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"Rapid diagnosis of *Cucumber mosaic virus* in banana plants using a fluorescence-based real-time isothermal reverse transcription-recombinase polymerase amplification assay"

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ABSTRACT

Cucumber mosaic virus (CMV) is a widespread plant virus infecting important vegetables, plantation and flower crops. Currently, CMV is detected by enzyme-linked immunosorbent assays (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) assays. ELISA requires polyclonal antibodies and is time-consuming. PCR requires skilled manpower and complex procedures of RNA isolation as well as a thermal cycler. To overcome these difficulties, a portable rapid, simple and visual fluorescence-based reverse transcription-recombinase polymerase amplification (portable RT-exo-RPA) assay for the detection of CMV was developed. A specific primer pair of 30–33 bp targeting a conserved region of the coat protein (CP) gene of CMV and a probe to function in the RT-exo-RPA assays were designed and synthesized. A total of 62 symptomatic as well as 58 asymptomatic banana plant samples, collected from banana orchards located in Jalgaon, Maharashtra, India, were evaluated for CMV infections using crude leaf extracts as templates by a reverse transcription-recombinase polymerase amplification (RT-RPA) assay as well as a real-time RT-exo-RPA assay and the results were compared with those of a reverse transcription-polymerase chain reaction (RT-PCR) assay using purified total plant RNAs as templates. CMV was as efficiently detected using the crude leaf extract template in the RT-RPA and real-time RT-exo-RPA assays as using the purified RNA template in the RT-PCR assay. To dispense with the use of real-time PCR, a portable RT-exo-RPA assay was developed and the alternative methods for the visualization of CMV detection using either a fluorometer or direct viewing with a UV transilluminator were evaluated. To our knowledge, this is the first report of the rapid and reliable diagnosis of CMV infections by a real-time RT-exo-RPA assay using a crude leaf extract as template.

1. Introduction

Cucumber mosaic virus (CMV), a member of the genus *Cucumovirus*, family *Bromoviridae,* is an economically important viral pathogen of crop and non-crop plants. CMV has a worldwide distribution and an extensive host range, infecting more than 1000 species of plants, including vegetables, fruits and ornamentals. It is transmitted by sap mechanically and by over 80 aphid species in a stylet-borne non-persistent manner (Palukaitis et al., 1992; Palukaitis and Garcia-Arenal, 2003). CMV is a multicomponent virus with a tripartite (+) single-stranded RNA genome along with a fourth subgenomic RNA and is encapsidated in icosahedral particles 28–30 nm in size (Peden and Symons, 1973; Palukaitis et al., 1992). CMV induces systemic infection in host plants causing a variety of symptoms in different species of plants with the most common being mild to severe mosaic. Once infected, plants cannot outgrow CMV. They harbor the virus throughout their life. There is no effective resistance to CMV and control is thus based mainly on the use of virus-free propagative materials, rouging of infected plants and implementation of quarantine barriers. Development of accurate and rapid detection methods for CMV is thus critical for managing the disease effectively.

Various serological and molecular methods were developed for the routine detection of CMV, such as enzyme-linked immunosorbent assay (ELISA) (Devergne et al., 1981; Zein et al., 2006; Eni et al., 2010), dot-blot hybridization (Hu et al., 1995), reverse transcription-polymerase chain reaction (RT-PCR) (Khan et al., 2011; Shetti et al., 2012),

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immuno-capture reverse transcription polymerase chain reaction (IC-RT-PCR) (Sharman et al., 2000) and tissue-print combined with molecular detection (Chen, 2010). However, most of these techniques are time-consuming and cumbersome and require sophisticated laboratories as well as technical expertise. RT-PCR is a commonly used molecular technique for the detection of CMV. However, it requires the isolation of RNA that is free from inhibitors which, in itself, is technically challenging and time-consuming and this limits its use for the analysis of large numbers of samples. With further advancement in molecular biology, techniques such as one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) and lateral flow assay were developed for the rapid detection of CMV infection (Peng et al., 2012; Yoon et al., 2014). Compared to the conventional RT-PCR assay, the RT-LAMP assay is simple, can be performed under isothermal conditions and, thus, dispenses the use of an expensive thermal cycler and has a shorter run time; but it requires the use of purified RNA and 4–6 pairs of primers. The serological assays, including the lateral flow assay, require expensive polyclonal/monoclonal antibodies which are often difficult to produce.

The recombinase polymerase amplification (RPA) technology, first introduced in 2006 (Piepenburg et al., 2006), is a simple isothermal nucleic acid amplification technique employed for the rapid detection of viruses and other pathogens (Daher et al., 2016; Lobato and O'Sullivan-Trac, 2018) and is suited for use in laboratory settings with limited resources. In contrast to the existing amplification techniques that rely on complex procedures, RPA employs a single primer pair and can be performed at a constant low temperature (37 °C–40 °C). Exponential amplification in the RPA reaction is achieved by two key proteins, *viz.*, the recombinase and single-strand DNA binding protein (SSB) and the strand-displacing DNA polymerase. In the first step, the recombinase combines with the oligonucleotide primers, forming recombinase-primer filaments that recombine with the homologous ds DNA to facilitate strand displacement. Then, the SSB binds to the displaced strand of DNA, preventing the dissociation of the primer. The final core enzyme, the strand-displacing polymerase, then initiates synthesis by adding bases to the 3′ end of the primer (Piepenburg et al., 2006). To date, begomoviruses and the *Banana bunchy top virus* have been detected using the basic RPA format in which the RPA products are detected by agarose gel electrophoresis (Londoño et al., 2016; Kapoor et al., 2017). RPA has led to a significant advancement in the development of portable nucleic acid tests using the probe-based detection approach. Real-time detection can also be achieved by adding an exo-probe to the reaction mixture, and device-free detection is now possible with lateral flow (LF)-RPA reactions which use LF-probes that allow the visualization of the RPA products directly on LF strip. A number of important plant viruses were successfully detected using the LF-RPA (Zhang et al., 2014; Mekuria et al., 2014) and the exo-RPA reactions (Silva et al., 2015, 2018; Babu et al., 2017).

