Journal of Virological Methods 263 (2019) 81–87

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Detection of *Mesta yellow vein mosaic virus* (MeYVMV) in field samples by a loop-mediated isothermal amplification reaction

Prabhu Narayan Meena[a,*], Lalit Laxman Kharbikar[a,b,*], Rajeev Singh Rana[a], Subrata Satpathy[a], Arti Shanware[c], Palaiyur Nanjappan Sivalingam[b], Shweta Nandanwar[d,1]

[a] ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, 700120, India
[b] ICAR-National Institute of Biotic Stress Management, Baronda, Raipur, 493225, India
[c] Rajiv Gandhi Biotechnology Centre, RTM Nagpur University, Nagpur, 440033, India
[d] Harper Adams University, Newport, Shropshire, TF10 8NB, United Kingdom

ARTICLE INFO

Keywords:
Mesta yellow vein mosaic virus
Kenaf
Roselle
Virus detection
LAMP
PCR

ABSTRACT

A loop-mediated isothermal amplification (LAMP) assay was optimized for the detection of *Mesta yellow vein mosaic virus* (MeYVMV) in diseased plants of mesta (*Hibiscus sabdariffa* L.& *H. cannabinus* L.). The LAMP assay was optimized using a set of six primers targeting the MeYVMV genome and could be completed in 30–60 min at 63 °C. The LAMP amplification results were visualized by adding 1 µl of hydroxy naphthol blue (HNB) dye in a 25 µl LAMP reaction mixture prior to amplification as well as by electrophoresis. The LAMP assay, which detected MeYVMV in a 10^{-5}-fold diluted total DNA, was more sensitive than the PCR assay (10^{-4}-fold dilution). The optimized LAMP assay was able to detect MeYVMV in different parts of the kenaf and roselle plants. Similarly, the optimized PCR assay was also capable of detecting MeYVMV in all the different parts of the kenaf plant but failed to detect the virus in the stem and flower buds of the roselle plant. Validation of the LAMP and LAMP with HNB dye assays revealed that the optimized reactions can be used successfully for the *in-situ* detection of MeYVMV in field samples and in virus quarantine programs. This is the first report of the detection of the begomovirus species, MeYVMV, in the mucilaginous plant species, kenaf and roselle, using a LAMP assay.

1. Introduction

Kenaf (*Hibiscus cannabinus* L.) and roselle (*H. sabdariffa* L.) are annual, herbaceous, lignocellulosic crops belonging to the family *Malvaceae*. These crops are grown in several countries in the world including India (Mahadevan et al., 2009). In India, both crops are grown for their bast fibres which provide traditional packaging material and a good quality pulp for the production of paper. Both these crops are also used as leafy vegetables and their seeds are rich in oil which has a low proportion of unsaturated fatty acids and has potential medicinal values (Duke, 1986; Pal and Jain, 1998; Wong et al., 2014)

Apart from infections by several pathogens, endemic infections of mesta by a monopartite *begomovirus*, identified as *Mesta yellow vein mosaic virus* (MeYVMV), have also been reported in different parts of India (Chatterjee et al., 2005a, 2005b, 2006, Chatterjee and Ghosh, 2007a, b; Das et al., 2008; Roy et al., 2009). The genome of this virus contains a single circular single-stranded DNA A of 2.6–2.7 kb in size which is encapsidated in a twinned quasi-icosahedral particle and is associated with betasatellites (Das et al., 2008; Roy et al., 2009). MeYVMV is transmitted by whiteflies (*Bemisia tabaci)* in a persistent manner. This virus reduces fibre and seed yields by 18 and 24%, respectively (Roy et al., 2008). The symptoms of MeYVMV infections are characterized by yellowing of the veins and veinlets and erratic chlorotic flakes in the veins and veinlets, leading to a complete chlorosis of the leaves, leaf laminae and stems (Chatterjee et al., 2006). When infected at an early growth stage, the plants do not bear flowers, whereas an infection at a late growth stage results in malformed flowers and fruits, causing low seed yields. In general, the infected plant shows stunted growth with reduced leaf size.

Both polymerase chain reaction (PCR)-based and non-PCR-based diagnostic methods had been used successfully for the detection of MeYVMV in host plants (Chatterjee et al., 2005a, 2007; Ghosh et al.,

* Corresponding authors.
E-mail addresses: pnshera@yahoo.co.in (P.N. Meena), lalitkharbikar@gmail.com (L.L. Kharbikar), rajeevrana08@gmail.com (R.S. Rana), satp1@rediffmail.com (S. Satpathy), artishanware@gmail.com (A. Shanware), pnsivalingam@gmail.com (P.N. Sivalingam), shwetanandanwar22@gmail.com (S. Nandanwar).
[1] Resigned

https://doi.org/10.1016/j.jviromet.2018.10.016
Received 23 January 2018; Received in revised form 5 October 2018; Accepted 20 October 2018
Available online 22 October 2018
0166-0934/

Table 1
Name of the virus species, isolates and their accession numbers used for designing primers.

Serial Number	Name of the virus species and isolates	NCBI GenBank Accession number
1	*Cotton leaf curl Bangalore virus* (CLCuBV)	AY705380
2	*Cotton leaf curl Multan virus* (CLCuMuV)	JN807763
3	*Eclipta* yellow *vein virus* (EcYVV)	KT390456
4	*Malvestrum* yellow *vein virus* (MaYVV)	AJ744881
5	*Mesta* yellow *vein mosaic Bahraich virus* (MeYVMBaV)	EU360303
6	*Okra enation leaf curl virus* (OELCuV)	GU111996
7	*Mesta* yellow *vein mosaic virus* (MeYVMV [IN:Bar2:06])	EF428256
8	*Mesta* yellow *vein mosaic virus* (MeYVMV [PK-CM-09])	FR715681
9	*Mesta* yellow *vein mosaic virus* (MeYVMV-Ben [IN: Bon-LC-07])	FJ345400

2007). Although the PCR-based methods are widely used, they have several intrinsic disadvantages, such as the requirement for rapid thermal cycling instruments and skilled technicians to operate them, occasional low amplification efficiency and time-consuming. Thus, a simple DNA amplification technique, known as loop-mediated isothermal amplification (LAMP), was adapted for the detection of MeYVMV in the present study.

