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SHORT COMMUNICATION

Colorimetric detection platform for banana bunchy top virus (BBTV) based on closed-tube loop mediated isothermal amplification (LAMP) assay

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Abstract A colorimetric closed-tube Loop mediated isothermal amplification (LAMP) assay was developed for rapid and sensitive detection of banana bunchy top virus (BBTV) from leaf and sucker tissues of infected banana plants. Six LAMP primers were designed targeting BBTV coat protein gene. Isothermal amplification was set at 65 °C and end point detection made by including hydroxy naphthol blue dye in the reaction where the positive samples showed colour change from violet to sky blue. Molecular characterization of LAMP amplicon was made with restriction digestion and sequencing. Restriction digestion with *Sau*3AI having single cut site within the LAMP internal primer flanking region yielded two fragments of expected size. Sequence analysis confirmed that amplification corresponded to BBTV coat protein gene. Comparison of LAMP assay with conventional PCR showed that LAMP assay was 1000 times more sensitive than conventional PCR in BBTV detection with a detection limit of 0.05 ng total DNA per reaction.

Keywords Banana bunchy top virus · Coat protein gene · HNB · LAMP

Banana (*Musa* spp.) is an important food and cash crop worldwide. Viral diseases are of great concern as far as banana cultivation is considered. The banana bunchy top disease, caused by banana bunchy top virus (BBTV, genus *Babuvirus,* family *Nanoviridae*), is the most economically destructive viral disease affecting banana, resulting in up to 100% yield loss [3]. The first written report on the disease came from Fiji during 1890 where it caused heavy destruction [13]. The disease was introduced to India from Sri Lanka during 1940 [14] and is prevalent in Kerala, Tamil Nadu, Andhra Pradesh, Karnataka, Maharashtra, Gujarat, Assam, West Bengal, Arunachal Pradesh, Meghalaya, Delhi and Andaman [22]. The disease is characterized by the development of "Morse code" pattern on the leaf veins, midribs and petioles, and marginal chlorosis [24]. The BBTV spreads through infected planting materials and is also transmitted by the banana aphid, *Pentalonia nigronervosa*. As banana is clonally propagated, using suckers, which do not have any quality check by farmers for large scale cultivation; the BBTV perpetuates season after season and spreads to new areas along with the infected planting material. The tissue culture techniques are now being used increasingly to get virus free planting materials in banana. Hence, accurate virus indexing of suckers and tissue culture plantlets is essential for supplying virus free planting materials for cultivation.

Enzyme Linked Immuno Sorbent Assay (ELISA), Polymerase Chain Reaction (PCR) and real time PCR techniques are commonly employed for the detection of BBTV [2, 7,9, 20]. These methods are cumbersome, time consuming, require well equipped laboratories and highly trained personnel to carry out the assay. A nucleic acid amplification method termed Loop-Mediated Isothermal Amplification (LAMP) enables rapid, sensitive and specific amplification of template DNA under isothermal condition using a strand

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displacing polymerase like *Bst* DNA polymerase producing 10^9 copies of the target in less than an hour [19]. This technique is highly specific as four specially designed primers bind to six positions on the template DNA. Two more primers, called loop primers, can be applied to accelerate the reaction making it more sensitive [16]. Of the different routes for monitoring the positive LAMP reaction, the colorimetric method based on Hydroxy Naphthol Blue (HNB) enables closed-tube detection with naked eye and evades sample cross contamination associated with post amplification sample handling [10]. The HNB based colorimetric LAMP assay has been employed for the simple visual detection of several plant pathogens [15, 17, 25].

Here, we report the optimization of a colorimetric closed-tube LAMP assay targeting coat protein gene of BBTV for rapid and sensitive detection of the virus from leaf and sucker tissues. Molecular characterization of the LAMP amplicons and validation of the assay with field collected banana samples is also described. The efficiency of the LAMP assay for the detection of BBTV was compared with conventional PCR.