In order to dispense with the use of gel electrophoresis, a fluorescence-based, reverse transcription-recombinase polymerase amplification (RT-exo-RPA) assay was standardized in a real-time PCR assay for the rapid and specific diagnosis of CMV. The sensitivity of a real-time RT-exo-RPA assay in detecting the virus was also compared with those of a reverse transcription-recombinase polymerase amplification (RT-RPA) assay and a standard RT-PCR assay. To dispense with the use of a real-time PCR instrument to monitor the fluorescence-based reaction, an attempt was made to directly visualize the fluorescent signal produced by the RT-exo-RPA reaction using an UV transilluminator and a fluorometer, respectively. Further, the RPA assays were evaluated for the detection of CMV infections in banana samples collected from different banana orchards in a major banana growing region in Maharashtra, India.

2. Materials and methods

2.1. Plants and viruses

CMV-infected and healthy banana plants maintained in the green-house at the Advanced Centre for Plant Virology (ACPV), Division of Plant Pathology, Indian Agricultural Research Institute, Pusa (IARI), New-Delhi, were respectively used as the positive and negative controls. CMV infection in the positive control banana plants was confirmed by RT-PCR (see below) followed by sequencing of the full-length CMV coat protein gene from the RT-PCR amplicon before being used for the development of RT-exo-RPA assay. The specificity tests were performed using the banana plants infected with *Banana bract mosaic virus* (BBrMV), *Banana streak Mysore virus* (BSMYV) and *Banana bunchy top virus* (BBTV), maintained in the green-house at ACPV, IARI, New-Delhi.

2.2. RT-PCR assay

Total RNAs were isolated from infected (3–4 banana plants) and healthy leaf tissue (100 mg/plant) using the RNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The RNA from each sample was eluted in 35 µl RNase-free water and its quality and quantity were assessed using the NanoDrop™One Spectrophotometer (Thermo Scientific, Wilmington, USA). The RNA was stored at −80 °C until further use. The presence of CMV infection was confirmed by RT-PCR using the CMV-specific primers carried out in two steps. First, single-stranded cDNA was synthesized from approximately 500 ng of the purified RNA using the CMV-specific reverse (5'-GAGCTCTCAAACTGGGAG CAC-3') primer and the Improm-II Reverse Transcriptase (Promega, Madison, USA), followed by PCR amplification of the coat protein (CP) gene from the cDNA using the CMV-specific primers, CMV forward (5′-GGATCCATGGACAAATCTGAATCA-3′) and the above CMV reverse primer. The PCR mixture contained 0.25 µM of each forward and reverse primer, 1.25 mM of $MgCl_2$, 0.5 mM of dNTP mix, 1X reaction buffer, 4 µl of cDNA template and 1 unit of DyNAzyme II DNA polymerase (Thermo Fisher Scientific, MA, USA), in a total volume of 25 µl. The PCR conditions used were: one cycle of initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and one cycle of final extension at 72 °C for 10 min. Corresponding RT-PCR were also performed using crude leaf extracts from infected and healthy banana plants as templates (2 µl per reaction). The crude leaf extracts were prepared by grinding approximately 100 mg of fresh leaf samples per plant in 500 µl of General Extraction Buffer 3 (GEB3, Agdia, Elkhart, USA) containing 2% Tween-20, followed by centrifugation at 12,000 rpm for 2 min at room temperature. The resulting RT-PCR amplicons (10 µl) were electrophoresed in 1.8% agarose gel containing ethidium bromide and visualized under UV illumination. The expected amplicon was gel-purified and cloned into the pGEM-T Easy vector (Promega, Madison, USA) by following the standard molecular biology procedures (Sambrook and Russell, 2001). Two CMV CP-positive clones were sequenced in the forward direction using the T7 universal primer. The sequences obtained were then analyzed by BLAST in the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/blast).

2.3. RT-RPA and real-time RT-exo-RPA assays

The primers and probe sequences (Table 1) for the RT-RPA and the RT-exo-RPA assays were designed and synthesized from the conserved sequence of the CP gene of CMV (Kapoor et al., 2014) based on the criteria suggested in the TwistDx manual. Preliminary RT-RPA assays were carried out with the same RNA samples used in the RT-PCR assay to assess the performance of the primers using the TwistAmp Basic RT kit (TwistDX, Cambridge, UK). The reaction was carried out in a total reaction volume of 25 µl according to the manufacturer's instructions. The reaction mixture, excluding the template, was prepared as

Table 1
Details of the designed RT-exo-RPA assay primers and probe used for CMV RT-RPA and RT-exo-RPA assays.

