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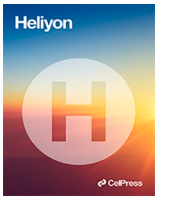

Research article

Quantitative detection of cassava common mosaic virus for health certification of cassava (*Manihot esculenta* Crantz) germplasm using qPCR analysis

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A B S T R A C T

Cassava (*Manihot esculenta* Crantz) is a crop of global economic and food safety importance, used for human consumption and in various industrial applications. The genebank of the Genetic Resources Program of the Alliance of Bioversity International and CIAT currently holds the world's largest cassava collection, with 5965 *in vitro* accessions from 28 countries. Managing this extensive collection involves indexing quarantine pathogens as a phytosanitary certification requirement for safely distributing cassava germplasm. The study therefore aimed to optimize a quantitative diagnostic protocol to detect cassava common mosaic virus (CsCMV) using quantitative PCR (qPCR) as a better alternative to other molecular techniques. This was done through designing primers and a probe in the RdRP region of CsCMV, and optimizing the qPCR conditions of the diagnostic protocol using primer concentration assays, and reaction amplification conditions such as volume and reaction time. We also evaluated the qPCR protocol by comparing the results of 140 cassava accession evaluations using three diagnostic methodologies (DAS-ELISA, end-point PCR, and qPCR) for CsCMV. Our protocol established that qPCR technique analysis is ten-times more sensitive in detecting CsCMV compared to end-point PCR, showing a maximum detection level of 77.97 copies/μL of plasmid, with 76 min of reaction time. The comparison allowed us to verify the level of CsCMV detection through the techniques evaluated, concluding that qPCR was more sensitive and allowed the quantification of viral concentration. The optimized qPCR protocol will be used to accelerate diagnostic screening of cassava germplasm for the presence or absence of CsCMV to ensure safe movement and distribution of disease-free germplasm.

1. Introduction

Cassava (*Manihot esculenta* Crantz) is considered the sixth-most important crop globally after wheat, rice, corn, potato, and barley, respectively. It is a basic primary staple for more than a billion people worldwide [1] Currently, global cassava production exceeds 300 million tons, with an annual yield of 10.61 ton/ha [2]. It is therefore a crop of economic importance, classified as the main crop for food security, human consumption purposes, and industrial applications as starch such as thickener, binder, and stabilizer that are

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widely used in the food industry [1]. Furthermore, cassava roots, stems, and leaves are used for animal feed [3].

Germplasm banks perform various functions for conserving and providing genetic resources in research, breeding, and improvement of seed supply, contributing to food security and nutrition [4]. The Genetic Resources Program (GRP) of the Alliance of Bioversity International and CIAT holds and distributes the world's largest cassava collection, made up of 5965 *in vitro* accessions from 28 countries. The main functions established by the genebank are conservation, distribution, duplication, and indexing of cassava germplasm [5]. Indexing is carried out by the GRP Germplasm Health Unit (GHU) using standardized and validated methodologies such as end-point PCR, qPCR, and LAMP [6]. Indexing cassava germplasm is an essential requirement for its safe distribution and must be fulfilled in accordance with international phytosanitary regulations. Samples must be accompanied by phytosanitary certification issued by the national institute authorized to issue sanitary certificates [4], which in Colombia is the Colombian Agricultural Institute (ICA).

Phytosanitary certification of the Alliance's cassava *in vitro* collection is currently carried out for three quarantine diseases, which are (i) Cassava frogskin disease [7], reported in association with four viral forms: Cassava frogskin-associated virus (CsFSaV), Cassava new alphaflexivirus (CsNAV), Cassava polero-like virus (CsPLV), Cassava torrado-like virus (CsTLV), and a phytoplasma of the 16SrIII-L group [8,9]; (ii) asymptomatic disease X of cassava caused by virus X (CsVX); and (iii) common mosaic disease caused by cassava common mosaic virus (CsCMV) [10,11].

CsCMV belongs to the genus *Potexvirus* in the Alphaflexiviridae family. The virus was first reported in southern Brazil [12,13] and in other Latin American countries such as Mexico [14], Colombia and Paraguay [15], Venezuela [16,17], Argentina [18–20], and Peru [21]. CsCMV has also been reported in Africa and Asia [22–24]. CsCMV was first detected using serology method with the Standard Double-Antibody-Sandwich Assay (DAS-ELISA) technique [11,25]. Currently, CsCMV is commonly detected using end-point PCR [26]. However, the technique involves additional steps in the reaction process, such as the use of gels, which is more time consuming and shows different levels of sensitivity in the diagnostic processes [27]. Thus, updating diagnostic methodologies has an important role to play in germplasm phytosanitary certification. This has allowed optimizing and improving diagnostic processes where conventional techniques such as end-point PCR are used.

