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RESEARCH ARTICLE

Development of reverse transcription recombinase polymerase amplification assay for rapid diagnostics of Peanut mottle virus

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Abstract

Peanut mottle virus (PeMoV) is a single-stranded RNA virus transmitted through seeds and aphids that affects peanut crops worldwide. Currently, Enzyme Linked Immune-Sorbent assays and Reverse-Transcription Polymerase Chain Reaction techniques are widely employed to detect PeMoV in infected plants. ELISA is labor-intensive and time-consuming, as it involves the preparation of buffers and the production of polyclonal antibodies. Even though RT-PCR bypasses the need for buffer preparation and antibody production, it demands trained professional's manpower, requires expensive equipment like thermal cyclers, and involves complex procedures such as RNA isolation and cDNA conversion. To avoid these constraints, there is a need for a fast, reliable, efficient, and economical method for detecting PeMoV to ensure the production of healthy seeds. This study optimized the Reverse Transcriptase Recombinase Polymerase Amplification (RT-RPA) method by eliminating the steps of RNA extraction, cDNA conversion, and the use of a thermal cycler. The optimized RT-RPA assay successfully detected PeMoV at concentrations as low as 10^{-6} and 10^{-7} dilutions (1 and 0.1 µg/µl) of both RNA an-6d crude sap templates, demonstrating high sensitivity comparable to the routine RT-PCR assay. The new RT-RPA technique was tested against other viruses that infect peanuts like the Peanut stunt Virus, Tomato spotted wilt virus and Peanut bud necrosis virus, this technique demonstrated great specificity and no cross-reactivity. The developed RT-RPA using a crude leaf sap template is time-saving, less laborious, not very complicated, high specificity, sensitivity, economical and efficient. Therefore, laboratories with limited resources can use the RT-RPA assay for preliminary screening of PeMoV in nurseries, farm and glasshouse conditions, and quarantine stations. The current study reports the development, optimization and validation of Reverse Transcriptase Recombinase Polymerase Amplification (RT-RPA) using crude sap as template for the onsite detection of PeMoV infection in peanut crops under field conditions for the first time.

Keywords Peanut mottle virus · Recombinase polymerase amplification assay · On-site detection · Point-of-care testing

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Abbreviations

PeMoV	Peanut mottle virus
ELISA	Enzyme linked immuno sorbent assay
DAS ELISA	Double antibody sandwich enzyme linked immuno sorbent assay
RT-PCR	Reverse transcription polymerase chain reaction
RT-RPA	Reverse transcription recombinase polymerase assay
LAMP	Loop mediated isothermal amplification
LOD	Limit of detection
qRT-PCR	Quantitative real time PCR

Introduction

The peanut crop is infected by more than 31 viruses, among which seed-borne viruses pose a significant threat to international trade and germplasm exchange. Peanut mottle virus (PeMoV) with a positive-sense single-stranded RNA genome, belonging to the genus *Potyvirus,* is one of the major viruses affecting peanuts that is transmitted by aphids in a non-persistent manner (Behncken et al. 1970) and through seeds (Elahinia et al. 2008). Since the first report of PeMoV in the USA in 1965 (Kuhn 1965), PeMoV has been reported in many peanut-growing areas worldwide (Bock 1973; Soumya et al. 2014). The yield loss due to PeMoV ranged from 20 to 70%. In 1977, the first reprot of Peanut mottle virus was reported on peanuts, soybeans, and peas in India (Reddy et al. 1978; Rao et al 1979). Besides, the virus infects other crops like French beans and *Cassia* sp., suggesting the wide host range of the virus (Adams et al. 1977). The seed transmission due to PeMoV ranged from 1 to 7% (Bock 1973; Chen et al. 2014, 2016). PeMoV is usually co-infected with strains of *Tomato-spotted wilt virus* (TSWV) (Hoffmann et al. 1998). Some strains of PeMoV are also capable of infecting bambara groundnut (Li et al. 1991). PeMoV induces typical symptoms like green mottling, vein banding, yellowing to dark islands, leaf curl, interveinal depressions on young leaves, reduction in pod size and weight, and pod and seed decolorization (Dey et al. 2018). Additionally, the seed coat of affected seeds may also show discoloration. PeMoV has quarantine significance due to its vertical transmission to the next generation through seeds (Adams et al. 1977) and seed transmission results in a 25% yield reduction (Kuhn 1965). PeMoV was successfully transmitted to experimental hosts like *Nicotiana benthamiana*, *N. clevelandii*, and *Phaseolus vulgaris,* among which *N. benthamiana* and *N. clevelandii* showed mottling symptoms in systemic tissues. In contrast, chlorotic local lesions were observed on *P. vulgaris* (Spiegel et al. 2008).