Primer	Sequence (5'-3')	Target Region	Primer Position (nucleotide coordinate)[a]	Expected product size (bp)
CMV F_1	CTTTCGCGACTTAATAAGACGTTAGCAGCTGGT	Coat protein gene	115-147	249 bp
CMV R_1	CACAGTAGAATCAAATTTCGGCAAGGGATT		363-334	
CMV exo probe	AACCTTTGTGGGTAGTGAGCGTTGTAAACC[F]G[Z]A[Q] ACACGTTCACGTCTA-Spacer C3		168-217	

F = FAM-dT(thymidine nucleotide carrying fluorescein); Z = tetrahydrofuran residue; Q = BHQ1- (dT thymidine nucleotide carrying Blackhole Quencher-1).
[a] Nucleotide coordinate is given with reference to the CMV Karnataka isolate (GenBank Accession no. - AM055602).

recommended for a 50 µl total volume, mixed with the corresponding freeze-dried pellet of TwistAmp Basic RT kit (comprising of the recombinase, SSB, strand-displacing DNA polymerase and reverse transcriptase) and then distributed equally into duplicate reaction tubes, followed by the addition of the RNA template (˜500 ng) and 1.25 µl of 280 mM magnesium acetate. Reactions were incubated at 40 °C for 25 min using a dry bath incubator. A similar reaction was also performed using 2 µl of crude leaf extract as the template. Crude leaf extract was prepared as described above. To visualize the RT-RPA amplicons, the reaction products (10 µl) were incubated at 65 °C for 10 min prior to loading on 1.8% agarose gel. For further confirmation, the RT-RPA amplicons were gel-purified, cloned into pGEM-T Easy vector and sequenced. Two of the positive clones were sequenced and the obtained sequences were then analyzed as described above.

The real-time RT-exo-RPA assay was performed using the RT-exo-RPA assay primer (CMV F_1/R_1) and probe using the TwistAmp exo RT kit (TwistDX, Cambridge, UK). The reaction was prepared as described above using purified total RNA (˜500 ng) and crude leaf extracts (2 µl) as templates. The tubes were centrifuged and incubated in a real-time PCR instrument (iQ5, Bio-Rad, California, USA) at 40 °C with fluorescence measurements recorded in the FAM channel every 1 min for 25 min. Total RNA and crude leaf extract from healthy leaf tissue were used as negative controls in the assay.

2.4. Portable RT-exo-RPA assay

For the portable fluorescence-based RT-exo-RPA assay, two methods of fluorescence detection, using either a UV transilluminator or a fluorometer, were investigated. The reactions were set up as described for the real-time RT-exo-RPA and carried out in a dry bath incubator at 40 °C for 25 min instead of a real-time PCR instrument, and the fluorescence was recorded in a fluorometer at 470 nm (Qubit 3.0, Invitrogen, California, USA) as well as a UV transilluminator (UVItec, Cambridge, UK) at the end of the incubation. The tests were carried out using purified total RNAs as well as crude leaf extracts from infected and healthy banana leaf tissue as templates.

2.5. Specificity assessment of the RT-RPA and real-time RT-exo-RPA assays

The specificity of the CMV primers (F_1/R_1) and exo probe (Table 1) used in the RT-RPA and real-time RT-exo-RPA assays, was confirmed by testing against other important viruses infecting banana, *viz.*, BBrMV, BSMYV and BBTV. These RPA assays were performed using the purified total RNAs of the virus-infected plants as templates. The corresponding RNAs from CMV-infected and healthy banana plants were used as positive and negative control, respectively.

2.6. Sensitivity assessment of the RT-PCR, RT-RPA and real-time RT-exo-RPA assays

The sensitivities of detection of the RT-PCR, RT-RPA and real-time RT-exo-RPA assays were evaluated using ten-fold serial dilution series of the purified RNA and crude banana leaf extract templates. The purified CMV-infected banana plant RNA (300 ng/µl) was diluted ten-fold serially to a final concentration of 300 fg/µl with a healthy banana plant RNA preparation (200 ng/µl). Each dilution was evaluated in the RT-PCR, RT-RPA and real-time RT-exo-RPA assay. Corresponding sensitivity assays with the CMV-infected crude leaf extract were performed using 100 mg banana leaf in GEB3 buffer, serially diluted ten-fold with a crude extract from healthy banana leaf (10°-10^{-6}). The RT-PCR, RT-RPA and real-time RT-exo-RPA assay reactions were carried out as described above, using the corresponding purified RNA and crude extract from healthy banana leaf tissue as negative control. All the experiments (RT-PCR, RT-RPA and real-time RT-exo-RPA assay) were repeated 2–3 times.

2.7. Validation of the RT-exo-RPA assay

For the RT-PCR and RT-RPA assays validation, a total of 120 (62 symptomatic and 58 asymptomatic) banana plant samples were collected from three different banana orchards in Jalgaon, Maharashtra and tested by RT-PCR, RT-RPA and real-time RT-exo-RPA assays. The RT-PCR was performed using purified RNAs as templates while the RT-RPA and real-time RT-exo-RPA assay reactions were carried out using crude leaf extracts as templates, all as described above. Purified RNA was not used in the RPA validation assays due to the cost and time of the assay. To validate the portable RT-exo-RPA assay, 30 symptomatic and 20 asymptomatic banana plant samples were randomly selected for the assay reactions which were carried out and the fluorescence scored in a UV transilluminator and a fluorometer. A CMV-infected and a healthy banana leaf extract were used as the positive and negative controls, respectively, in all the validation experiments.