LAMP, developed by Notomi et al. (2000), is based on the principle of auto-cycling, strand displacement DNA synthesis performed by the *Bacillus stearothermophilus* (*Bst*) DNA polymerase. Since four to six primers are used to amplify six to eight regions of the target DNA, the technique is considered to be highly specific (Nagamine et al., 2002; Notomi et al., 2000). The amplification can be performed under isothermal temperatures between 60 °C and 65 °C which results in a large number of copies of the target DNA (Notomi et al., 2000). The reaction shows a high tolerance to biological contaminants (Kaneko et al., 2007). Like PCR, the LAMP amplification products can be detected by agarose gel electrophoresis and, for field application, hydroxy naphthol blue (HNB) dye can be used to detect the amplification products through a visual colourimetric assay (Goto et al., 2009). LAMP assays had been used successfully for the detection of different viruses infecting plants and animals (Fukuta et al., 2003a & 2003b; Yoshikawa et al., 2004; Hong et al., 2004; Parida et al., 2004; Kaneko et al., 2005; Nie, 2005; Pham et al., 2005; Varga and James, 2006; Dukes et al., 2006; Kuan et al., 2010). In the present study, for the first time, a LAMP assay was optimized for the detection of MeYVMV in field samples of kenaf and roselle plants and can be used in quarantine programs.

2. Material and methods

2.1. Plant materials and viruses

A population of whiteflies was collected in 2013 from kenaf and roselle plants showing severe symptoms of MeYVMV infection in the experimental fields maintained at ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, West Bengal, India. These whiteflies were released on healthy seedlings of kenaf and roselle growing in pots under controlled environmental conditions at 28–30 °C. After 2–3 weeks, leaves of the whitefly-inoculated plants showing symptoms typical of MeYVMV infection were harvested and stored at −80 °C. These samples were confirmed infection of MeYVMV by PCR and sequence before using into LAMP assay. Similarly, leaves from healthy plants, maintained under the same environmental conditions without whitefly inoculated, were also harvested and stored at −80 °C for use as controls. Tomato leaves with Tomato leaf curl disease (ToLCD) symptoms, mungbean leaves with yellow mosaic disease (MYMD) symptoms, bhendi leaves with yellow vein mosaic disease (BYVMD) symptoms and cotton leaves with leaf curl disease (CLCD) symptoms were also included in this study.

2.2. DNA extraction

Total DNA was extracted from each 150 mg samples of leaves, stem and flower buds of kenaf and roselle according to the method described by Doyle and Doyle (1987) with some minor modifications. During DNA extraction, 2 µl of RNase A was added to each sample and incubated at 37 °C for 10 min to remove RNA. After incubation, one millilitre of dichloromethane was added to each of the samples and centrifuged at 12,000 × g for 15 min. The DNA was precipitated from the supernatants and washed. Finally, the DNA pellets were dried and re-suspended in 1x TE buffer (10 mM Tris − HCl, 1 mM EDTA, pH 8). The same method was used to extract DNA from leaves of field samples of kenaf and roselle and, DNA from tomato leaves with ToLCD symptoms, mungbean leaves with YMD symptoms, bhendi leaves with YVMD symptoms and cotton leaves with CLCD symptoms were tested. The DNA of the whiteflies was extracted as described previously (Fukuta et al., 2003a).

2.3. LAMP and PCR primer design

The nucleotide sequences of the DNA A of various begomovirus species infecting *H. cannabinus* and *H. sabdariffa* (Brown and Zerbini, 2015; Malathi et al., 2017), were compared with the DNA A of various MeYVMV isolates, MeYVMV [IN:Bar2:06] (EF428256), MeYVMV [PK-CM-09] (FR715681) and MeYVMV-Ben [IN:Bon-LC-07] (FJ345400) (Table 1) (Supplementary Fig. 1). Three pairs of primers were designed on the basis of consensus nucleotide sequences among the MeYVMV isolates with the aim of amplifying all the isolates of MeYVMV (Table 2). For the LAMP assay, totally three pairs of primers; one pair of each inner/internal primers (FIP and BIP), outer/external primers (F3 and B3) and loop primers (B-Loop [LB] and F- Loop [LF]) were designed using the Primer Explorer V4 server (http://primerexplorer.jp/e/). For the PCR assay, only one pair; outer/external primers, *viz.*, F3 and B3, were used as the forward and backward primers, respectively.

2.4. Optimization of the LAMP assay

The specific reagents and enzymes used in the LAMP assay, Thermopol buffer (New England Biolabs, Ipswich, Massachusetts, USA), betaine (Sigma Chemicals, Kolkata, India), Bst DNA polymerase (large fragment; New England Biolabs), template DNA, dNTPs, $MgSO_4$ and primers, were first optimized by testing different concentrations and volumes of these components in the LAMP reaction (Supplementary Table 1 and 2).

The amplification temperature was then optimized by testing the reaction at 61 °C, 62 °C, 63 °C and 65 °C for 60 min and, finally, the amplification time was optimized by testing the reaction at the optimized temperature for 30, 45, 60 and 75 min, respectively. All the reactions were terminated by heat inactivation at 80 °C for 10 min. The reactions were performed in a gradient thermal cycler (Eppendorf, India) as well as in a water bath (JetoTech, India) in order to evaluate the suitability of the LAMP assay for field application.

Table 2
Oligonucleotide primers used in polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) assays to detect *Mesta yellow vein mosaic virus* (MeYVMV).

