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Rapid detection of *Piper yellow mottle virus* and *Cucumber mosaic virus* infecting black pepper (*Piper nigrum*) by loop-mediated isothermal amplification (LAMP)

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The loop-mediated isothermal amplification (LAMP) assay for *Piper yellow mottle virus* and the reverse transcription (RT) LAMP assay for *Cucumber mosaic virus* each consisted of a set of five primers designed against the conserved sequences in the viral genome. Both RNA and DNA isolated from black pepper were used as a template for the assay. The results were assessed visually by checking turbidity, green fluorescence and pellet formation in the reaction tube and also by gel electrophoresis. The assay successfully detected both viruses in infected plants whereas no cross-reactions were recorded with healthy plants. Optimum conditions for successful amplification were determined in terms of the concentrations of magnesium sulphate and betaine, temperature, and duration. The detection limit for both LAMP and RT-LAMP was up to 100 times that for conventional PCR and up to one-hundredth of that for real-time PCR. The optimal conditions arrived at were validated by testing field samples of infected vines of three species from different regions.

1. Introduction

Black pepper (*Piper nigrum* L.) is used for a variety of purposes including medicinal purposes. Two viruses, namely *Cucumber mosaic virus* (CMV) (genus *Cucumovirus*) and *Piper yellow mottle virus* (PYMoV) (genus *Badnavirus*) have been detected in black pepper, which originated in the tropical evergreen forests of the Western Ghats in India. The two viruses, often found together, are the cause of 'stunted disease' in black pepper (Lockhart et al., 1997; Sarma et al., 2001; de Silva et al., 2002). Of the two viruses, CMV is an isometric ssRNA virus with a broad host range whereas PYMoV is a bacilliform dsDNA virus with a very narrow host range. The disease has been reported from all the countries that grow black pepper commercially: Brazil, India, Indonesia, Malaysia, Philippines, Sri Lanka, Thailand, and Vietnam. Black pepper is a perennial vine propagated vegetatively through stem cuttings, a method that favours the build-up of virus concentration over time. The current approach to manage the disease is to remove severely infected vines and replant with virus-free stock. Identifying virus-free plants based on external symptoms alone is not reliable because many infected vines remain symptomless either in particular seasons or for a few years. A sensitive, reliable, and quick method for early detection of viruses is therefore necessary, and molecular techniques have proved superior to conventional methods in this regard.

Currently available methods for detecting viruses that affect black pepper are as follows: PCR for PYMoV, RT-PCR for CMV, and multiplex RT-PCR (mRT-PCR) for simultaneous detection of both (Bhat and Siju, 2007; Siju et al., 2007; Bhat et al., 2009). These methods are valuable for identifying latent infections and early stages of the disease. However, PCR-based methods are expensive in terms of time and equipment, and hence impractical for large-scale use; loop-mediated isothermal amplification (LAMP), on the other hand, is not only sensitive and relatively inexpensive but can also be carried out at a constant temperature of 60–65 °C in a water bath. The technique can also be used for amplifying RNA by adding reverse transcriptase to the LAMP reaction mixture at the same time (often referred to as reverse-transcription LAMP or RT-LAMP). Both LAMP and RT-LAMP use four to six primers and a DNA polymerase with strand-displacing activity to generate amplification products (Notomi et al., 2000; Nagamine et al., 2002), and the products can be detected by agarose gel electrophoresis or visually by turbidity or colour changes (Mori et al., 2001; Tomita et al., 2008). The products of both LAMP and RT-LAMP are of different lengths and contain alternately inverted repeats of the target sequence, visible as ladder-like patterns on a gel. Both the methods have been used successfully for detecting many RNA and DNA viruses of plants (Fukuta et al., 2003, 2004, 2005; Nie, 2005; Varga and James, 2006;

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Liu et al., 2010; Peng et al., 2012). The present paper describes the design and optimization of an assay based on LAMP and RT-LAMP to detect PYMoV and CMV respectively in infected vines of black pepper.

2. Materials and methods

2.1. Plant material and virus isolates

Two black pepper plants, one healthy and one known to be infected by both the viruses (PYMoV and CMV), were used for standardizing the LAMP and RT-LAMP assays. The status of the plants was confirmed by using mRT-PCR (Bhat and Siju, 2007). The same two plants were used for determining the detection limits of the assays and for comparing the results with those obtained by using conventional and real-time RT-PCR. For validating the LAMP and RT-LAMP assay, 80 samples of black pepper vines (64 with and 16 without the characteristic symptoms) and 5 samples each of betelvine (*Piper betle*) and Indian long pepper (*Piper longum*) – all with the symptoms – from different regions of Karnataka and Kerala, the two major black-pepper-growing states of India, were used.

2.2. Isolation of total nucleic acid

The combined total RNA and DNA was isolated from the black pepper plants following the procedure described by Bhat and Siju (2007): 100 mg of young tissue was ground in extraction buffer [4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium N-lauryl sarcosine, 0.1% β-mercaptoethanol, and 0.5% sodium sulphite], water-saturated phenol was added, and the mixture was centrifuged at 12,000 × *g*. The aqueous phase containing RNA was aspirated into a fresh tube, and DNA was isolated from the interface and the organic phase after increasing the pH of the mixture using Tris–HCl, pH 10.5 and adding Tris–saturated phenol. Finally, both the phases, one containing RNA and the other containing DNA, were pooled and the mixture precipitated to obtain both RNA and DNA in the same tube. The yield of nucleic acid was determined using a spectrophotometer (Biophotometer plus, Eppendorf, Germany).