3. Results

3.1. Detection of CMV by the RT-PCR, RT-RPA and RT-exo-RPA assays

The RT-PCR of purified RNAs from CMV-infected banana plant samples using the CMV-CP gene-specific primers produced the desired amplicon of 657 bp. No amplification was obtained with either crude leaf extract from CMV-infected banana leaf tissue or with RNA and crude leaf extract from healthy banana leaf tissue (Fig. 1(a)). In the RT-RPA evaluations, using either the purified CMV-infected total plant RNA or the CMV-infected crude leaf extract as templates, positive amplifications of the target CMV sequences were observed using the CMV-specific F_1/R_1 primers, generating the expected amplicon size of 249 bp. No amplification was seen with either the RNA or crude leaf extract from healthy banana plant samples (Fig.1(b)). The amplicon obtained from the RT-RPA reaction using CMV-infected crude leaf extract template was successfully cloned into pGEM-T Easy vector and sequenced. The BLASTN analysis revealed a 98% sequence identity with the CP gene sequences of known CMV isolates from cucumber, basil and hot pepper (Acc. No. FM999065, KM651842 and AY560556).

In the portable RT-exo-RPA reactions, the fluorescence signal generated under the UV transilluminator could be clearly seen when the crude extract from CMV-infected plant was tested but when the corresponding CMV-infected purified RNA was used as a template, the fluorescent signal generated was low and could not be clearly

