

Development of Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) for rapid detection of viruses infecting patchouli (*Pogostemon cablin*)

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Research Article

Keywords: BBWV2, PSTV, Isothermal amplification, RT-LAMP, Hydroxy-naphthol Blue

Posted Date: November 14th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3590921/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Archives of Microbiology on January 23rd, 2024. See the published version at <https://doi.org/10.1007/s00203-023-03798-0>.

Abstract

Patchouli (*Pogostemon cablin*) a highly valued medicinal plant, suffers significant economic losses following infection with *Broad bean wilt virus 2* (BBWV2) and *Peanut stripe virus* (PStV). In this study, a field-based isothermal technique called reverse transcription loop-mediated isothermal amplification (RT-LAMP) was established for an early and specific identification of BBWV2 and PStV. The oligo primers for RT-LAMP assay were designed from the conserved domains of the coat protein genes of PStV and BBWV2. The reaction temperature and time duration were optimised to 65 °C for 60 min, followed by incubation at 85 °C for 10 min in a water bath. The LAMP amplicons positive for PStV and BBWV2 revealed characteristic ladder-type bands when analysed through gel electrophoresis. Further, a colorimetric assay using a metal ion-based indicator (Hydroxy-naphthol Blue, HNB) was conducted to visualize the amplified products with the naked eye, thus facilitating accessibility to field practices. The assays developed in this study were virus-specific as no cross-amplification occurred with other viruses, and were 100 times more sensitive than RT-PCR. Thus, the RT-LAMP assays established in this study are quick, reliable, and cost-effective for the accurate identification of BBWV2 and PStV. It will facilitate screening of patchouli planting materials. Thus, may reduce the risk of virus spread and could be helpful in phytosanitary programs.

1. Introduction

Patchouli (*Pogostemon cablin*), an aromatic perennial herb in the family Lamiaceae, is regarded as one of the most valuable medicinal plants because of the oil extracted from its leaves. Patchouli alcohol (PA), which is present in the extracted oil, has several health benefits including anti-inflammatory, antioxidant, ulcerogenic, colitis and mucositis, as well as preventive properties that protect against lung and brain damage (Yu et al. 2015; Lee et al. 2020). Due to its distinctive qualities, such as its long-lasting camphoraceous and earthy aroma, patchouli oil plays a significant role in industries thriving on perfume and soap businesses (Hasegawa et al. 1992).

Cultivation of patchouli crop is compromised following infection with multiple pathogens and pests, such as, viruses, fungi and nematodes. Several viruses badly affect the quantity of its production. However, only *Peanut stripe virus* (PStV) [Singh et al. 2009] and *Patchouli mild mosaic virus* (PaMMV), an isolate of *Broad bean wilt virus 2* (BBWV2) [Natsuaki et al. 1994] have been partially or fully characterised from patchouli plants. Some preliminary studies have reported the occurrence of a few more viruses in patchouli plants, such as, *Patchouli virus X* (PatVX) [Filho et al. 2002], *Patchouli mottle virus* (PaMoV) [Natsuaki et al. 1994] and yellow mosaic of Patchouli [Sastry & Vasanthakumar. 1981; Zaim et al. 2013]. But viruses belonging to the genera *Potyvirus* and *Fabavirus* are the most common threats that negatively impact the productivity of *P. cablin* (Noveriza et al. 2012).

PStV (genus *Potyvirus*, family *Potyviridae*) is known to be a peanut-infecting strain of *Bean common mosaic virus* (Vetten et al. 1992). The virions are linear, measuring approximately 680–780 nm in length, having monopartite, positive-sense RNA genome (~ 10 kb). Potyviruses are transmitted non-persistently

by aphids and also through mechanical means (Singh et al. 2009; Hou et al. 2011). Symptoms induced by potyviruses in *P. cablin* include typical mosaic with their severity ranging from mild to severe.

On the other hand, BBWV2 (genus *Fabavirus*, family *Secoviridae*) affects vast variety of crops (Kobayashi et al. 1999). The virions of the genus *Fabavirus* are icosahedral in shape and approximately diameter of about 30 nm having two types of proteins i.e., large coat protein (LCP) and small coat protein (SCP). The virus has positive sense single-stranded, bipartite, RNA genome, namely RNA 1 (~ 6 kb) and RNA 2 (~ 4kb), that are encapsidated and polyadenylated separately (Panno et al. 2014).

Since patchouli crop grows vegetatively from cuttings, the viruses may persist in varying seasons and disperse to new locations along with the propagating materials. The most effective way to restrict the spread of viruses is to use virus-free patchouli germplasm. To accomplish this, it is crucial to develop a simple, sensitive, affordable field-based diagnostic technique which can reliably detect viruses infecting patchouli plants. The most commonly used techniques for virus detection are enzyme-linked immunosorbent assay (ELISA) and PCR. Although ELISA is highly efficient for large-scale testing of field samples, it is always carried out in a laboratory. Amplification of viral gene via PCR is another sensitive approach. However it requires expensive equipments. In addition, mutagenic reagents such as ethidium bromide need to be used to visualise the PCR results. Inference shows that both the lab techniques (ELISA and PCR) have some disadvantages and require costly equipment, thus, making them unsuitable for virus detection under the field conditions (Okiro et al. 2019). During the past years, several efficient isothermal techniques have been developed for virus detection. The main advantage and essential commonality of the isothermal technique is to eliminate the use of thermal cycling, which is necessary in PCR assays (Gill & Ghaemi. 2008). Among isothermal amplifications, loop-mediated isothermal amplification is the most commonly used approach (Notomi et al. 2000). The ease of access and rapid performance shown by LAMP makes it quite suitable for on-site diagnosis as well as laboratories which are less equipped. Addition of a reverse transcriptase helps detect RNA and also supports the LAMP assay (Fukuta et al. 2003). This approach requires a DNA polymerase, *Bacillus stearothermophilus* having strand displacement activity at isothermal temperature. It employs four/six primer pairs that could specifically detect six/eight different target nucleotide sequences (Notomi et al. 2000). RT-LAMP being cost-effective eliminates the use of expensive thermocycling apparatus, and can be conducted in a simple setup such as water bath. Furthermore, amplified products can be visualised by naked eye for colour change following the addition of an intercalating dye such as SYBR Green I, calcein and hydroxy naphthol blue (HNB) to the amplicons. Among them metal ion HNB is the most simplest and commonly used where detection and amplification of a target gene are combined in one processing step. And thus, it reduces contamination risk and detection cost (Goto et al. 2009; Tomlinson et al. 2010). The mechanism of the LAMP reaction is based on the interaction of pyrophosphate ions with metal indicators, which results in a white precipitate. As a result, HNB can be employed as a metal ion in the LAMP process, and the results can be observed following colour change. Because of its simplicity, specificity and low cost, the RT-LAMP method has gained popularity for virus detection under the field conditions (Fukuta et al. 2003; Varga & James. 2006; Kamala et al. 2015; Anandakumar et al. 2020; Wani et al. 2023). In present study, RT-LAMP assay was developed for the detection of PSTv for the first time. Though a LAMP assay