Serological techniques like double-antibody sandwich Enzyme-Linked Immuno-Sorbent Assay (DAS ELISA), Dot Enzyme Linked Immuno Sorbent assay (Dot ELISA), Direct-Tissue Blot-Immunoassay (DT-BIA) and Immune Chromato-Graphic Strip (ICP) developed for PeMoV detection involves the use of polyclonal and monoclonal antibodies produced against the virus proteins (Bharathan et al. 1984; Ahlawat et al. 2003; Penchalaiah et al. 2008; Bin et al. 2015; Zhen et al. 2017; Bin et al. 2018). Nucleic acid-based detection assays like Reverse Transcriptase Polymerase Chain Reaction (Chen et al. 2014; Meena et al. 2016; Meena et al. 2019), Quantitative RT-PCR (Chen et al. 2016), Loop Mediated Isothermal Amplification and nested PCR are also available for detecting PeMoV (Gillaspie et al. 2000). But all the aforementioned methods are laborious, complicated, requires proficient personnel and well-equipped laboratories. The lowest rate of seed transmission and lower outcome of symptoms of PeMoV is difficult to detect in growing out tests and it requires more time during RNA extraction, cDNA conversion, and PCR amplification (Bharathan et al. 1984). In recent times, isothermal amplification techniques like Recombinase Polymerase Amplification (RPA) methods can be utilized for detecting pathogens with DNA or RNA genomes in a relatively short time, approximately an hour (Euler et al. 2012). The working principle of RT-RPA is based on amplification of targeted conserved region by the combination of proteins and enzymes (TwistDx, Cambridge, United Kingdom) under isothermal condition (anywhere from 37℃ to 42℃). The whole reaction can be carried out in 20–30 min in a dry bath, dispensing the requirement of sophisticated equipment. After amplification, RPA products can be confirmed and visualized on agarose gel under UV illumination system (Chen et al. 2010; Mekuria et al. 2014; Zhang et al. 2014; Silva et al. 2015; Babu et al. 2017; Srivastava et al. 2019; Jiao et al. 2020; Kumar et al. 2021; Kishan et al. 2023). Several studies have used RT-RPA technique for the rapid detection of viruses/viroids with RNA genomes by adding reverse transcriptase (RT) to the RPA reaction mixture (Faggioli et al. 2017; Silva et al. 2018). Despite, the importance, RT-RPA assay for PeMoV detection is still unavailable. In this current study, we developed an RT-RPA assay that was optimized and validated for detection of PeMoV in peanut crops using crude leaf sap template for the first time from field samples.

Materials and methods

Sample source and inoculum maintenance

Young symptomatic peanut leaves in PeMoV infected plants were collected from the farmers' fields between October 2023 and March 2024 in Kadiri, Satya Sai district of Andhra Pradesh. PeMoV infection in the symptomatic peanut plants was confirmed primarily through RT-PCR amplification of partial Coat Protein (CP) gene of PeMoV and Sanger sequencing of amplicons. Further, the viral presence was confirmed through Direct Antigen Coated Elisa (DAC-ELISA) (American Type Culture Collection, Virgina, USA). Crude sap was obtained from PeMoV-positive samples by grinding in 0.1 molar Phosphate Buffer (pH 7.0) that was rubbed over the young leaves of 10 days-old seedlings of French bean (*Phaseolus vulgaris*) injured with celite powder (0.22 µm). The inoculated Frech bean plants were

maintained in the glasshouse facility at NBPGR Regional Station, Hyderabad.

Designing of primers for RT-PCR and RT-RPA

Two sets of primers designated as PeMoV 1F/1R and PeMoV 2F/2R (Table 1) were designed from the conserved CP sequences of different PeMoV isolates available from GenBank (NCBI) for RT-PCR and RT-RPA assays. The RPA primers (30–35 nucleotides long) were custom-designed based on the guidelines of manufacturer (Twist-Dx, Cambridge, United Kingdom) and synthesized by Integrated DNA Technologies (IDT) (Iowa, USA). The initial optimisation was performed using gradient RT-PCR at temperatures ranging from 52 to 58 °C The primer pairs which showed high specificity in RT-PCR assays were subsequently employed in the RT-RPA methods.

RNA isolation and RT-PCR based detection

Total RNA from the leaves of PeMoV suspected plants was isolated using Qiagen, RNeasy Plant-Mini Kit, (Germany) as per the manufacturer's guidelines. Isolated RNA from all sample was eluted in 25 µL nuclease-free water individually and its quality and quantity were determined using the BioTek Take 3™ Micro Volume Plate Spectrophotometer (BioTek® Instruments, USA). Isolated RNA was stored at -80℃ for future use. PeMoV infection was verified through a two-step RT-PCR protocol. Initially, complementary DNA (cDNA) was synthesized using the G-BIOSCIENCES cDNA synthesis kit and oligo dT as the reverse primer. The 25 µL polymerase chain-reaction mixture containing 2.5 µL of 500 ng cDNA as-template, 12.5 µL of Master mix (G-BIOSCIENCES. St. Louis, USA), 2.5 µL of 10 µM of both the primers, and 5 µL of nuclease-free water was used. Subsequently, polymerase chain reaction (PCR) amplification of the coat protein (CP) gene was performed using PeMoV-specific primers (PeMoV1 and PeMoV2) (Table 1). The 25 µL PCR reaction mixture contained 2.5 µL of 500 ng cDNA template, 12.5 µL of the Master mix (G-BIOSCIENCES. St. Louis, USA), 2.5 µL of 10 µM each forward and reverse primers, and 5 µL of nuclease free water. The PCR conditions used were as follows: one cycle of initial denaturation at 94 ℃ for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °Cfor 35 s, extension at 72 °C for 1 min and one cycle of final extension at 72 °C for 10 min. The amplified product was seen using a gel-imager (Syngene, UK) after being electrophoresed in a 1% agarose gel with lab-safe stain (G-BIOSCIENCES, St. Louis, USA). After being refined, the amplified product was cloned in to the pGEM-T easy-vector (Promega, USA). Transformation of *E.coli* DH5α competent-cells with the recombinant vector

Table 1 List of primers used for detection of Peanut mottle virus

S.No	Primers	Nucleotide sequence (5´-3´)	Bases	Target Position on coat protein gene	Position on Accession Number in NCBI GenBank PP998001	GC (%)	Tm (°C)	Amplicon length (bp)	References
	Coat protein gene specific primers								
1	PeMoV1	5'-GCTGTGAATTGTTGTTGAGAA-3'	21	_	_	30.1	60.4 °C	913 bp	Soumya et al. (2014)
	PeMoV2	5'-ACAATGATGAAGTTCGTTAC-3'	20	_	_	35	54 °C		
	RPA primers								
2	PeMoV 1F	GGCTGAGGCGTACATTGAGATGAGAAATGCAG	32	545–576	29–59	50	77.6 °C	230 bp	This study
	PeMoV 1R	CCTGGGTTCCGACGTTACCATCAAGACCAAATGTC	35	739–774	211–240	51.4	80.6 °C		
3	PeMoV 2F	GGGCTGATGGTTTGGTGCATTGAGAATGGCAC	32	388–419	1–32	53.1	82.1 °C	300 bp	This study
	PeMoV 2R	CGCTTTAGCTGATGTACGCGACGTGATTTC	30	657–686	271–300	50	76.1 °C		

was carried out. Transformants were selected on Luria Agar plates containing 23.8 mg/mL IPTG, 20 mg/mL Xgal and 25 mg/mL ampicillin through Blue-white screening. The transformed colonies were further confirmed by restriction digestion with *EcoR* 1 enzymes and visualized in agarose gel after electrophoresis under a gel documentation system. Three PeMoV-positive clones were further confirmed through colony PCR using the T7 and SP6 universal primers followed by sequencing at Eurofin Genomics, Bengaluru. The obtained sequences were further confirmed by BLAST analysis (http://www.ncbi.nlm.nih.gov/blast).