2.3. Primer design and synthesis

LAMP primers were designed for PYMoV based on the conserved region in the ORF III region. RT-LAMP primers for CMV were designed based on the conserved region in the coat protein gene identified by multiple-sequence alignment of coat protein gene sequences of CMV subgroup I B available in GenBank (Benson et al., 1999). Primer design was carried out using a software package, namely Primer Explorer version 4 (http://primerexplorer.jp/e/). Two LAMP external primers, F3 and B3, two internal primers FIP and BIP, and the loop primer (B-loop or F-loop), were designed for both viruses. Desalted F3, B3, FL, and BL and HPLC-purified FIP and BIP primers, the sequences of which are shown in Table 1, were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

2.4. Optimization of LAMP and RT-LAMP reactions

Reaction components for LAMP (for detecting PYMoV) and RT-LAMP (for detecting CMV) were optimized in 0.2 ml tubes using total nucleic acids extracted from the infected plant (the positive control) and from the healthy plant (the negative control). The third control was the 'water control' (a negative control, in which water was used instead of nucleic acids). The LAMP reaction mixture (25 μl) contained 1 μl (about 60 ng) of the template nucleic acids, 2× thermopol buffer (New England Bio Labs, Ipswich, MA, USA), 1.4 mM each of dNTPs, 0–14 mM $MgSO_4$ and 0.4–2.4 M betaine (Sigma Chemicals, Bangalore, India), 200 nM each of the external primers F3 and B3, 2 μM each of the internal primers FIP and BIP, 1 μM of the loop primer (B-loop), 1 mM $MnCl_2$ and 50 μM calcein (Sigma Chemicals), and 8 U of *Bst* DNA polymerase (New England Bio Labs). In determining the conditions optimal for the reaction (incubation temperatures, duration, and the concentration of $MgSO_4$ and betaine), the reaction was carried out at 65 °C for 75 min followed by incubation at 80 °C for 10 min to inactivate the *Bst* polymerase. In determining the optimum concentration of $MgSO_4$, betaine was maintained at 0.8 M in both LAMP and RT-LAMP whereas in determining the optimum concentration of betaine, $MgSO_4$ was maintained at 6 mM in the case of LAMP and 4 mM in the case of RT-LAMP.

Table 1
Primers used for loop-mediated isothermal amplification (LAMP) of *Piper yellow mottle virus* (PYMoV) and reverse transcription (RT) LAMP of *Cucumber mosaic virus* (CMV).

Primer name	Sequence (5′–3′)
CMV-F3	TGTGGGTGACAGTCCGTAA
CMV-B3	ACGGCGTACTTTCTCATGTC
CMV-FIP	AACCAGTACTGGTGAGGCTCCGTGCCTCCTCGGACTTGTC
CMV-BIP	ATCAGTATGCCGCGTCCGGACGCATCACCGAAAGATCGTA
CMV-FL	GCAGAGATGGCGGAAACG
PYMoV-F3	AAAATGCACCTGCTGTGT
PYMoV-B3	TCCCAGGAAATCAATAGTTCC
PYMoV-FIP	TCATGTTTTCTGAGAACACCAGAATAGAAAAATGGACAACTGTTTCA
PYMoV-BIP	AACACTTAGTCGCAATGCTGGAGCCCAATTTTCATTTTTGTTG
PYMoV-BL	AGTATGCAAGAAGAATGGGCTTATC

To find out the optimum temperature, the reaction was carried out for 75 min at different temperatures: 56 °C, 59 °C, 62 °C, 65 °C, and 68 °C; to find out the optimum duration, the reaction was carried out at 65 °C for different durations (15 min, 30 min, 45 min, 60 min, and 75 min). Initially, the LAMP reaction was optimized using a thermo cycler (Master cycler gradient, Eppendorf, Germany) for maintaining a constant reaction temperature. Subsequently, four other options were evaluated to ascertain their suitability for the purpose: a heat block (Henry Troemner, LLC, USA), a water bath (Plastocrafts, Mumbai, India), an incubator (Thermo Scientific, USA), and real-time PCR (Rotor Gene Q system, Qiagen, Hilden, Germany).

The RT-LAMP reaction was optimized the same way except that the reaction mixture, in addition to the above components, also contained 1.5 U of thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

2.5. Visual detection of LAMP and RT-LAMP products

In both LAMP and RT-LAMP reactions, DNA is synthesized in large quantities, with pyrophosphate ion (magnesium pyrophosphate) as a by-product, also produced in large quantities. Because the by-product is insoluble, the liquid in the reaction tube becomes turbid. The degree of turbidity can be measured by different methods and provides an indirect measure of the yield of the reaction. In the first method, the reaction tube was examined visually in normal daylight, and only the presence or absence of turbidity was noted. The second method involved adding Mn in ionic form and calcein to the reaction solution, which induces fluorescence: the reaction tube was then observed under UV light for the presence or absence of green fluorescence. In the third method, following LAMP and RT-LAMP amplification, the tubes were centrifuged for 3 min at 8000 rpm in a microcentrifuge and observed for the presence or absence of a white pellet. In the fourth method, amplification products were made visible on 2% agarose gel through electrophoresis.