for BBWV2 detection has been established in China, its host was *Pseudostellaria heterophylla* rather than *P. cablin* (Liang et al. 2022).

2. Materials and methods

2.1. Virus samples

Symptomatic patchouli leaves exhibiting mild to severe mosaic symptoms were collected from patchouli-cultivating regions of India such as Assam, Karnataka and Palampur in 2018 (Supplementary Fig. S1). The leaf samples were kept at -80 °C for future experiments.

2.2. Total RNA extraction and RT-PCR

Total RNA was extracted from healthy and infected leaf tissues (~ 100 mg) following option 2 protocols developed by Jordon et al. (2015). Extracted RNA was re-suspended in nuclease free water. Its purity and concentration were determined using spectrophotometer (Eppendorff Biophotometer Plus, Hamburg, Germany), electrophoresed on formaldehyde gel (1.5% w/v) and kept at -20 °C until further use.

cDNA was synthesised using Verso cDNA kit following the manufacturers instruction (Thermo fisher Scientific, US). For initial screening, RT-PCR was conducted using PStV/ BBWV2- specific primers and cDNA as the template as described earlier (Bano & Khan. 2023). As expected the primers amplified the 1300 and 1032 bp fragments of the two viruses. Initially, leaf tissues from RT-PCR- positive PStV/ BBWV2- patchouli plants were taken to develop the RT-LAMP assays for the respective viruses.

2.3 Designing of RT-LAMP primers

The oligo primers for RT-LAMP assay were designed from the CP gene of PStV (GenBank accession number ON152322) and BBWV2 (GenBank accession number ON638918) using the default settings of Primer Explorer version 5.0 software (<http://primerexplorer.jp/lamp5e/>). For PStV, five sets of primers consisting of two outer (F3 and B3), two inner (FIP and BIP) and one type of loop primers (BL) were designed. Whereas for BBWV2, six sets of primers were designed including two outer (F3 and B3), two inner (FIP and BIP), and two loop primers (LF and BL). Primer were synthesized from Integrated DNA Technologies (IDT, Coralville, IA, USA). Sequence are given in Table 1, Supplementary Fig. S2 and S3.

Table 1
Sequences of oligo primers employed for detection of PStV and BBWV2 by RT-LAMP

Primer names	Length(bp)	Position	Sequence (5'→3')
F3-PstV	21	856–876	GACAAAAATCTAGCTCGCTAC
B3-PstV	18	1034–1051	TCTGATTGACGTCCCTTG
FIP-PstV	42(F2-F1c)	F1c:917–936; F2:877–898	TGCTACTGCTTCCCTTGAC- GCTTTCGACTTCTATGAAGTGA
BIP-PstV	41(B1c-B2)	B1c:950–970; B2:1011–1030	CAGCCCTCAGCAATGTTAACG- TGTGCCTTTCAGTATTCTCG
BL-PstV	23	971–992	GCAAGTTGTTTGGACTTGATGGG
F3-BBWV2	21	378–398	TGTATCAGGTAATTTCTGTGCT
B3-BBWV2	20	544–563	TCAGGATCAAGTGATCTTCC
FIP-BBWV2	43(F2-F1c)	F1c:458–478; F2:403–424	TGATATCTCCAGACAAGCGGG- CAGAGAGATGCAATCTCTAGTG
BIP-BBWV2	39(B1c-B2)	B1c:484–503; B2:525–543	ACAGGAGTCAGTGCAGGTTA- AATGACTTTGCCTTTCCT
FL-BBWV2	23	425–447	AAATGTGAGTTCACCTTCCAGAG
BL-BBWV2	20	505–524	CCAACCACAATAACGCGCAT

2.4. Optimization of RT-LAMP reaction conditions

RNA from PStV/ BBWV2-infected patchouli leaves was used as the template in RT-LAMP assays. It was carried out in a reaction mixture of 25 µl that contained template RNA of 1 µl (10 ng), isothermal amplification buffer of 2.5 µl (10X) (New England Biolabs, USA), 0.2 µM each of outer primers (F3 and B3), 1.6 µM each of inner primers (FIP and BIP), 0.4 µM each of loop primers (LF and BF), 1.4 mM of dNTP, 6 mM of MgSO₄, 0.6 M betaine, 8U of *Bst* 3.0 polymerase (New England Biolabs, USA). Initially, the mixture was incubated at 65 °C for 1 hr followed by heating at 85 °C for 10 min to bring the enzyme in inactive state. In order to determine the ideal temperature for PStV detection, RT-LAMP mix were incubated at different temperatures ranging from 59, 61, 63, 65, 67 and 69 °C for 60 min. Similarly, for BBWV2 detection reaction mixture was subjected to different temperature range 57, 59, 61, 63, 65 and 67 °C.