Molecular techniques are among the most promising diagnostic methodologies, offering a safe, rapid, and reliable pathology diagnosis; specifically qPCR, which allows real-time detection of any fragment of interest using specific probes labeled with fluorophores [28]. The benefits of qPCR diagnosis are the real-time reading of the amplified product, shortening the detection process; quantifying the virus' concentration; and using a specific probe that enhances diagnostic sensitivity and reliability [29].

In recent years, molecular diagnostics for CsCMV using end-point PCR with different sets of primers has been reported specifically in Brazil, China, and Colombia [24,26,30]. However, there are no reports for CsCMV diagnosis using qPCR.

For this reason, a robust qPCR protocol requires a good primer and probe design, both previously verified, which contributes to the robustness of the diagnostic technique [31], as well as establishing the amplification profile, standard curve, and verification of percentages of sensitivity and specificity compared to other methodologies. Therefore, the objective of this research was to perform the quantitative detection of the CsCMV virus by optimizing a protocol based on qPCR for the phytosanitary certification of cassava germplasm, contributing to the continuous improvement of diagnostic processes for safely moving germplasm.

2. Materials and methods

2.1. Primer and probe design

Primers and a probe for detection of cassava common mosaic virus (CsCMV) were designed in the coding region for the RNA-dependent RNA Polymerase protein (Replicase) described by Lozano et al. [26] as a conserved region used to classify species of viruses of the genus *Potexvirus*. For designing the primers and the probe, the sequence KT002437.1 belonging to the replicase region for CsCMV was used, from nucleotide 1 to 609 of the sequence.

For this design we used the PrimerQuest software Tool from Integrated DNA Technologies (IDT) [32]. The process followed quality parameters such as GC content, salt concentration, product size, melting temperature (Tm), size of the primers and probe, ΔG of hairpins, self-completion, and crossover dimers [28].

Verification of the primers' and probe specificity was performed using the primer BLAST tool available in the National Center for Biotechnology Information (NCBI) database: https://www.ncbi.nlm.nih.gov/tools/primer-blast/. The primers and probe designed were evaluated with the database of the genus *Potexvirus* and *Manihot esculenta*, to verify the non-binding to specific sequences such as cassava and other viruses belonging to the described genus [33].

Additionally, each ΔG sequence was evaluated using the PREMIER Biosoft Beacon designer free edition program, available at: http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1. Once the best result was selected, the primers and probe sequences were synthesized in IDT [32], using a 6-FAM reporter fluorophore at the 5′ end and two Quenchers for the probe — one called ZEN (internal) and another one at the 3′ end called Iowa Black FQ.

2.2. Reaction concentrations and amplification profile for qPCR

Through four standardization trials, the diagnostic protocol for CsCMV was established. The first was performed by evaluating a gradient with four hybridization temperatures: 50, 54, 57, and 60 °C. The reaction was performed using 1X GoTaq® Green Master Mix (Promega©), 0.2 μM of previously-designed qPCR primers, 0.5 ng/μL of plasmid and water at a final volume of 25 μL. The amplification profile used was denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, hybridization for 45 s, extension at

72 °C for 30 s, and a final extension at 72 °C for 10 min in an end-point PCR thermocycler. The results were visualized in a 1 % agarose gel stained with GelRed®. The second assay was conducted by optimizing the primer and probe concentrations using a concentration matrix: 100, 200, and 300 nM of the primers, and 150, 200, and 250 nM of the probe. The reaction was completed with 1X QuantiNova Probe Master Mix (QIAGEN©) and water to a final volume of 25 μL [31,34,35].

Nine concentration combinations between the primers and probe were performed with the following reaction profile: Denaturation at 95 °C for 2 min, 40 denaturation cycles at 95 °C for 15 s, annealing and extension at 50 °C for 60 s with reading of signal in this last step using the Green channel (FAM) in the QIAGEN© Rotor-Gene Q equipment. In the nine combinations, 0.5 ng of positive control was used, 0.5 ng of negative control corresponding to a healthy plant, and a reaction blank with water.

The third trial consisted of optimizing the reaction volume from 25 to 20 μL, using the reaction, amplification, and control conditions described in trial 2, with the defined concentration of the primers and probe. The fourth assay was performed for qPCR reaction time improvement. Assay reaction and amplification were carried out using the conditions described in assay 2, changing the reading time from 1 min to 30 s in the annealing cycle, using 20 μL of the final volume in the reaction, a positive control (0.5 ng), negative (0.5 ng), and no-template control.

All the results in the qPCR assays were visualized using the QIAGEN© Rotor-Gene Q series software. They were analyzed according to the controls used in each of the assays; positive control previously verified with PCR, negative control, and blank reaction, each of the reactions was performed in duplicate. The established protocol for CsCMV was used for sensitivity and specificity assays.