Crude sap preparation for use as template in RT-RPA assay

Crude sap was obtained by grinding leaves in ten various lysis-buffers viz., nuclease free water, CTAB 5%, CTAB 10%, phosphate-buffer, TRIzol-TM reagent (G-BIOSCIENCES. St. Louis, USA), RLT-buffer, RLC-buffer (RNAeasy-Plant Mini Kit, Qiagen, Germany), EDTA, 1N-NaOH, and NaOH: EDTA (1:1). PeMoV-suspected leaf tissue (approximately 100 mg) was subjected to homogenization with 1 ml of each buffer with an auto-claved, pre-cooled mortar and pestle to yield crude-sap. The sap was transferred to a 1.5 ml microcentrifuge tubes and was subjected to centrifugation for 2 min at 10,000 g. The supernatant was transferred to a new micro centrifuge tube and used as a template-in the RT-RPA assay.

RT-RPA based detection of PeMoV

RT-RPA technique was carried out using the specific primers PeMoV 2F and PeMoV 2R using RNA and crude sap as template by adopting the guidelines given in Twist-Amp® Basic-Twist-Dx Limited. The reaction-mixtures were arranged by adding 2.1 µL of primers each PeMoV 2F/2R (10 µ-mol μl^{-1}), 29.5 microlitre of rehydration-buffer, 0.5 µL of RNAse-inhibitor, 1 µL of reverse-transcriptase and 8.3 µL of nuclease-free-water (NFW), totalling 43.5 µL. This mixture was added to the frozen pellets and was mixed thoroughly using pipette. The final reaction mixture was then transferred equally into two 1.5 ml capacity microcentrifuge tubes (21.75 µL each). Subsequently 1 µL of template (RNA, crude sap, or plasmid DNA) and 1.25 µL of 280 µM magnesium-acetate to each tube (1.5 mL capacity). The final reaction mixture was quick-spinned in a mini spin (Mini -10 k+, BR Biochem Life Science pvt.ltd.) to facilitate proper mixing and to initiate the amplification. The RT-RPA assay was incubated at 40 °C for 30 min, then heat-inactivation at 65 °C for 10 min using dry-bath (BT-MINIB-100, BT Lab Systems). The amplified product along with loading dye was mixed properly and loaded in 1% agarose gel electrophoresis and visualized in UV-Gel imager (IG-618GD, iGene Labserve pvt. ltd.). The positively amplified reaction mixture was purified using a PCR purification kit (QIAquick, QIAGEN, USA) and cloning was done in the pGEM-T-vector (Promega, Madison, USA) as per the manufacture protocol. RT-RPA PeMoV clones were sequenced bidirectionally using T7 and SP6 primers. Sequence identity was confirmed via BLAST analysis.

Comparison of different lysis buffers used in extracting the crude sap as template in RT-RPA assay

After molecular confirmation, the PeMoV infected leaves were homogenized separately using ten different lysis buffers as previously described. Among the ten buffers, the optimal buffer yielding a clear, specific amplicon on 1% agarose gel was used for further investigations using ribonucleic acid, plasmid and crude-sap templates. PeMoV samples and healthy peanut leaves served as positive and negative controls respectively in RT-RPA technique.

Optimisation of different incubation periods and temperature for the detection of PeMoV using crude sap as template in RT-RPA assay

The best lysis buffer which gave precise, thick, and specific amplicon was used to homogenize PeMoV infected leaf tissue and the crude sap, thus obtained, was used as template for the optimisation of incubation period and temperature. The period of incubation was standardized by carrying out the RT-RPA assay at different incubation times ranging from 5 to 50 min to determine the best incubation time. Similarly, to standardize the best amplification temperature, RT-RPA reaction mixture was incubated at different temperatures ranging from 5 to 50 °C.

Determination of limit of detection (LOD) of RT-PCR and RT-RPA assays using RNA, crude sap, and plasmid templates and specificity assay of the designed RPA primer pairs

Before checking the limit of detection in PeMoV the specificity among the two RPA primer (PeMoV 1F/1R, PeMoV 2F/2R) was evaluated in PCR using cDNA as a template. The PeMoV detection limits were evaluated using several serial-dilutions of purified ribonucleic acid (initial-stock: 10^2 (100 ng/µL), Crude-Sap of infected leaf (100 ng/µL of Lysis-Buffer) and plasmid-DNA (initial-stock: 10^2 (100 ng/µL -final stock up to 10^{-18}). These templates were tested in both RT-PCR and RT-RPA techniques. To find out maximum threshold of LOD while using crude sap as template, the best lysis buffer was obtained after gel electrophoresis followed by ascertaining the Limit of

Detection (LOD). To detect the maximum threshold of LOD, different serial dilutions were prepared for RNA and plasmid DNA as crude sap templates. For comparative analysis of the sensitivity of RT-RPA with RT-PCR technique, all diluted templates (crude sap, RNA, and plasmid) were concurrently checked in RT-PCR also. For the comparison of specificity of the RT-RPA technique in detecting PeMoV, cross reactivity of the developed RT-RPA technique was conducted with crude-sap from peanut leaf samples infected with other common viruses like Peanut bud necrosis virus (PeBNV), Peanut stunt virus (PeSV), and Tomato spotted wilt virus (TSWV). All RT-RPA assays were repeated thrice and those found reproducible were only shown in the results. Further, to validate the viability of the RT-RPA for PeMoV diagnostics in the field, around 51 peanut samples showing PeMoV-like- symptoms were detected using RT-RPA and RT-PCR respectively.