For determination of minimum reaction time to generate sufficient amount of DNA amplicons reaction time at various intervals were also standardized having 65 °C as the incubation temperature. RT-LAMP

reaction components for PStV amplification were incubated for 20, 30, 40, 50, 60 and 70 min, while those for BBWV2 at 10, 20, 30, 40, 50 and 60 min.

2.5 Detection of RT-LAMP amplicons

DNA amplification was analysed by agarose gel electrophoresis (2% w/v) with ethidium bromide staining (1 µg/ml), and visualized directly following the addition of 120 µM of HNB to the RT-LAMP reaction mixture before incubation. After the incubation, the samples were spun down to analyse the results.

2.6 RT-LAMP specificity

Specificity of RT-LAMP for detection of PStV was determined using RNA as template from PStV-infected patchouli leaves. RNA isolated from unrelated viruses, such as, BBWV2, *Papaya ringspot virus* (PRSV), *Apple mosaic virus* (ApMV) and *Prunus necrotic ringspot virus* (PNRSV) were included as negative controls. In a similar manner, specificity for BBWV2 RT-LAMP assay was evaluated using RNA from BBWV2- infected patchouli leaves. Negative controls included RNA from unrelated viruses such as PStV, PNRSV, and ApMV. Also, a closed-tube colorimetric assay using HNB was included to assess the specificity of the PStV as well as BBWV2. For this, the reaction parameters were similar as specified in Section 2.4 or following the addition of 120 µM of HNB to the mixture before incubation, which was briefly centrifuged after incubation.

2.7 Comparative analysis of RT-LAMP and RT-PCR

The RNA from PStV/BBWV2- infected patchouli leaves was taken to assess the comparative sensitivity of RT-LAMP and RT-PCR assays. Template RNA (100 ng) was 10 – fold serially diluted to 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg and 10 fg/ µl. The diluted RNA directly served as the template in RT-LAMP, whereas in RT-PCR experiments, it was cDNA synthesised from subsequently diluted RNA was used as the template. The reaction condition for both the assays remained same as mentioned earlier (section 2.4 and 2.2, respectively). Detection limit of PStV/BBWV2 was verified following agarose gel electrophoresis and colorimetric detection through HNB, as stated earlier (section 2.5).

3. Results

3.1. Optimization of RT-LAMP reaction conditions

For optimising the ideal temperature for RT-LAMP assays, RT-LAMP temperature 65 °C was considered as the best for detection of PStV and BBWV2 (Fig. 1A & 2A, lane 1–6).

Similarly, incubation of RT-LAMP mixture for 60 minutes was considered as optimal for the amplification of both the viruses (Fig. 1B & 2B, lane 1–6).

3.2 Visualization of RT-LAMP amplicons

RNA from PSTv and BBWV2- infected plants yielded characteristic ladder-like patterns on the agarose gel (Fig. 3A & 4A, lane 1), whereas negative samples exhibited no band (Fig. 3A & 4A, lanes 2 & 3). The HNB-based colorimetric results for both the viruses were in line to those of the agarose gel. It showed sky blue colour for the positive samples (Fig. 3B & 4B, tube 1) and violet for negative samples (Fig. 3B & 4B, tube 2 & 3).

3.3 RT-LAMP specificity

Specificity test of PSTv RT-LAMP assay displayed a typical ladder like multiple DNA fragments when electrophoresed on agarose gel (Fig. 5A, lane 1), while unrelated viruses and the negative controls failed to do so (Fig. 5A, lanes 2–7). Similarly, colorimetric results using HNB, were consistent with those of agarose gel. While PSTv- infected samples turned sky blue (Fig. 5B, tube 1), negative reaction tubes (PRSV, BBWV2, PNRSV and ApMV) remained violet (Fig. 5B, tubes 2–7)

The BBWV2 RT-LAMP specificity test showed a typical ladder-like DNA fragments after electrophoresis (Fig. 6A, lane 1), while samples infected with different viruses did not amplify (Fig. 6A, lanes 2–6). This was further supported by visual detection by the HNB test. Only BBWV2 could yield sky blue colour (Fig. 6B, tube 1), whereas others remained violet (Fig. 6B, tubes 2–6).

3.4 Comparative analysis of RT-LAMP and RT-PCR

Comparative analysis of RT-LAMP and RT-PCR using different concentration of the total RNA revealed that RT-LAMP was able to detect PSTv RNA templates up to 1 pg (Fig. 7A, lanes 1–7), whereas it was 100 pg for RT-PCR (Fig. 7C, lanes 1–7). The visual assessment of RT-LAMP amplification in reaction tubes containing HNB dye was consistent with the agarose gel electrophoresis. The reaction tubes turned sky blue up to 1 pg concentration of RNA (Fig. 7B, tubes 1–7).

Similarly, when the detection limit of BBWV2 by the two assays was compared, RT-LAMP could amplify BBWV2 upto the concentration of 1 pg of total RNA (Fig. 8A, lanes 1–7), however it was 100 pg for RT-PCR (Fig. 8C, lanes 1–7). Also, the pre-indicator HNB dye test demonstrated the same sensitivity result (Fig. 8B, tubes 1–7). Thus for both the viruses, RT-LAMP assay has higher sensitivity and could detect the viruses with 100-fold higher sensitivity.

4. Discussion

In the present study, RT-LAMP assay was developed for detection of PSTv and BBWV2 in fast, specific and sensitive manner under the field conditions. These viruses infect not only patchouli plants but also to a broad range of Leguminosae, Chenopodiaceae and Solanaceae plant species (Kwak et al. 2013). Patchouli has significant importance in pharmaceutical industries, and hence its preservice is of great importance. Since it is propagated by vegetative means, the chances of virus survival in infected plants remain high. Therefore, for controlling further spread of viruses detection at an early stage and selection of healthy mother plants is of utmost importance. Earlier techniques such as ELISA and RT-PCR have

been used for the detection of patchouli- infecting viruses, however these require tedious-post amplification analysis and costly equipments. In contrast, RT-LAMP, tackles these issues using simple devices like heating block, incubator and waterbath, thereby reducing the need of costly equipment in fields (Siljo et al. 2014).

