REVERSE TRANSCRIPTION LOOP MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) FOR DIAGNOSIS OF *Banana bract mosaic virus* IN BANANA (*Musa* spp.)

By

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(2019-11-002)

THESIS

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DECLARATION

I, Midhuna Madhu K. (2019-11-002) hereby declare that the thesis entitled **"Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) for diagnosis of *Banana bract mosaic virus* in Banana (*Musa* spp.)** is a *bona-fide* record of research done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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2019-11-002

CERTIFICATE

Certified that this thesis entitled **"Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) for diagnosis of *Banana bract mosaic virus* in Banana (*Musa* spp.)"** is a record of research work done independently by **Ms. Midhuna Madhu K.** (2019-11-002) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Midhuna Madhu K.

Dedicated to my family for their endless love, support and encouragement

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ABBREVIATIONS

%	Percentage
BLAST	Basic Local Alignment Search Tool
BBTD	Banana Bunchy Top Disease
BBTV	*Banana bunchy top virus*
BBrMV	*Banana bract mosaic virus*
BSV	*Banana streak virus*
bp	Base pair
Bst	*Bacillus stearothermophilus*
CMV	*Cucumber mosaic virus*
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DIBA	Dot Immuno Binding Assay
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
CP	Coat Protein
HNB	Hydroxy Naphthol Blue
dNTPs	Deoxyribo Nucleoside Triphospate
ELISA	Enzyme Linked Immuno Sorbent Assay
KAU	Kerala Agricultural University
Kb	Kilo base pairs
L	Litre
LAMP	Loop Mediated Isothermal Amplification
RT-LAMP	Reverse Transcription Loop Mediated Isothermal Amplification
M	Molar
mg	Milligram

$MgSO_4$	Magnesium sulfate
ml	Millilitre
mM	Millimolar
ng	Nanogram
NTC	No Template Control
NHB	National Horticulture Board
nm	Nanometer
°C	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PVP	Poly vinyl pyrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
Taq	*Thermus aquaticus*
TAE	Tris Acetate EDTA
U	Unit
V	Volts
µg	Microgram
µl	Microlitre

Introduction

1. INTRODUCTION

Banana (*Musa* spp.) is one of the most important fruit crops of the world belonging to family Musaceae. Banana is cultivated in the tropics and is valued worldwide for its flavour, availability and nutritional value. India tops banana production in the world producing 29 million tons per year (FAOSTAT, 2020). In India, banana is cultivated in 0.9 million hectares with a production of 30.8 million tonnes (NHB, 2020).

Banana as both ripe and raw fruit is widely used due to its high nutritional value, easy availability and low price. It is a rich source of vitamins and carbohydrates. It is also an important source of Mg, Ca and K. The fruit is free from cholesterol and fat and is very easy to digest. It is also used in powder form for feeding babies. It is recommended for people suffering from various diseases, kidney disorders, ulcer and arthritis (NHB, 2013).

Several processed products including wine, chips, halwa etc can be made from the fruit. The tender stem bearing the inflorescence is taken by removing the leaf sheaths from the pseudostem and is used as vegetable. Good quality fibres obtained from the plant is used to make wall hangings, ropes, pots etc. Best quality paper can be made from wastes of banana. Banana leaves are used for making hygienic and healthy eating plates (NHB, 2013).

Bananas is one of the most produced and consumed foods globally. Despite its importance, growers are not in a position to realize high productivity due to the prevalence of various biotic and abiotic stresses, especially diseases like panama wilt, moko wilt and several viral diseases. Banana is mainly infected by four types of viruses. These include *Banana bract mosaic virus* (BBrMV), *Banana bunchy top virus* (BBTV), *Banana streak virus* (BSV) and *Cucumber mosaic virus* (CMV).

The BBrMV, a single stranded RNA virus, of the genus *Potyvirus*, causes bract mosaic disease in banana. In India, several states including Andra Pradesh, Karnataka, Tamil Nadu and Kerala have reported the occurrence of the disease

(Cherian *et al*., 2002; Selvarajan and Jeyabaskaran, 2006). About 40 percent reduction in yield due to this disease has been reported. The virus is readily transmitted through vegetative propagules and insect vectors like *Aphis gossypi, Pentalonia nigronervosa*, and *Rhopalosiphum maidis* (Magnaye and Valmayor, 1995). Internal quarantine is necessary to prevent the viral spread. To develop stringent quarantine measures, highly sensitive diagnostic methods are a pre-requisite to certify planting materials. According to National Horticulture Board (2010), 70 per cent of farmers use suckers for planting. There is no quality control for banana suckers used for planting. So, there should be a platform for efficient virus indexing, so that farmers can get good quality virus free planting materials.

The available detection methods for BBrMV are the Enzyme Linked Immuno-Sorbent Assay (ELISA) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The ELISA is time consuming while the RT- PCR needs post PCR sample handling and hence there exists a chance of sample cross contamination. The LAMP (Loop Mediated Isothermal Amplification) is an auto-cycling strand displacement DNA synthesis method. The LAMP is an amplification reaction which amplifies a target DNA sequence with high level of specificity and sensitivity under constant temperature conditions (Notomi *et al*., 2000). The RT-LAMP (Reverse Transcription Loop Mediated Isothermal Amplification) is a specific, highly sensitive and relatively faster diagnostic method for RNA viruses. It does not involve thermal cycling as amplification takes place under isothermal condition.

Development of molecular diagnostics for BBrMV based on RT-LAMP will enable rapid screening of tissue culture plants and suckers and will help in the distribution of disease free planting materials to farmers. Hence, the present study was taken up with the objective to develop a closed tube colorimetric RT-LAMP assay for detection of BBrMV in banana.

Review of Literature

2. REVIEW OF LITERATURE

2.1. Banana and its economic importance

The banana is one of the most widely traded tropical fruits. Several species of the genus *Musa* have been cultivated and the name 'banana' refers to all plants of the family Musaceae. They thrive in a variety of environments and serve a wide range of human purposes, from tropical fruits like bananas and plantains to cold-hardy fibre and ornamental plants. Bananas have been a part of the human diet since the beginning of time. The wide adoption of banana is ascribed to the availability of a wide range of diversity, varied production strategies, and suitability to different agro-climatic conditions (Jyotsna *et al*., 2019).

The banana is native to the tropics, and while it is most commonly consumed there, it is prized globally for its nutritional content, flavour, and year-round availability. India produces the most bananas in the world, with 29 million tonnes per year (FAOSTAT, 2020). In India, banana is cultivated on 0.9 million hectares, yielding 30.8 million tonnes (NHB, 2020).

2.2. Diseases affecting banana

Independent of region and also production system, diseases and pests have been considered as the main constraint which is responsible for low productivity and yield losses in bananas. Banana is propagated vegetatively and produces fruit throughout the year. After fruiting, the suckers emerging from the underground rhizome take the place of main shoots which gradually withers and this process of succession continues (Morton, 1987). To build new fields, farmers typically use suckers in early stages cut from old orchards. This approach has been linked to the increase in spread of a several banana pests and diseases across the globe (Jones, 2002), particularly viral diseases spreading along with the planting material.

Apart from the viral diseases, bacterial diseases like moko wilt, Xanthomonas wilt, and the fungal diseases like Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*)

and black leaf streak disease (known as black sigatoka- *Mycosphaerella fijiensis*) are considered as the major diseases affecting banana and plantain (Blomme *et al*., 2017).

2.2.1. Viral diseases of banana

Viral diseases have a major impact on banana production due to their effects on quality and yield. They spread through planting materials. There have been reports of about 20 distinct viral species representing five different families infecting banana all over the world. The important ones are *Banana bract mosaic virus* (BBrMV, genus *Potyvirus*), *Banana streak virus* (BSV, genus *Badnavirus*), *Banana bunchy top virus* (BBTV, genus *Babuvirus*), and *Cucumber mosaic virus* (CMV, genus *Cucumovirus*). Other viruses include *Banana virus* X and *Abaca bunchy top virus* (ABTV, genus *Babuvirus)* (Balasubramanian and Selvarajan, 2014).

2.3. *Banana bract mosaic virus* (BBrMV)

2.3.1. The BBrMV in India

Banana is one of the widely cultivated and consumed fruit crops worldwide. Fungal, bacterial and viral diseases reduce banana production across the globe especially in India. The BBrMV is an important virus affecting banana. The banana bract mosaic disease was first detected in early 1979 in the Philippines by Magnaye and Espino (1990) and subsequently, reported from Sri Lanka, India, Thailand, Vietnam, and Ecuador (Magnaye and Espino, 1990; Bateson and Dale, 1995). Samraj *et al*. (1966) recorded bract mosaic disease for first in India in 1966 in a cultivar Nendran and named as Kokkan disease in Kerala, but the etiology was not known. Later, the causal organism of the disease was confirmed as BBrMV (Rodoni *et al*., 1997; Singh and Selvarajan, 2000).

2.3.2. Occurrence and symptoms

The BBrMV is a single stranded RNA virus (ssRNA), of the genus *Potyvirus*. It is found to occur in several banana cultivating states including Andhra Pradesh, Maharashta, Tamil Nadu, Kerala and Karnataka (Cherian *et al*., 2002; Selvarajan and Jeyabaskaran, 2006). The disease incidence ranges from 5 to 30 per cent and is more

in the cultivar Nendran in Kerala (Selvarajan and Jeyabaskaran, 2006). In India, yield losses up to 40 per cent have been found (Cherian *et al*., 2002; Thangavelu *et al*., 2002). The disease has a significant effect on the yield of the banana crop with maximum yield loss in cultivar Robusta and in cultivar Nendran (Cherian *et al.,* 2002).

Disease causes prominent spindle shaped, reddish streaks on midribs, peduncles, fruits, pseudostem and mainly on bracts (Rodoni *et al*., 1997; Thomas *et al*., 1997). In severe stages, the leaves show discontinuous streaks throughout the veins. Another symptom is the scattered yellowish to white streaks from the midrib to the margin of leaf (Selvarajan and Jeyabaskaran, 2006).

Towards the base of the pseudostem, reddish brown and even necrotic discontinuous streaks are also seen. The infected plants produce small bunches of brittle fruits. Plants may also die by stunted growth if affected severely and necrosis of pseudostem is also observed. Gradually it fails to flower. The male buds have mosaic patches and are dark purple in colour. Symptomatology of the disease varies with the varieties (Balasubramanian and Selvarajan, 2014).

2.3.3. Disease transmission and host range

The BBrMV has been reported to spread throughout the fields in a very short period of time. It is mainly transmitted through infected planting material or vegetative propagule. The BBrMV, in a non-persistent manner is transmitted by aphids, (*Rhopalosipum maidis, Pentalonia nigronervosa* and *Aphis gossypii)* (Magnaye and Espino, 1990). The transmission of virus particles by aphids *Pentalonia nigronervosa* was reported by Munez, 1992 and also through A*phis craccivora* by Selvarajan *et al*. (2006). Due to its potential spread along vegetative plant parts and also through aphids, the virus has a considerable quarantine risk (Rodoni *et al*., 1997).

The main host of BBrMV is banana (*Musa* spp.) (Sharman *et al*., 2000). Other natural hosts include cardamom especially small cardamom (Siljo *et al*., 2011) in

India and also flowering ginger, *Alpinia purpurata*, a cut flower in tropical regions (Wang *et al*., 1997).