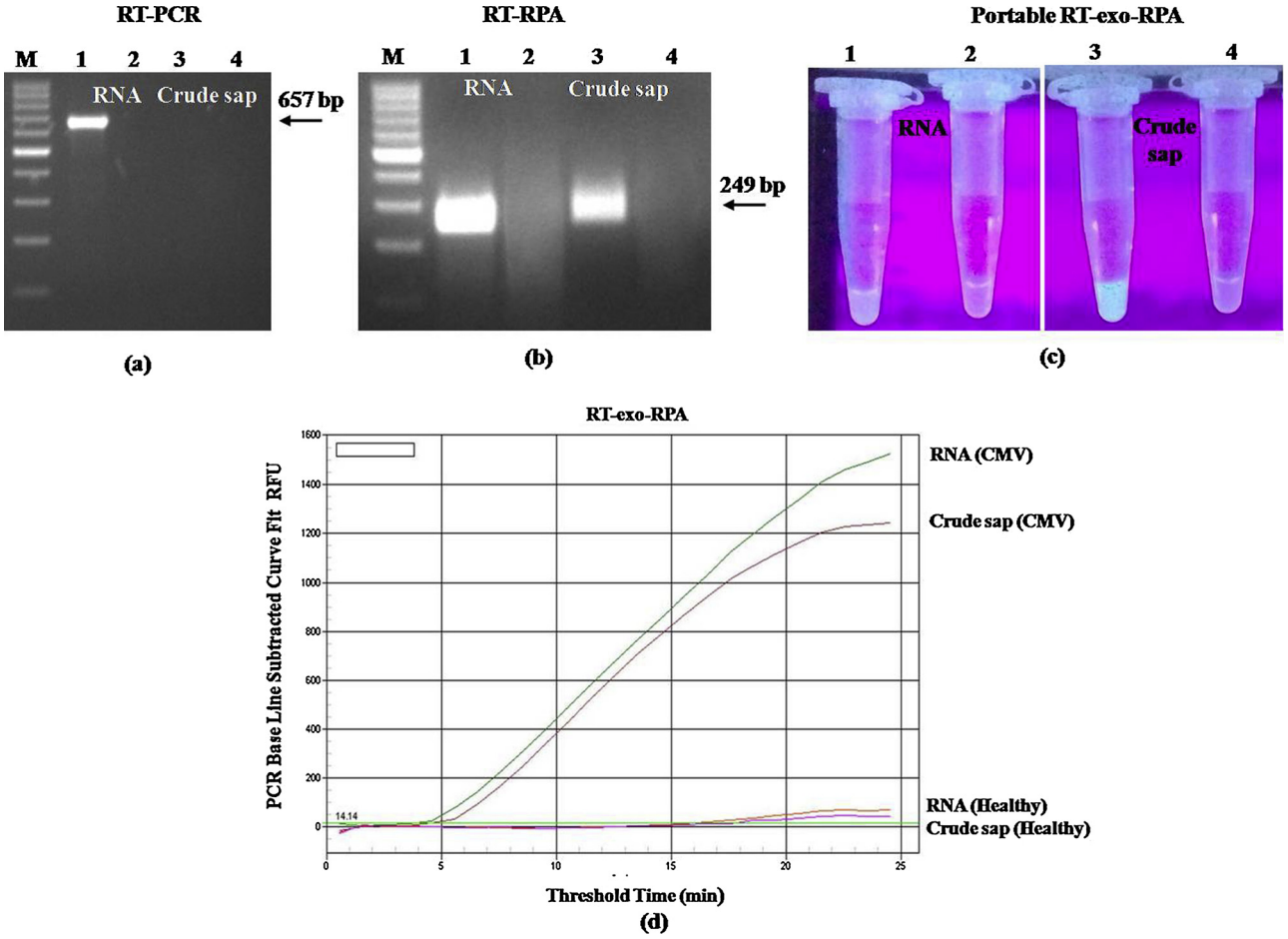

Fig. 1. Detection of CMV in CMV-infected banana plant samples using the purified total RNA and crude leaf extract templates, respectively, by the (a) RT-PCR assay, (b) RT-RPA assay, (c) portable RT-exo-RPA assay showing fluorescence in an UV-transilluminator and (d) real-time RT-exo-RPA assay. For (a) to (c), lanes 1 and 2: purified RNAs from infected & healthy banana leaf, respectively; lanes 3 and 4: crude leaf extracts from infected and healthy banana leaf, respectively. Lane M:100 bp DNA ladder (Thermo Scientific, EU, Lithuania).

distinguished from the negative control. As expected, no fluorescence was observed with the purified RNA and crude extract from healthy banana leaf tissue (Fig. 1(c)). In portable RT-exo-RPA assay, the purified RNA and crude extract from healthy banana leaf control showed relative fluorescence unit (RFU) less than 800 and the CMV-infected purified RNA and crude extract showed RFU twice the healthy control, indicating a positive reaction. In the real-time RT-exo-RPA assay, positive amplification signals above the threshold were obtained in less than 10 min using the purified RNA and crude leaf extract from the infected banana plant sample while the corresponding templates derived from the healthy banana plant sample did not produce any signal (Fig. 1(d)).

3.2. Specificity of the RT-RPA and real-time RT-exo-RPA assays

Specificity studies showed that only the purified RNA from the CMV-infected banana plant sample produced a positive amplification in the RT-RPA (Fig. 2 (a)) and real-time RT-exo-RPA assay (Fig. 2 (b)). In contrast, the corresponding BBrMV-, BSMYV- and BBTV-infected banana RNA samples all tested negative, indicating a high specificity of the primers developed for the RT-RPA as well as the real-time RT-exo-RPA assay for CMV detection. No amplification was observed in either of the RPA assays with the healthy control (Fig. 2).

3.3. Sensitivity of the RT-PCR, RT-RPA and real-time RT-exo-RPA assays

The detection limits of the RT-PCR, RT-RPA assay and real-time RT-exo-RPA assay were compared by testing a series of 10-fold dilutions of the purified RNA preparation and crude banana leaf extract from a CMV-infected banana plant sample. The results showed that the CMV amplicon of 657 bp could be positively detected at RNA concentrations down to 3 pg/µl by RT-PCR using the CP gene-specific primers (Fig. 3 (a)). However, no CMV RNA amplification by RT-PCR was obtained from any dilutions of the crude leaf extract (Fig. 3(b)). Comparatively, with the RT-RPA, the CMV CP amplicon of 249 bp was clearly visible at dilutions down to 3 pg/µl of RNA and to 10^{-5} dilution of the crude leaf extract template (Fig. 3 (c) and (d)).

For the real-time RT-exo-RPA assay, positive amplification signals were also observed from the infected sample at RNA dilutions down to 3 pg/µl, but with the crude extract, 10^{-4}dilution was the limit of detection. No positive amplification was seen in any of these assays for the healthy controls (Fig. 4). These results were consistently obtained when the experiments were repeated. (Data not shown).

3.4. Validation of the RT-exo-RPA assay with field samples

Out of the 120 banana plant samples, consisting of 62 symptomatic and 58 asymptomatic samples, all the 62 symptomatic and 21 asymptomatic samples tested positive in RT-PCR using the purified RNA as the template, each generating the expected amplicon of 657 bp of the CMV coat protein gene (Table 2). In the RT-RPA assay of these samples using the crude leaf extract as the template, all the 62 symptomatic and only 8 asymptomatic banana plant samples were found positive for CMV, each generating the expected size band of ˜249 bp from the CMV CP