Leaf and sucker tissues from BBTV symptomatic and asymptomatic banana plants were collected from Banana Research Station, Kannara in Thrissur, Kerala, India. Samples were also obtained from tissue culture raised healthy banana plants at the Centre for Plant Biotechnology and Molecular Biology, Kerala Agriculture University, Thrissur, Kerala, India. Leaf samples from banana plants showing symptoms of banana streak virus (BSV), cucumber mosaic virus (CMV) and banana bract mosaic virus (BBrMV) were also sourced from Banana Research Station, Kannara. Total genomic DNA was purified from 3.0 g tissue using the Doyle and Doyle [6] protocol followed by RNase treatment. The quality and quantity of the isolated DNA was checked by electrophoresis on agarose gel and through NanoDrop spectrophotometer reading. The DNA samples were diluted to 25 ng/µl and we used 2 µl of the sample for optimizing the LAMP assay.

The BBTV isolate Kerala coat protein gene sequence retrieved from NCBI (GenBank accession number GU125413.1) was used as the template for picking a set of six LAMP primers using the software Primer Explorer version 5.0. (http://primerexplorer.jp/lampv5e/index.html). The external and internal primers were designed initially and that sequence information file was used as input for designing the loop primers. All the six primers were validated using BLASTN and synthesized with Sigma Aldrich Pvt. Ltd.

The LAMP assay was initially standardized using one BBTV symptomatic and one healthy sample. The assay was optimized by varying the concentration of different components in the LAMP reaction like dNTPs (1.4 to 1.6 mM), $MgSO_4$ (4 to 8 mM) betaine (0.8 to 1 M) and HNB (120 µM to 150 µM). The final optimized reaction mixture contained 50 ng template DNA, 1.6 mM each dNTP, 0.8 µM each primer FIP and BIP, 0.2 µM each primer F3 and B3, 0.4 µM each primer LF and LB, 1 M betaine (Sigma), 6 mM $MgSO_4$ (New England BioLabs), 1xThermopol buffer with 2 mM $MgSO_4$, 4U *Bst* polymerase large fragment (New England BioLabs) and 120 µM HNB dye (Sigma) in 25 µl reaction volume. Molecular biology grade water (Sigma) was used to make up the volume to 25 µl. The reaction was prepared in 0.2 ml tubes and incubated at 65 °C for 60 min for isothermal amplification followed by enzyme inactivation at 80 °C for 20 min on a dry bath (NeuationiTherm D150-2). Along with each assay, a no template control (NTC) was also run. The LAMP products were initially observed on 2% agarose gel stained with ethidium bromide. Colorimetric determination for LAMP positives was done based on the colour change at the end of the reaction in the presence of 120 µM HNB dye (Sigma) in the reaction mixture.

Molecular typing of LAMP amplicons was carried out to test the fidelity of the assay. Restriction enzyme having a single cut site within the LAMP internal primer flanking region was identified using NEB cutter version 2.0. (http://nc2.neb.com/NEBcutter2/) software. The enzyme *Sau*3AI was selected and 10 µl of the LAMP product was digested with restriction enzyme *Sau*3AI (Thermo Fischer Scientific) as per the manufacturer's instructions. Restriction fragments were analyzed by electrophoresis on 2.5% agarose gel stained with ethidium bromide.

For sequence confirmation, conventional PCR using the LAMP external primers F3 and B3 was performed. The 15 µl PCR reaction contained 50 ng of DNA template, 0.6 µM of each primer, 150 µM of each dNTP, 0.5U of *Taq* DNA polymerase and 1 × PCR buffer with 1.5 mM $MgCl_2$. The thermal profile consisting of an initial denaturation at 95 °C for 1 min followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C and 1 min primer extension at 72 °C followed by a final extension at 72 °C for 8 min was programmed in the Agilent thermocycler. The products were electrophoresed on agarose gel, amplicons of expected size purified with MachereyNagel gel extraction kit and sequenced with BBTVF3 primer. The sequence was subjected to similarity search using the local alignment search algorithm, BLASTN [1].

The optimized LAMP assay was validated with field collected banana samples, taking a tissue culture raised healthy banana sample as the negative control. We tested leaf samples from 15 BBTV symptomatic banana plants and two asymptomatic banana plants and one sample each from plants showing symptoms of other viruses commonly infecting banana like the CMV, BBrMV and BSV. Leaf samples from asymptomatic plants in the disease sick garden were tested to check for latent infection. Tissue culture raised healthy banana plant was used as the negative control. Banana suckers from symptomatic plants were also

tested to check the suitability of the assay for detecting BBTV from sucker samples.