2.3.4. Viral genome and diversity

The BBrMV is from the family *Potyviridae* and genus *Potyvirus* (Francki *et al*., 1991). The potyviral genome includes a ssRNA molecule of size 10 kb and is polyadenylated at the 3' (Li *et al*., 1997). The BBrMV particles have single-stranded positive-sense RNA genome that is flexuous and filamentous particle of 75011 nm (Bateson and Dale, 1995).

The BBrMV was found mostly alike to *Maize dwarf mosaic virus* (MDMV). Atreya *et al*. (1991) confirmed that BBrMV coat protein is involved with aphid transmission. Recombination and genetic diversity studies in the coat protein gene of BBrMV isolates indicated high variation among them (Balasubramanian and Selvarajan, 2014).

Recombination events were found to generate considerable genetic diversity in a study conducted among eleven putative recombinants of 49 BBrMV isolates (Suzuki *et al*., 1998; Varsani *et al*., 2006) and these events are important source of genetic variation for *potyviruses* (Gibbs and Ohshima, 2010).

2.3.5. Management strategies for BBrMV

Viruses pose a significant threat to banana cultivation because of their effects on quality and yield and also due to the quarantine issues in the flow of Musa germplasm. For BBrMV, no sources of natural resistance has been identified till now. The removal and killing of afflicted plants, as well as the rhizome, are standard control procedures, just as the use of virus-free planting material. In some commercial banana cultivars, fertiliser application has been reported to help alleviate BBrMV symptoms (Selvarajan *et al*., 2009).

In the banana tissue-culture sector, viral infections are also a severe hindrance. Shoot-tip culture, which is the industry's method for mass replication, will not be able to eradicate them. Most banana viruses live in the host in latent form, meaning they

do not show any visible symptoms for a long time (Drew *et al*., 1989). Symptoms of viral diseases are most alike with nutrient deficiency symptoms.

Early and accurate virus diagnosis is critical for making appropriate management decisions. This will aid in the prevention of infection spread, particularly in new areas. The key strategy to control them is to discover them early and use sensitive diagnostic methods. When non-indexed plants are utilised as mother plants in tissue culture, viruses spread, resulting in a significant decrease in production. It is necessary to confirm that producers have access to good quality mother plants that have been virus-indexed in order to produce healthy planting material in order to sustain and reap the benefits of tissue-culture in banana (Singh *et al.*, 2011).

2.4. Diagnostic methods for plant RNA viruses

The RNA viruses are common plant pathogens, posing a substantial danger to agriculture and food security. Most plant RNA viruses have ssRNA genome that encodes the proteins needed for translation, replication, cell-to-cell migration etc (Joshi and Haenni, 1984).

Virus infected crops and weeds that act as inoculum sources are removed and destroyed as an agronomic strategy to reduce virus dissemination. This necessitates the use of speedy and specific detection techniques capable of analysing more number of samples from the field, such as Enzyme Linked Immuno-Sorbent Assay (ELISA) and molecular detection (Rubio *et al*., 2003; Rubio *et al*., 1999).

To manage plant viruses, it is necessary to develop a safe and reliable diagnostic technique. The ELISA, molecular hybridization, polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR) and microarrays are only a few of the approaches that have been developed (Rubio *et al*., 2020).

2.4.1. Microscopy

When electron microscopes are readily available, electron microscopy provides highly useful information on the morphology of viral particles and is widely employed for virus detection (Milne, 1992). In leaf-dip preparations, rod-shaped and

filamentous viruses such as *Tobamoviruses, Potexviruses*, and *Potyviruses* can be distinguished more easily than other viruses. Viruses found in plant sap in low concentrations are difficult to see and hence the virus in the test sample is concentrated first (Edwardson and Christie, 1978). When screened under a Jeol CX-100 electron microscope, the leaf dip technique preparation of BBrMV revealed distinct flexuous rod shaped viral particles. Virus particles were 750-780 x 10-15 mm in size (Suresh, 2004).

2.4.2. Serological techniques

The ELISA relies on binding of viral proteins specifically with antibodies (Clark and Adams, 1977; Hull and Al-Hakim, 1988). The ELISA and its derivatives grew more prominent in India's plant virus laboratory during the 1990s. During those times, Indian scholars used additional serological approaches to detect plant viruses, including the Dot Immuno-Binding Assay (DIBA), Immuno-Sorbent Electron Microscopy (ISEM) and fluorescent antibody technique (Bhat, 2017). The antiserum specificity and titre of *Potyviruses*, such as *Sorghum mosaic virus* (SrMV) and BBrMV were determined using the direct antigen coating ELISA (DAC-ELISA) approach (Balamuralikrishnan *et al*., 2002). The virus was detected in *in vitro* and *ex vitro* cultures using the ELISA and PCR. The ELISA detected BBTV, BBrMV, and SCMV with infection rates of more than 50 per cent and even higher, up to 100 per cent (Landicho, 2014). Using plate-trapped ELISA, serological relationships were found between BBrMV and other members of the family (Thomas, 1997).

About 83.33 percent BSV-free and 93.33 percent CMV-free plantlets were detected using enzyme linked immunosorbent assay (DAS-ELISA), whereas 86.67 percent BBTV-free and 83.33 percent BBrMV-free plantlets were detected using a PCR-based approach (Punyarni and Kshetrimayum 2013).

To detect BBrMV, a lateral flow immunoassay (LFIA) was developed. The LFIA could take anywhere from 5 to 10 minutes to complete detection depending on the tissue extracts. The LFIA had a detection limit of 10 ng of expressed CP of BBrMV and a 1:20 crude extract dilution (Selvarajan, 2020).

2.4.3. Molecular detection methods

2.4.3.1. Hybridization techniques

Membrane hybridization with radioactive ^{32}P labelling is still commonly employed today, while non-radioactive techniques for the diagnosis of plant viruses also have been developed (Hull and Al-Hakim, 1988). Herranz *et al*. (2007) proposed a new technique for viral detection using non-isotopic molecular hybridization. Here, 2, 4 and 6 viral sequences were transcribed to produce unique riboprobes and named as 'polyprobes'. A molecular hybridization with non-isotopic approach was devised for the diagnosis of five RNA viruses affecting carnation using construction of cDNA clones and hybridization with labelled probes (Sánchez-Navarro *et al*., 1999).

To identify distinct *Potyviruses* in a single test, a method comparable to gene identification using microarrays was created (Gerhold *et al*., 1999). The virus was detected by reverse dot blot hybridization (Martin *et al*., 2000) utilising the DIG-labelled RT-PCR product obtained from the diseased tissue using species-specific probes mounted on nylon membrane. For the diagnosis of distinct RNA viruses in tomato seedlings, a dot-blot hybridisation technique using a variety of individual single digoxigenin-labelled probes (riboprobes) was created (Saldarelli *et al*., 1996). This method, which involves a cocktail of multiple riboprobes, has also been used to identify plant viruses in geranium crops (Ivars *et al*., 2004) and ornamentals (Sanchez-Navarro *et al*., 1999).

2.4.3.2. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Bateson and Dale (1995) used *Potyvirus* specific degenerate PCR primers to characterise the BBrMV. The *Potyvirus* degenerate primers were used to amplify virus-specific cDNA from field samples using RT-PCR. Rodoni *et al*. (1997) sequenced the full coat protein (CP) region as well as the 3' UTR of various BBrMV samples. To detect BBrMV, one-step RT-PCR and immunocapture RT-PCR (IC-RT-PCR) were developed. Primers were developed targeting the coat protein coding region giving 320 bp amplification products. IC RT-PCR was more labour-saving and cost-effective than one-step RT-PCR (Dassanayake, 2001). A method for obtaining

BBrMV RNA from infected banana pseudostems and bracts has been devised. For detecting the pathogen from affected tissues, a set of primers were designed which amplifies 699-bp product of the CP coding region (Sankarlingam, 2006).

A real-time fluorescence RT-PCR approach based on particular primers and TaqMan probes constructed with conserved sequences of the coat protein genes of various BBrMV isolates was developed. A specificity analysis revealed that typical amplification curves could be generated for the two BBrMV strains, with Ct values of 26.65 and 27.52, but not for *Potato virus Y* (PVY), *Peach rosette mosaic virus*, or other viruses (Weigang, 2009). Similarly, to increase the diagnosis of BBrMV in large number of banana samples, IC RT-PCR test was devised. The assay was devised as a new standard to monitor BBrMV and allowed diagnosis of BBrMV in leaf extract diluted up to 1×10^{-10} and in bulked samples of ten plants (Iskra-Caruna, 2005).

Later on, for the simultaneous detection of two RNA viruses, BBrMV and SCMV, a multiplex RT-PCR test was devised. As an internal control, primer sets were developed to amplify SCMV coat protein, BBrMV coat protein and *Musa* sp. elongation factor (219 bp). Cloning and sequencing of the RT-PCR results validated the specificity of each primer pair. When compared to uniplex RT-PCR detection for single virus, the multiplex RT-PCR devised has the same degree of specificity and sensitivity (Galvez, 2020). A multiplex, IC-PCR was devised for rapid diagnosis of simple RNA viruses affecting banana and plantain (Sharman *et al*., 2000).

Following the identification of conserved regions utilising all full-length *Potyvirus* genomes, a pair of universal primers was created to detect *Potyvirus* species. The innovative primers are expected to detect isolates from all main groups of viruses within the genus *Potyvirus*, and their dependability makes them suited for use as an assay for routine diagnosis (Zheng, 2009).

2.4.3.3. Isothermal amplification

Isothermal amplification techniques such as, Nucleic Acid Sequence-Based Amplification (NASBA), Helicase-Dependent Amplification (HDA), LAMP (Loop Mediated Isothermal Amplification), and Recombinase Polymerase Amplification

(RPA) allow nucleic acid amplification at a single temperature, allowing for on-site diagnosis (Rubio *et al*., 2020).

When paired with an appropriate reverse transcriptase, LAMP and RPA-DNA amplification techniques, RT-LAMP and RT-RPA can be developed for pathogens with an RNA genome. Among these, LAMP is the most accurate process in terms of its sensitivity and reliability.

2.5. Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP)

The LAMP is an isothermal amplification reaction that amplifies a target DNA sequence with high level of sensitivity and specificity under isothermal conditions. The mechanism of the LAMP amplification reaction is described in three steps which include binding of primers, the amplification and elongation (Mori and Notomi, 2009). Using a strand displacing polymerase like *Bst* DNA polymerase and two pairs of specifically designed primers that recognise a total of six unique sequences in target DNA, the LAMP assay can amplify nucleic acid under isothermal conditions in the range of 60 to 65 ºC (Notomi *et al*., 2000).

The RT-LAMP is an amplification method used to diagnose diseases caused by RNA viruses. Viruses such as CMV, *Chrysanthemum virus B* (CVB), as well as *Chrysanthemum stunt virus* (CSV), were detected using a RT-LAMP approach (Fukuta, 2005). Using LAMP, a simple, quick, specific, sensitive, and visible technique for detecting BaYMV (*Barley yellow mosaic virus*) was created (Chen *et al*., 2020). The LAMP method amplifies the DNA at the rate of producing 10^9 copies of the target in less than one hour (Notomi *et al*., 2000). The RT-LAMP is the process of incorporating a reverse transcription step to the procedure of LAMP for the diagnosis of RNA viruses.