The conserved CP gene nucleotide sequences of the of BBWV2 and PStV were used to make four sets of oligo primers that recognize six different sites on the target genes, thus adding specificity to the RT-LAMP (Notomi et al. 2000). Further loop primers not only increase the specificity but also accelerate the LAMP reactions (Nagamine et al. 2002). In the present study, without loop primers amplification was shown to be poor, but their subsequent addition increased the amplification efficiency of the viruses being tested. Although the standard RT-PCR protocol typically takes 3–4 hours to complete, it is only 30 min for RT-LAMP based detection of viruses by RT-LAMP (Wani et al. 2023). The RT-LAMP specifically detected both PStV and BBWV2 specifically as negative controls showed no amplification. In addition, a comparative analysis of the conventional RT-LAMP and RT-PCR assays demonstrated that the LAMP had higher sensitivity for PStV and BBWV2. This goes in line with the sensitivity of RT-LAMP assays reported for other plant viruses (Liu et al. 2010; Kamala et al. 2015).

Colorimetric methods employing SYBR Green I as DNA intercalating dyes (Tomlinson et al. 2010), calcine and metal ion indicator HNB in the pre-reaction mixture cause a change in colour of the reaction, thus proving to be more accessible for field practises (Mori et al. 2001; Tomita et al. 2008; Nkere et al. 2018; Wani et al. 2023). Taking into account the field practises, this study has also contributed to the development of a colorimetric assay utilising HNB dye that yielded sky blue in the positive samples and violet in the negative samples. In this study, RT-LAMP could detect PStV for the first time though this approach has been for BBWV2 in *Pseudostellaria heterophylla* (Liang et al. 2022). In conclusion, developed RT-LAMP assay could be utilized to screen a large number of patchouli germplasms for early and rapid detection of BBWV2 and PStV under the field conditions. Thus, it may of great help to the farmers and growers engaged in cultivation of patchouli crop.

Declarations

Funding: The author would like to thanks Department of Biotechnology (Government of India), New Delhi for financial grant to JAK (Research grant number No.BT/PR2293/BPA/118/126/2016). HB acknowledges University Grant Commission (UGC) for fellowships (JRF & SRF) to pursue PhD.

Author Contributions: Conceptualization, JAK; Experiments performed by HB, Data curation by HB; Resource generation JAK; Formal analysis, JAK; original draft written by HB; Revision, review & editing by JAK.

Conflict of interest: Authors declares that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

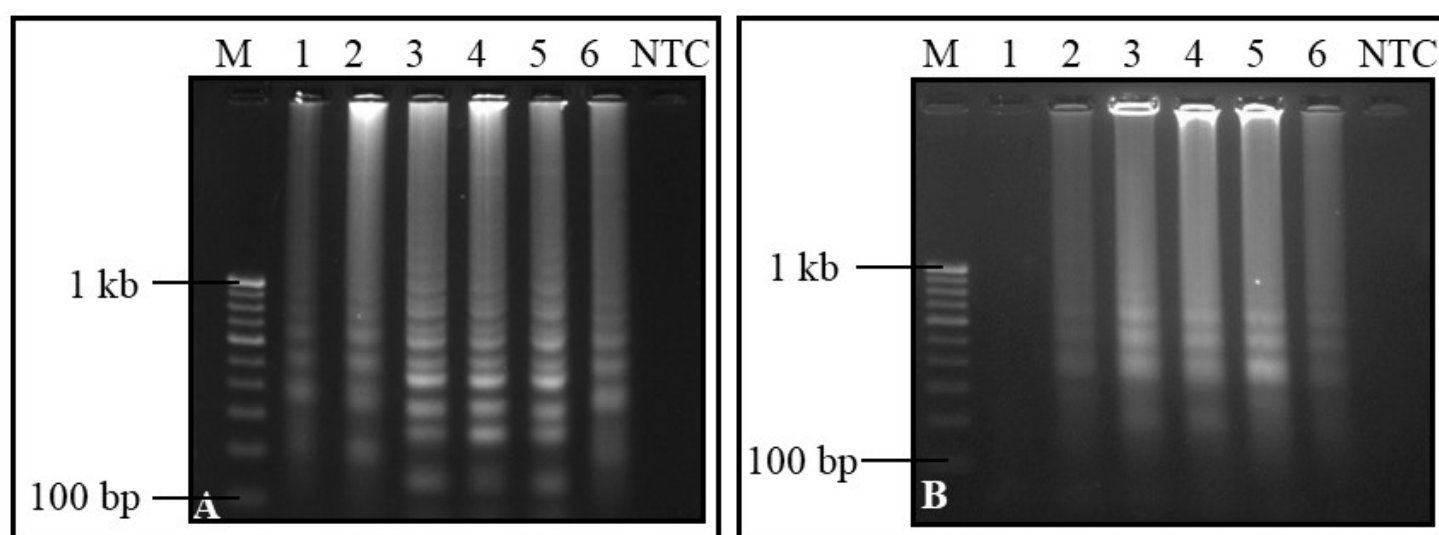


Figure 1

Optimisation of the reaction conditions for detection of *Peanut stripe virus* by RT-LAMP. (A) Agarose gel showing different incubation temperatures; Lanes 1 to 6 represent 59, 61, 63, 65, 67 and 69 °C, respectively. (B) Agarose gel showing different incubation durations; Lanes 1 to 6 represent 20, 30, 40, 50, 60 and 70 min respectively. M =100 bp DNA ladder. NTC = non-template control