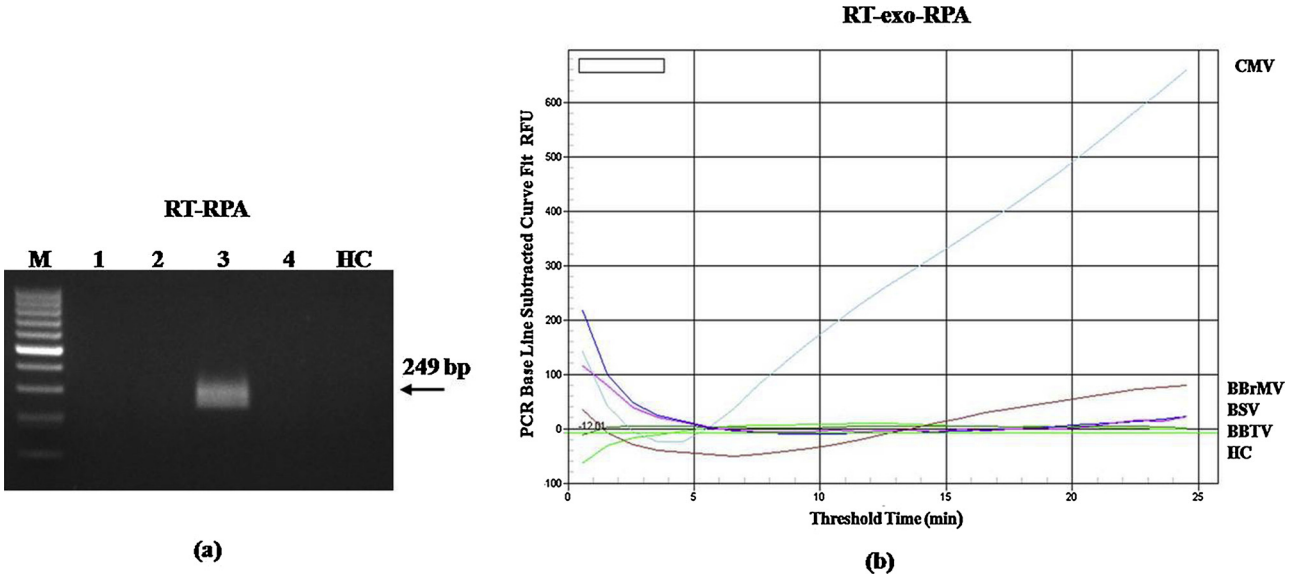

Fig. 2. Specificity of the (a) RT-RPA assay and (b) real-time RT-exo-RPA assay using primer pair CMV F_1/R_1 and CMV exo-probe. (a) Lane 1: BSMYV RNA; Lane 2: BBTV RNA; Lane 3: CMV RNA; Lane 4: BBrMV RNA; Lane M: 100 bp DNA ladder (Thermo Scientific, EU, Lithuania); HC: RNA from healthy banana leaf control.

gene (Table 2). Further, the real-time RT-exo-RPA assay done on these samples confirmed the results of the RT-RPA assay, showing positive amplification signals above the threshold limit in less than 15 min in the same 62 symptomatic (threshold-reaching time ranging from 3.5–6.5 min) and in 8 asymptomatic samples (threshold-reaching time ranging from 12.8–14.8 min). No amplifications were detected in the healthy banana plant samples even after the reaction time exceeded 25 min. Of the 21 asymptomatic samples that tested positive for CMV infection by RT-PCR, 13 samples showed very faint amplification. These same samples were in bad condition due to long storage and did not test positive in both RT-RPA and RT-exo-RPA assays. When the portable RT-exo-RPA fluorescence-based assay was used to test 30 symptomatic and 20 asymptomatic samples, only 27 of the symptomatic samples showed a positive fluorescence at 470 nm (RFU ranging from 2000 to 4500) as well as a bright fluorescence in the UV transilluminator. Out of the 20 asymptomatic samples, three showed a positive fluorescence at 470 nm (RFU ranging from 1600 to 1800) as well as a mild fluorescence in the UV transilluminator (Table 2). All the remaining three symptomatic and 17 asymptomatic banana plant samples, along with the healthy controls, showed no fluorescence in either the UV transilluminator or the fluorometer (RFU ranging from 500 to 800).

4. Discussion

This study describes the development and evaluation of a fluorescence-based isothermal RT-exo-RPA assay for the rapid, sensitive and specific detection of CMV-infected banana plants. The assay can be used as a real-time RT-exo-RPA assay to detect CMV using the purified plant