Primer designing is one of the most important factors for establishment of any LAMP assay for proper amplification and to avoid non-specific detection (Wei *et al.,* 2012; Boubourakas *et al.,* 2009). The RT-LAMP uses two sets of primers and additional loop primers for specific amplification. The proper design of primers is

crucial for gene amplification utilising the LAMP technique. Several softwares are available for LAMP primer designing like Primer explorer, Premier Biosoft etc. Six primers named external primers F3 and B3, internal primers FIP and BIP, and loop primers LF and LB are used for RT-LAMP assay. The primers F3-B3 and FIP-BIP specifically bind to six distinct regions on the target DNA sequence. Forward and reverse internal primers binds at end region of the target sequence which in turn produces a intermediate stem-loop structure and this acts as template for further rounds of amplification. The essence of the reaction is an internal primer with two separate regions that correspond to the sense and antisense sequences of the target DNA. One is for initial priming and the other is for self-priming during subsequent amplification (Notomi *et al.,* 2000). The four types of primers targeting 6 distinct regions of the target gene: the F3c, F2c and F1c regions at one end and the B1, B2 and B3 regions at another end are shown in the Fig 2.1.

The loop primers (either loop primer F or loop primer B), containing complementary sequences to the loop region on the 5' end of the stem loop structure provides more number of origin for synthesis of DNA using LAMP method. With the inclusion of loop primers, the assay sensitivity enhanced even more, and the time required could be reduced to 30–60 minutes (Li and Ling, 2014). Loop primers, also known as LF (Loop forward) and LB (Loop backward) primers, can be used to further speed up the reaction (Nagamine *et al*., 2002).

The schematic representation of LAMP amplification is shown in Fig 2.2 (Notomi *et al*., 2000). As given in the figure, through the activity of the *Bst* DNA polymerase with strand displacement activity, the 3' end of F2 region in FIP becomes the origin to synthesise the complementary strand. The F3 primer later anneals to the outside region of internal primer, and it becomes the origin to synthesize a new strand and concurrently displaces the previously synthesised DNA strand. The DNA strand synthesized by external primer together forms a double stranded DNA with the template DNA strand. Since the FIP linked strand contains self complementary sequences, it anneals and forms a loop like structure. This structure acts as the artificial template for further rounds of amplification. The loop like DNA structure unfolds the loop at 5' end to extend DNA synthesis. In a similar fashion, the BIP

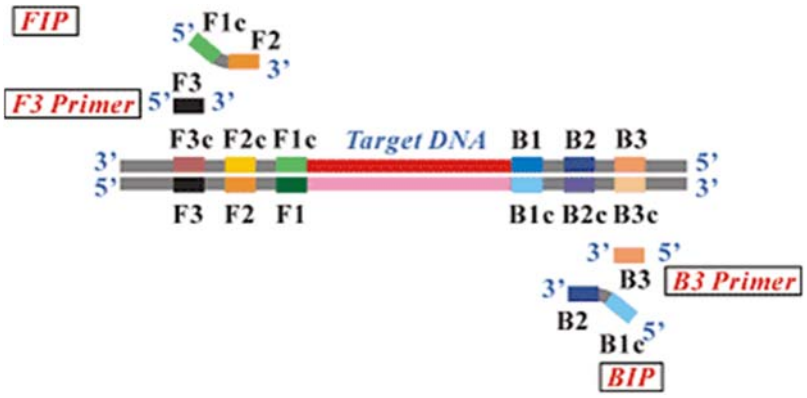


Fig 2.1. Primers used in LAMP reaction

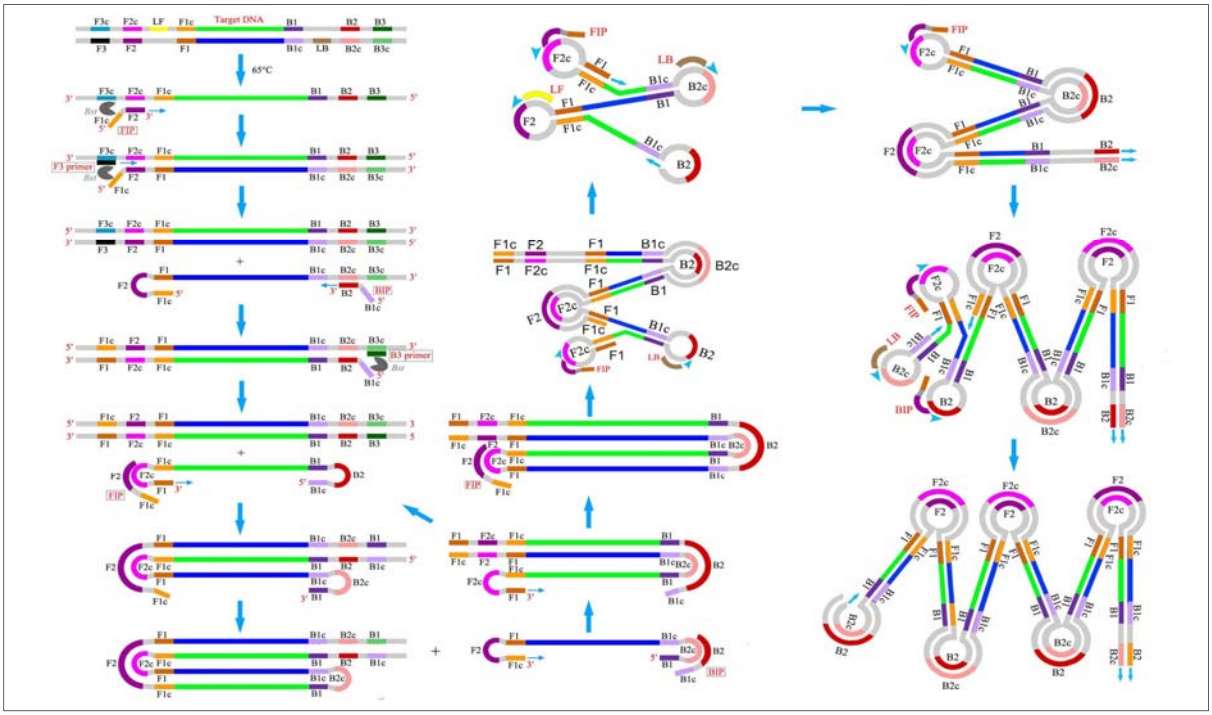


Fig 2.2. The schematic representation of LAMP amplification (Notomi *et al*., 2000)

anneals to the single stranded region in the newly synthesised strand and starts DNA synthesis, and releases the previously synthesized strand. Thus in a similar way, the artificial template formed leads to further rounds of amplification. As a result of this process, the inverted repeats of the target sequence are formed, and it appears as ladder like bands in agarose gel (Notomi *et al.*, 2000).

2.5.1. Two-step RT-LAMP

The two-step RT-LAMP process uses cDNA as a template. The reverse transcription LAMP test has been found to be a rapid, easy, sensitive, and low-cost method of detecting RNA in plant virus detection (Notomi *et al*., 2000; Fukuta *et al*., 2004; Huang *et al*., 2010). In this method, total RNA is reverse transcribed to cDNA. When there is a potential that more than one virus will show up in the sample, two-step RT-LAMP is used. Also for initial standardisation procedures, two-step method is ideal. Gawande *et al*. (2019) published the first report of TSV diagnosis using two-step RT-LAMP on cotton and soybean. Colorimetric detection was used to improve the methodology for quick diagnosis of infected samples of TSV utilising RT-LAMP. A two-step RT-LAMP assay was established for the detection of *Apple stem pitting virus* (ASPV) an important virus affecting pear trees (Lu *et al*., 2018).

2.5.2. One-step RT-LAMP

When paired with reverse transcription, the LAMP assay can be used to diagnose pathogens with RNA genomes (Notomi *et al*., 2000). Despite this, only a few cases of plant RNA viruses have been detected using this technique (Fukuta *et al*., 2003; Varga and James, 2006). One-step RT-LAMP could easily detect total RNA or crude RNA isolated from leaf or seed samples. The RT-LAMP assay had hundreds of times the sensitivity of conventional RT-PCR (Li *et al*., 2013). One-step RT-LAMP is generally used for routine detection of RNA viral samples. This assay requires only one step of master mix preparation and amplification at constant temperature for 60 minutes to detect virus effectively. When RNA template is directly used in case of one-step RT-LAMP assay, a process of reverse transcription is also involved. For the

detection of plant RNA viruses, one-step RT-LAMP assay can be used as it completes the process within an hour (Fukuta *et al.*, 2004).

2.5.3. Detection of RT-LAMP products

Simple visual detection methods such as turbidity can be used, due to large scale amplification in the process (Tomita *et al*., 2008). For detection, the turbidity of magnesium pyrophosphate as well as fluorescent chelation reagents are used. Real-time detection and visual examination are possible with both methods. Isothermally amplified DNA can be visualised using UV light and by gel electrophoresis and staining with ethidium bromide solution, and it can also be detected using other post-amplification detection methods which needs opening the reaction tubes, that might increase the chances of sample cross contamination (Zanoli and Spoto, 2013). The addition of indicator to the reaction mixes before the reaction, such as Hydroxy Naphthol Blue (HNB), GenefinderTM, or calcein, can help identify LAMP amplification products (Tomita *et al*., 2008; Goto *et al*., 2009). Calcein, which is a metal indicator that produces bright fluorescence, forms complexes with metal ions like calcium and magnesium. It is utilised in a variety of tests (Diehl and Ellingboe, 1956; Demertzis, 1988).

The change in colour after the reaction, generated by the pH shift shows successful amplification with HNB (Goto *et al*., 2009). Under alkaline circumstances, the large fragment of *Bst* DNA polymerase synthesises DNA (pH 8.8). When the solution has 8 mM Mg^{2+} ions with no dNTPs, the colour was magenta at pH 8.6–9.0 and violet at pH 8.4.

Colorimetric detection of *Maize chlorotic mottle virus* was devised with Hydroxy Napthol Blue dye (HNB) using RT-LAMP assay. The reaction was devised to amplify in a single tube at the optimum conditions. It helps in detection of positive samples without the need for expensive instruments (Liu *et al*., 2015). Positive reaction is indicated by a colour change from violet to sky blue (Goto *et al*., 2009). Ahmadi *et al*. (2013) used HNB for detection of *Potato leaf roll virus* using RT-LAMP. Colorimetric LAMP assay based on HNB has been optimized for the

detection of arecanut yellow leaf disease and coconut root wilt disease (Nair *et al*., 2016).

The real time monitoring of RT-LAMP reaction could be done by using a fluorescent reporter molecule in the reaction. A real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay in single tube has been developed by Lenarcic *et al.* (2013) for detecting Potato spindle tuber viroid. In a closed-tube real-time RT-LAMP assay for fluorescent detection of PVY, specific RT-LAMP primers were developed to target the conserved region of the coat protein gene. The assay was highly specific and sensitive, allowing detection at 65° C (Przewodowska *et al*., 2015). A real time RT-LAMP performed to detect root wilt disease of symptomatic coconut plants and yellow leaf disease of arecanut plants generated signals within 30 min of reaction (Nair *et al*., 2016).