Assay	Primer	Type	Length of primer	Sequence (5' - 3')[a]	Genome position
PCR	F3	Forward	21 nt	AGTCATTGTATTTTGTTGGCA	626-646
	B3	Backward	18 nt	AAAACGATCACGCTGGTC	802-819
LAMP	F3	Forward outer	21 nt	AGTCATTGTATTTTGTTGGCA	626-646
	B3	Backward outer	18 nt	AAAACGATCACGCTGGTC	802-819
	FIP	Forward inner	46 nt	ACGCCTATCTCTAACAATCCAAA-TGGATGGACGAGAACATCAAGAC	701-723, 654-674
	BIP	Backward inner	40 nt	CTTCAGGAACCCCAAACGATTT-TCTTCACAGTAGCCGTAG	725-746, 782-799
	LF	Loop Forward	16 nt	TTGGATGGACGAGAAC	651-666
	LB	Loop Backward	25 nt	GTTCAATGTTTATGATAACGAGCCC	756-780

[a] Nucleotide sequence of MeYVMV (GenBank accession number: EF428256) available in NCBI database was used for designing the primers.

The optimized assay was used to test the specificity of the LAMP primers. Total DNA samples (100 ng) from MeYVMV-infected kenaf and roselle plants and other plant samples were tested. Reactions containing DNA from the respective healthy leaves and those without any template DNA were used as controls. The assays were visualized by adding 1 µl of HNB dye in a 25 µl LAMP reaction mixture prior to amplification. The results were also assessed by electrophoresis of the LAMP products in 2% agarose gels.

2.5. Optimization of the PCR assay

PCR assay was optimized with various concentrations of different components: 0.5, 1.0, 1.5 U Taq DNA polymerase, 0.5, 1.0, 1.5 mM $MgCl_2$ and 50 ng,100 ng, 150 ng, 200 ng of DNA extracted. The optimized concentrations of PCR components were used in this study. The final reaction was carried out in a 25 µl reaction and each reaction contains 1x PCR buffer (Qiagen, India), 0.2 mM of the total dNTPs (Invitrogen, India), 1 U of Taq DNA polymerase (Qiagen, India), 0.2 µM of each of the forward and reverse primer 1 mM $MgCl_2$, 100 ng of DNA and final volume made to 25 µl with nuclease-free sterile double-distilled water. The reaction consisted of an initial incubation at 94 °C for 2 min, followed by 30 cycles of melting at 94 °C for 15 s, annealing at 53 °C for 30 s and extension at 68 °C for 3 min, with a final extension at 72 °C for 10 min. The reactions were performed in a gradient thermal cycler (Eppendorf, India) and the products were resolved in 1.6% agarose gels stained with ethidium bromide.

2.6. Comparison of the sensitivities of the LAMP and PCR assays

The limits of detection of MeYVMV by the LAMP, a LAMP with HNB dye and the PCR assays were compared using a 10° to 10^{-5} serial dilutions of a DNA sample (*ca.* 100 ng) purified from MeYVMV-infected kenaf leaves. These reactions were performed using the respective optimized conditions and visualized as described.

2.7. Evaluation of LAMP for the detection of MeYVMV in different plant parts

DNA (*ca.* 100 ng) extracted from each plant part of an infected plant was used for MeYVMV detection by the optimized LAMP, a LAMP with HNB dye and PCR assays, respectively. DNA (*ca.* 100 ng) from the corresponding uninfected plant parts were used as negative controls. The assays were done in duplicates.

2.8. Field validation of the LAMP assay

The optimized LAMP and LAMP with HNB dye assays were validated using DNA extracted from leaf samples harvested from field plants potentially infected with MeYVMV based on the visual symptoms and from whiteflies. The plant samples were obtained from field trials of kenaf conducted in 2015–2016 at three geographical locations in India. Ten samples each were harvested from the Advanced Varietal Trial - I (AVT-I) and Advanced Varietal Trial – II (AVT-II) conducted at the main farm of ICAR-CRIJAF, Barrackpore (22.76 °N 88.37 °E) and five samples were harvested from each of the field trials conducted at Cooch Behar (26.33 °N, 89.44 °E) and Amadalavalasa (18.41 °N 83.90 °E). The whiteflies were collected from the AVT-I and AVT-II field trials as well as from a field trial conducted at the south farm of ICAR-CRIJAF which was severely infected with MeYVMV in 2016.

3. Results

3.1. Optimization of the LAMP assay for the detection of MeYVMV

The optimal amounts of reagents in the assay (25 µl final volume) were 0.5 µl (*ca.* 100 ng) of template DNA, 2× Thermopol buffer (New England Biolabs, Ipswich, Massachusetts, USA), 1.4 mM each of the dNTPs, 2 mM $MgSO_4$, 1 M betaine (Sigma Chemicals, Kolkata, India), 0.2 µM of each of the external primers F3 and B3, 2 µM of each of the internal primers FIP and BIP, 1 µM of each of the loop primers (LB and LF) and 8 U of *Bst* DNA polymerase (large fragment; New England Biolabs) (Supplementary Table 2).

LAMP products were detected at 61, 62, 63 and 65 °C using as little as 50 ng DNA from MeYVMV-infected leaves of kenaf (Fig. 1A). Increasing or decreasing the temperature beyond this range (61–65 °C) resulted in no amplification. Very little difference was observed in the intensity of bands obtained at these temperatures. Therefore, the reactions were carried out at 63 °C for further optimization of the time required for amplification. All the reactions carried out for different amplification times (30, 45, 60 and 75 min, respectively) could produce bands at 63 °C (Fig. 1B). Irrespective of temperature, all the positive LAMP reactions changed colour from violet to sky blue and could be easily visualized by the naked eye at 60 min amplification time which was considered optimal for the detection of MeYVMV using the LAMP. A similar result was obtained while the same procedure was repeated three times with different samples of MeYVMV-infected leaves of kenaf.