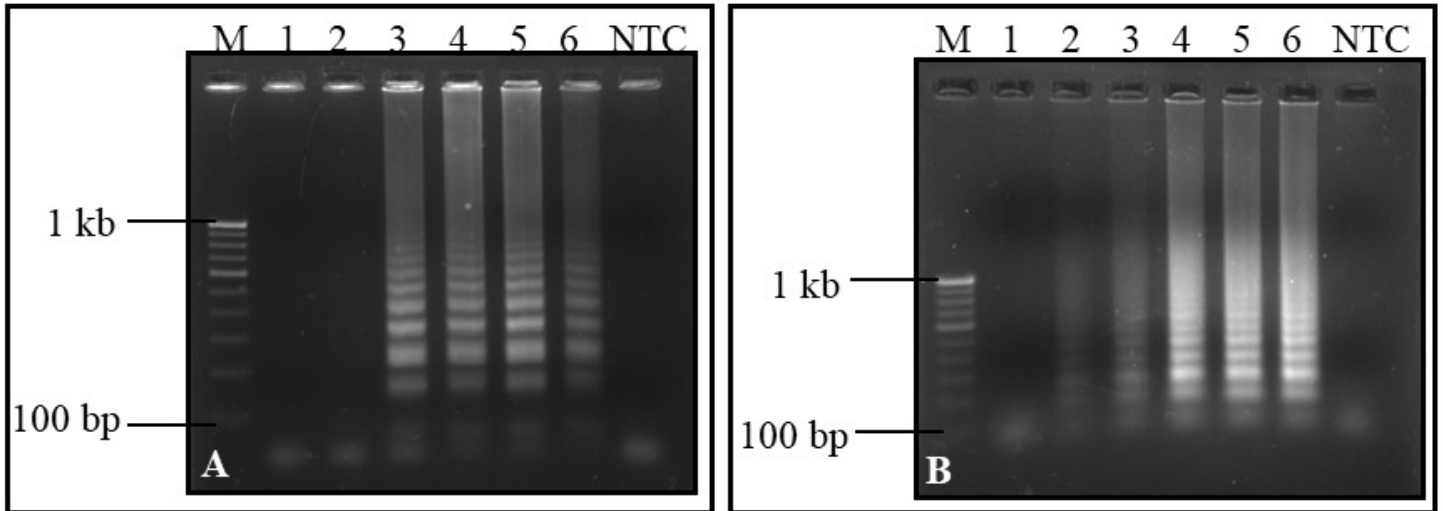


Figure 2

Optimisation of the reaction conditions in RT-LAMP for *Broad bean wilt virus 2* detection. (A) Agarose gel showing incubation at different temperatures; Lanes 1 to 6 represent 57, 59, 61, 63, 65, and 67 °C, respectively. (B) Agarose gel showing incubation at different durations; Lanes 1 to 6 represent 10, 20, 30, 40, 50 and 60 min respectively. M = 100 bp DNA ladder marker, NTC = non-template control

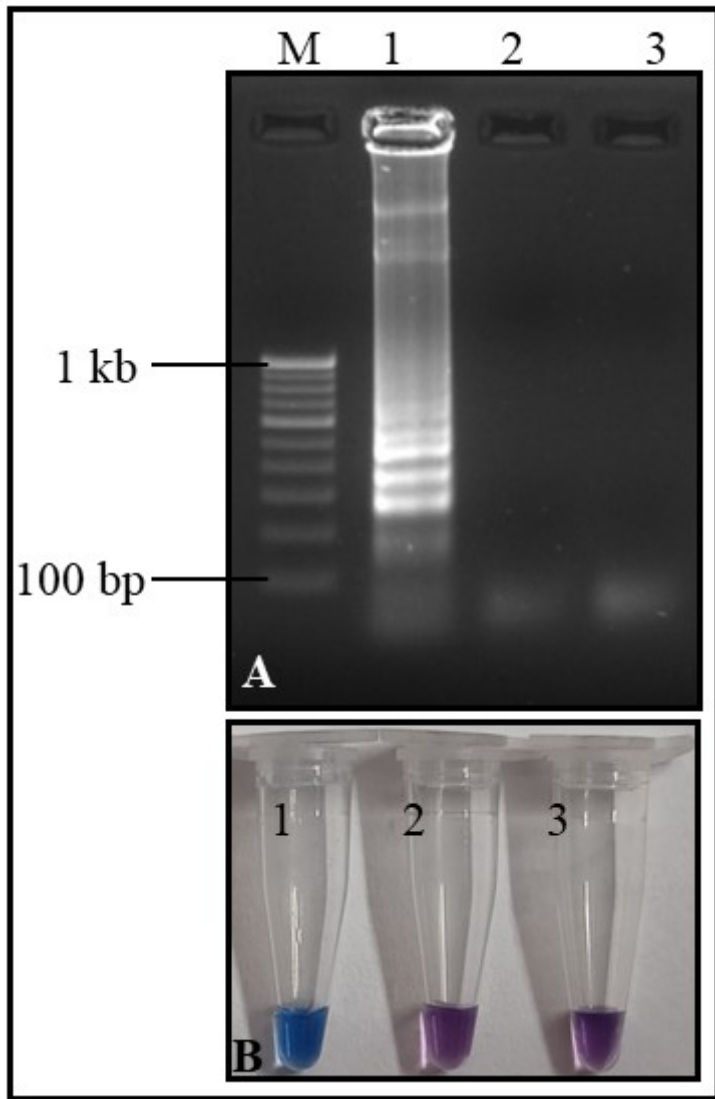


Figure 3

RT-LAMP-mediated detection of *Peanut stripe virus*(PStV) in patchouli. (A) Agarose gel electrophoresis. (B) Visual detection of the RT-LAMP products using hydroxyl naphthol blue (HNB). lane M: 100-bp DNA ladder; total RNA from PStV -infected (lane 1, tube 1) & healthy (lane 2, tube 2) leaves; negative control water (lane 3, tube 3)