Results

Confirmation of PeMoV infection in the symptomatic peanut plants

Peanut leaves suspected of PeMoV infection were collected from farmers' fields located in the Kadiri, Satya Sai district of Andhra Pradesh. The virus inoculum from positive samples was maintained in the French bean host in the glasshouse at NBPGR-RS, Hyderabad (Fig. 1A; a-c). The presence of PeMoV infection was confirmed by amplifying the expected 920 nt amplicon from the CP gene using PeMoV1/2 primer pair (Fig. 1B). Two RPA primers were designed within the coat protein gene of PeMoV i.e., PeMoV 1F/1R (amplicon size: 230nt) and PeMoV 2F/2R (amplicon size 300nt). Among the two pairs of RPA primers used, the primer pair PeMoV 2F/2R yielded a precise single band (300 nt in size). Further, the amplicons were sequenced through

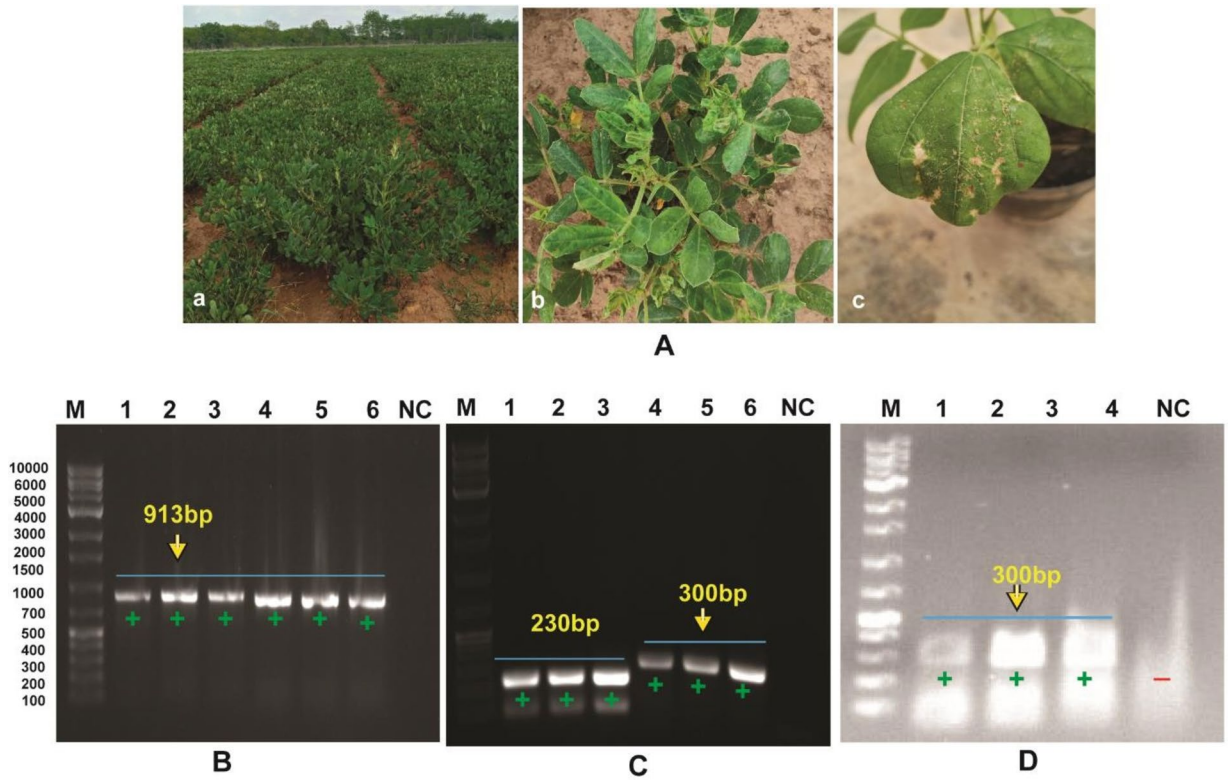

Fig. 1 RT-PCR based detection of Peanut mottle virus and optimization of recombinase polymerase amplification (RPA) assay for PeMoV. **A**. Collection and maintenance Peanut mottle virus in glass house. **a** Peanut farm in Kadiri district of Andhra Pradesh. **b** Infected plants showing mottling and mosaic symptoms. **c** PeMoV inoculated French bean plant maintained in glass house of NBPGR-RS, Hyderabad. **B**. Molecular confirmation of PeMoV infection in the groundnut leaf samples by RT-PCR using PeMoV CP-specific primers, Lane M: GeneRulerTM1kb ladder (G-BioSciences), Lane 1–6: symptomatic leaf samples and lane NC, negative control. **C**. PCR based specificity checking among the two designed RPA primer sets (PeMov 1F/1R and PeMov 2F/2R), Lane M: GeneRuler TM 1 Kb ladder (G-BioSciences), Lane 1–3: amplicons produced by RPA primer pair PeMov 1F and 1R lane 4–6 amplicons produced by RPA primer pair PeMov 2F and 2R from three symptomatic samples collected from peanut farm in Kadiri district of Andhra Pradesh. **D**. RT-RPA based confirmation of PeMoV using the selected RPA primers of PeMov 2F and 2R

sanger method and the obtained sequence was submitted to GenBank (accession number, PP998001). BLASTn analysis of obtained sequence showed its maximum identity of 99.17% with CP gene sequence of a PeMoV isolate (MG640414) The primer pair PeMoV 2F/2R was then analyzed in RT-RPA assay using cDNA template derived from three diseased individuals that yielded desired amplicons in all the three samples tested. In contrast, no amplification was obtained using cDNA template derived from a healthy plant (Fig. 1D).