Experiment was conducted to compare the efficiency and ease of LAMP assay with conventional PCR in the detection of BBTV. All the BBTV symptomatic and healthy banana samples tested in the LAMP assay were tested using the conventional PCR with BBTV coat protein gene specific primers (BBTVF15'GGTATCCGA AGAAATCCATCAA 3' and BBTVR1 5'ATTCTTCCT CAACACGGTTGTC 3' amplifying 416 bp fragment of the coat protein gene). The PCR assays were carried out in 15 µl reaction mixture containing 50 ng of DNA template, 0.6 µM of each primer BBTVF1 and BBTVR1, 150 µM of each dNTP, 0.5 U of *Taq* DNA polymerase and 1 × PCR buffer with 1.5 mM $MgCl_2$. The reaction was set with an initial denaturation at 95 °C for 1 min followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C and 1 min primer extension at 72 °C followed by a final extension at 72 °C for 8 min in the Agilent thermocycler. The amplicons were observed by agarose gel electrophoresis.

Experiments were further conducted to determine the detection limit of LAMP and PCR using serial dilutions of total DNA prepared from diseased plant. The reactions were set with 50 ng, 5 ng, 0.5 ng, 0.05 ng and 0.005 ng of total DNA per reaction.

A set of six LAMP primers complimentary to eight regions on the BBTV coat protein gene was designed (Table 1, Supplementary File 1). The LAMP primers amplified the BBTV coat protein gene from all symptomatic leaf and sucker samples tested. No amplification was observed for the healthy banana samples and the NTC. The positive LAMP products on agarose gel produced a series of ladder like bands representing the stem-loop DNA with inverted repeats of the target sequence (Fig. 1, Supplementary File 2, 3, 4). The LAMP reaction mixture with 120 µM of HNB dye had a violet colour. The positive samples could be distinguished from negative samples on the basis of colour change

Table 1 Oligonucleotides for loop mediated isothermal amplification of BBTV coat protein gene

Primer type	Primer name	Position[a]	Sequence	Length (bases)
Forward external	BBTVF3	146–169	CTACTGATAAAACATTACCCAGAT	24
Reverse external	BBTVB3	329–347	TCCCCACTACATACCAGTT	19
Forward internal	BBTVFIP	F1c: 224–248	CTCTTGATCATAGCCCAATGAAGTA-	48
	(F1c+F2)	F2: 178–200	TGGAAAATGTTTATGCTTCTTGT	
Reverse internal	BBTVBIP	B1c: 250–270	TCTTGGGAAATCAACCAGCCG-	42
	(B1c+B2)	B2: 308–328	TAACCAGATGGCTATGTTCAG	
Forward loop	BBTVLF	201–217	CGGGCTTCACCTTGCAC	17
Reverse loop	BBTVLB	279–296	TCTGGAAGCCCCAGGTTT	18

[a] Position is shown with reference to BBTV coat protein gene (GenBank Acc. No. GU125413.1)