2.6. Sensitivity of detection in plants and its comparison with PCR and real-time PCR

In order to compare the degree of sensitivity of different methods, serial dilutions of the original nucleic acid extract from the virus-affected plant were subjected to LAMP and RT-LAMP. The following dilutions were used, using1 μl of each dilution: undiluted extract, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. The original extract from the virus-free plant was used as a diluent. All the dilutions were also subjected to conventional PCR and SYBR Green real-time PCR (for PYMoV) and to RT-PCR and SYBR Green real-time-RT-PCR (for CMV). Both PCR and RT-PCR were carried out using 1 μl of each of the above dilutions using the respective F3 and B3 primers as described by Bhat et al. (2009) and Siju et al. (2007). For real-time RT-PCR, which was carried out using the Rotor Gene Q system (Qiagen, Hilden, Germany), the final volume of 25 μl contained 12.5 μl of 2× QuantiFast™ SYBR Green PCR Master mix, 1.0 μl each of the forward and reverse primers (1 μM/μl), 50 U of Revert Aid reverse transcriptase, and 1 μl of the template. The thermocycling steps were as follows: initial cDNA synthesis at 42 °C for 45 min, denaturation at 95 °C for 5 min, and 35 cycles of 95 °C for 15 s and 60 °C for 45 s. For real-time PCR, the procedure was identical except that the reaction mixture did not contain reverse transcriptase and cDNA synthesis was not a part of the thermocycling process.

2.7. Validation of detection by LAMP and RT-LAMP

In order to validate the detection of PYMoV and CMV in the field samples, total nucleic acids (DNA and RNA) isolated from each of the field samples were subjected to conventional PCR and SYBR Green real-time PCR (for detection of PYMoV), and conventional RT-PCR and SYBR Green real-time RT-PCR (for the detection of CMV) and subsequently to LAMP and RT-LAMP along with the positive and negative controls.

3. Results

3.1. Optimization of LAMP and RT-LAMP reactions

Products of LAMP and RT-LAMP obtained using 1 μl of the nucleic acids from the infected black pepper plant showed turbidity, green fluorescence under UV light, a white pellet in the reaction tube, and the typical ladder-like pattern in agarose gel electrophoresis characteristic of LAMP and RT-LAMP in both the cases, PYMoV and CMV, whereas none of the four manifestations (turbidity, fluorescence, pellets, and ladder-like patterns) was observed either in the negative control (the virus-free vine) or in the 'water control' (Fig. 1). Specificity of the primers was confirmed by carrying out LAMP and RT-LAMP reactions using the nucleic acids isolated from black pepper plants infected only with CMV and only with PYMoV as templates (data not shown).

The LAMP and RT-LAMP primers we used at the concentrations recommended by Tomita et al. (2008) and Tomlinson et al. (2010). The optimum concentrations of $MgSO_4$ and betaine in the present experiment were, respectively, 6 mM and 0.8 M for the detection of PYMoV by LAMP and 4 mM and 0.8 M for the detection of CMV by RT-LAMP (Fig. 2).

As to the optimum temperature when the duration was held constant for 75 min, no amplification was seen either at 56 °C or at 68 °C but was evident at 59 °C, 62 °C, and 65 °C, in the case of both viruses (Fig. 3A). Similarly, when the temperature was held constant at 65 °C, amplification was very slight at 45 min but adequate at 60 min and at 75 min (Fig. 3B). The intensity of bands obtained at 60 min differed little from that obtained at 75 min. All the four ways of incubating the LAMP and RT-LAMP reactions (a heat block,