2.3. Limit of detection and lack/presence of non-specific amplification of end-point PCR and qPCR

The qPCR limit of detection (LOD) was evaluated using a standard curve of 10 serial dilutions of a positive control plasmid of previously known concentration (4.2 ng/μL). The plasmid used for this assay belonged to the pGEM®-T Easy Vector Systems kit with an insert of 650 bp corresponding to the RdRP region of the CsCMV virus. Each dilution was carried out with two replicates, two negative controls, and two no-template controls. These dilutions were also compared with end-point PCR to determine the level of LOD between both techniques [36]. The number of copies of the serial dilutions was calculated with an equation described by Shirima et al. [37]. The serial dilutions were obtained from a concentrated plasmid with 1.05×10^9 copies/μL. The calculated copies were added to the Rotor-Gene Q series software before starting the run.

Lack/presence of non-specific amplification of the qPCR protocol was evaluated using plasmids and DNA from different viruses such as CsFSaV, CsNAV, CsPLV, CsTLV, Cassava virus X (CsVX), bacterial DNA (*Xanthomonas* sp. (Xt), *Pseudomonas savastanoi* pv. *phaseolicola* (Ps), and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cb8)) and fungal DNA (*Phoma* sp. (P3), *Trichoderma* sp. (T1), and *Alternaria* sp. (A2)), with positive (C+), negative (C-), and no-template controls (B), with two replicates by reaction [35]. This assay was also carried out in end-point PCR using two reactions for each plasmid and DNA evaluated with the respective reaction controls mentioned above.

2.4. Plant material and RNA extraction

All samples used in this research were cassava plants established under greenhouse conditions at the *in vitro* conservation laboratory of the Alliance of Bioversity International and CIAT's Genetic Resources Program (GRP). The materials used were the young leaf tissue of 70 accessions, according to 2020 historical results for this virus; 35 positive accessions and 35 negative accessions with end-point PCR performed by the GHU. Furthermore, 35 additional accessions were randomly selected from the Argentinian (Arg) collection of the same greenhouse with unknown results. The collection of plant material was carried out using the methodology described by Cuervo et al. [6]. This protocol describes the method for collecting young leaf tissue from the establishment plant, using a previously-disinfected scalpel. The samples were covered and labeled in aluminum sheets and stored in a Styrofoam cooler during transportation. From each sample, 200 mg of tissue was selected and organized in 2.0 mL Eppendorf tubes. The tubes were stored at −80 °C until processing.

The RNA extraction process was carried out following the CTAB methodology (N-Cetyl-N, N, N-Trimethyl-Ammonium bromide, Merck) described by Lopez et al. [38], with modifications in the buffer solution, solvents, and reagents used for the RNA extraction. For preparing the buffer solution, the following stock solutions were used: 2 M NaCl (4 M), 25 mM EDTA (0.5 M), 100 mM TRIS HCl (1 M), 2 % W/V CTAB and 2 % W/V for PVP. Additionally, at the beginning of the extraction process, 0.2 % β-Mercaptoethanol was added to the extraction buffer.

RNA extraction was performed using 200 mg of young leaf tissue previously macerated with liquid nitrogen, which was mixed with the extraction buffer mentioned above. The steps for the isolation of nucleic acids involved the following order of reagents: 1) Chloroform (1:1), 2) Isopropanol (1:1), and 3) 70 % Ethanol. Finally, to obtain the total RNA, a treatment with type I DNAse from Promega's RQ1 RNase-Free DNase kit was used for DNA degradation. Once the enzymatic treatment was completed, the total RNA was precipitated overnight with lithium chloride at 2 M. The next day, a second wash was performed with 70% ethanol, the samples were centrifuged, and the ethanol was discarded and left to dry in an extractor chamber for 1 h. The total RNA was resuspended in 50 μL of nuclease-free water. The quality and quantity of total RNA was measured in a spectrophotometer before the cDNA synthesis step [6].

2.5. Comparison of diagnostic techniques: DAS-ELISA, end-point PCR and qPCR

Evaluation of CsCMV was carried out from a new collection of plant material to establish the same conditions of physiological state and sampling time of the accessions to be evaluated in the comparative diagnostic. We evaluated 105 accessions collected from the

cassava bonsai greenhouse, using two diagnostic techniques currently available to detect CsCMV virus: DAS-ELISA test and end-point PCR [6], plus an evaluation with qPCR using the optimization of the protocol carried out in this research work, in order to determine the protocol's sensitivity, according to the number of positive and negative results for each diagnostic technique.

2.5.1. CsCMV diagnosis using DAS-ELISA

Evaluation of CsCMV with the DAS-ELISA technique was carried out using a sandwich-type serological kit. The diagnosis was made using 200 mg of fresh tissue from the 105 accessions to be evaluated. The capture and conjugate antibody concentrations used in this work were 1/200 for both, in accordance with the manufacturer's instructions.

All the accessions were evaluated in duplicate, and the protocol was evaluated by adding 1X of PNP substrate, performing a first reading at 30 min and a second reading at 60 min with a 405 nm wavelength in a Biotek Epoch 2 spectrophotometer. Positive results are those that present absorbance of more than twice the value of the negative control.