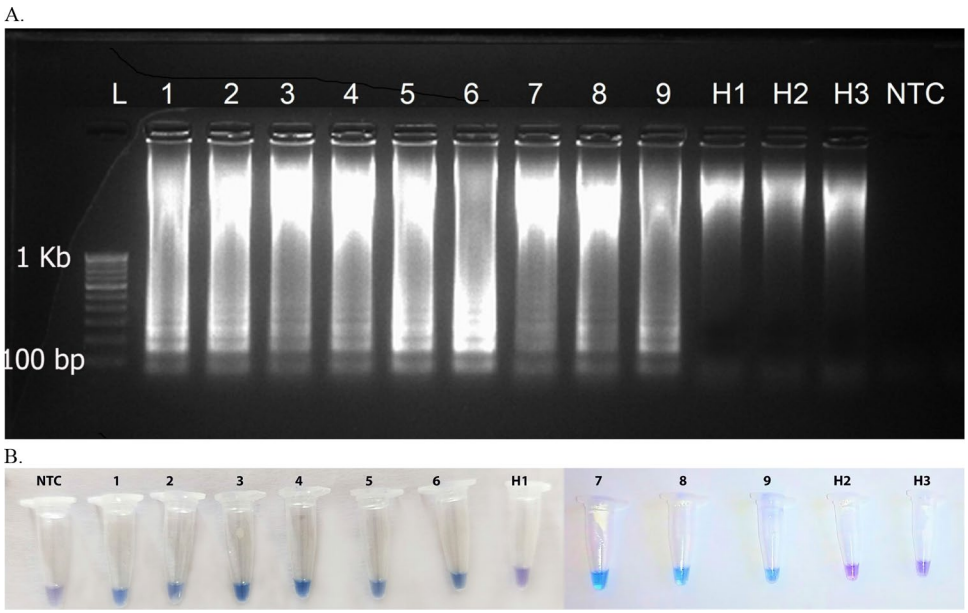

Fig. 1 LAMP assay for BBTV detection. Agarose gel profile of LAMP amplicons (**A**). Lanes L-100 bp DNA Ladder (GeNei); 1 to 9—BBTV symptomatic banana samples; H1, H2—field collected healthy plants; H3—tissue culture raised healthy banana sample; NTC—No template control. Colorimetric detection of LAMP positives using HNB dye (**B**). NTC—No template control, 1 to 9—BBTV symptomatic banana samples, H1, H2- field collected healthy banana sample, H3—tissue culture raised healthy banana sample. The positive samples show blue colour, the negative samples (NTC and healthy) remain violet

form violet to sky blue after the amplification. The negative samples remained violet (Fig. 1). The developed LAMP assay was also validated by testing banana plants showing symptoms of other viruses commonly infecting banana like the BSV, BBrMV and CMV. However, no amplification was observed with nucleic acid purified from the leaf samples collected from these plants. An asymptomatic plant in the disease affected garden also tested positive in the assay, thus detecting latent infection before symptom development.

Molecular identity of LAMP products was confirmed using restriction analysis and sequencing. The restriction enzyme *Sau*3AI, with a single recognition site within the LAMP product was identified using NEB cutter software. The enzyme cuts the 151 bp region flanked by BBTVF2/BBTVR2 in to two fragments of 62 bp and 89 bp size. The LAMP product on actual restriction digestion produced fragments of the expected sizes for the diseased samples as observed by electrophoresis on 2.5% agarose gel (Fig. 2). Hence, we could confirm the accuracy of the LAMP assay developed. Sequencing with LAMP external primer, BBTVF3 and sequence analysis using BLASTN revealed that the sequence had 100% identity with BBTV isolate KOL1 coat protein gene (GenBank Accession number MT174330.1). A representative sequence has been deposited in NCBI (GenBank Accession number—OL757511).

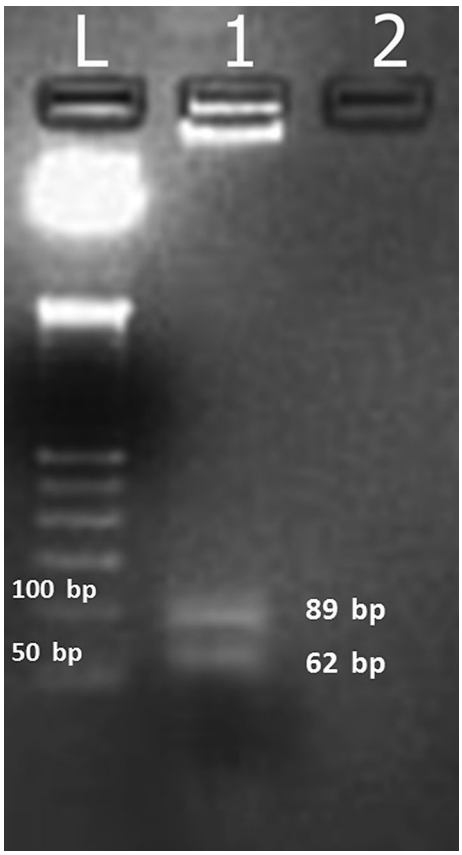

Fig. 2 Restriction analysis of LAMP product. Lanes L- 50-bp DNA ladder (GeNei), 1—amplified product of BBTV sample digested with enzyme *Sau*3AI, 2—NTC digested with enzyme *Sau*3AI. Two fragments of size 62 and 89 bp are indicated in the positive sample