All the three primer pairs designed for the LAMP assay were found to produce the expected amplicons (size ranging between 0.1 kb and 1.0 kb) with the DNA from MeYVMV-infected kenaf and roselle plants (Fig. 2A) but not with the DNA of the corresponding healthy plants. These results were also confirmed by the LAMP with HNB dye assay (Fig. 2B). When the HNB dye was added to the LAMP reaction containing DNA of the infected kenaf and roselle plants, the colour of the reaction changed from violet to sky blue. However, the addition of HNB dye to the LAMP assays of healthy kenaf and roselle plants did not change the reaction colour. Similarly, there was no positive amplification of the DNA of ToLCD, MYMD, BYVMD and CLCD by the LAMP primers, indicating that these primers were specific to MeYVMV.

3.2. Optimization of the PCR assay for the detection of MeYVMV

The PCR optimisation results revealed that an amplification product

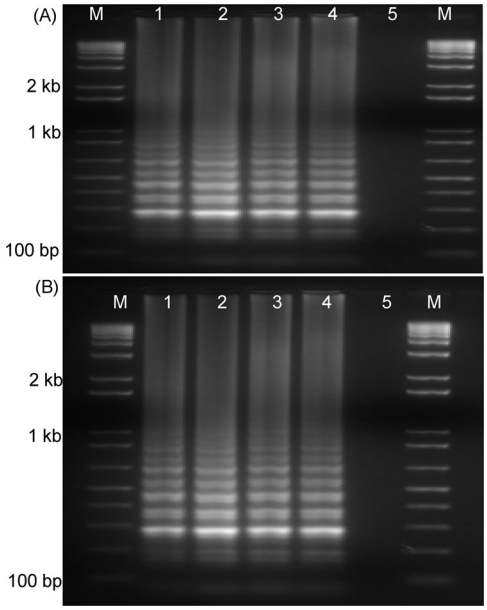

Fig. 1. Loop-mediated isothermal amplification (LAMP) reaction products of MeYVMV, infecting kenaf and roselle plants, at different amplification temperatures (A) and time (B) respectively. Very little difference was observed in the intensity of bands obtained at amplification temperatures of 61 °C (Lane 1), 62 °C (Lane 2), 63 °C (Lane 3) and 65 °C (Lane 4). Therefore, the reactions were carried out at 63 °C for different amplification timings of 30 min (lane 1), 45 min (lane 2), 60 min (lane 3) and 75 min (lane 4). Lane 5: LAMP with DNA from healthy plants (negative control) and Lane M: 1 KB Plus DNA ladder.

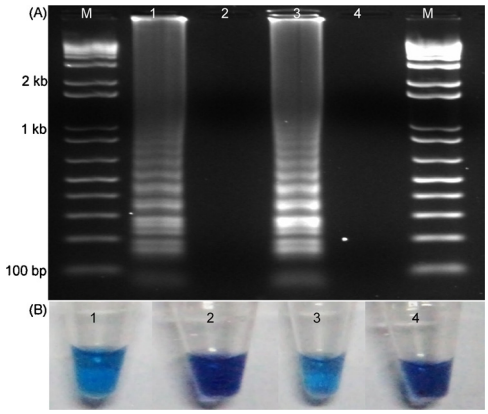

Fig. 2. Specificity of primers designed for LAMP assay in the present study. The primers were found to be specific for MeYVMV only but not for its hosts, kenaf and roselle, respectively. Amplified products were electrophoresed on 2% agarose gel (A) and also visualized by adding HNB dye prior to amplification (B). In electrophoresis, lane 1: amplified DNA extracted from MeYVMV infected kenaf plant, lane 2: amplified DNA extracted from healthy kenaf plant (negative control), lane 3: amplified DNA extracted from MeYVMV infected roselle plant, lane 4: amplified DNA extracted from healthy roselle plant (negative control), lane M: 1KB plus DNA ladder. In LAMP with HNB dye assay, tube 1: amplified DNA extracted from MeYVMV infected kenaf plant, tube 2: amplified DNA extracted from healthy kenaf plant (negative control), tube 3: amplified DNA extracted from MeYVMV infected roselle plant, tube 4: amplified DNA extracted from healthy roselle plant (negative control).

of 190 bp was produced at the annealing temperatures of either 53, 54 or 55 °C in assays of MeYVMV-infected samples. However, the intensity of the amplification product under the UV-transilluminator was highest at 53 °C (data not shown). The negative control did not produce any band (data not shown).