2.5.2. CsCMV diagnosis using end-point PCR

Firstly, cDNA synthesis was performed according to the methodology described in Cuervo et al. [6], using the Invitrogen synthesis kit (M-MLV Reverse Transcriptase - 200 U/μL). Subsequently, the cDNA was verified with an internal control PCR using the primers described by United States Department of Agriculture (USDA) (Nad5.656.f and Nad5.835.r) that amplify for a region corresponding to the endogenous gene of the Nad5 plant, with the following reaction conditions: 1X GoTaq Green Master Mix (2X) and a primer solution at 0.2 μM each, following the amplification profile described by Cuervo et al. [6].

All PCR products were visualized in 1 % agarose gels stained with GelRed® (Biotium); in this way the confirmed cDNA presented a PCR product of 200 base pairs [39]. The samples were evaluated for the CsCMV virus using the CsCMV_3269F/CsCMV_3896R primers described by Lozano et al. [26] for CsCMV, with the following reaction conditions: 1X GoTaq Green Master Mix (2X), 0.2 μM forward primers and reverse, and 2 ng of cDNA previously verified with internal control PCR. The amplification profile reported by Cuervo et al. [6] was used for the amplification of the reaction.

2.5.3. CsCMV diagnosis using qPCR

Accessions were evaluated with qPCR using the new primers and probe described in section 2.1. Five positive samples were selected and visualized in 1 % agarose gels stained with GelRed® (Biotium) in a transilluminator. The bands were cut and purified according to the protocol described by QIAGEN [40] using the QIAquick Gel Extraction purification kit. The purified samples were sent to Korea for the Sanger sequencing service at Macrogen. The received sequences were edited in Bioedit [41] and later analyzed with the NCBI https://blast.ncbi.nlm.nih.gov/Blast.cgi nucleotide BLAST tool [42] to confirm and verify the qPCR amplified product.

2.6. Analysis of the results

Analysis of the concentration matrix, volume, and time assays was performed with the normalized fluorescence emitted by the QIAGEN© Rotor-Gene Q series software at 40 cycles, using ANOVA and Tukey's means test. The results of the comparison of methodologies (ELISA, PCR, and qPCR) were analyzed using a Chi-square test (X2). The variables used for the comparison were the total number of positive and negative accessions obtained in the three diagnostic techniques of the virus (CsCMV).

All analyzes were performed using the statistical software SAS® Studio (https://welcome.oda.sas.com/login) with a significance value of 0.05 %. The reactions were visualized and interpreted according to the Ct obtained from the positive controls in each of the assays. The bar graphs for the comparison of methodologies were generated using the statistical software R (https://www.r-project.org/).

3. Results

3.1. Design of primers, probes, and in-silico validation

Primer and probe design performed to detect the CsCMV virus using IDT's PrimerQuest Tool software showed five primer set options for amplification of the RNA-dependent RNA polymerase (RdRP) domain region of the RNA polymerase gene. The primers and probe used in option one — CsCMV-q5F (5′–CAAAGCTAGGCTCGTGATAAG– 3′), CsCMV-q5R (5′–GGATGGGATCTTCTGGTAAATG–3′), and CsCMV-q5P (5′–CCGTCCAGTTGTGTTCCTTAT– 3′) — were those that showed the best results according to the *in-silico* analysis performed (Table S1). These analyses showed primers and the probe between 21 and 22 bases in length, with a Tm of 54 °C for the primer Forward (F) and Reverse (R), and 56 °C for the probe, with a final product of 125 bp.

Other parameters such as the percentage of GC values were between 45 and 47 % and showed some secondary structures within the acceptable ranges (less than 15) with ΔG values maintained between 0 and -3.5 in structures such as crossed dimers, self-complementarity, and hairpins.

Additionally, the selected primers and probe presented favorable results in terms of the specificity evaluation carried out *in silico* in Primer Blast, with the *Manihot esculenta* database and the *Potexvirus* genus, where they did not present pairing with cassava genome sequences and virus sequences belonging to this genus, thus demonstrating the specificity required to perform the diagnosis of CsCMV by qPCR.

3.2. Protocol optimization using qPCR for detecting CsCMV

In the first assay of qPCR primer optimization for detecting CsCMV, the annealing temperature of 50 °C showed strong amplification of the positive control. Dissimilarly, the other temperatures of 54, 57, and 60 °C showed amplification, but in a reduced band intensity from 54 °C upwards. Also, the results showed that the negative controls and the no-template controls did not present non-specific results and dimer formation (Fig. S1).

On the other hand, the second assay ANOVA for the optimization of primer and probe concentrations showed that the different combinations evaluated presented significant differences in at least one of them. Furthermore, the Tukey means test showed that the best combinations were C7, C8, C9, and C4, presenting the highest value in their respective means, and that the C7 and C8 combinations were statistically equal, but different from C9 and C4 (Table S2).