2.5.4. The RT-LAMP for plant virus detection

The RT-LAMP assay was used to detect *Tomato chlorosis virus* using total RNA from infected plants using a water bath within one hour (Karwitha *et al*., 2014). The assay has also been optimized for the detection of *Citrus tristeza virus* from infected plant samples (Warghane *et al*., 2017). One-step reverse transcription loop mediated isothermal amplification of *Tomato torrado virus* showed ten-fold higher sensitivity than conventional RT-PCR (Budziszewska *et al*., 2016). Zhang *et al*. (2016) developed an efficient and robust IC-RT-LAMP assay to monitor this virus in the field.

The *Tomato spotted wilt virus* (TSWV) from chrysanthemum was detected using an IC/RT-LAMP. The TSWV was detected from plants using this approach, which was sensitive, repeatable, and specific (Fukuta, 2004).

For the detection of PVY, RT-LAMP assay was devised. A set of four primers was developed in this assay targeting six sequences of the PVY CP gene. The RNA samples from several PVY samples were used in two-step and one-step RT-LAMPs, and the findings were similar to two-step RT-PCR for PVY detection. Furthermore, using a time-saving spectrophotometric approach, the turbidity

generated by the precipitation of magnesium pyrophosphate created in positive amplicons was used for detection (Nie, 2005).

Viruses such as CMV, *Chrysanthemum virus B*, were detected using a RT-LAMP approach. Fukuta, (2005) created LAMP primers for CMV and CVB coat protein genes, as well as CSV genomic RNA. The sensitivity of RT-LAMP for detection of CSV was comparable to that of RT-PCR, and it was found more rapid than RT-PCR.

Using LAMP, a rapid, easy, specific, sensitive, and visible technique for detecting BaYMV (*Barley yellow mosaic virus*) was created. To amplify the gene encoding BaYMV's coat protein, two pairs of oligonucleotide primers (inner and outer primers) were constructed. In the sensitivity test, the RT-LAMP method was 10 times more sensitive than the RT-PCR approach (Chen *et al*., 2020).

The RT-LAMP was found 100 times more sensitive than conventional RT-PCR in the detection of *Wheat yellow mosaic virus* (Zhang *et al*., 2011). Detection using RT-LAMP provides a platform where the virus is effectively and easily detected in infected plants, and there was no cross-reaction with healthy plants. The RT-LAMP had a detection limit up to 100 times that of conventional RT-PCR and was comparable to real-time RT-PCR (Siljo and Bhat, 2014).

Materials and Methods

3. MATERIALS AND METHODS

The research work on "Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) for diagnosis of *Banana bract mosaic virus* in banana (*Musa* spp.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur. This chapter describes the materials used for the study and the methodologies involved in the research.

3.1. Materials

3.1.1. Plant materials

The BBrMV symptomatic plant samples were collected from Banana Research Station, Kannara, Kerala. Leaf samples from twelve symptomtic plants were collected from field in RNA Later solution after wiping with RNase ZAP and stored at -20 ºC till RNA isolation. Healthy leaf samples were collected from asymptomatic plants in the field and from tissue culture plantlets produced at Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Vellanikkara. Banana plant samples showing symptoms of other viruses such as BBTV, BSV and CMV were also collected from Banana Research Station, Kannara, Kerala.

3.1.2. Laboratory chemicals and equipment

The research was carried out using the available facilities at Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Vellanikkara. The instruments like electronic balance (Shimadzu), icematic (F 100 compact), water bath (Rotek), high speed refrigerated centrifuge (KUBOTA 3500, Japan), pH meter (EUTech instrument pH tutor), thermocycler (Agilent and BioRad), heat block (Neuation i Therm D150-2) and micropipettes from Eppendorf were used. NanoDrop spectrophotometer (NanoDrop ND-1000) was used and for quality check and analysis, gel electrophoresis unit (BioRad) and gel documentation unit (BioRad Gel Doc™ XR+) were used. The plastic wares were obtained from Tarsons India Ltd., glassware from Borosilicate.

The reagents for RNA isolation like RNA Later solution, RNase ZAP were purchased from Sigma Aldrich Pvt. Ltd., TRI reagent and Purelink^{TM} plant RNA reagent from Invitrogen, 2x RNA loading dye, Riboruler RNA low range ladder, RevertAid^{TM} first strand cDNA synthesis kit from Thermofischer Scientific, RNeasy plant mini kit from Qiagen, and chemicals for RT-LAMP such as *Bst* polymerase, *Bst* thermopol buffer, and $MgSO_4$ were purchased from New England Biolabs Pvt. Ltd. Nuclease free water from Hi-Media. The other components for RT-LAMP like betaine, PCR grade water, dNTPs and Hydroxy Naphthol Blue dye were brought from Sigma Aldrich Pvt. Ltd.

3.2. Methods

3.2.1. General precautions for RNA isolation

All the materials for RNA isolation such as centrifuge tubes, microtips, mortar and pestle were treated with 0.1 per cent DEPC overnight and then double autoclaved. Double autoclaved DEPC water was used for preparation of TAE buffer and 75 per cent ethanol. Work bench, micropipettes and gel electrophoresis unit was wiped with 75 per cent ethanol and RNase ZAP before use.

3.2.2. Isolation of total RNA

For isolating good quality RNA from leaf tissues for RT-LAMP assay, three different methods were tried. The detailed procedure for RNA isolation using different methods are given below.

3.2.2.1. Isolation of total RNA using TRI reagent

The RNA isolation was performed as per the manufacturer's protocol.

Reagents used

1. TRI reagent (Invitrogen)
2. Chloroform
3. Ice-cold isopropanol
4. 75 per cent chilled ethanol (prepared in DEPC water)

5. Nuclease free water (Hi-Media)

Procedure

1. Leaf samples (100 mg) were ground to fine powder using with liquid nitrogen using DEPC treated pre-chilled mortar and pestle.
2. One ml of TRI reagent was added and homogenate was then transferred to 1.5 ml DEPC treated microcentrifuge tube after complete thawing.
3. Sample was homogenised by vortexing for 15 seconds.
4. Tube was incubated for 10 min at room temperature.
5. To the tube, 200 µl chloroform was then added.
6. Content was mixed by shaking the tube vigorously by hand for 15 seconds.
7. Tube was incubated at room temperature for 5 min.
8. Content was centrifuged at 12,000 g for 15 min at 4° C.
9. Clear supernatant was transferred to 1.5 ml microcentrifuge tube.
10. To this, 500 µl ice-cold isopropanol was added.
11. Tube was kept for 10 min at room temperature to precipitate RNA.
12. Again the content was centrifuged at 12,000 g for 10 min at 4° C and the pellet saved.
13. Pellet was washed with 75 per cent ethanol.
14. Dried pellet was then dissolved in 30 µl nuclease free water.
15. The RNA samples were stored at -80° C.

3.2.2.2. Isolation of total RNA using Purelink™ Plant RNA reagent

The RNA isolation was performed as per the manufacturer's protocol.

Reagents used

1. Purelink™ plant RNA reagent(Invitrogen)
2. Chloroform
3. 1.5 M NaCl
4. Nuclease free water
5. Ice-cold isopropanol

6. 75 per cent DEPC treated chilled ethanol

Procedure

1. Leaf samples (100 mg) were ground well with liquid nitrogen using DEPC treated mortar and pestle.
2. One ml of plant RNA reagent was added and mixed well and the homogenised sample was transferred to DEPC treated 1.5 ml micro centrifuge tube.
3. Sample was vortexed for two min.
4. Tubes were incubated at room temperature for 5 min horizontally.
5. Centrifugation was done at 11,400 rpm at room temperature for 2 min.
6. The supernatant was transferred to a 2 ml centrifuge tube.
7. Then, 100 µl of 1.5 M NaCl was added to the supernatant and mixed well by inverting the tube eighty times.
8. Then, 300 µl chloroform was added to the content and mixed by inverting eighty times.
9. Tubes were centrifuged at 11,400 rpm for 10 min at 4 ºC.
10. Upper phase was recovered and transferred to a new tube.
11. Thereafter, 300 µl of chloroform was added and above procedure was repeated.
12. Supernatant after centrifugation was extracted and 500 µl isopropanol was added and inverted eighty times.
13. Tube was incubated for 10 min at room temperature.
14. Precipitate was recovered by centrifugation at 11,400 rpm at 4 ºC for 10 min.
15. Pellet was then washed with one ml of 75 per cent ethanol by centrifuging at 11,400 rpm for 1 min at room temperature.
16. Supernatant was discarded and the pellet was dried under laminar air flow.
17. Pellet after drying was then dissolved in 40 µl of nuclease free water.
18. The RNA sample was stored at -80 ºC.

3.2.2.3 Isolation of total RNA using RNeasy plant mini kit, Qiagen

The RNA isolation was performed as per the manufacturer's protocol using the reagents provided with the kit.

Reagents used

1. Buffer RLC (RNeasy plant mini kit, Qiagen)
2. Buffer RW1 (RNeasy plant mini kit, Qiagen)
3. Buffer RPE (RNeasy plant mini kit, Qiagen)
4. β- mercaptoethanol
5. Liquid nitrogen
6. 96 per cent Ethanol
7. RNase free water

Procedure

1. The tissue was ground with liquid nitrogen to fine powder and transferred to a 1.5 ml tube.
2. Then, 450 µl of buffer RLC was added to the tube and vortexed.
3. The lysate was transferred to a QIAshredder spin column (lilac) placed in a 2 ml collection tube. Contents were centrifuged for 2 min at full speed. The supernatant was transferred to a new microcentrifuge tube without disturbing the cell-debris pellet.
4. Half the volume of ethanol was added to the cleared lysate, and immediately mixed by pipetting.
5. The sample was transferred, to an RNeasy Mini spin column in a 2 ml collection tube and centrifuged for 15 s at 10000 g. Flow-through was discarded.
6. Then, 700 µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at 10000 g. Flow-through was discarded.
7. To this, 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at 10000 g. Flow-through was discarded.
8. Again, 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 2 min at 10000 g. Flow-through was discarded.

9. RNeasy spin column was placed in a new 1.5 ml collection tube. 30-50 µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at >8000 g to elute the RNA.
10. Storage at -80 °C.

3.2.3. Analysis of total RNA

The quality and quantity of extracted RNA was checked using NanoDrop spectrophotometer and agarose gel electrophoresis.

3.2.3.1. Agarose gel electrophoresis

Reagents used

1. Agarose
2. Ethidium bromide (10 mg/ ml stock)
3. 50X TAE buffer, pH 8.0 (Tris buffer 242.2 g, 100ml 0.5 M EDTA and, 57.1 ml Glacial acetic acid made up to 1 L)
4. RiboRuler^{TM} low range RNA ladder (Thermo Scientific^{TM})
5. RNA Loading dye (Thermoscientific)
6. Autoclaved DEPC treated water

Procedure

1. 1X TAE buffer was made from 50X stock using autoclaved DEPC treated water and to 60 ml 1X TAE buffer, 0.6 g of agarose was added to prepare 1.0 per cent gel. This mixture was heated in a microwave oven till a clear solution was obtained.
2. The solution was allowed to cool down to room temperature.
3. Thereafter, 3.0 µl ethidium bromide was added to the solution and mixed well.
4. The gel casting tray was placed in the gel caster by closing both the sides and the tray and the comb was wiped with 70 per cent ethanol. The comb was placed on the grooves of the casting tray appropriately.
5. The warm gel after adding ethidium bromide was poured into the casting tray with the comb avoiding air bubbles and allowed to solidify for few min.