Evaluation of various lysis-buffers used in template preparation for RT-RPA assay for PeMoV detection

Different lysis buffers were exploited for template preparation (crude leaf sap) and concurrently tested for diagnostics of the PeMoV coat-protein gene in the RT-RPA technique. Among all the lysis buffers used, the target-specific amplification and thick bands were observed only when the Crude-leaf-sap extracted with 1 Normal Sodium hydroxide and Sodium hydroxide: Ethylenediaminetetraacetic acid, (1:1) lysis buffers were used as template. The specific bands that appeared in the RT-RPA assay were consistently reproducible, indicating that 1N NaOH followed by NaOH (1:1) was the best lysis buffer among all the lysis buffers tested. However, a thick band was observed only when 1N NaOH was used as a lysis buffer in comparison to that produced using NAOH: EDTA (1:1) buffer. Thus, 1N NaOH lysis buffer was used for further experiments (Fig. 2A).

Incubation temperature and period optimization for RT-RPA technique using crude-sap as a template for PeMoV

RT-RPA was performed with crude-leaf-sap extracted with the help of 1N NaOH lysis buffer to optimize the

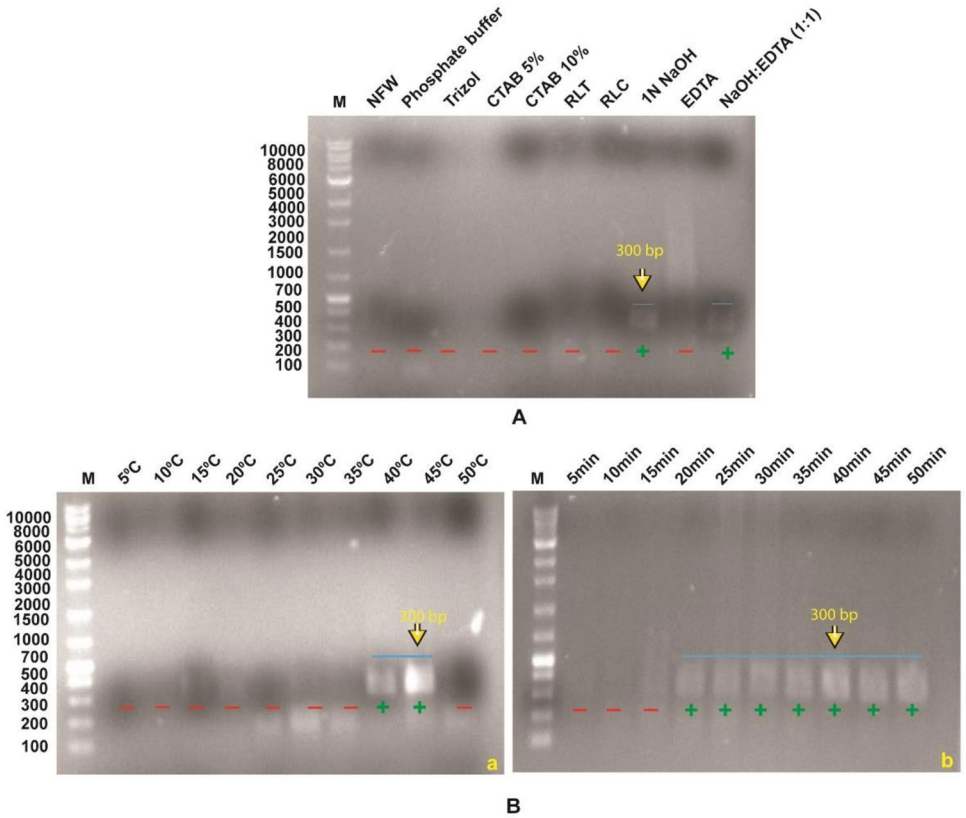

Fig. 2 Optimization of incubation temperature and time for the detection of PeMoV through RT-RPA using crude sap template. **A** RT-RPA based detection of PeMoV infection in peanut leaf sample using PeMoV 2F and PeMoV 2R amplifying 300 bp of amplicon from crude sap template. Lane M: GeneRuler™ 1 kb ladder (G-BioSciences), Crude sap obtained using Lane 1: Nuclease free water, Lane 2: Phosphate buffer, Lane 3, TRIzol TM Reagent (Invitrogen, Thermofisher Scientific, Wilmington, USA), Lane 4: CTAB 5%, Lane 5: CTAB 10%, Lane 6: RLT, Lane 7: RLC, Lane 8: NaOH: EDTA (1:3), Lane 9: NaOH: EDTA (1:1), **B** Optimization of RT-RPA based detection of PeMoV infection in crude leaf sap of peanut using primer set RPA 2F/2R amplifying 300 bp of amplicon at different temperatures and time periods. Lane M: GeneRuler^{TM} 1 kb ladder (G-BioSciences). **a** RT-RPA detection assay at different incubation temperatures: 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C. **b** RT-RPA detection assay for different incubation times: 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 min.

incubation time and temperature. Out of various incubation temperatures tested from 5–50 °C (5 °C increment in temperature), the desired PeMoV amplicon (300 nt) was visualized in gel only at two different temperatures i.e., 40 and 45 °C but disappeared at 50 °C and afterwards (Fig. 2B–a). The bright bands observed at 45 °C were used in further assays. The efficiency in detecting PeMoV through RT-RPA technique was also tested at 10 different incubation-times starting from 5 to 50 min. Among the incubation times tested, incubation for 40 min gave the ideal amplification result (Fig. 2B–b). However, the visible amplified band of 300 nucleotides started appearing from 20 min incubation time and was seen till 50 min incubation time. The optimized incubation temperature of 45 °C and time (40 min) standardized in this study were used for PeMoV based detection in further experiments.