The PCR primers, BBTVF1 and BBTVR1, amplified 416 bp fragment of the BBTV coat protein gene from symptomatic banana samples. Healthy sample and NTC did not amplify. Sequencing and BLASTN analysis showed that the sequence had 100% identity with BBTV isolate KOL1 coat protein gene (GenBank Accession number MT174330). However, among the 15 BBTV symptomatic samples tested in the PCR assay, only 13 samples showed positive amplification in PCR, while all the 15 symptomatic samples had amplified in the LAMP assay (Supplementary File 5, 6, 7, 8). The presence of inhibitors in the DNA preparation from diseased plants often interferes with PCR amplification [4]. As compared to conventional PCR, the LAMP assay identified a higher proportion of positives from the same tested samples. The *Bst* polymerase used in LAMP is less sensitive to inhibitors in the DNA samples when compared to the *Taq* DNA polymerase used in PCR [5].

While determining the sensitivity of LAMP assay, we observed that LAMP could detect BBTV from 0.05 ng of total DNA isolated from diseased samples while the PCR method could not detect BBTV even in 5 ng total DNA preparation from the same sample (Supplementary File 9, 10). The LAMP assay for BBTV, targeting the BBTV replication initiator protein gene, was reported to be 100-fold more sensitive than PCR [21]. Our LAMP assay was 1000 times more sensitive than conventional PCR and the use of loop primers has contributed to the increased sensitivity. In the LAMP assay reported previously for the detection of BBTV in abaca samples, the virus could be detected from 10 pg of DNA [8]. The LAMP assay optimized to detect mosaic virus on wheat was reported to be 100 times more sensitive than PCR [26]. In another report, the LAMP assay developed for the diagnosis of early blight in potato had shown ten-fold more sensitivity than PCR [11].

We devised a colorimetric closed-tube LAMP assay for rapid screening of banana planting materials for the BBTV. A sensitive platform for quick virus indexing of tissue culture plants and planting materials is essential for the distribution of disease-free planting materials [3]. Isothermal amplification methods are preferred alternative to PCR in routine pathogen detection and the LAMP assay for detection of several plant pathogens has already been reported [11, 12, 18]. A LAMP assay targeting BBTV replicase gene and using the SYBR Green fluorescence for end point detection has been reported from China [21]. However, the assay did not use loop primers. We have targeted the BBTV coat protein gene in our LAMP assay and the six primers including the loop primers make the assay highly sensitive, as the loop primers further increase the amplification

efficiency [16]. Moreover, the HNB enables closed-tube simple visual detection by adding the dye in the reaction tube prior to amplification and observing color change from violet to sky blue at the end of the reaction [10]. We could visually differentiate the positives from negative by including 120 µM of HNB in the reaction. The concentration of $MgSO_4$ and dNTPs in the reaction had to be optimized to get a clear colour change. The dNTPs at 1.6 mM and the $MgSO_4$ at 8 mM were needed in the reaction mixture. In the LAMP assay devised for the detection of abaca bunchy top virus and BBTV in abaca, end point detection was based on GelRed or SYBR Green staining which was added to the reaction tube post amplification [8]. The LAMP assay devised here can be combined with rapid nucleic acid extraction method reported earlier [23] to reduce the time for DNA sample preparation.

The LAMP test always raises the risk of carryover contamination due to strong target amplification. Closed-tube detection methods combined with proper sample handling, on the other hand, ensure contamination-free reactions. We used a separate sterile, UV-enabled PCR work station for setting up the LAMP reaction. A different laboratory room was used for sample preparation. Furthermore, after the initial optimization, sequence confirmation and molecular typing, solely the closed-tube assays should be carried out for routine diagnostics and the HNB based detection enables this. Assuring virus free planting materials is the principal approach for managing BBTV infections all over the world and the LAMP assay reported here will enable quick virus indexing of tissue culture plantlets and suckers aiding in the distribution of virus free planting materials to farmers.

Author contributions SN conceptualized the idea, designed the experiments and edited the MS. NB conducted the experiments and drafted the manuscript. VL identified the diseased plants and provided samples for the experiments.

Data availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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