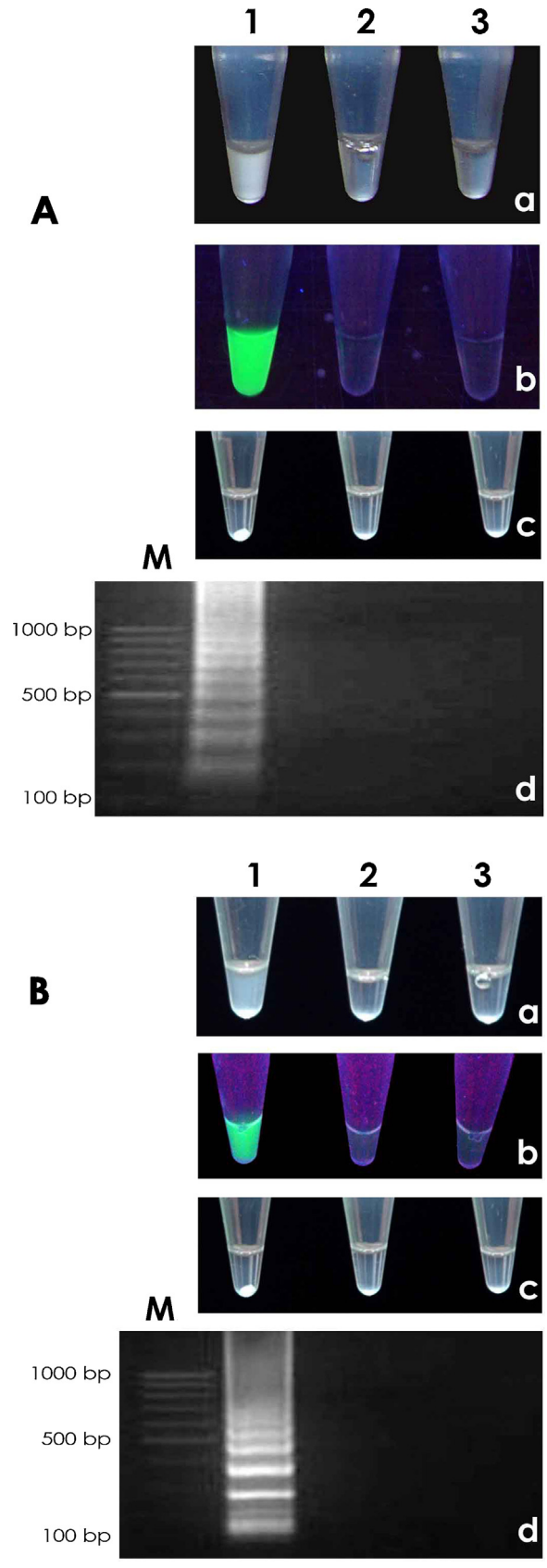

Fig. 1. Loop-mediated isothermal amplification (LAMP) for detecting *Piper yellow mottle virus*·(A) and reverse transcription (RT) LAMP for detecting *Cucumber mosaic virus*·(B) infecting black pepper. Lane 1: Infected black pepper, Lane 2: healthy black pepper, Lane 3: control (with water instead of nucleic acids). Making the products visible (a) under normal light, (b) under UV light, (c) centrifugation, and (d) gel electrophoresis. Lane M shows a 100 bp DNA ladder.

Table 2
Components of the optimized loop-mediated isothermal amplification (LAMP) and reverse transcription (RT) LAMP reaction mixture for detection of *Piper yellow mottle virus* (PYMoV) and *Cucumber mosaic virus* (CMV).

Component	Stock conc.	Required concentration		Required volume per reaction (μl)	
		LAMP (PYMoV)	RT-LAMP (CMV)	LAMP (PYMoV)	RT-LAMP (CMV)
Thermopol reaction buffer	10×	2×	2×	5.0	5.0
$MgSO_4$	50 mM	6 mM	4 mM	3.0	2.0
dNTPs	10 mM	1.4 mM	1.4 mM	3.5	3.5
F3 primer	10 μM	0.2 μM	0.2 μM	0.5	0.5
B3 primer	10 μM	0.2 μM	0.2 μM	0.5	0.5
FIP	100 μM	2 μM	2 μM	0.5	0.5
BIP	100 μM	2 μM	2 μM	0.5	0.5
BL primer/FL primer	100 μM	1 μM	1 μM	0.25	0.25
Betaine	5 M	0.8 M	0.8 M	4.0	4.0
Bst polymerase	8 U/μl	8 U	8 U	1.0	1.0
Thermoscript RT	1.5 U/μl	–	1.5 U	–	1.0
$MnCl_2$	20 mM	1 mM	1 mM	1.25	1.25
Calcein	1 mM	50 μM	50 μM	1.25	1.25
Sterile water	–			2.75	2.75
Template nucleic acid	60 ng/μl			1.0	1.0
Total reaction volume	–			25.0	25.0

Reaction mix incubated at 65 °C for 60 min followed by 80 °C for 10 min in an incubator.

a water bath, an incubator, and real-time PCR) resulted in successful amplification (data not shown). The optimal LAMP and RT-LAMP reaction mixture and conditions are given in Table 2.

3.2. Sensitivity of detection in plants and its comparison with PCR and real-time PCR

The detection limits for PYMoV were 10^{-1} using LAMP and 10^{-3} for SYBR-Green-based real-time PCR whereas PCR failed to detect the virus at any dilution: it could detect the virus only in undiluted samples (Fig. 4). The detection limits for CMV were as follows: 10^{-4} using RT-LAMP, 10^{-2} using RT-PCR, and 10^{-5} using SYBR-Green-based real-time RT-PCR (Fig. 5). Thus the LAMP and RT-LAMP assays were 10 times as sensitive as conventional PCR and 100 times as sensitive as conventional RT-PCR but only one-hundredth as sensitive as real-time PCR and one-tenth as sensitive as real-time RT-PCR. All the four methods of examining the products of LAMP and RT-LAMP visually (turbidity, UV light, centrifuging, and electrophoresis) led to similar results in terms of detection limits although using turbidity was subjective to some extent especially in borderline cases (Figs. 4 and 5).

3.3. Validation of LAMP and RT-LAMP for detection of PYMoV and CMV

When tested by using conventional PCR and RT-PCR, 14 of the 64 samples from vines with the characteristic symptoms tested positive for both PYMoV and CMV whereas all the remaining samples tested positive only for PYMoV. The results with the 64 samples subjected to real-time PCR, real-time RT-PCR, LAMP and RT-LAMP assays were similar (Fig. 6). Of the 16 samples of black pepper without the symptoms subjected to conventional PCR and RT-PCR, only 2 tested positive for PYMoV initially and none tested positive for CMV. When these 16 samples were subjected to real-time PCR, real-time RT-PCR, LAMP and RT-LAMP, 6 (including the two that had tested positive for PYMoV in conventional PCR) tested positive for PYMoV but none tested positive for CMV. All the samples of Indian long pepper tested positive for both the viruses in all three types of assays. Of the 5 samples of betelvine, 3 tested positive for both viruses and 2 tested positive only for PYMoV, either through conventional PCR or through LAMP or through real-time PCR. The Ct values of positive samples in real-time PCR varied from 13 to 33 for black pepper, 11 to 24 for betelvine and 13 to 23 for Indian long pepper used for validation. The Ct values of positive samples in real-time RT-PCR varied from 9 to 27 for black pepper, 11 to 26 for betelvine and 11 to 24 for Indian long pepper used for validation.