6. Comb was gently removed after solidification of the gel and gel along with tray was placed in the electrophoresis tank containing 1X TAE buffer with wells placed at the cathode (negative electrode) end.
7. RNA ladder (4.0 µl) was loaded on the first well after mixing it with 2.0 µl loading dye.
8. The samples to be analysed were loaded into the wells after mixing 4.0 µl of RNA sample with 3.0 µl loading dye.
9. Electrophoresis was done at 70 V.

The gel after electrophoresis was documented under UV with BioRad GelDoc™ XR system using Quantity One software.

3.2.3.2. Quantity and quality analysis using NanoDrop spectrophotometer

Quantity and quality analysis of isolated RNA was done using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Purity of sample was checked using OD_{260}/OD_{280} ratio since absorption maxima of proteins and nucleic acids are at 280 nm and 260 nm respectively. To initialize the instrument, 1.0 µl of nuclease free water was used as blank. The pedestal and sampling arm were wiped properly before loading blank and the sample. Also 1.0 µl of RNA was loaded for analysis.

3.2.4. Designing RT-LAMP primers

Primers for RT-LAMP were developed using the software Primer Explorer version 5.0 (primerexplorer.jp/lampv5e/index.html) with default parameters. Three different target regions of viral genome were selected for deigning RT-LAMP primers. Selected targets were

1. BBrMV coat protein gene (GenBank accession no. MK139143.1)
2. BBrMV replicase gene (GenBank accession no. MH253670.1)
3. BBrMV movement protein gene (GenBank accession no. MH476482.1)

`Sequences were retrieved from NCBI. Initially two sets of RT-LAMP primers (External primers BrF3 and BrB3, internal primers BrFIP and BrBIP) were

designed. The output file was used as the input for picking loop primers (BrLF and BrLB). All the designed primers were validated using BLASTN. One set of primers was finally selected for the RT-LAMP assay. The primers were synthesised with Sigma-Aldrich.

3.2.5 The RT-LAMP reaction

Both one-step RT-LAMP assay and two-step RT-LAMP assay was standardized.

3.2.5.1. Two-step RT-LAMP

Two-step RT-LAMP was performed in two steps; the cDNA synthesis form RNA sample and isothermal amplification using the cDNA sample.

3.2.5.1.1. First strand cDNA synthesis

RevertAid™ first strand cDNA synthesis kit by Thermo Scientific™ was used for first strand cDNA synthesis. The reaction was performed according to the protocol given by manufacturer.

Reagents used

1. RNA sample
2. RevertAid M-MuLV RT (200 U/µl)
3. Ribolock RNase inhibitor (20 U/µl)
4. Oligo(dT) primer
5. 5X reaction buffer
6. 10 mM dNTP mix
7. Nuclease free water

Procedure

After thawing, the components of the kit were briefly centrifuged and placed on ice.

1. Following components were added into a sterile nuclease free PCR tube on ice.

Components	**Volume**
Template RNA	2 µg
Oligo(dT) primer	1 µl
Nuclease free water	To make up the volume to 12 µl

2. After adding these components, the tube was mixed gently and centrifuged briefly.
3. The tube was incubated at 65 °C for 5 min in a thermal cycler and placed back on ice after incubation.
4. Thereafter, remaining components were added in the order

Table 3.1. Reaction cocktail for cDNA synthesis

Components	**Volume**
5X reaction buffer	4 µl
RevertAid M-MuLV RT	1 µl
Ribolock RNase inhibitor	1 µl
10 mM dNTP mix	2 µl
Total volume	**20 µl**

5. Mixture was centrifuged briefly.
6. The tube was kept in the thermal cycler for cDNA synthesis with reaction programmed for 1 hour at 42 °C and final heating for 5 min at 70 °C for terminating the reaction.
7. The cDNA sample was stored at -20 °C.

3.2.5.1.2. The RT-LAMP reaction

1. The RT-LAMP assay was standardised for the detection of BBrMV using cDNA prepared from a BBrMV symptomatic sample. Initially, optimisation of RT-LAMP assay was done by varying the concentrations of reagents such as betaine, dNTPs, *Bst* polymerase and $MgSO_4$. Several sets of reactions were

carried out to optimise the conditions for RT-LAMP. Different concentrations of $MgSO_4$ (4-8 mM), dNTP (1.4-1.6 mM each), betaine (0.8-1 M) and *Bst* polymerase (4-8 U) were tried for optimisation. The reaction was incubated at different temperatures ranging from 60 ºC to 65 ºC for one hour. Initially, a primer mix was made containing all the six RT-LAMP primers in following concentration.

Primer	**Concentration (µM)**
BrFIP	20
BrBIP	20
BrF3	5
BrB3	5
BrLF	10
BrLB	10

Reagents used

1. RT-LAMP primer mix
2. cDNA sample
3. *Bst* polymerase large fragment
4. 10x thermopol buffer
5. Betaine
6. dNTP
7. $MgSO_4$
8. PCR grade water

Procedure

1. Primer mix was made to 50 µL stock and 1 µL primer was taken from the stock for each reaction.
2. The final optimised reaction mixture contained 40 ng cDNA, 1.6 mM each dNTP, 0.2 µM each primers BrF3 and BrB3, 0.8 µM each primers BrFIP and BrBIP and 0.4 µM each primers BrLF and BrLB, 1 M betaine (Sigma), 4 mM $MgSO_4$ (New England BioLabs), 1x Thermopol buffer with 2 mM $MgSO_4$, 120 µM HNB and 4 U *Bst* polymerase large fragment (New England BioLabs) in 25 µl reaction volume.

3. Reaction was set up using following components and was then incubated at 65 ºC for one hour in a dry bath.

Table 3.2. Reaction cocktail for two step RT-LAMP

Reagents	Final conc.	Volume (µl)
RT-LAMP Primer mix	BrF3/BrB3 -0.2 µM, BrFIP/BrBIP-0.8 µM, BrLF/BrLB - 0.4 µM	1
cDNA	40 ng	2
Bst polymerase	4 U	0.5
Thermopol buffer	1x	2.5
Betaine	1 M	5
dNTP	1.6 mM each	4
$MgSO_4$	4 mM	1.5
Water		8.5
Total volume		**25**

2. Along with the samples, a no template control (NTC) and a healthy sample were also run.

3.2.5.2. One-step RT-LAMP

One-step RT-LAMP was performed using total RNA as template RNA. The optimized RT-LAMP reaction contained 2 µg RNA, 100 U M-MLV reverse transcriptase (Promega), 20 U RNAsin® plus RNAse inhibitor (Promega), 1.6 mM each dNTP, 0.2 µM each primers BrF3 and BrB3, 0.8 µM each primers BrFIP and BrBIP and 0.4 µM each primers BrLF and BrLB, 1 M betaine (Sigma), 4 mM $MgSO_4$ (New England BioLabs), 1x Thermopol buffer with 2 mM $MgSO_4$ and 8 U *Bst* polymerase large fragment (New England BioLabs) in 25 µl reaction volume. Molecular biology grade water (Hi-Media) was used to make up the volume to 25 µl. A no template control was also run. The reaction was set for 60 min incubation at 65 ºC on a dry bath.

Reagents

1. RNA sample

2. Reverse transcriptase enzyme (Promega)
3. RNase inhibitor (Promega)
4. RT-LAMP Primer mix
5. *Bst* polymerase
6. 10x thermopol buffer
7. Betaine
8. dNTP
9. $MgSO_4$
10. PCR grade water

Procedure

1. Initially primer mix was made from all the six primers as described in 3.5.1.2.2.
2. Following components were then added to 0.2 ml tube.

Table 3.3. Reaction cocktail for one step RT-LAMP

Reagents	Final conc.	Volume (µl)
RT-LAMP Primer mix	BrF3/BrB3 -0.2 µM, BrFIP/BrBIP-0.8 µM, BrLF/BrLB - 0.4 µM	1
RNA sample	2 µg	2.5
Reverse transcriptase	100 U	0.5
RNase inhibitor	20 U	0.5
Bst polymerase	8 U	1
Thermopol buffer	1x	2.5
Betaine	1 M	5
dNTP	1.6 mM each	4
$MgSO_4$	4 mM	1.5
PCR grade water		6.5
Total volume		**25**

3. The tube was then incubated at 65 ºC for one hour on a dry bath and then results were analyzed.

3.3. Analysis of RT-LAMP amplicons

3.3.1. Agarose gel electrophoresis

The RT-LAMP amplicons were initially analyzed based on agarose gel profiling. The amplicons were run on 2 per cent agarose gel stained with ethidium bromide along with 1 kb plus DNA ladder (GeNei).

3.3.2. Colourimetric detection of amplicons

The RT-LAMP amplicons were detected using Hydroxy Naphthol Blue dye (HNB) (Goto *et al*., 2009). Different concentration of HNB (120 µM-150 µM) was tried for optimisation. Finally, the HNB dye (Sigma) was added at a concentration of 120 µM to the RT-LAMP reaction mixture prior to the amplification. Then colour change was observed after the reaction.

3.4. Molecular characterization of RT-LAMP products

3.4.1. Restriction profiling of RT-LAMP product

The RT-LAMP products were digested with a restriction enzyme specific for the product to demonstrate fidelity of the RT-LAMP assay. For restriction profiling, restriction enzyme having internal cut site in the BrF2-BrB2 flanking region was identified using NEBcutter (http://nc2.neb.com/NEBcutter2/) software. Restriction digestion of RT-LAMP product was done using identified restriction enzyme *Sau3*AI (Thermofischer Scientific) cutting between BrF2 and BrB2 region of the sequence. The detailed procedure is given below.

1. The restriction digestion was set as given below

Components	**Volume (µl)**
RT-LAMP product	10
Restriction enzyme (*Sau3*AI)	2
10x Buffer	5
Water	33
Total volume	**50**

2. Reaction was set in a 0.2 ml tube and incubated at 37 ºC for 8 hours and terminated with heat inactivation at 65 ºC for 20 min.
3. Restricted product was then analyzed on 2.5 per cent agarose gel stained with ethidium bromide along with 50 bp DNA ladder (GeNei).

3.4.2. Sequence analysis

The PCR with RT-LAMP primers BrF3 and BrB3 was carried out and sequencing was done to confirm the specificity of RT-LAMP assay.

3.4.2.1. Polymerase Chain Reaction

The PCR reaction mixture contained 40 ng template cDNA, 0.6 µM of each primer, 150 µM of each dNTPs, 0.5 U of *Taq* DNA polymerase and 1x PCR buffer with 1.5 mM $MgCl_2$ and final volume was made to 15 µl using molecular biology grade water. The PCR program was set with an initial denaturation at 95 ºC for 1 min followed by 35 cycles of 30 sec denaturation at 95 ºC, 30 sec annealing at 55 ºC and 30 sec of primer extension at 72 ºC followed by a final extension at 72 ºC for 8 min and was performed in the BioRad thermocycler.

