tafesse FG, Messer WB, Reif JH. A lyophilized colorimetric RT-LAMP test kit for rapid, low-cost, at-home molecular testing of SARS-CoV-2 and other pathogens. Sci Rep 2022;12(1):7043. https://doi.org/10.1038/s41598-022-11144-5.
[30] Selvaraj V, Maheshwari Y, Hajeri S, Yokomi R. A rapid detection tool for VT isolates of Citrus tristeza virus by immunocapture-reverse transcriptase loop-mediated isothermal amplification assay. PLoS One 2019;14(9):e0222170.
[31] Shao Y, Zhu S, Jin C, Chen F. Development of multiplex loop-mediated isothermal amplification-RFLP (mLAMP-RFLP) to detect Salmonella spp. and Shigella spp. in milk. Int J Food Microbiol 2011;148(2):75–9.
[32] Wang DG, Brewster JD, Paul M, Tomasula PM. Two methods for increased specificity and sensitivity in loop-mediated isothermal amplification. Molecules 2015;20(4):6048–59.
[33] Kim SH, Lee SY, Kim U, Oh SW. Diverse methods of reducing and confirming false-positive results of loop-mediated isothermal amplification assays: a review. Anal Chim Acta 2023:341693.
[34] Jain NK, Roy I. Trehalose and protein stability. Curr Protoc Protein Sci 2010;59(1). https://doi.org/10.1002/0471140864.ps0409s59. 4.9.1-4.9.12.
[35] Zhang Y, Ren G, Buss J, Barry AJ, Patton GC, Tanner NA. Enhancing colorimetric loop-mediated isothermal amplification speed and sensitivity with guanidine chloride. BioTechniques 2020;69(3):178–85. https://doi.org/10.2144/btn-2020-0078.