4. Discussion

Viral disease, which debilitate the plant and reduce its yield, are ranked third among the most serious diseases of black pepper (Sarma et al., 2001). Early detection of the pathogen at the incubation stage is crucial to effective management of the disease. Black pepper is a perennial crop propagated vegetatively, and viruses that infect black pepper spread primarily through planting material. Identification and use of virus-free mother plants is therefore particularly important because black pepper is propagated through either stem cuttings or tissue culture. Because virus-affected plants are often without symptoms, virus-free plants cannot be identified from external symptoms alone (Bhat et al., 2009); more reliable and sensitive methods for identifying virus-free plants are therefore essential. Currently, PCR-based methods are used for selecting virus-free mother plants.

Of the two viruses associated with the disease, CMV is an ssRNA virus whereas PYMoV is a dsDNA virus. Therefore, total RNA as well as total DNA need to be isolated for detecting both viruses by RT-PCR, PCR and mRT-PCR (Bhat and Siju, 2007). The present study was successful in achieving the same result through alternative means: PYMoV was detected using LAMP and CMV using RT-LAMP. Fukuta et al. (2005) and Peng et al. (2012) were able to detect CMV through RT-LAMP from chrysanthemum in Japan and from banana in China respectively. In the present study, the RT-LAMP assay for CMV from black pepper was developed using primers to the conserved region of coat protein gene. Besides, the presence of virus was detected by two other means, observing for green fluorescence (after adding manganese and calcein) under UV light and centrifuging to form a pellet. The sensitivity of RT-LAMP was also compared with that of RT-PCR and real-time RT-PCR. To our knowledge, this is the first report of a LAMP assay for PYMoV.

Compared to the conventional PCR, LAMP/RT-LAMP is not only less expensive in terms of equipment required but also more sensitive, and has been used for detecting not only viruses but also various other pathogens. Developing a LAMP/RT-LAMP procedure for each virus requires appropriately designed primers, standardization of the reaction mixture (especially the concentration of $MgSO_4$, betaine, and the template), and standardization of optimal temperature and duration. The present experiment served to standardize all these parameters for detecting PYMoV and CMV.

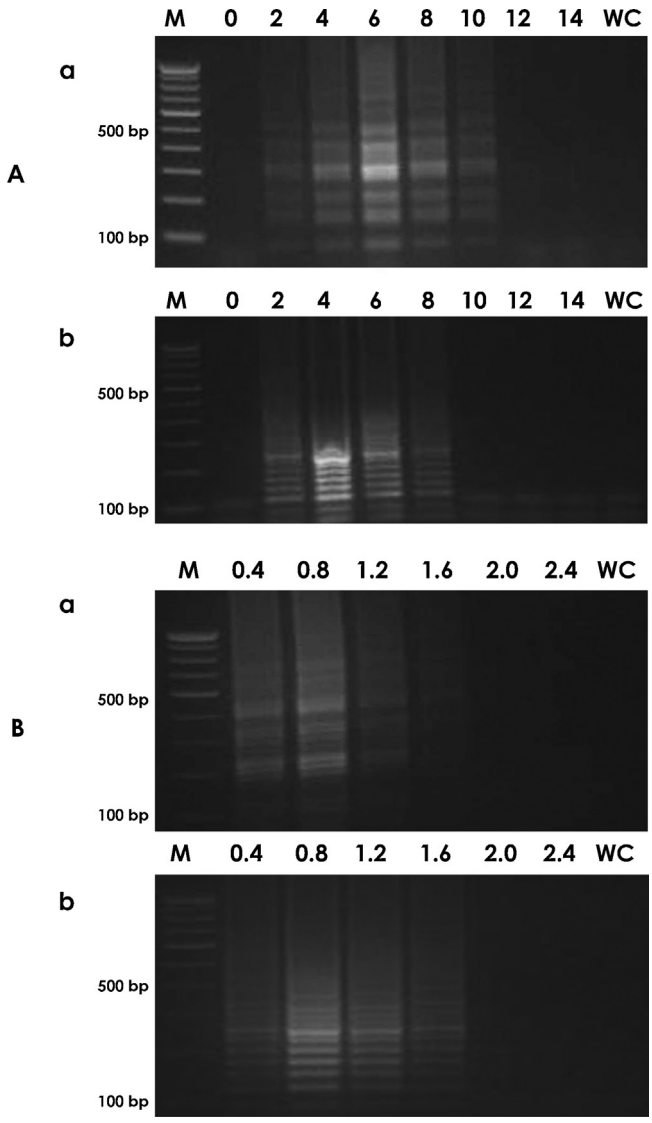

Fig. 2. Agarose gel electrophoresis showing the effect of different concentrations of $MgSO_4$ (A) and of betaine (B) on the detection of *Piper yellow mottle virus* (PYMoV) by loop-mediated isothermal amplification (LAMP) and of *Cucumber mosaic virus* (CMV) by reverse transcription (RT) LAMP. (A) $MgSO_4$. Lanes 0, 2, 4, 6, 8, 10, 12, and 14 loaded with products of LAMP (a) and RT-LAMP (b) carried out using different concentrations of $MgSO_4$. (B) Betaine. Lanes 0.4, 0.8, 1.2, 1.6, 2.0, and 2.4 loaded with products of LAMP (a) and RT-LAMP (b) carried out using different concentrations of betaine. Lane WC is for water control. Lane M shows a 100 bp DNA ladder.