3.4.2.2. Gel purification, sequencing and sequence analysis

The PCR products were electrophoresed on 1 per cent agarose gel and PCR amplicons of expected size (196 bp) were purified from agarose gel using Macherey-Nagel™ Nucleospin™ gel and PCR clean-up kit. The procedure of gel elution is given below:

Materials required

- Buffer NT1 (Provided with the kit)
- Buffer NT3 (Provided with the kit)
- Nuclease free water

Procedure

1. The DNA fragment was excised from the gel and gel slice was solubilised by adding 200 µl of buffer NT1 for each 100 mg of agarose gel in a 2 ml tube and incubated for 10 min at 50 ºC.
2. The sample was vortexed briefly every 3 min until the gel slice was completely dissolved.
3. A gel clean up column was placed into a collection tube of 2 ml and sample was loaded in the column.
4. Sample was centrifuged for 30 seconds at 11000 g.
5. Flow-through was discarded and column was placed back to the collection tube.
6. To this, 700 µl of buffer NT3 was added followed by centrifugation for 30 seconds at 11000 g. Flow-through was discarded, and this step was repeated.
7. To dry silica membrane, the tube was centrifuged for 1 min at 11000 g to remove NT3 completely. The column was incubated for 2-5 min at 70 ºC.
8. The column was placed in 1.5 ml tube, 10 µl of nuclease free water was added and centrifuged for 1 min at 11000 g.
9. The above step was repeated by adding same amount of nuclease free water.
   The eluted sample was checked on 0.8 per cent agarose gel. The sample was sequenced using BrF3 primer at AgriGenome Labs Pvt. Ltd., Kochi. Sequence was analysed using BLASTN search against NCBI database (Altschul *et al*. 1997).

3.5. Validation of RT-LAMP assay with field collected samples

The RT-LAMP assay was validated using 12 BBrMV symptomatic field collected banana samples along with the one sample each from plants showing symptoms of other viruses commonly infecting banana such as BBTV, BSV and CMV. Healthy samples tested in the assay include asymptomatic field collected sample and tissue culture banana sample. The NTC was run along with each assay.

Results

4. RESULTS

The results of the research work on "Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) for diagnosis of *Banana bract mosaic virus* in banana (*Musa* spp.)" carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur, during 2019-2021 are presented in this chapter.

4.1. Collection of plant samples

Leaf samples were collected from banana plants showing symptoms of BBrMV from Nendran and Kadali varities. Samples were collected from Banana Research Station, Kannara. Samples were stored in RNA later (Sigma) solution and stored at -80 ºC till RNA extraction. Leaf samples from twelve banana plants (Kannara 1- Kannara 12) showing symptoms of BBrMV were collected (Plates 4.1, 4.2). Healthy leaf samples (H1 and H2) were collected from tissue culture plantlets produced at Centre for Plant Biotechnology and Molecular Biology, and from fields of College of Agriculture, Vellanikkara. For validation of RT-LAMP results, leaf samples of banana plants showing symptoms of other viruses such as BBTV, BSV and CMV were collected from Banana Research Station, Kannara.

4.2. Isolation of total RNA

For isolating good quality RNA from leaf tissues for RT-LAMP assay, three different methods were tried.

4.2.1. Isolation of total RNA using TRI reagent

Initially, RNA isolation from banana leaves was tried using TRI reagent. No bands were observed on the agarose gel Plate 4.3).

4.2.2. Isolation of total RNA using Purelink^{TM} Plant RNA reagent

The RNA isolation was then tried using Purelink^{TM} plant RNA reagent. The quantity and quality of RNA was not satisfactory using this method.

4.2.3. Isolation of total RNA using RNeasy plant mini kit by Qiagen

The RNA isolation was performed using RNeasy plant mini kit, Qiagen using which intact bands were obtained. This method yielded good quality and quantity of RNA.

4.3. Analysis of total RNA

4.3.1. Agarose gel electrophoresis

In agarose gel electrophoresis RNA samples produced intact bands for all the samples isolated using RNeasy plant mini kit. Distinct bands corresponding to 28S and18S rRNA were obtained (Plate 4.4).

4.3.2. NanoDrop spectrophotometer

Purity of isolated RNA was checked using NanoDrop spectrophotometer by OD_{260}/OD_{280} ratio, the ratio was between 1.80 and 1.99 indicating pure RNA (Table 4.1).

Table 4.1. Concentration and purity of collected RNA samples

Sample	A 260/280	Conc. ng/µl
Kannara1	1.85	716.28
Kannara 2	1.82	836.94
Kannara 3	1.891	1011.20
Kannara 4	1.826	940.33
Kannara 5	1.80	634.36
Kannara 6	1.991	936.83
Kannara 7	1.816	1006.23
Kannara 8	1.92	892.67
Kannara 9	1.96	903.42
Kannara 10	1.812	756.84
Kannara 11	1.863	843.69
Kannara 12	1.844	886.93
Healthy tissue culture sample (H1)	1.92	908.80
Healthy field collected sample (H2)	1.90	1020.10

A

B

C

D

E

F

Plate 4.1. Banana plants showing symptoms of BBrMV-reddish streaks on pseudostem

A- Kannara 1; B- Kannara 2; C- Kannara 3; D-Kannara 4; E-Kannara 5; F-Kannara 6

Plate 4.2. Sample collection from BBrMV symptomatic banana plant

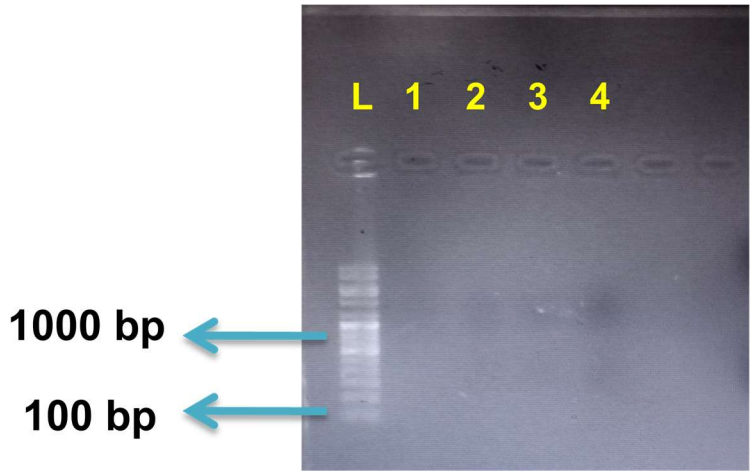


Plate 4.3. Total RNA sample purified from banana leaves using TRI reagent (L-Ladder; 1- Kannara 1; 2- Kannara 2)

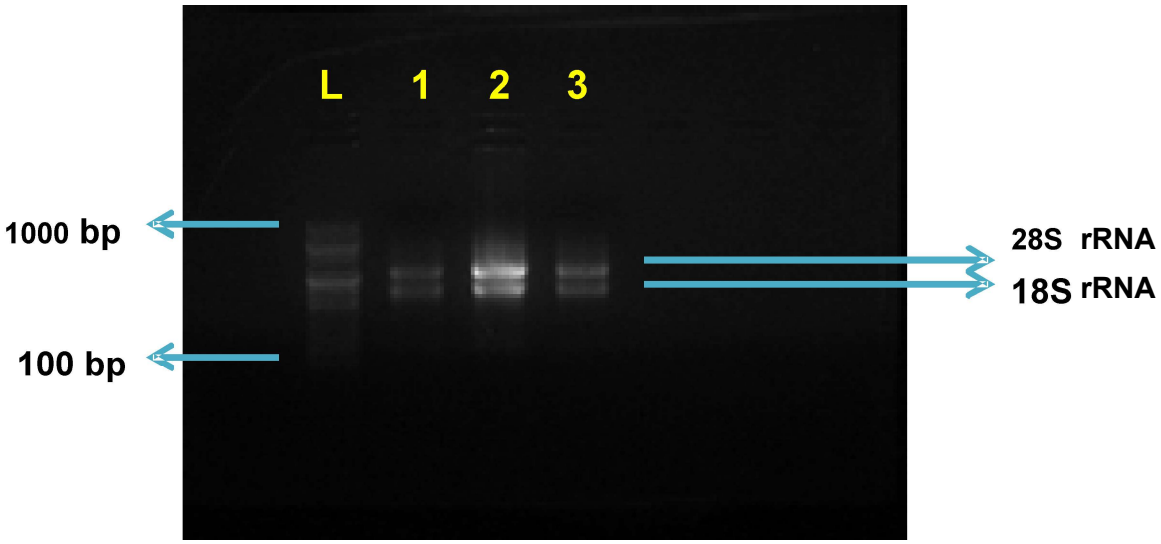


Plate 4.4. Total RNA sample purified from banana leaves using RNeasy plant mini kit, Qiagen (L-Ladder 1- Kannara 5; 2- Kannara 6; 3- Kannara 7)

4.4. Designing of RT-LAMP primers

The RT-LAMP primers were designed using the software Primer Explorer version 5.0 (primerexplorer.jp/lampv5e/index.html). The sequences of selected targets for primer designing were retrieved from NCBI GenBank. Primers were designed for selected targets such as coat protein gene, movement protein gene and replicase gene. Loop primers were unavailable for the movement protein gene and replicase gene sequences (Fig 4.1, 4.2, 4.3). Hence, six RT-LAMP primers (Table 4.2) were designed targeting coat protein gene of BBrMV (GenBank accession no. MK139143.1). The location of six primers binding to eight target regions of BBrMV coat protein gene is shown in Fig 4.4.

Table 4.2. Primers for RT-LAMP reaction

Name of the primer	**Sequence**	**Length of the primer**
BrF3	TTGATGTTGGGTAGTACAAAC	21
BrB3	TTTTACAGCATCACACCATG	20
BrFIP	GCAGTTTTTCCTCGATACCTTTGG-ATCCCTTCGAATTAAACCGAT	45
BrBIP	GTCGAGTTCTTGCTTCAATATAAGC-ATATTGCTCCCTAGTTGCA	44
BrLF	GGAGACGCATTTTCCCAGTC	20
BrLB	CTGATCAGTTCGATTTGTCAAATGC	25

4.5. Establishment of the RT-LAMP assay

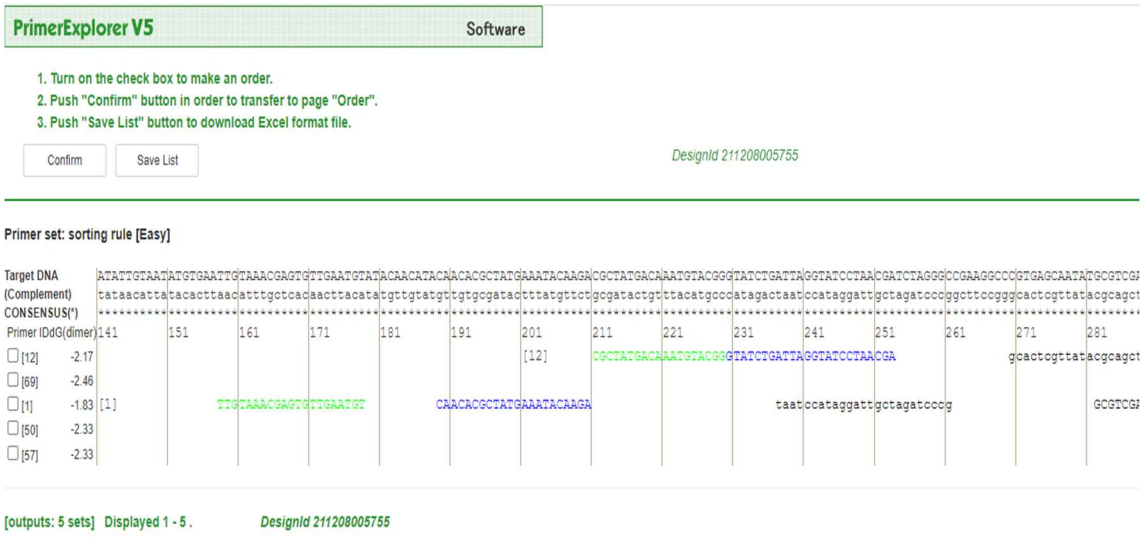
Both one-step and two-step RT-LAMP assay was standardised for diagnosis of BBrMV.