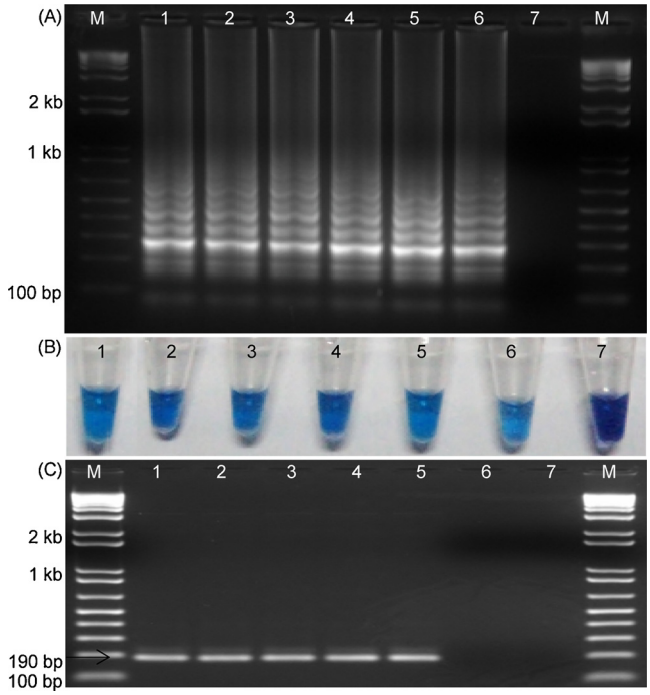

Fig. 3. Comparison of sensitivities of LAMP (A), LAMP with HNB dye (B) and PCR (C) for MeYVMV detection. The serial dilutions (from 1 to 10^{-5}) of DNA (*ca.* 100 ng) extracted from MeYVMV infected leaves of kenaf were analyzed with LAMP and PCR and electrophoresed on 2% and 1.6% agarose gels, respectively (Lane 1: 100 × 1, Lane 2: 100 × 10^{-1}, Lane 3: 100 × 10^{-2}, Lane 4: 100 × $10^{-3,}$ Lane 5: 100 × 10^{-4}, Lane 6: 100 × 10^{-5}, Lane 7: Reactions with DNA from healthy plants (negative control) in both (A) and (C) and Lane M: 1KB plus DNA ladder in (A) and (C). In LAMP with HNB dye assay, tube 1: 100 × 1, tube 2: 100 × 10^{-1}, tube 3: 100 × 10^{-2}, tube 4: 100 × 10^{-3}, tube 5: 100 × 10^{-4}, tube 6: 100 × 10^{-5} and tube 7: Reaction with DNA from healthy plants (negative control).

3.3. Comparison of the sensitivities of the LAMP and PCR assays

As little as the 10^{-5} concentration of genomic DNA of MeYVMV infected plant (0.01 ng) was detectable by both LAMP and LAMP with HNB dye assays, respectively (Fig. 3A and B). However, the highest dilution of MeYVMV genomic DNA detectable by a PCR assay was 10^{-4} (Fig. 3C).

3.4. Evaluation of the LAMP assay with different parts of a MeYVMV–infected plant

The optimized LAMP assay was capable of detecting MeYVMV in leaves, stems and flower buds of inoculated kenaf and roselle plants (Fig. 4). The expected products of the LAMP reactions were seen on the agarose gel (Fig. 4A). Similarly, all the positive LAMP reactions changed colour from violet to sky blue and could be easily visualized by the naked eye (Fig. 4B). However, the PCR assay was capable of detecting MeYVMV in leaves, stems and flower buds of inoculated kenaf and leaves of inoculated roselle only, but not the stem and flower buds of inoculated roselle (Fig. 4B). The result was reproducible when the same procedure was repeated three times with different samples of MeYVMV-infected plant parts.

3.5. Validation of the LAMP assays for field samples

The results of the validation of the LAMP assay using a total of 25 samples revealed that the optimized reaction successfully detected MeYVMV in field samples (Fig. 5A). All the naturally infected leaf samples from the different geographical locations in India produced the

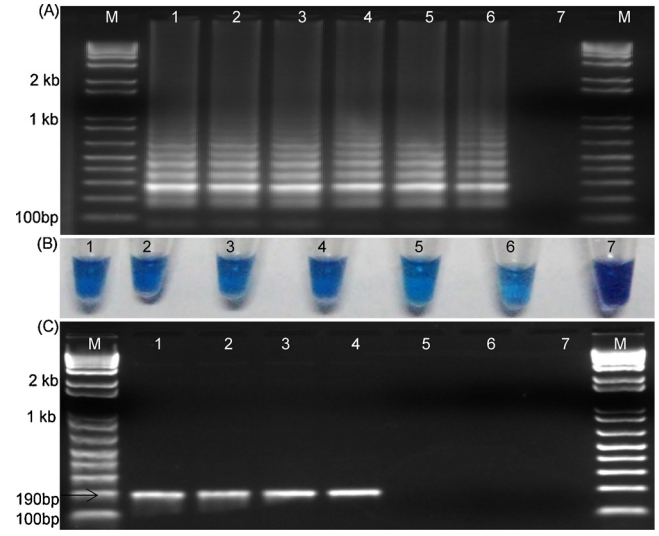

Fig. 4. Evaluation of LAMP assay with different plant parts infected by MeYVMV. The LAMP assay (A) was capable of detecting MeYVMV in leaves (lane 1 and lane 4), stems (lane 2 and lane 5) and flower buds (lane 3 and lane 6) of inoculated kenaf and roselle plants. The LAMP with HNB dye assay (B) was capable of detecting MeYVMV in leaves (tube 1 and tube 4), stems (tube 2 and tube 5) and flower buds (tube 3 and tube 6) of inoculated kenaf and roselle plants. The PCR assay (C) was capable of detecting MeYVMV in leaves (lane 1), stems (lane 2) and flower buds (lane 3) of inoculated kenaf and leaves (lane 4) of roselle, but not in stems (lane 5) and flower buds (lane 6) of roselle plants. Lane 7: Reactions with DNA from healthy plants (negative controls) in both (A) and (C), tube 7: Reaction with DNA from healthy plants (negative control) in (B), lane M: 1 kb plus DNA ladder in (A) and (C).

expected amplicons of the LAMP reaction and confirmed the presence of MeYVMV (lanes 1, 2, 3, 4, 5 for Cooch Behar; lanes 6, 7, 8, 9, 10 for AVT-I and lanes 11, 12, 13, 14, 15 for AVT-II in Barrackpore and lanes 16, 17, 18, 19, 20 for Amadalavalasa). The healthy leaf samples, used as negative controls, did not produce the expected amplicons in the LAMP reactions (lane 24). The whiteflies collected from AVT-I, AVT-II and the south farm also produced the expected amplicons in the LAMP reactions and confirmed for the presence of MeYVMV (lanes 21, 22 and 23). The laboratory-reared virus-free whiteflies, used as negative control, did not produce the expected amplicons (lane 25). The samples which produced a positive amplification in the LAMP assays also showed a colour change reaction from violet to sky blue in the presence of the HNB dye (Fig. 5B).