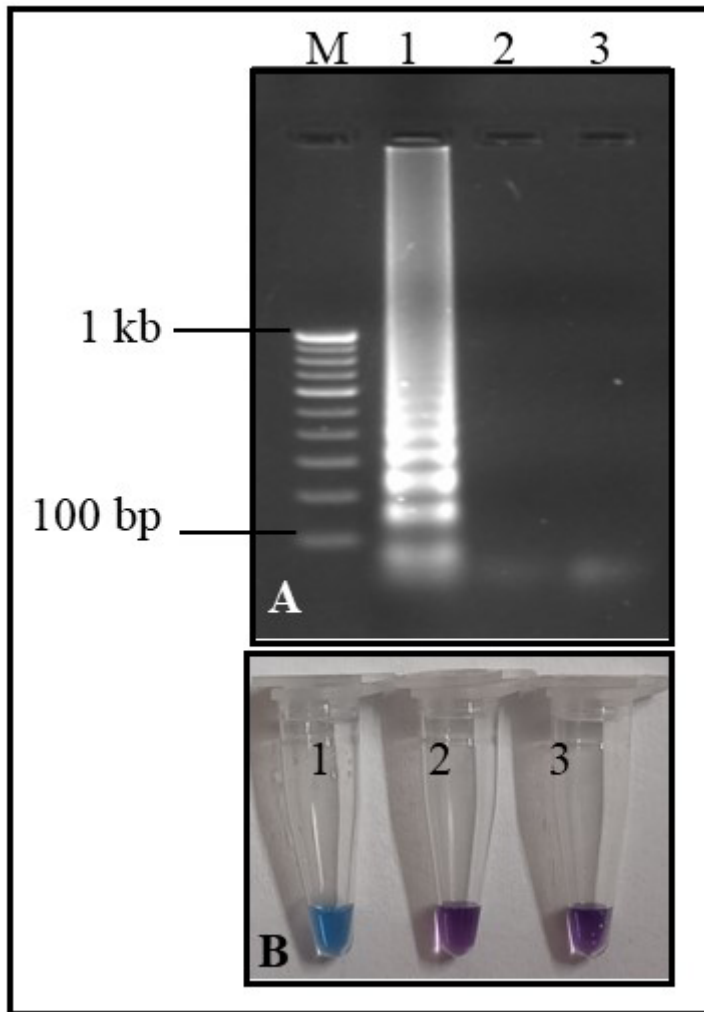


Figure 4

RT-LAMP-mediated detection of *Broad bean wilt virus 2* (BBWV2) in patchouli, following (A) Agarose gel electrophoresis. (B) Visual detection of the RT-LAMP products using hydroxynaphthol blue (HNB). lane M: 100-bp DNA ladder; total RNA from BBWV2-infected (lane 1, tube 1) & healthy patchouli (lane 2, tube 2) leaves; negative control water (lane 3, tube 3)

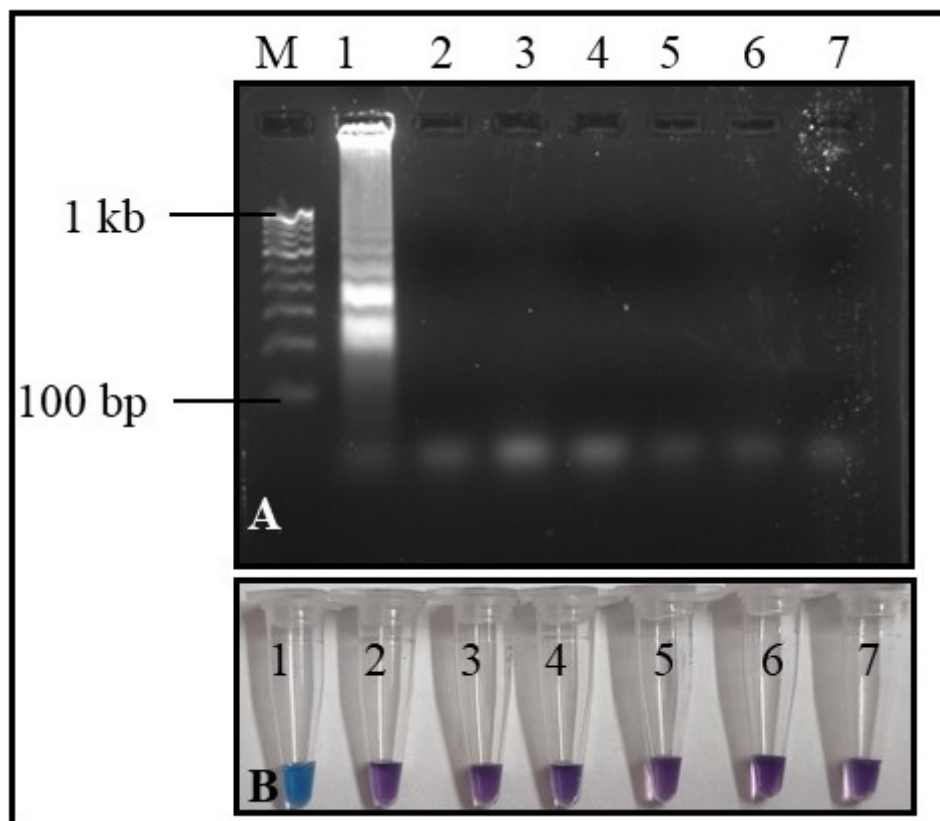


Figure 5

Specificity of RT-LAMP-mediated detection of *Peanut stripe virus* (PStV). (A) Agarose gel electrophoresis of the RT-LAMP products. (B) Specificity of the RT-LAMP assay detected using pre indicator HNB test. Lane M: 100 bp DNA marker; lane 1 & tube 1: leaf samples infected with PStV; lane 2 and tube 2: infected with *Broad bean wilt virus 2*; lane 3 and tube 3: infected with *Papaya ringspot virus*; lane 4 and tube 4: infected with *Prunus necrotic ringspot virus*; lane 5 and tube 5: infected with *Apple mosaic virus*; lane 6 and tube 6: healthy leaf sample. Lane 7 and tube 7: negative control (water)