4.5.1. Two-step RT-LAMP

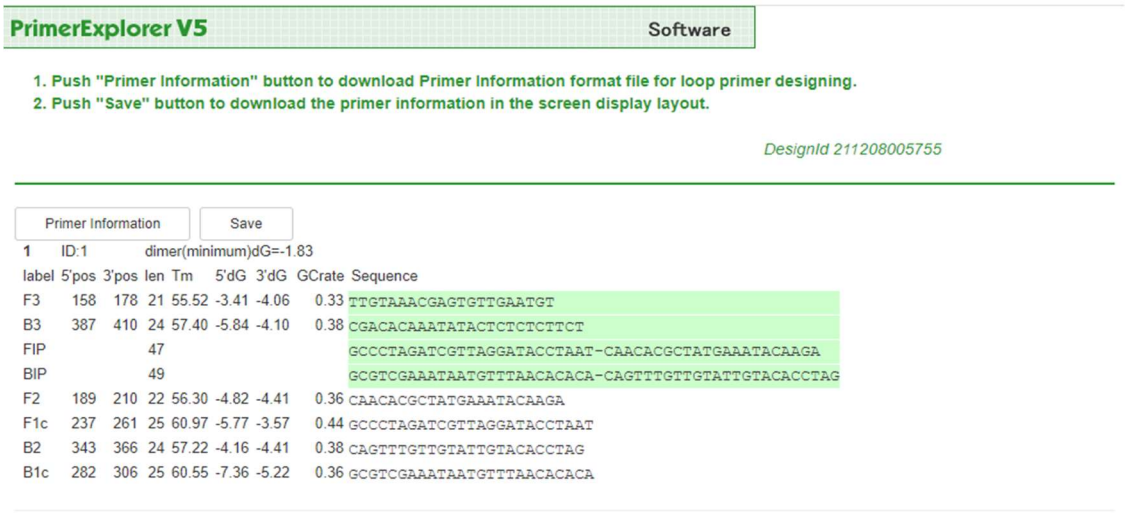
Two-step RT-LAMP assay was performed using cDNA as template. In this study, isolated RNA samples from 12 symptomatic banana plants, healthy tissue culture plantlet and from field collected healthy plant were converted to cDNA using RevertAid^{TM} first strand cDNA synthesis kit (ThermoScientific$^{TM)}$. The cDNA was then used for isothermal amplification. Initial optimisation of the assay was done by varying the concentration of the reagents. The final optimised reaction mixture contained 40 ng cDNA, 1.6 mM each dNTP, 0.2 µM each primers F3 and B3, 0.8 µM each primers BrFIP and BrBIP and 0.4 µM each primers BrLF and BrLB, 1 M betaine (Sigma), 4 mM $MgSO_4$ (New England BioLabs), 1x Thermopol buffer with 2 mM $MgSO_4$, 4 U *Bst* polymerase large fragment (New England BioLabs) and 120 µM HNB in 25 µl reaction volume. Incubation temperature at 65° C was found optimum. The RT-LAMP primers amplified the specific region of the coat protein gene in BBrMV positive samples.

4.5.2. One-step RT-LAMP assay

In this study, one-step RT-LAMP assay was standardised using RNA as template and the optimised reaction mixture contained 2 µg RNA, 1.6 mM each dNTP, 0.2 µM each primers BrF3 and BrB3, 0.8 µM each primers BrFIP and BrBIP and 0.4 µM each primers BrLF and BrLB, 1 M betaine (Sigma), 4 mM $MgSO_4$ (New England BioLabs), 1x Thermopol buffer with 2 mM $MgSO_4$, 8 U *Bst* polymerase large fragment (New England BioLabs), 100 U M-MLV reverse transcriptase (Promega) and 20 U RNasin® plus RNase inhibitor (Promega) in 25 µl reaction volume.



(a)

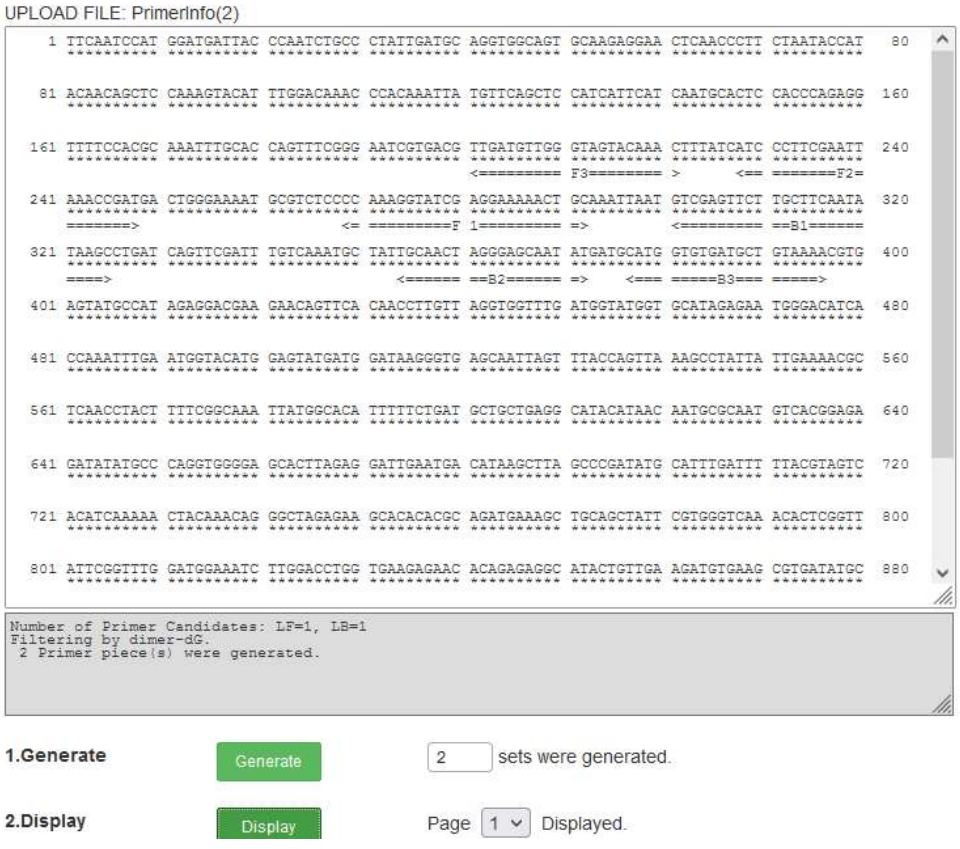


(b)

Fig 4.1(a) and (b). Primer explorer version 5.0. window displaying output for RT- LAMP primers targeting movement protein gene of BBrMV

(a) Regular primers specific to movement protein gene

(b) Regular primer set

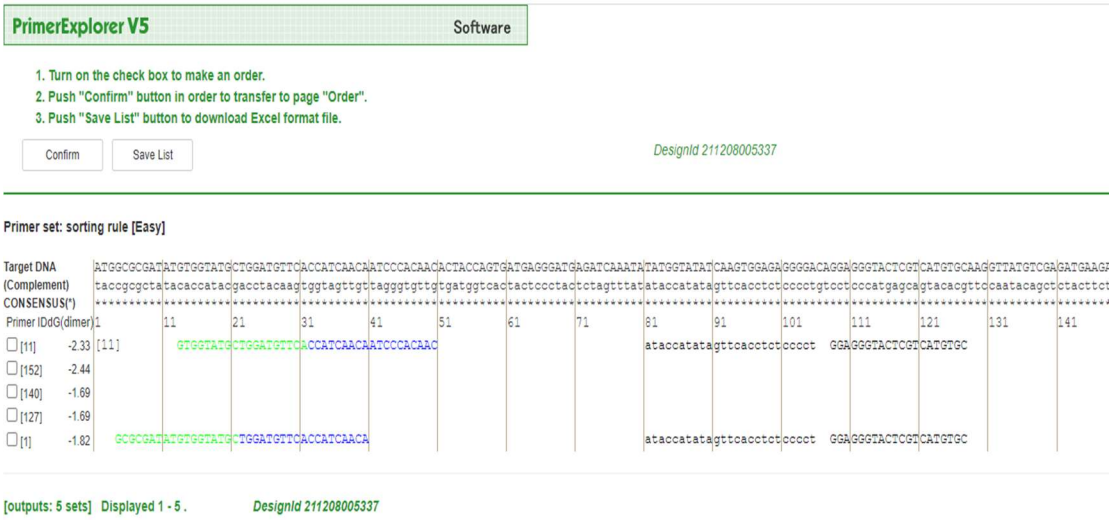


(c)

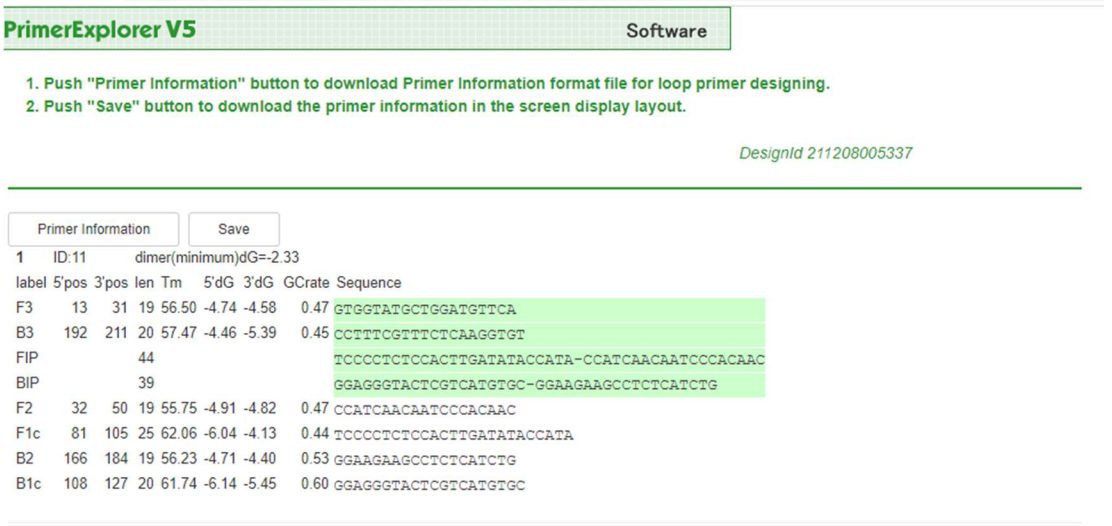
Fig 4.1(c). Primer explorer version 5.0. window displaying output for RT-LAMP primers targeting movement protein gene of BBrMV