4. Discussion

Kenaf and roselle plants having a rigid cell wall and high amounts of mucilage. Comparatively, PCR could amplify only up to a four-fold dilution of the 100 ng MeYVMV DNA sample. Since, mucilage often binds to secondary metabolites such as phenolics, tannins and alkaloids and co-precipitates with DNA its presence in kenaf and roselle makes the DNA extraction difficult (Roy et al., 2009) which results in a low yield of DNA. In the present study, a LAMP assay was optimized and evaluated for the detection of MeYVMV in infected parts of kenaf and roselle plant. However, a low yield of DNA, as in the present study, was found to be suitable for the detection of MeYVMV using the LAMP assay. This indicated that the LAMP assay could detect a lesser quantity of the target virus DNA compared to normal PCR. Therefore, the LAMP assay is useful for plants yielding low quantities of the target DNA, such as due to difficulties in extraction. Indeed, the LAMP assay successfully amplified the target DNA from a five-fold dilution of a 100 ng of MeYVMV infected kenaf and roselle plant DNA in this study.

Successful primer design is an important limiting factor for the general use of LAMP in virus diagnosis. More non-specific reactions are usually observed in LAMP assays compared to conventional PCR when the primers used are not well designed (Boubourakas et al., 2009; Notomi et al., 2000; Wei et al., 2012). In the present study, the two outer primers (F3 and B3) used in LAMP assay were also successfully utilized in the PCR assay. Therefore, separate primers for PCR assay is not required as described in previous studies (Almasi et al., 2013; Keizerweerd et al., 2015; Zhao et al., 2016).

The optimum amplification temperature of the LAMP assay for the detection of MeYVMV ranged between 61 and 65 °C. This might be due to the inactivation of the DNA polymerase or the instability in the reaction mixture caused by too high or low temperatures (Fukuta et al., 2004; Wei et al., 2012). The optimum amplification temperatures observed in various LAMP assays were 61 to 65 °C for the detection of *Peach latent mosaic viroid* (Boubourakas et al., 2009), 61 to 67 °C for *Squash leaf curl virus* (Kuan et al., 2010) and 61 to 65 °C for the detection of MeYVMV (in the present study). This could be due to the different sets of primer pairs used for the amplification of the DNA from the different viruses. When determining the optimum reaction time for the LAMP assay using a thermal cycler, very little difference was observed in the intensity of bands obtained with the different time durations. The objective of this study was to develop a LAMP assay suitable for the *in-situ* detection of MeYVMV in field samples, therefore it is recommended that the 60 min time is suitable for the HNB dye to detect the virus.

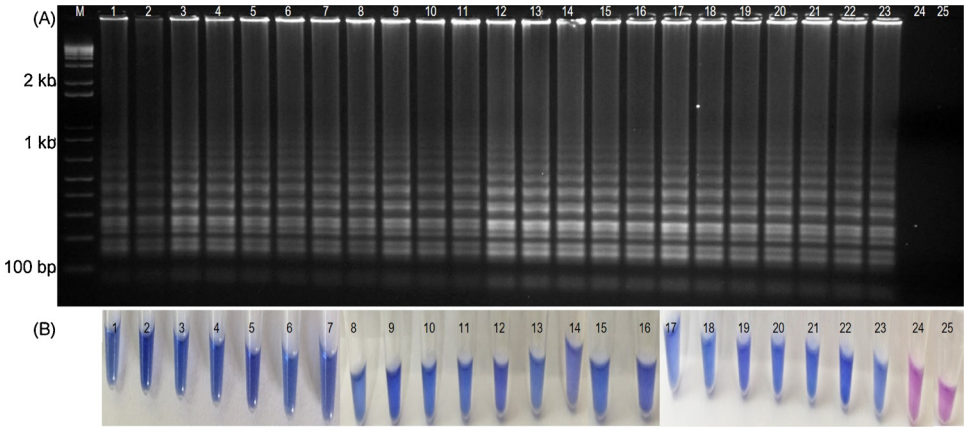

Fig. 5. Validation of LAMP (A) and LAMP with HNB dye (B) assays optimized in the present study to confirm the presence of MeYVMV in naturally infected samples. All the leaf samples obtained from different geographical locations in India produced the expected amplicons of LAMP reactions and positive results with HNB dye. In LAMP, lanes 1, 2, 3, 4 and 5: Samples from Cooch Behar, lanes 6, 7, 8, 9 and 10: samples from AVT-I at Barrackpore, lanes 11, 12, 13, 14 and 15 samples from AVT-II at Barrackpore, lanes 16, 17, 18, 19 and 20 samples from Amadalavalasa. The whiteflies collected from AVT-I (lane 21), AVT-II (lane 22) and the south farm (lane 23) also produced the expected amplicons of LAMP reactions. In LAMP with HNB dye assay, tubes 1, 2, 3, 4 and 5: Samples from Cooch Behar, tubes 6, 7, 8, 9 and 10: samples from AVT-I at Barrackpore, tubes 11, 12, 13, 14 and 15 samples from AVT-II at Barrackpore, tubes 16, 17, 18, 19 and 20 samples from Amadalavalasa. The whiteflies collected from AVT-I (tube 21), AVT-II (tube 22) and the south farm (tube 23) also produced the positive results with HNB dye. The healthy leaf samples (lane 24 and tube 24) as well as healthy whiteflies (lane 25 and tube 25), used as negative controls, did not show positive results for the presence of MeYVMV. Lane M: 1 kb plus DNA ladder.

The LAMP assay for the detection of MeYVMV was found to be specific for the virus as these primers could not detect the other tested begomoviruses infecting kenaf and roselle. Although the possibility of the detection by the MeYVMV LAMP assay of another begomovirus species, Mesta yellow vein mosaic Bahraich virus (MeYVMBaV), infects kenaf and roselle could not be ruled out. However, this is unlikely as nucleotide sequence identity in the corresponding genomic region between MeYVMV and MeYVMBV was only 76% (Supplementary Fig. 1). Therefore, these primers would not detect MeYVMBV in these hosts.