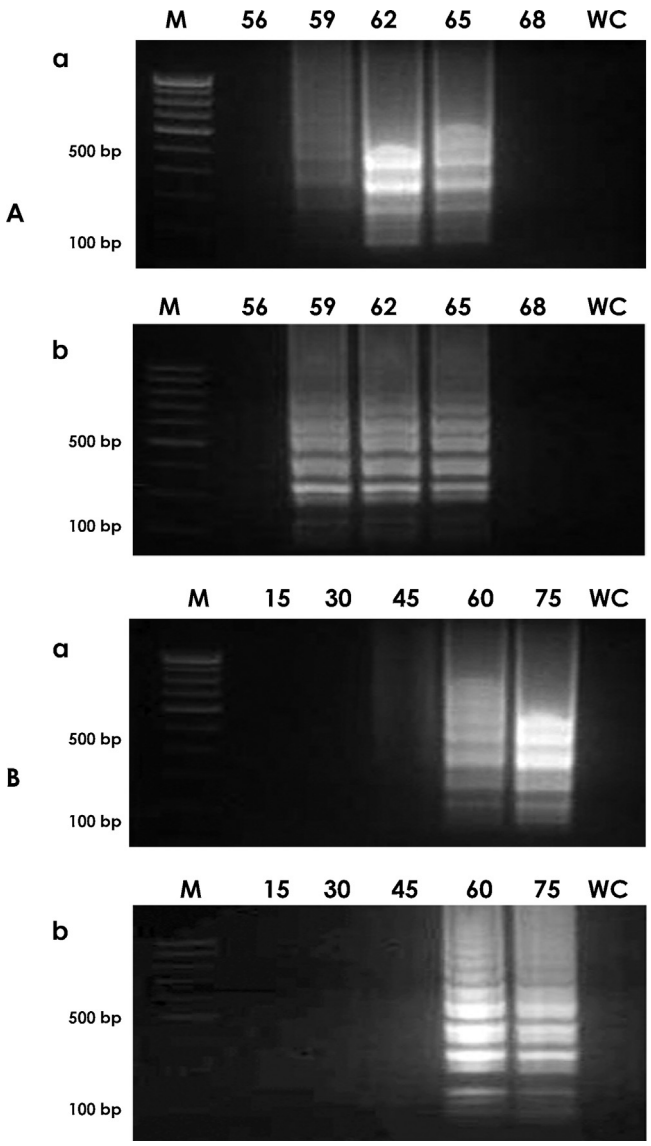

Fig. 3. Agarose gel electrophoresis showing the effect of amplification temperature (A) and duration (B) on the detection of *Piper yellow mottle virus* (PYMoV) by loop-mediated isothermal amplification (LAMP) and of *Cucumber mosaic virus* (CMV) by reverse transcription (RT) LAMP. (A) Temperature. Lanes 56, 59, 62, 65, and 68 loaded with products of LAMP (a) and RT-LAMP (b) carried out at different temperatures. (B) Time. Lanes 15, 30, 45, 60, and 75 loaded with products of LAMP (a) and RT-LAMP (b) carried out over different durations. Lane M shows a 100 bp DNA ladder; Lane WC is for water control.

As reported earlier (Ravindran et al., 2012), thermopol buffer at 2× proved better than 1×. Both the assays, LAMP and RT-LAMP, were successfully carried out using several alternative items of equipment such as an incubator, a heating block, and a water bath, which served to confirm that any device that can maintain a set temperature accurately is adequate for LAMP/RT-LAMP amplification.

The products of LAMP and RT-LAMP were confirmed through several means including a simple visual examination for turbidity as well as through agarose gel electrophoresis. As reported by several researchers (Tomita et al., 2008; Ravindran et al., 2012), the present experiment also showed that LAMP products could be detected through direct observation by examining for turbidity or for green fluorescence (by adding manganese chloride and calcein to the reaction tube). The present study also included another method, which consisted of centrifuging the tubes and examining them for the presence or absence of a pellet, the pellet being the proof of a positive reaction and the size of the pellet being proportional to the intensity of reaction. All the four visual methods (turbidity, green fluorescence, pellet, and gel electrophoresis) gave similar results in terms of detection limits, although turbidity was to some extent subjective, especially in borderline cases. However, the presence or absence of a pellet serves as a simple and objective proof. Our results show that such simple indications as turbidity, a pellet, or green fluorescence are adequate for performing LAMP and RT-LAMP in laboratories with minimum facilities.

With respect to dilution limits, our results are consistent with those obtained by many researchers (Fukuta et al., 2003; Varga and James, 2006; Boubourakas et al., 2009; Liu et al., 2010; Ravindran et al., 2012): the sensitivity of LAMP in detecting PYMoV was up to 10 times that of conventional PCR but only one-hundredth of that of SYBR-Green-based real-time PCR; the sensitivity of RT-LAMP in detecting CMV was up to 100 times that of conventional RT-PCR but only a tenth of that of SYBR-Green-based real-time RT-PCR.