Finally, C4 and C9 combinations were the same among themselves, but different from the other combinations (C5, C6, C1, C2, and C3). These results suggest that the best combination is C4 because the combinations C7, C8, and C9 require a higher concentration of primers and the probe. Furthermore, C4 presents the second-best average value in normalized fluorescence, therefore selecting these conditions will allow the detection of CsCMV by qPCR allowing the correct optimization of reagents (Table S2).

Assays three and four, for optimizing the volume and reaction time, respectively, showed that the changes made did not present significant differences in the amplification of the positive control of each trial (normalized fluorescence) (Table S3). Therefore, the reduction of the final volume to 20 μL and the 20-min reduction in the reaction profile allowed optimization of the qPCR protocol for CsCMV diagnosis.

According to the assays carried out, the reaction mixture preparation by qPCR defined for the diagnosis of CsCMV was: 1X QuantiNova Probe Master Mix (QIAGEN©), 0.2 μM of primers, 0.15 μM of the probe, 0.5 ng of sample and water to final volume 20 μL. The amplification conditions established in the equipment were: denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 50 °C for 30 s using the Green channel in this last step (FAM) of the QIAGEN© Rotor-Gene Q for reading the fluorescent signal during amplification, obtaining a final reaction time of 76 min.

3.3. LOD of the qPCR technique and end-point PCR for detecting CsCMV

Serial dilutions evaluated with the qPCR technique for CsCMV showed amplification of the positive control up to the 10^{-7} dilution of ten serial dilutions (Fig. S2), with a threshold of 0.0248. The average Ct of the 10^{-7} dilution was 37.34 and is the last value detected by the technique, indicating that the maximum reading for the diagnosis of CsCMV will be made up of this Ct value (Table 1). Additionally, the negative controls and the no-template controls did not show non-specific amplifications. Also, the calculation of the average number of copies (Rep. Cal. Conc.) quantified by the qPCR technique showed the first reading in the 10^{-1} dilution with 70,327,206.46 copies/μL up to the 10^{-7} dilution with 77.97 copies/μL of the CsCMV virus. In the other dilutions (10^{-8} to 10^{-10}) no quantification of the virus was found (Table 1).

Quality parameters of the standard curve for the diagnosis of CsCMV showed an R^2 value of 0.9973, a slope value (M) of −3.088,

Table 1
Results of absolute quantification of the standard curve for CsCMV.

N.	Name	Ct	Given Conc (copies/μL)	Calc Conc (copies/μL)	Rep. Ct	Rep. Ct Std. Dev.	Rep. Cal. Conc (copies/μL).
1	10^{-1}	18.90	104,647,236.98	72,966,077.26	18.95	0.07	70,327,206.46
2	10^{-1}	19.00	104,647,236.98	67,783,772.33			
3	10^{-2}	21.30	10,464,723.70	12,146,218.20	21.29	0.03	12,309,016.54
4	10^{-2}	21.27	10,464,723.70	12,473,996.91			
5	10^{-3}	24.42	1,046,472.37	1,189,162.11	24.27	0.22	1,334,017.07
6	10^{-3}	24.11	1,046,472.37	1,496,517.18			
7	10^{-4}	27.74	104,647.24	100,250.56	27.59	0.20	111,691.37
8	10^{-4}	27.45	104,647.24	124,437.83			
9	10^{-5}	30.57	10,464.72	12,107.49	30.52	0.07	12,592.49
10	10^{-5}	30.47	10,464.72	13,096.93			
11	10^{-6}	33.83	1046.47	1064.66	33.81	0.04	1085.17
12	10^{-6}	33.78	1046.47	1106.08			
13	10^{-7}	37.26	104.647237	82.606304	37.34	0.11	77.97523
14	10^{-7}	37.41	104.647237	73.603782			
15	10^{-8}	–	10.464724	–	–	–	–
16	10^{-8}	–	10.464724	–			
17	10^{-9}	–	1.046472	–	–	–	–
18	10^{-9}	–	1.046472	–			
19	10^{-10}	–	0.104647	–	–	–	–
20	10^{-10}	–	0.104647	–			
21	Neg Cont.	–	–	–	–	–	–
22	Neg Cont.	–	–	–			
23	NTC	–	–	–	–	–	–
24	NTC	–	–	–			

Ct: Threshold cycle; **Conc:** Concentration; **Calc Conc:** Calculated concentration; **Rep:** Repetition; **Std. Dev.:** Standard deviation; **Neg Cont.:** Negative control; **NTC:** No-template control.

and an efficiency value of 1.11. These values are within the parameters suggested by the literature [43]. Thus, analysis of the sample evaluation results using this curve would show a reliable result in CsCMV copy number quantification.

On the other hand, when evaluating the previous standard curve by end-point PCR, the technique only detected the first six dilutions (from 10^{-1} to 10^{-6}), showing very clear bands in the last dilution (10^{-6}), indicating the LOD of the end-point PCR (Fig. S3). The results shown in Figs. S2 and S3, and Table 1, suggest that the qPCR technique LOD is 10 times more sensitive than the end-point PCR. This is because qPCR presented an additional dilution reading to those detected by the end-point PCR, indicating the technique's greater capacity to detect and quantify the CsCMV viral concentration, and therefore greater reliability in the results obtained.