The LAMP assay was capable of detecting MeYVMV in all the tested parts of the kenaf and roselle plants, while the PCR assay failed to detect the virus in the stem and flower buds of roselle. The reason for this might be due to the varying concentrations of MeYVMV in the different parts of the mesta species and the virus concentrations in the stems and flower buds of roselle were below the limit of detection by PCR.

Validation of the LAMP and LAMP with HNB dye assays showed that they can be successfully used for the *in-situ* detection of MeYVMV in field samples similar to *Tomato yellow leaf curl virus* (TYLCV) in infected tomato plants (Fukuta et al., 2003b). These assays could also be extended to virus indexing in quarantine programs. Measurement of the turbidity of magnesium pyrophosphate produced by the LAMP assays has been used for detecting various pathogens (Mori and Notomi, 2009; Nakao et al., 2010; Yamazaki et al., 2010). However, expensive equipment, such as a real-time turbidimeter, was required. Such equipment is not always available, especially in developing countries. Colourimetric detection provides the simplest and most cost-efficient way of identifying a positive or negative LAMP reaction. For better distinguishability in calorimetric detection, a DNA intercalating dye, SYBR Green, can be used after amplification (Hill et al., 2008; Parida et al., 2005). Colourimetric measurements of the LAMP assay can also be done by using an HNB dye before amplification. In the present study, the detection sensitivity of LAMP assay using HNB dye was similar to that of the assays using SYBR green (data not shown).

The LAMP assay optimized in this study was very useful for early detection of low titer levels of MeYVMV in field samples of kenaf and roselle. This assay will provide a simple, efficient and highly specific tool for the detection of MeYVMV in virus quarantine programs. This is the first report of detection of begomovirus in mucilaginous species like kenaf and roselle using the LAMP.

Acknowledgements

The first author is grateful to the Director and Head, Division of Crop Protection, ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, for providing support to undertake the study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.10.016.

References

Almasi, M.A., Jafary, H., Moradi, A., Zand, N., Ojaghkandi, M.A., Aghaei, S., 2013. Detection of coat protein gene of the Potato Leafroll Virus by reverse transcription loop-mediated isothermal amplification. Plant Pathol. Microbiol. 4, 156–161.

Boubourakas, I.N., Fukuta, S., Kyriakopoulou, P.E., 2009. Sensitive and rapid detection of *Peach latent mosaic viroid* by the reverse transcription loop-mediated isothermal amplification. J. Virol. Methods 160, 63–68.

Brown, J.K., Zerbini, et al., 2015. Revision of Begomovirus taxonomy based on pairwise sequence comparisons. Arch. Virol. 160, 1593–1619.

Chatterjee, A., 2007. Characterization of Yellow Vein Mosaic Disease of *Hibiscus cannabinus* and *H. Sabdariffa* and Its Etiological Agent. PhD Thesis. pp. 184.

Chatterjee, A., Ghosh, S.K., 2007a. A new monopartite begomovirus isolated from*Hibiscus cannabinus* L. in India. Arch. Virol. 152, 2113–2118.

Chatterjee, A., Ghosh, S.K., 2007b. Association of a satellite DNA b molecule with mesta yellow vein mosaic disease. Virus Genes 35, 835–844.

Chatterjee, A., Roy, A., Ghosh, S.K., 2006. Yellow vein mosaic disease of kenaf. In: In: Roa, G.P., Paul Khurana, S.M., Lenardon, S.L. (Eds.), In Characterization, Diagnosis and Management of Plant Viruses, vol. 1. Studium Press, Houston, TX, pp. 497–505 Industrial crops.

Chatterjee, A., Roy, A., Padmalatha, K.V., Malathi, V.G., Ghosh, S.K., 2005a. Yellow vein mosaic disease of Kenaf (*Hibiscus cannabinus*) and Roselle (*H. sabdariffa*) – a new disease in India caused by a *Begomovirus*. Indian J. Virol. 16, 55–56.

Chatterjee, A., Roy, A., Padmalatha, K.V., Malathi, V.G., Ghosh, S.K., 2005b. Occurrence of a *Begomovirus* with yellow vein mosaic disease of mesta (*Hibiscus cannabinus* and *Hibiscus sabdariffa*). Australas. Plant Pathol. 34, 609–610.

Das, S., Roy, A., Ghosh, R., Paul, S., Acharyya, S., Ghosh, S.K., 2008. Sequence variability and phylogenetic relationship of betasatellite isolates associated with yellow vein mosaic disease of mesta in India. Virus Genes 37, 414–424.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19, 11–15.

Duke, J.A., 1986. Isthmian Ethnobotanical Dictionary. Scientific Publisher, Jodhpur, India.

Dukes, J.P., King, D.P., Alexanderson, S., 2006. Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot and mouth disease virus. Arch. Virol. 151, 1093–1106.

Fukuta, S., Iida, T., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., Ishimoto, Y., 2003a. Detection of *Japanese yam mosaic virus* by RT-LAMP. Arch. Virol. 148, 1713–1720.

Fukuta, S., Kato, S., Yoshida, K., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., Ishimoto, Y., 2003b. Detection of *tomato yellow leaf curl virus* by loop-mediated isothermal amplification reaction. J. Virol. Methods 112, 35–40.

Fukuta, S., Ohishi, K., Yoshida, K., Mizukami, Y., Ishida, A., Kanbe, M., 2004. Development of immunocapture reverse transcription loop-mediated isothermal amplification for the detection of *Tomato spotted wilt virus* from chrysanthemum. J. Virol. Methods 121, 49–55.

Ghosh, R., Paul, S., Roy, A., Mir, J.I., Ghosh, S.K., Srivastava, R.K., Yadav, U.S., 2007. Occurrence of *begomovirus* associated with yellow vein mosaic disease of kenaf (*Hibiscus cannabinus*) in north India. Plant Health Prog. https://doi.org/10.1094/PHP-2007-0508-01-RS.