Comparison of the limit of detection (LOD) of RT-PCR, RT-RPA and RPA assays

The limit of Detection (LOD) of the RT-RPA assay was compared with that of RT-PCR using different dilutions of crude leaf sap extracted with 1N NaOH lysis buffer, purified RNA, and plasmid (containing the desired PeMoV insert) as templates. The initial concentration of infected-tissue derived RNA was 100 ng/µL for RT-PCR and RT-RPA assays and positive amplification was obtained up to 10^{-7} and 10^{-6} dilution, respectively, which is equivalent to 0.1 and 1 µg/µL of RNA, respectively (Fig. 3A–a and 3A–b). When 1 µL of crude leaf sap (prepared using 1N NaOH lysis buffer) was used as template in RT-RPA, confirmed amplification was obtained up to 10^{-7} dilution (Fig. 3A–c). Whereas in RT-PCR, there was no amplification while using the same crude leaf sap as template (Fig. 3A–d). RT-PCR and RT-RPA assays performed using plasmid (containing the desired PeMoV insert) as template showed positive amplification up to 10^{-16} and 10^{-12} dilutions, respectively, which is equivalent to 0.0001 and 1 pg/µL (Fig. 3A–e, f). In addition, the developed RT-RPA assay showed high specificity in detecting PeMoV even in samples co-infected with other peanut crop-infecting viruses like PeSV, PeSWV and PeBNV (Fig. 4).

Standardization of the developed RT-RPA assay using field samples

To validate the developed RT-RPA assay for PeMoV diagnostics in the field, 51 peanut samples with PeMoV symptoms were collected from Andhra Pradesh, Telangana, and Tamil Nadu from different regions and evaluated by RT-RPA and conventional RT-PCR techniques, respectively (Supplementary Table 1). The findings indicated that 45 samples tested positive for PeMoV with RT-RPA assay, whereas only 42 samples were positive by RT-PCR (Supplementary Table 1). Nevertheless, all three samples that tested negative for PeMoV in the RT-PCR assay also quickly displayed typical symptoms, suggesting that they were infected with PeMoV but the technique was unable to identify it. Furthermore, the PeMoV-positive samples analysed with RT-RPA test exhibited distinct bands in the agarose gel electrophoresis akin to those observed in RT-PCR (Supplementary Fig. 1), indicating its comparable efficacy to RT-PCR. The results indicated that the developed RT-RPA assay was an efficient and sensitive method for detecting PeMoV and could be effectively utilised on field-collected materials.

Discussion

Peanut crop is infected by more than 31 viruses representing 14 genera in different parts of the world (Sreenivasulu et al. 2008). Among these, Peanut mottle virus (PeMoV), Peanut bud necrosis virus (PeBNV), Tomato spotted wilt virus (TSWV), Tobacco streak virus (TSV), Groundnut rosette assistor virus (GRAV), Groundnut rosette virus (GRV), satellite RNA associated with GRV and/or GRAV, Peanut clump virus (PCV), Peanut stripe virus (PStV), a strain of Bean common mosaic virus (BCMV), and Cucumber mosaic virus (CMV) have contributed significant economic losses to groundnut worldwide (Sreenivasulu et al. 2008). PeMoV has quarantine significance due to its transmission through seeds. India ranks second in terms of groundnut production next to China. Early detection of PeMoV under field conditions is important to avoid the inevitable persistence of this virus transmission through seeds.

Conventional nucleic acid-based assays like RT-PCR (Chen et al. 2014; Meena et al. 2016; Meena et al. 2019) real-time PCR (Chen et al. 2016), nested PCR and serological assays like ELISA and its variants are routinely used for indexing of PeMoV. However, these methods are time-consuming and demand skilled manpower and costlier equipment and reagents viz., thermal cycler, RNA extraction kits, spectrophotometer and probes. Other isothermal amplification methods were also employed for detection of peanut viruses e.g. loop-mediated isothermal amplification (LAMP) assay for PeBNV detection (Raigond et al. 2023). However, LAMP assay requires 4–6 primers, which increases the overall cost, besides the false positive results it yields due to the formation of multimeric products (Bakheit et al. 2008). RPA assay is an isothermal amplification assay that is relatively more specific and faster than LAMP (Devi et al. 2023). RT-RPA assay was successfully exploited for early detection of citrus yellow vein clearing virus, and the LOD of RNA and crude leaf sap were up to 1 pg/µl and 10^{-7}, respectively (Gupta et al. 2024). Similarly, RT-RPA assay for little cherry virus detection could detect the virus from