3.4. Lack/presence of non-specific amplification of the qPCR technique and end-point PCR for detecting CsCMV

The qPCR assay results for CsCMV indicated that the nucleic acids evaluated were not detected with the primers and the CsCMV probe, except for the amplification of the positive control (Fig. S4). This result indicated that the primers and the probe designed in this work for the diagnosis of CsCMV by qPCR are specific for the diagnosis.

Additionally, the primers test performed by end-point PCR described by Lozano et al. [26] for CsCMV, showed only the amplification of the positive control, confirming that the primers are specific for virus amplification (Fig. S5) and non-specific amplifications were not observed in the negative controls, no-template controls, and DNAs of other pathogens. Thus, both methodologies proved to be specific for the detection of CsCMV.

3.5. Comparison of methodologies, DAS-ELISA, end-point PCR, and qPCR in the diagnosis of CsCMV

Chi-square analysis carried out in the comparison of the three methodologies for the diagnosis of CsCMV showed that there are significant differences in at least one of the diagnostic methods used ($p < 0.05$) (Table S4). In this way, the null hypothesis is rejected, indicating that there is no difference in the results according to the diagnostic method used. These results were verified through a comparative analysis between the evaluated techniques, showing that, in the case of the DAS-ELISA methodology, all results (100%) were negative. Additionally, in the case of the end-point PCR technique, 9 (8.57%) samples were positive and 69 (65.71%) were positive for qPCR (Fig. 1), demonstrating that the lowest detection rate was obtained through DAS-ELISA and the highest through qPCR.

All the positive results with the end-point PCR coincided with the positive results of the qPCR, and the five positive samples with qPCR selected were confirmed with Sanger sequencing showing identity percentages greater than 94% for this virus, indicating the reliability of the qPCR-amplified product.

On the other hand, the comparison of techniques between end-point PCR and qPCR diagnosis, according to the groups of samples selected and evaluated at different times, showed that in the case of the CsCMV group, of the 35 accessions positive by end-point PCR in 2020, 27 (77%) accessions tested positive for this virus using qPCR (Fig. 2). In addition, the 35 accessions of this group were evaluated again by end-point PCR, finding only two positive samples (5.7%) for CsCMV.

Additionally, in the group of negative accessions called Neg, we found that, of the 35 accessions that tested negative for CsCMV by end-point PCR in 2020, 17 (48.5%) accessions tested positive using qPCR for CsCMV (Fig. 2). Also, the new diagnosis made with end-point PCR for this group of samples did not show positive results for this virus.

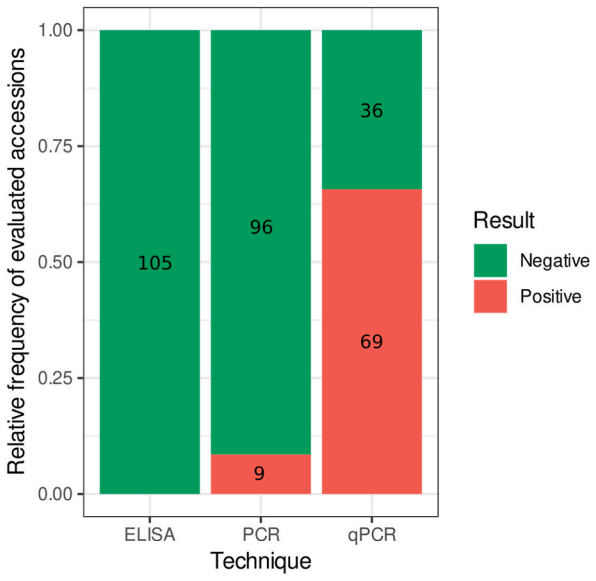

Fig. 1. Diagnostic results of CsCMV with DAS-ELISA, PCR, and qPCR.

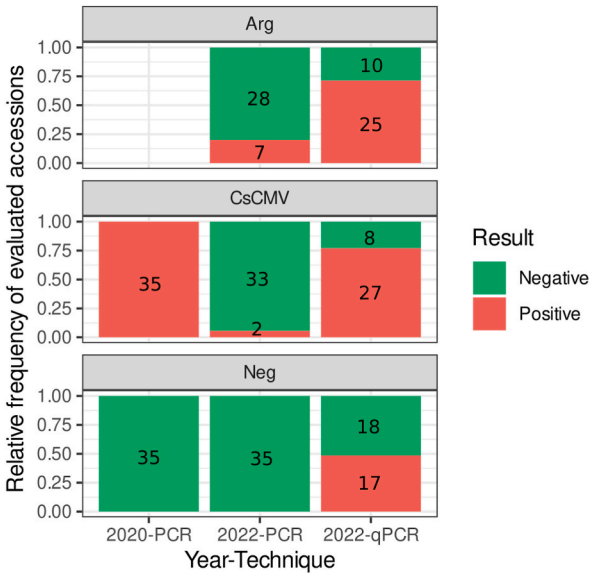

Fig. 2. Comparative diagnostic analysis between PCR and qPCR in different years, with three different groups of samples. **Arg:** 35 accessions from the Argentine collection (Arg) without a previous diagnosis in the year 2020; **CsCMV:** 35 positive accessions by end-point PCR for CsCMV (2020); **Neg:** 35 negative accessions by end-point PCR for CsCMV.