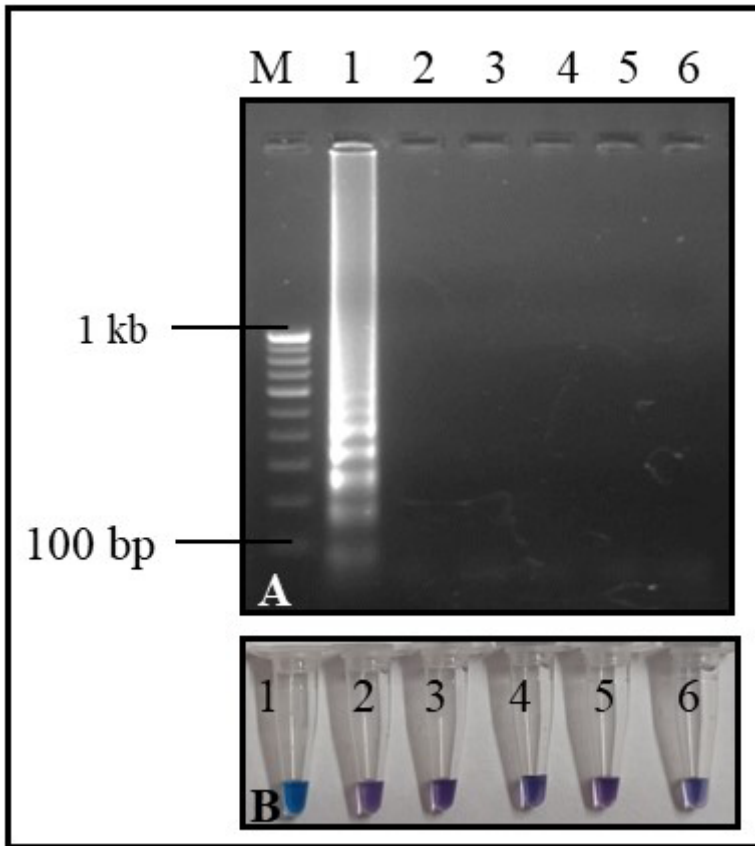


Figure 6

Specificity of RT-LAMP-mediated detection of *Broad bean wilt virus 2* (BBWV2). (A) Agarose gel electrophoresis of the RT-LAMP products. (B) Specificity of RT-LAMP assay detected using pre indicator HNB test. Lane M: 100 bp DNA marker; lane 1 and tube 1: leaf samples infected with BBWV2; lane 2 and tube 2: infected with *Peanut stripe virus*; lane 3 and tube 3: infected with *Prunus necrotic ringspot virus*; lane 4 and tube 4: infected with *Apple mosaic virus*; lane 5 and tube 5: healthy leaves; Lane 6 and tube 6: negative control (water)