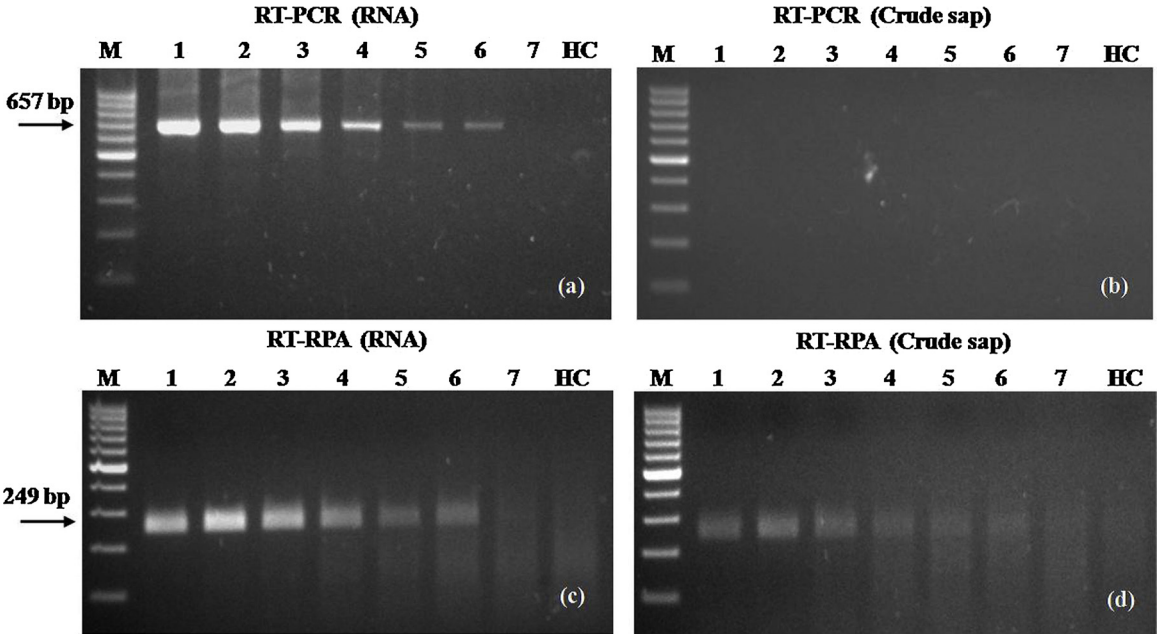

Fig. 3. Sensitivity studies using ten-fold serial dilutions of the purified RNA (a and c) and crude leaf extract (b and d) of a CMV-infected plant sample in the RT-PCR assay (a and b) and the RT-RPA assay (c and d). In each panel, lanes 1–7 correspond to a serial dilution of either the RNA (300 ng/μl-300 fg/μl) or the crude sap (10°-10^{-6}); Lane M: 100 bp DNA ladder (Thermo Scientific, EU, Lithuania); HC: corresponding healthy banana leaf RNA or crude extract controls.

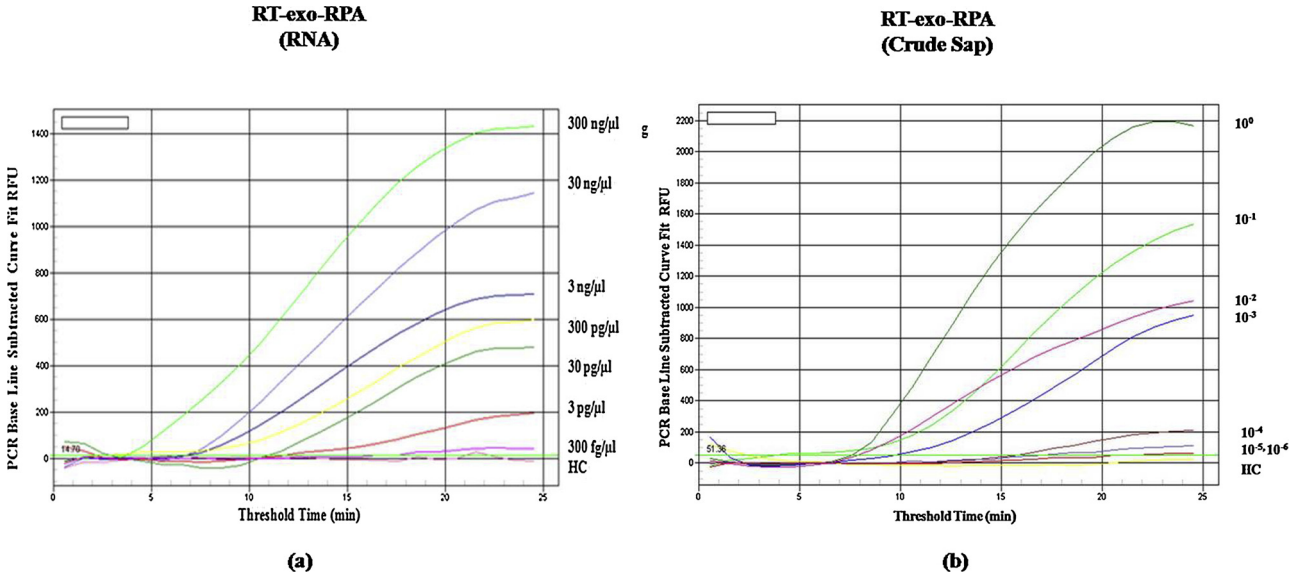

Fig. 4. Sensitivity test of the real-time RT-exo-RPA assay showing the fluorescence development from 0 to 25 min observed with each of the ten-fold serial dilutions of the purified RNA (300 ng/µl-300 fg/µl) (a) and the crude extract (10°-10^{-6}) (b) of a CMV-infected sample; HC: corresponding healthy controls.

RNA as well as the crude extract of infected banana leaf tissue by monitoring the fluorescence signal using a real-time PCR instrument. This fluorescence-based RPA assay was also adapted as a portable assay by carrying out the reactions in a portable dry bath incubator and detecting the fluorescence signals with a fluorometer or a UV transilluminator. The primers designed for the fluorescence-based assay worked equally well in a gel-based RT-RPA assay. Both of these assays did not produce any non-specific or cross reactions against the uninfected control or leaf samples of banana plants infected with other viruses (BBrMV, BBTV and BSMYV), suggesting a high level of specificity of the designed primer set and exo probe.