Finally, the group of samples from Argentina (Arg) – 35 accessions without prior evaluation with PCR in 2020 – found 25 (71.4%) positive accessions with qPCR for CsCMV (Fig. 2). These results seem to indicate that the plants belonging to this group of accessions could present a degree of susceptibility to CsCMV and its detection was possible with qPCR because in the evaluation with end-point PCR (2022) only seven (20%) accessions were detected as positive (Fig. 2).

All the techniques compared in this diagnosis presented different execution times and different operational costs (Table 2). The cost and time described are based on the evaluation of 140 cassava samples. This comparison showed that the qPCR technique has a running time of 2 h, with an approximate cost per reaction of US$2.23. In contrast, ELISA and PCR techniques took two days and 6 h of execution with an approximate cost of US$2.5 and US$1, respectively. Although the qPCR technique has some limitations, it showed greater advantages compared to the ELISA and PCR (Table 2), allowing a complete and safe diagnosis in phytosanitary certification in the evaluated germplasm. Additionally, the operational cost of qPCR can be lower in duplex reactions for the simultaneous evaluation of different pathogens, reaching an approximate cost of US$1.16 per reaction.

4. Discussion

DAS-ELISA technique had been successfully used at the Germplasm Health Unit of the Alliance of Bioversity International and CIAT

Table 2
Comparison of time, cost, advantages, and limitations of the three diagnostic techniques evaluated for CsCMV detection.

Technique	Execution time	Approximate cost (US$) per reaction	Advantages	Limitations
DAS-ELISA	2 days	2.5	• Shorter diagnosis time • Availability of controls in commercial kits	• Low sensitivity • Requires specialized infrastructure and equipment • Risk of false positive • Longer diagnosis time
End-point PCR	6 h	1	• Amplification of a specific region • Easy to execute	• Contamination risk • Longer diagnosis time • Qualitative results • Results depend on the sensitivity of the contrast dye
qPCR	2 h	2.23	• Specific • Sensitive • Allows quantitative analysis • Allows duplex or multiplex reactions that reduce the cost • Easy to analyze results	• Requires specialized equipment • Requires staff training • High cost in single pathogen detection

*This time does not include the sample processing (solutions preparation, sample grinding, RNA extraction, and cDNA synthesis).

for the phytosanitary certification of the cassava collection for CsCMV for many years. However, this technique relied on the antisera produced by the CIAT Virology Laboratory in the 1980s, which was running out. Thus, more recently, the DAS-ELISA protocol was replaced with an alternative available molecular technique, end-point PCR, for this germplasm certification.

At the same time, the implementation of quantitative methodologies such as qPCR for cassava germplasm certification has raised interest for its applicability in the quantification of viral concentration. However, to obtain a reliable protocol that would speed up the evaluation process, the qPCR protocol needed to be refined, through the optimization of primers, reaction conditions, amplification, and by verifying if the protocol is sensitive and specific.

This work designed a set of primers and a probe for CsCMV detection using qPCR analysis following the parameters indicated for the design of the qPCR protocol, such as the primer and probe length, which indicates a direct relationship with the specific amplification, stability, and cost [28]. Additionally, the correct selection of the Tm (less than 60 °C) avoids the unspecific binding of the primers and the formation of secondary structures, which should not be greater than 5 °C between the designed primers [44].

Furthermore, in the formation of dimers and secondary structures, the primers must not present sequences with internal homology and at the 3′ end, because they could lead to the formation of hairpins and self-complementarity structures, generating interferences with the alignment of the DNA and with the formation of the final reaction product [45]. Furthermore, acceptable ΔG values for primers and probe should range from 0 to −3.5 to avoid any non-specific structure formation, such as dimers [44].

Advancement in molecular methods and the widening range of techniques have contributed to the GRP Germplasm Health Unit's advancement of the protocol established for CsCMV diagnosis by qPCR. This allows updating, refining, and using more efficient techniques such as qPCR when detecting any virus of interest [46]. The safe movement and distribution of germplasm are strictly linked to the requirement of diagnostic tests to determine the germplasm's phytosanitary status, because one of the risks involved in the transfer of plant material is the possible movement of pests and diseases to unreported sites [47]. For this reason, methodologies must contribute to a safe and reliable diagnosis for safely acquiring and distributing germplasm.