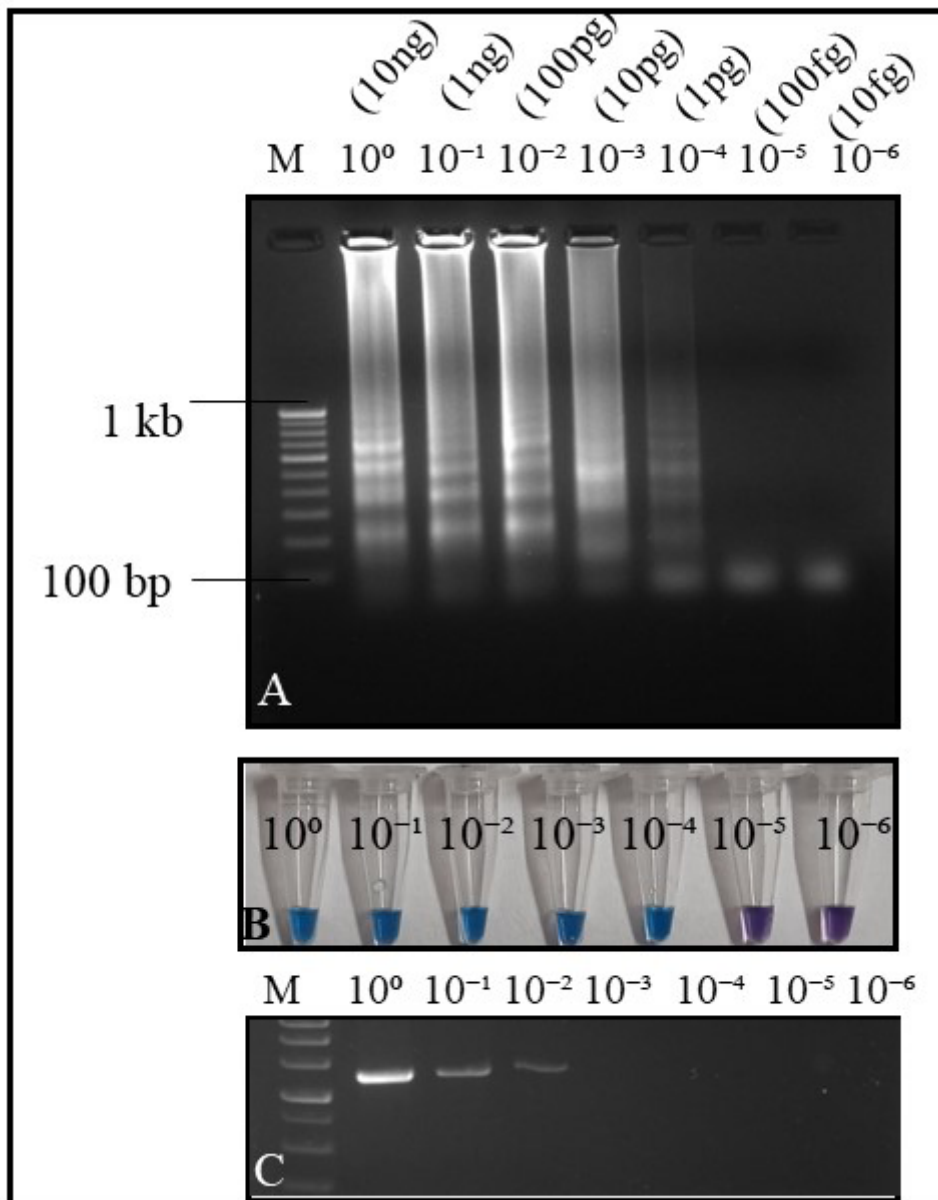


Figure 7

Comparison of the sensitivities of RT-LAMP and RT-PCR assays for the detection of *Peanut stripe virus*. (A) Sensitivity of RT-LAMP assay detected by agarose gel electrophoresis using a serial dilution of total RNA (10ng/μl). lane M: 100 bp DNA ladder, lanes 10⁰ -10⁻⁶ : amplification products with 10-fold serially diluted total RNA. (B) Sensitivity of RT-LAMP assay by pre indicator dye HNB. Tube 1 to 7 represents serially diluted total RNA 10⁰ -10⁻⁶ (C) Sensitivity of RT-PCR assay determined by agarose gel electrophoresis using a serial dilution of total RNA (10 ng/μl)

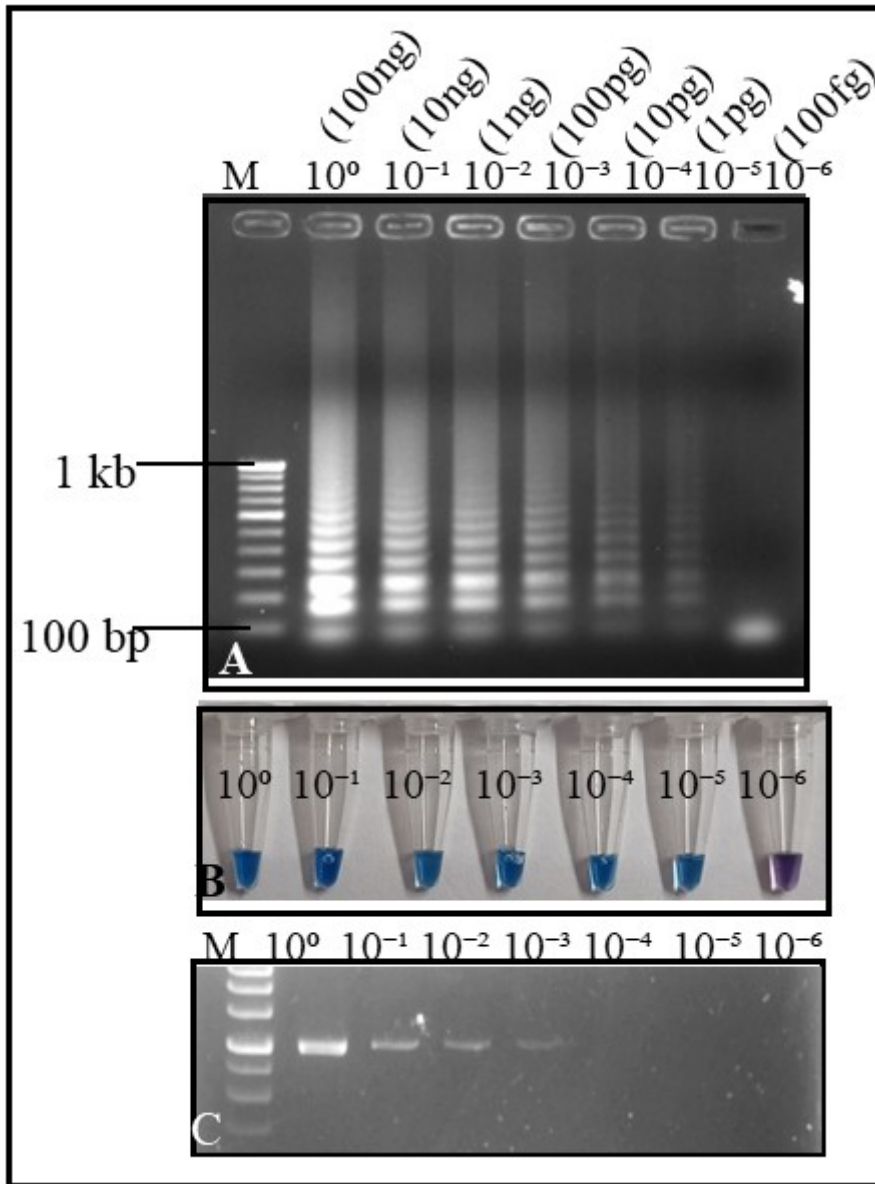


Figure 8

Comparison of the sensitivities of RT-LAMP and RT-PCR assays for the detection of *Broad bean wilt virus 2*. (A) Sensitivity observed by agarose gel electrophoresis using a serial dilution of total RNA (100 ng/ μ l). lane M: 100 bp DNA ladder, lanes 10^0 - 10^{-6} : amplification products with 10-fold serially diluted total RNA. (B) Sensitivity determined by pre indicator dye HNB. Tube 1 to 7 represent serially diluted total RNA 10^0 - 10^{-6} : (C) Sensitivity of RT-PCR assay detected by agarose gel electrophoresis using a serial dilution of total RNA (100 ng/ μ l)

Supplementary Files

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