The ELISA and RT-PCR are the two most commonly used diagnostic tests for CMV detection. RT-PCR has been shown to be 100 times more sensitive than double antibody sandwich-ELISA (DAS-ELISA) (Berniak et al., 2009). ELISA could only detect CMV in undiluted preparations of plant extracts or in preparations diluted not more than 100 times. The isothermal RT-LAMP assay for CMV was shown to be 100 times more sensitive than the RT-PCR, with a detection limit as little as 1 pg/µl of plasmid DNA (Peng et al., 2012). In comparison, the real-time RT-exo-RPA, RT-RPA and RT-PCR assays developed in this study all showed a detection limit of 3 pg/µl of purified plant RNA. The comparable sensitivities of virus detection by the real-time RT-exo-RPA assay and the RT-PCR assay were also demonstrated in the detection of *Yam mosaic virus* and *Rose rosette virus* (Silva et al., 2015; Babu, et al., 2017). In contrast to the RT-RPA assays, the RT-PCR assay could not amplify the CMV CP gene in crude leaf sap. The failure to detect CMV in the crude sap by RT-PCR could be due to the presence of inhibitors in the leaf extract (Mekuria et al., 2014). Also, with crude saps, the sensitivity of the real-time RT-exo-RPA assay was lower (10^{-4} dilution) compared to the RT-RPA (10^{-5} dilution). The fluorescent signals generated from positive amplifications by the portable RT-exo-RPA reactions could be successfully measured by a portable fluorometer or visualized using a UV transilluminator. The fluorescent signals of the positive reactions ranged from 1600 to 4500 RFU which were at least twice of those obtained with the healthy control and asymptomatic, uninfected samples (600–800 RFU). Similarly, healthy control and asymptomatic uninfected plant samples with RFU reading of less than 800 showed no fluorescence in an UV transilluminator. Sensitivity assessment of the portable RT-exo-RPA assay using CMV-infected crude leaf extracts (dilutions 10^{0}-10^{-6}) and measuring the fluorescence in a fluorometer produced reproducible results until 10^{-1} dilution (Data not shown).

The efficiency of detection of CMV in the symptomatic plant

Table 2
Validation of RT-PCR, RT-RPA and RT-exo-RPA assays for the detection of CMV in symptomatic and asymptomatic banana plant leaf samples collected from banana orchards.

Location	Total Positive samples/Total samples indexed									
Jalgaon, Maharashtra,India	Purified RNA		Crude leaf extract							
	RT-PCR		RT-RPA		Real time RT-exo-RPA assay		Portable RT-exo-RPA assay			
							UV transilluminator		Fluorometer	
Orchards	[a]S	[b]AS	[a]S	[b]AS	[a]S	[b]AS	[a]S	[b]AS	[a]S	[b]AS
1	34/34	10/31	34/34	4/31	34/34	4/31	13/15	2/12	13/15	2/12
2	0/0	0/10	0/0	0/10	0/0	0/10	0/0	0/0	0/0	0/0
3	28/28	11/17	28/28	4/17	28/28	4/17	14/15	1/8	14/15	1/8
Total	62/62	[c]21/58	62/62	8/58	62/62	8/58	27/30	3/20	27/30	3/20

[a] S-Symptomatic banana plant samples.
[b] AS-Asymptomatic banana plant samples.
[c] Out of 58 asymptomatic banana plant samples, 21 were identified as positives in RT-PCR assays using RNA as a template, the amplicons were very faint in 13 of these samples which were in bad conditions and did not test positive in RT-RPA and RT- exo-RPA (real time and under UV light detection and in fluorometer) using crude sap as template.

samples by RT-PCR, RT-RPA and real-time RT-exo-RPA assays was identical. RT-PCR, however, detected CMV in a greater number of asymptomatic banana plant samples than the RT-RPA and RT-exo-RPA assays. This could be explained by the bad conditions of some of the RT-PCR positive asymptomatic samples due to deterioration during transport and storage, since these were the same samples that gave negative reactions in the RT-RPA and RT-exo-RPA assays using crude saps. However, the RT-PCR used purified plant RNA as templates which could enable amplification more efficiently. Whether using purified RNA as templates for the RT-RPA assays would identify more CMV-positive samples remains to be seen. In addition to banana plant samples, an attempt was made to detect CMV infections in long pepper (*Piper longum)* samples collected from Bhubaneswar, Odisha, India. Of the 15 samples tested, nine tested positive for CMV by the real-time RT-exo-RPA assay using crude leaf extracts as templates. This result was identical to those of the RT-PCR using purified RNA template and the RT-RPA assay using crude leaf extract as template (Data not shown). The uninfected long pepper sample used as negative control in these experiments did not show any amplification/fluorescence. These results suggested that the designed primer set/probe could also be used to detect CMV infection in other hosts. While the RT-RPA and real-time RT-exo-RPA assays produced consistent results with the fresh symptomatic and asymptomatic samples, with efficiencies of CMV detection comparable with that of the standard RT-PCR, the corresponding efficiencies of CMV detection by the portable RT-exo-RPA assay, where the fluorescence signals were analysed using an UV transilluminator or a fluorometer, were much lower. Thus, at this stage, it is not recommended for reliable diagnosis of CMV infection in field situations until further tests are done.

In summary, the developed real-time RT-exo-RPA assay is superior to the standard RT-PCR in terms of its simplicity, ease of sample preparation and short run time (10–20 min s) while the latter requires 4–8 h for nucleic acid isolation, thermal cycling and gel imaging. Although, there is not much of a difference in the cost of reagents involved in the RT-exo-RPA and RT-PCR assays but, overall, the former is more economical as it requires much less time and much less intensive labor. The RT-exo-RPA assay will be extremely useful in diagnostic laboratories for the rapid and specific detection of CMV. It will also be useful in certification programmes where mass indexing of samples is required, thereby decreasing the further spread of the virus.

Conflict of interest statement

All the authors declare that there are no financial or other relationships that might lead to a conflict of interest. All the authors have seen and approved the manuscript and have contributed significantly to the work.

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