Based on some reports, the qPCR technique offers greater diagnostic sensitivity compared to other techniques [48]. This coincides with our diagnostic protocol, which found qPCR to be 10 times more sensitive than end-point PCR. In addition, qPCR offers the advantage of quantifying the emitted fluorescence proportional to the concentration of the template sequence of interest [49]. In this way, qPCR protocols are an option for the quantification of viruses in germplasm testing to guarantee the phytosanitary status of the material, and also contribute to the indexing of germplasm for both the GRP and for breeding programs [37,47]. An additional advantage of qPCR is that it can be used as a tool to verify the effectiveness of virus-cleaning protocols currently applied by the germplasm bank.

Fluorescence techniques such as qPCR work best with TaqMan type probes — also known as hydrolysis probes — that provide a specific and reliable reaction [48]. This was evidenced by the results of the test performed for the qPCR, where non-amplifications were not obtained with the DNA, plasmids, negative controls, and no-template controls evaluated. Likewise, end-point PCR has been reported as specific in protocols for detecting *Ralstonia solani*, *Fusarium oxysporum*, and *Alternaria alternata*, showing specificity levels of 100% [50]. This was confirmed with the test performed for the end-point PCR, demonstrating 100% specificity in CsCMV detection. Nonetheless, this technique did not show greater detection of positive samples than qPCR.

Some research papers report qPCR as a specific and sensitive method for virus detection, such as in the *Cassava brown streak virus* (CBSV) model of the genus *Ipomovirus* for the certification of cassava germplasm [37], and in the detection of viral models such as baculoviruses for the quantification of virus concentration in biological products at an industrial level [49].

Additionally, the comparison between the diagnostic techniques also confirmed that qPCR can detect lower viral concentrations, allowing a reliable safety diagnosis. In contrast, techniques such as DAS-ELISA incur similar costs (~USD 2.5 per sample), but are not as sensitive. They also present the risk of omitting virus-contaminated accessions, which could cause long-term quarantine problems. Additionally, qPCR i) offers a better cost/benefit ratio, since the investment made ensures the diagnosis; ii) avoids cross-contamination, (corroborated by Hendling et al. [28]); iii) allows viral quantification, and iv) contributes to rapidly obtaining results.

Comparing the evaluation of the three accession groups, according to the diagnosis conducted at different times, results for the CsCMV, Neg, and Arg group tested with qPCR and end-point PCR carried out in 2022 showed that (i) the highest detection rate for CsCMV is obtained with qPCR and that (ii) even though qPCR did not achieve total detection for the 35 accessions of the positive group for this virus (2020), qPCR continues to be the tested technique that provides the greatest detection of positive samples. However, this result may also be because the samples evaluated in 2022 presented physiological changes generated by the agronomic management practices carried out in the greenhouses during the time interval between the two evaluations (2020 and 2022), which may have generated a change in the viral concentration.

Management practices applied prior to testing in 2022 include fertilization and pruning, where fertilization for greenhouse accessions includes products with a high content of macroelements such as potassium (K), which — according to the literature — induces resistance to bacteria, fungi, and viruses due to the generation of high molecular weight compounds such as cellulose and lignin, creating barriers and making it difficult for pathogens to move [51].

Additionally, potassium deficiency generates susceptibility in the plant to the presence of viruses, generating foliar chlorosis due to the response mechanism generated by the activation of reactive oxygen species as a signal for the positive regulation of transcriptional genes such as *HAK5* involved in the uptake of potassium ions [52]. Thus, the application of this macroelement would reduce the effect generated by the virus in the movement from cell to cell, affecting the viral infection. These reasons could explain the change in viral concentration in the plants evaluated. Additionally, the tissue collected in 2022 differed from that initially evaluated in 2020 due to pruning that was conducted, which could also be an important factor in reducing the viral load.

5. Conclusions

This study has allowed designing new and effective primers and a probe for efficient and rapid detection of CsCMV generating the amplification of the region conserved RdRP. Also, the optimized amplification conditions allowed establishing a protocol for accurately diagnosing CsCMV, with optimal concentration values of primers and a probe for correct amplification. This achieving shortened diagnostic time and optimized reagents use.

Additionally, this qPCR protocol allowed us to achieve absolute viral quantification, favoring the LOD calculations for both the Ct and the maximum number of copies of the technique. On the other hand, the comparative analysis between end-point PCR and qPCR showed that the latter is 10-times more sensitive than the former for detection of CsCMV. Additionally, the study determined that both techniques and the DAS-ELISA were specific detecting CsCMV. Our results verify that qPCR provides greater detection of positive cassava germplasm samples, thus contributing to the sanitary certification of CsCMV in cassava germplasm for safe distribution processes.

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Ethics declarations

Review and/or approval by an ethics committee was not needed for this study because this work was applied to plant assays and their phytopathogens.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Diana-Patricia Niño-Jimenez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Karina López-López:** Writing – review & editing, Supervision, Methodology, Investigation. **Maritza Cuervo-Ibáñez:** Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors 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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Appendix A. Supplementary data

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