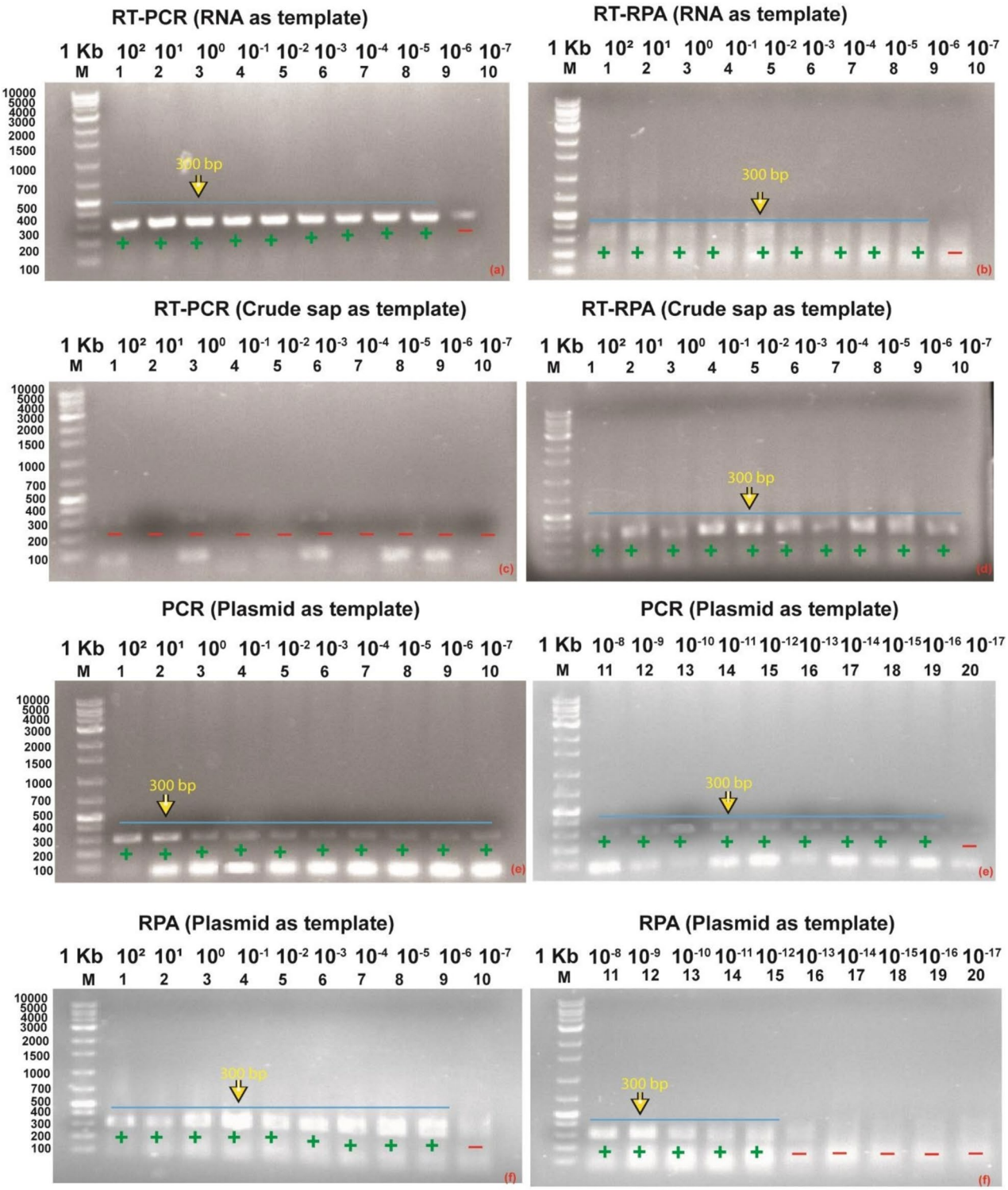

Fig. 3 Comparative analytical sensitivity of RT-PCR (crude sap/RNA as template), RT-RPA (crude/RNA as template) and RPA (Plasmid as template). Lane M: GeneRuler™ 1 kb ladder (G-Bio-Sciences), Lane NC: negative control, Lane 1–11: shows different dilutions i.e. 10^{2}, 10^{1}, 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}; RT-PCR. **a** Initial concentration of RNA used is 100 ng and it shows the sensitivity up to 10^{-8} dilutions. **b** Initial concentrations of RNA used is 100 ng and it shows the sensitivity up to 10^{-7} dilutions; Crude RT-PCR. **c** Initial 1:1 volume of crude sap prepared by 0.5N NaOH lysis buffer was used and no amplification in any dilutions, **d** Initial 1:1 volume of crude sap prepared by 0.5N NaOH lysis buffer was used and it shows the sensitivity up to 10^{-7} dilutions Plasmid-PCR. **e** Initial concentration of plasmid having PeMoV insert used is 100 ng and it shows the sensitivity up to 10^{-18} dilutions in PCR, **f** Initial concentration of plasmid having PeMoV insert used is 100 ng and it shows the sensitivity up to 10^{-12} dilutions in RPA

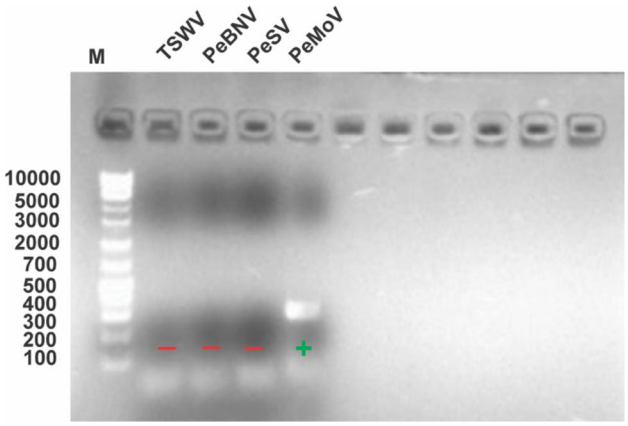

Fig. 4 Validation of RT-RPA assay for its detection and specificity towards PeMoV infected plants and absence of amplification from the plants and absence of amplification from the plants infected with other viruses viz., Tomato spotted wilt virus (TSWV), Peanut bud necrosis virus (PeBNV) and Peanut stunt virus (PeSV) using 2F and 2R amplifying 300 bp of amplicon where, Lane M: GeneRulerTM 1 kb DNA ladder (Gene BioScience). 1: TSWV (Negative amplification) 2. PeBNV (Negative amplification) 3: PoSV (Negative amplification) and 4: PeMoV (Positive amplification)

crude sap of infected leaf up to 10^{-2} dilution (Mekuria et al. 2014). An RT-RPA assay detected plum pox virus infection using RNA and crude leaf sap templates of up to 1 fg/µL and 10^{-4} dilution, respectively through lateral flow-based RT-RPA assay (Zhang et al. 2014). Similar crude leaf sap template-based RT-RPA assay could detect citrus tristeza virus up to 10^{-8} dilution (Sharma et al. 2023). Despite the importance of PeMoV, RT-RPA-based assay for its rapid detection is still lacking. Thus, in the present study, we developed an RT-RPA assay for rapid detection of PeMoV for the first time. Like other RT-RPA assays, the main power of the established RT-RPA method for PeMoV diagnostics is that it is simple and can be performed in a low resource setting, specific and robust even with crude leaf sap as template instead of RNA. RT-RPA assay excludes laborious RNA isolation, nucleic acid concentration optimization, and cDNA preparation, which are prerequisites in the conventional nucleic acid-based assays.