Hill, J., Beriwal, S., Chandra, I., Paul, V.K., Kapil, A., 2008. Loop-mediated isothermal amplification assay for rapid detection of common strains of Escherichia coli. J. Clin. Microbiol. 46, 2800–2804. https://doi.org/10.1128/JCM.00152-08.

Hong, T.C., Mai, Q.L., Cuong, D.V., Parida, M., Minekawa, H., Notomi, T., 2004. Development and evaluation of a novel loop-mediated isothermal amplification method of rapid detection of severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. 42, 1956–1961.

Kaneko, H., Iida, T., Aoki, K., Ohno, S., Suzutani, T., 2005. Sensitive and rapid detection of herpes simplex virus and varicella-zoster virus DNA by loop-mediated isothermal amplification. J. Clin. Microbiol. 43, 3290–3296.

Kaneko, H., Kawana, T., Fukushima, E., Suzutani, T., 2007. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J. Biochem. Biophys. Methods 70, 499–501.

Keizerweerd, A.T.1, Chandra, A.2, Grisham, M.P.3, 2015. Development of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of Sugarcane mosaic virus and Sorghum mosaic virus in sugarcane. J. Virol. Methods 212, 23–29.

Kuan, C., Wu, M., Lu, Y., Huang, H., 2010. Rapid detection of *Squash leaf curl virus* by loop-mediated isothermal amplification. J. Virol. Methods 169, 61–65.

Mahadevan, N., Shivali, Kamboj, P., 2009. *Hibiscus sabdariffa* Linn. An overview. Nat. Prod. Radiance 8, 77–83.

Malathi, V.G., Renukadevi, P., Chakraborty, S., Biswas, K.K., Roy, A., Sivalingam, P.N., Venkataravanappa, V., Mandal, B., 2017. Begomoviruses and their satellites occurring in India: distribution, diversity and pathogenesis. In book- A Century of Plant Virology in India. Springer, Singapore, pp. 75–177.

Mori, Y., Notomi, T., 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J. Infect. Chemother. 15, 62–69.

Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16, 223–229.

Nakao, R., Stromdahl, E.Y., Magona, J.W., Faburay, B., Namangala, B., Malele, I., 2010. Development of Loop-Mediated Isothermal Amplification (LAMP) assays for rapid detection of Ehrlichia ruminantium. BMC Microbiol. 10, 296.

Nie, X., 2005. Reverse transcription loop-mediated isothermal amplification of DNA for detection of Potato virus Y. Plant Dis. 89, 605–610.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, 63.

Pal, D.C., Jain, S.K., 1998. Tribal Medicine. Naya Prakash, Calcutta, India.

Parida, M., Horioke, K., Ishida, H., Dash, P.K., Saxena, P., Jana, A.M., Islam, M.A., Inoue, S., Hosaka, N., Morita, K., 2005. Rapid detection and differentiation of Dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay. J. Clin. Microbiol. 43, 2895–2903. https://doi.org/10.1128/JCM.43.6.2895-2903.2005.

Parida, M., Posadas, G., Inoue, S., Hasebe, F., Morita, K., 2004. Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. J. Clin. Microbiol. 42, 257–263.

Pham, H.M., Nakajima, C., Ohashi, K., Onuma, M., 2005. Loop-mediated isothermal amplification for rapid detection of Newcastle disease virus. J. Clin. Microbiol. 43, 1646–1650.

Roy, A., Acharyya, S., Das, S., Ghosh, R., Paul, S., Srivastava, R.K., Ghosh, S.K., 2009. Distribution, epidemiology and molecular variability of the begomovirus complexes associated with yellow vein mosaic disease of mesta in India. Virus Res. 141, 237–246.

Roy, A., De, R.K., Ghosh, S.K., 2008. Diseases of bast fiber crops and their management in jute and allied fibres. In: Karmakar, P.G., Hazara, S.K., Subramanian, T.R., Mandal, R.K., Sinha, M.K., Sen, H.S. (Eds.), Updates Production Technology. Central Research Institute for Jute and Allied Fibres, Barrckpore, West Bengal, India, pp. 327.

Varga, A., James, D., 2006. Use of reverse transcription loop-mediated isothermal amplification for the detection of Plum pox virus. J. Virol. Methods 138, 184–190.

Wei, Q., Yu, C., Zhang, S., Yang, C., Miriam, K., Zhang, W., Dou, D., Tao, X., 2012. One-step detection of *Bean pod mottle virus* in soybean seed by the reverse-transcription loop-mediated isothermal amplification. Virol. J. 9, 187.

Wong, Y.H., Tan, W.Y., Tan, C.P., Long, K., Nyam, K.L., 2014. Cytotoxic activity of kenaf (*Hibiscus cannabinus* L.) seed extract and oil against human cancer cell lines. Asian Pac. J. Trop. Biomed. 4 (Suppl 1), S510–S515. https://doi.org/10.12980/APJTB.4.2014C1090.

Yamazaki, W., Kumeda, Y., Misawa, N., Nakaguchi, Y., Nishibuchi, M., 2010. Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of the tdh and trh genes of Vibrio *parahaemolyticus* and related Vibrio species. Appl. Environ. Microbiol. 76, 820–828.

Yoshikawa, T., Ihira, M., Akimoto, S., Usui, C., Miyake, F., Suga, S., 2004. Detection of human herpesvirus 7 DNA by loop-mediated isothermal amplification. J. Clin. Microbiol. 42, 1348–1352.

Zhao, M.M., Shi, Y.H., Wu, L., Guo, L.C., Liu, W., Xiong, C., et al., 2016. Rapid authentication of the precious herb saffron by loop-mediated isothermal amplification (LAMP) based on internal transcribed spacer 2 (ITS2) sequence. Sci. Rep. 6, 25370. https://doi.org/10.1038/srep25370.