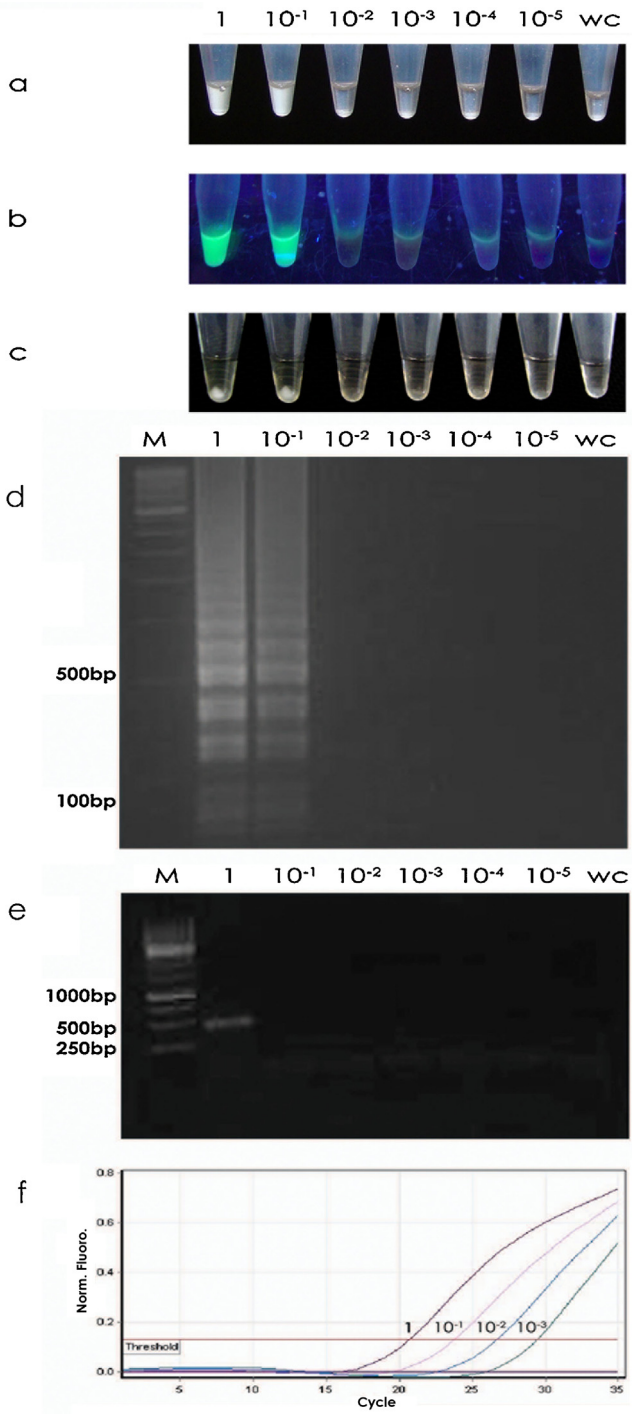

Fig. 4. Comparison of sensitivity of detection of *Piper yellow mottle virus* (PYMoV) by loop-mediated isothermal amplification (LAMP) (a–d), PCR (e), and real-time PCR (f). Lanes 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} show different dilutions of the original extract of total nucleic acids; Lane M shows a 100 bp DNA ladder; Lane WC is water control. (a–d) Making the products of LAMP visible: (a) under normal light, (b) under UV light, (c) centrifuging, and (d) gel electrophoresis. (e) Agarose gel electrophoresis of PCR products. (f) Amplification curve obtained with real-time PCR carried out using different dilutions of total nucleic acid.