The primer set (PeMoV 2F/2R) used in the detection assay is highly target-specific and did not show any cross-reaction with other viruses infecting peanut crop. Specific amplification was observed for all the symptomatic suspected plants. Besides, the developed assay could detect PeMoV in a few asymptomatic samples, which was further confirmed through RT-PCR. The developed RT-RPA and RT-PCR assays showed confirmatory positive amplification when dilutions were made from PeMoV infected plants up to 10^{-7} (0.1 µg/µl) and 10^{-6} (1 µg/µl) dilutions, respectively. Interestingly, RT-RPA assay using crude sap as template was equally sensitive as RT-RPA assay, whereas RT-PCR could not detect PeMoV while using crude leaf sap template. Thus, the developed RT-RPA assay can be reliably exploited and used for screening peanut crops for PeMoV infection in field conditions and quarantine stations. RT-PCR requires significantly more time and cost compared to RT-RPA using crude sap because the traditional RT-PCR method involves several intermediate steps such as RNA extraction, cDNA preparation, gel electrophoresis, and documentation. It also requires costly equipment like a thermal cycler (Londoño et al. 2016) and takes approximately 4–5 h from the preparation of the sample to the completion of the assay. On the other hand, when RT-RPA assay is put to use with crude sap of leaf as template, the entire process is completed within 40 min to 1 h, and is found to be less expensive and portable. The ability of RT-RPA to detect PeMoV in crude sap could be attributed to the 1 N NaOH lysis buffer used in extracting crude leaf extract preparation that results in more stability in template preparation and tolerance to RNA inhibitors present in crude leaf sap of infected plants and the chemicals in the RPA kit (TwistDx, Cambridge, United Kingdom) that preventing fluctuation in pH leading to more RNA retention and amplification (Tomar et al. 2022). Conventional RT-PCR did not amplify PeMoV from crude sap, possibly because of the presence of RNAse in plant extracts. Thus, RT-RPA is sensitive and is relatively cheaper than RT-PCR when crude leaf extract is used as template (Devi et al. 2023). While comparing RT-PCR and RT-RPA assay, the total time required from sample preparation to obtaining final results in RT-RPA is one to one and half hours and costs about 290 Rupees/sample, which is five times cheaper than RT-PCR that requires 3.5 h' time and about 1300 Rupees/sample (Table 2). Therefore, the developed RT-RPA can be used for the detection of PeMoV using crude sap as template which is cheaper and consumes less time when compared to RT-PCR. To further increase the efficiency of the RT-RPA assay, ssDNA reporter can be added to check the virus presence through naked eye, which would further reduce the reaction time. However, the procedure needs to be standardized by performing the same assay on different PeMoV RNA concentrations.

Conclusion

In comparison with the conventional plant viral detection assays, the advantage of Recombinase Polymerase Amplification (RPA) lies in the requirement of a minimal resource setting dispensing off the need for sophisticated equipment like thermal cycler and template preparation, thereby reducing the associated cost and time. Even though PeMoV infected crude leaf sap obtained using 1N NaOH lysis buffer is a simple template that can be used in RT-RPA reaction, is reliable, robust, portable, affordable, and shows great potential for large-scale screening of

Table 2 Comparative study of RT PCR and RT RPA technique in terms of equipment and reagents required, time consumed, limit of detection and total cost per reaction in Indian rupees (INR)

	RT PCR			RT-RPA		
	Crude Sap as template	RNA as template	Plasmid containing PeMoV insert as template	Crude Sap of leaf as template	RNA as template	Recombinant plasmid with PeMoV template
Required instruments	Biometra trio analytical Jena Thermal cycler (~1,00,000–2,00,000 INR)			Dry bath or water bath (~42,000 INR)		
Required kits	RNA isolation kit (~43,696.93INR/50 reaction) and cDNA preparation kit (~12,305.46 INR/30 reaction), GSURE plasmid mini kit (~55,022.64 INR/250 reaction)			RPA kit (~56,000 INR/96 rxn: only ½ reaction is used to sample to be processe		
Completion time	~3.5 h	~4–6 h	~24 h	~1–1.5 h	~2–2.5 h	~24 h
Limit of detection	Nil	Up to 10^{-7} dilution (0.1 µg/µl)	Up to 10^{-16} dilution (0.0001 pg/µl)	Up to 10^{-7} dilution (0.1 µg/µl)	Up to 10^{-6} dilution (1 µg/µl)	Up to 10^{-12} dilution (0.0001 pg/µl)
Cost (in INR)	~1300 INR/Reaction		~1300 INR/ reaction	~290 INR/Reaction	~1160 INR/reaction	~1200 INR /reaction

PeMov samples in laboratories with limited resources. The standardized RT-RPA assay showed high specificity without cross-reacting with other viruses infecting peanut with a Limit of Detection (LOD) up to 10^{-7} dilution of crude sap. RT-RPA assay showed resistance to the RNA inhibitors found in the crude leaf sap, and thus the developed assay is highly useful for the diagnosis of PeMoV in onsite detection in farm and glasshouse conditions, preliminary screening, and quarantine stations. Overall, the developed assay is valuable for onsite detection and management of PeMoV in India and elsewhere where PeMoV is common.

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Data availability Sequence obtained in this study was submitted to NCBI GenBank with accession number: PP998001. The data that support the findings of this study are openly available in this manuscript and in the Supporting Information attached.

Declarations

Conflict of 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.

Ethical statement This article does not contain any studies with human or animal subjects performed by any of the authors.

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