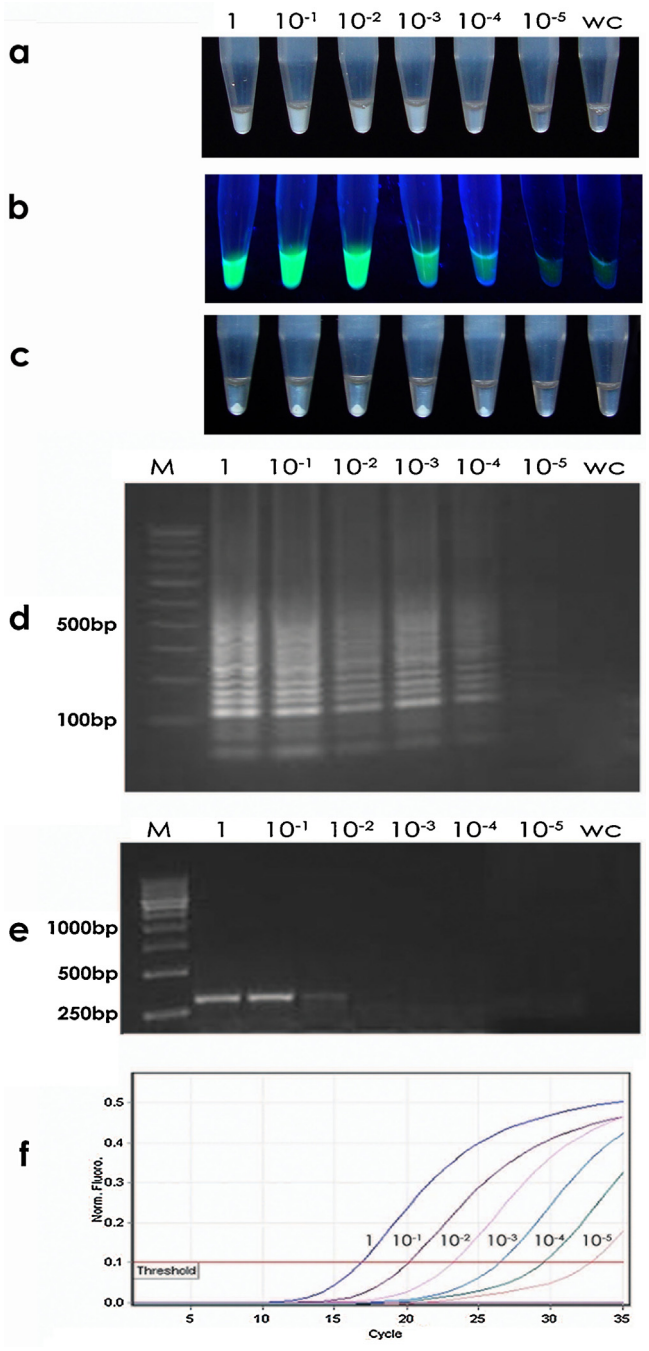

Fig. 5. Comparison of sensitivity of detection of *Cucumber mosaic virus* (CMV) by reverse transcription loop-mediated isothermal amplification (RT-LAMP) (a–d), RT-PCR (e), and real-time RT-PCR (f). Lanes 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} show different dilutions of the original extract of total nucleic acids; Lane M shows a 100 bp DNA ladder; Lane WC shows water control. (a–d) Making the products of RT-LAMP visible: (a) under normal light, (b) under UV light, (c) centrifuging, (d) gel electrophoresis. (e) Agarose gel electrophoresis of RT-PCR product. (f) Amplification curve obtained with real-time RT-PCR carried out using different dilutions of the original extract of total nucleic acids.

That the optimized LAMP and RT-LAMP procedures proved satisfactory even with a large number of samples shows that the method is suitable for rapid detection of different isolates of CMV and PYMoV. Because LAMP made it possible to detect the virus in more non-symptomatic plants than that was possible with PCR, LAMP can certainly be regarded as a more sensitive and suitable method than PCR for identifying virus-free plants; besides, LAMP and RT-LAMP require no special equipment and prove adequate even in minimally-equipped field laboratories and are therefore particularly suitable not only for identifying virus-free plants on a large scale – whether propagated from stem cuttings or through tissue culture but also for epidemiological studies and for screening *Piper* germplasm against viruses to identify resistant sources.

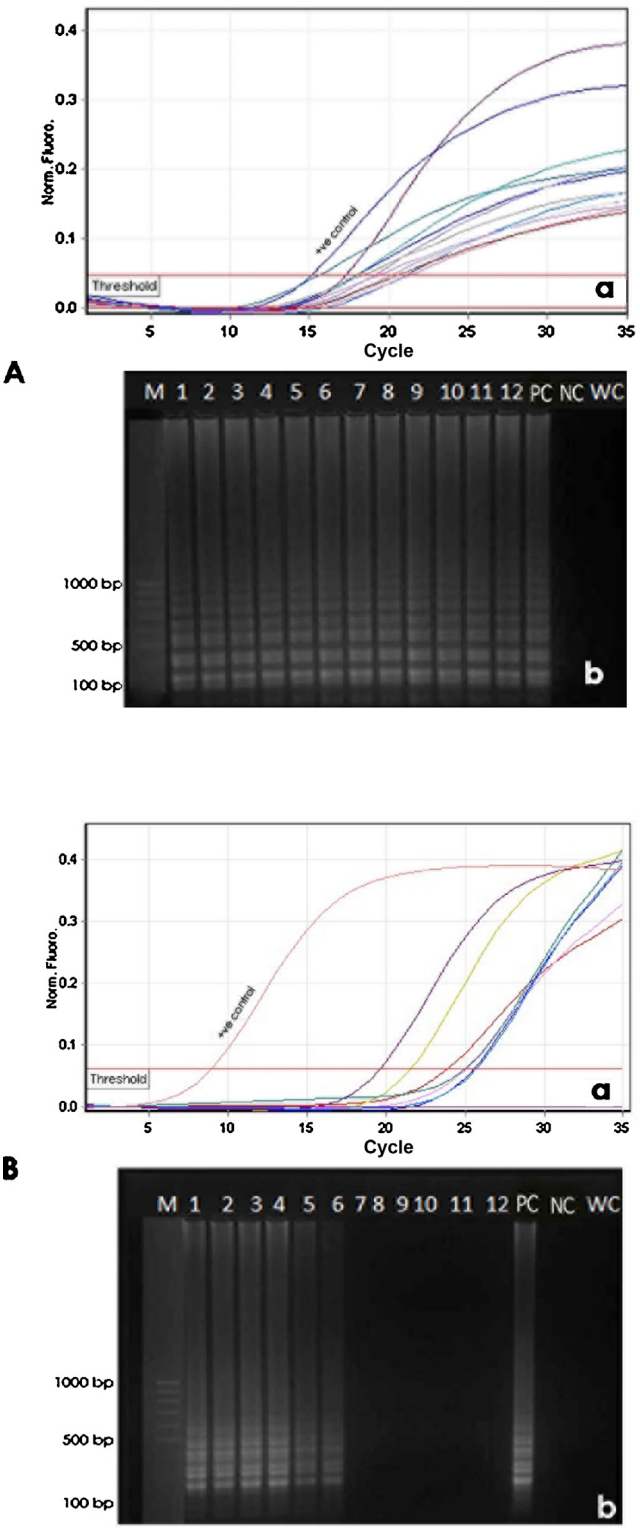

Fig. 6. Validation of LAMP and RT-LAMP for detecting *Piper yellow mottle virus* (PYMoV) (A) and *Cucumber mosaic virus* (CMV) (B) in field samples of black pepper collected from different regions. Aa (real-time PCR) and Ba (real-time RT-PCR) show amplification curves and Ct value obtained for different samples. No amplification curve was seen in negative control. (Ab) and (Bb) show results of LAMP and RT-LAMP respectively. Lane M, molecular size markers; Lane 1–12, test samples of black pepper; PC, positive control; NC, negative control; WC, water control.

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