(c) Output for loop primer design

No loop primers obtained for movement protein gene



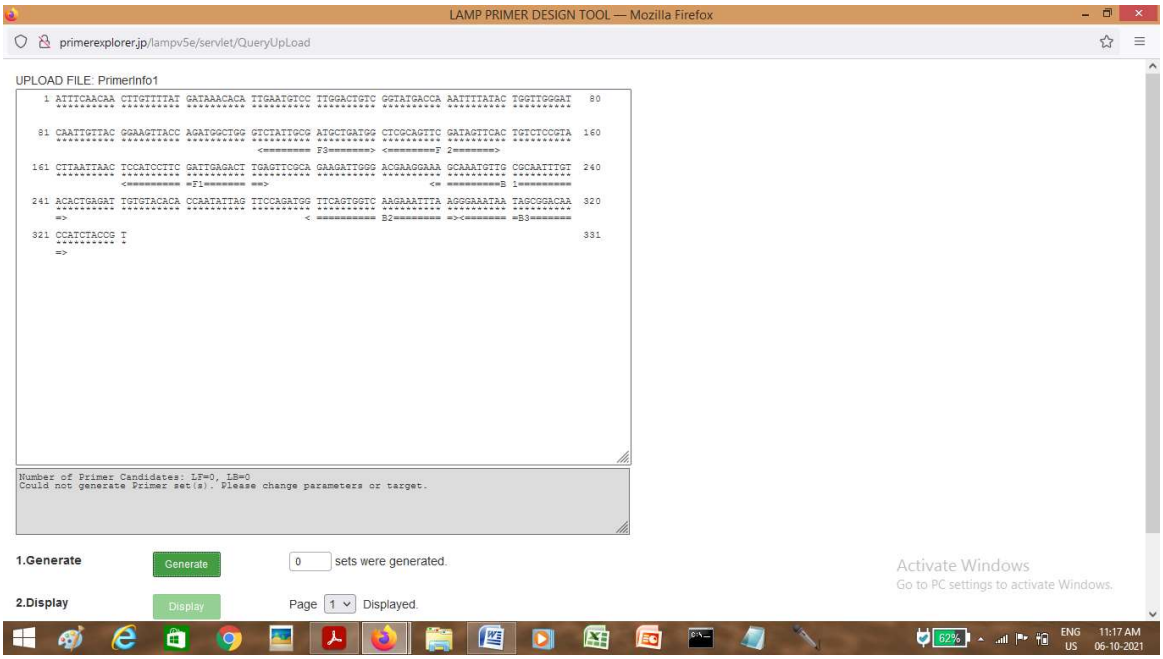
(a)



(b)

Fig 4.2(a) and (b). Primer explorer version 5.0. window displaying output for RT-LAMP primers targeting replicase gene of BBrMV

(a) Regular primers specific to replicase gene (b) Regular primer set selected



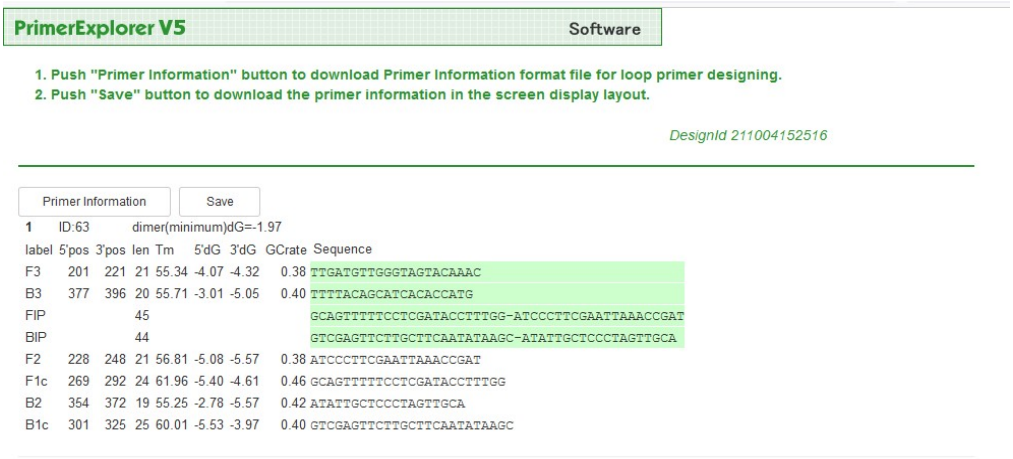
(c)

Fig 4.2(c). Primer explorer version 5.0. window displaying output for RT-LAMP primers targeting replicase gene of BBrMV

(c) Output for loop primer design

No loop primers obtained for replicase gene

(a)

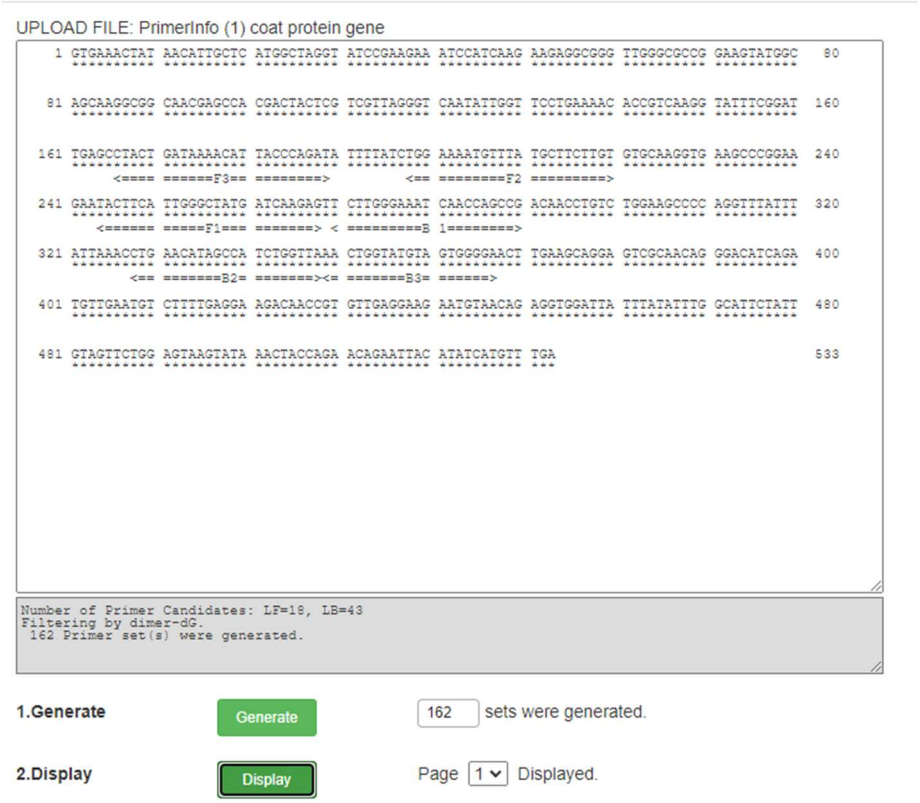


(b)

Fig 4.3(a) and (b). Primer explorer version 5.0. window displaying output for RT-LAMP primers targeting coat protein gene of BBrMV

(a) Regular primers specific to coat protein gene

(b) Regular primer set selected



(c)

Fig 4.3(c). Primer explorer version 5.0. window displaying output for RT-LAMP primers targeting coat protein gene of BBrMV

(c) Loop primers obtained for coat protein gene

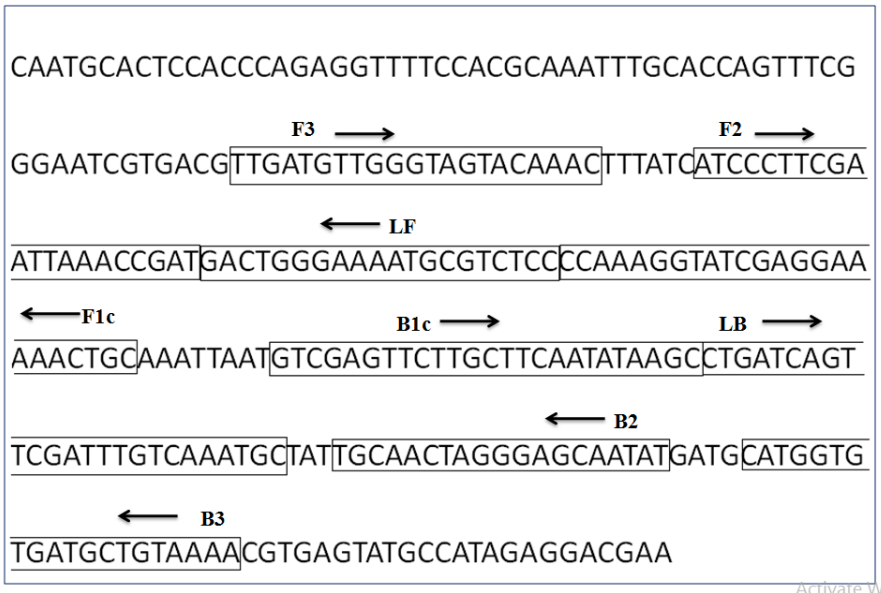


Fig 4.4. Position of RT-LAMP primers in BBrMV coat protein gene (GenBank accession no. MK139143.1)

4.6. Analysis of RT-LAMP amplicons

4.6.1. Agarose gel electrophoresis

The positive amplicons showed ladder like bands in 2 per cent agarose gel representing the stem-loop DNA indicating inverted repeats of target sequence whereas healthy sample and no template control did not show any amplification (Plates 4.5, 4.6).

4.6.2. Colourimetric detection

Hydroxy Naphthol Blue dye was used for colourimetric detection of RT-LAMP amplicons. The colour of the reaction mixture turned violet after addition of 120 µM HNB dye with 6 mM $MgSO_4$ before the start of the reaction. Positive amplicons showed a colour change from violet to sky blue after the RT-LAMP reaction while negative samples remained violet (Plate 4.7). When 150 µM HNB was added, no colour change observed in amplified sample.

4.7. Molecular characterization of RT-LAMP products

4.7.1. Restriction profiling of RT-LAMP product

For restriction profiling, the enzyme *Sau3*AI having internal cut site in the BrF2-BrB2 flanking region was used. The enzyme has single cut site generating fragments of size 100 bp and 45 bp. The products after restriction digestion were run on 2.5 per cent agarose gel and produced two bands of expected size bp (Plate 4.8). This confirmed the specificity and accuracy of the developed RT-LAMP assay.

4.7.2. Sequence analysis

The RT-PCR amplification of diseased samples using the RT-LAMP external primers F3 and B3 gave amplicons of the expected size (196 bp). A PCR product was gel purified and sequenced at AgriGenome Labs Pvt. Ltd., Kochi. The sequence was subjected to BLASTN analysis. The sequence showed 97.78 % identity with query coverage of 99 % with BBrMV isolate BRS2 coat protein mRNA, complete cds (GenBank Acc no: MK139142.1) (Fig 4.5). A representative sequence was deposited in the GenBank database (GenBank Acc no: OL757512).

4.8. Validation of RT-LAMP assay with field collected samples

The RT-LAMP assay was validated using twelve BBrMV symptomatic samples along with no template control and healthy samples which included a tissue culture banana and an asymptomatic field collected banana sample. Banana samples showing symptoms of other viruses such as BBTV, BSV and CMV were also tested using the optimised assay. The results showed that all the BBrMV symptomatic samples produced ladder like bands on 2 per cent agarose gel and positive colour change using HNB dye. No amplification was observed in the healthy banana samples and NTC. Also, no amplification was observed in RNA samples of plants showing symptoms of BSV, BBrMV and CMV (Plate 4.9).

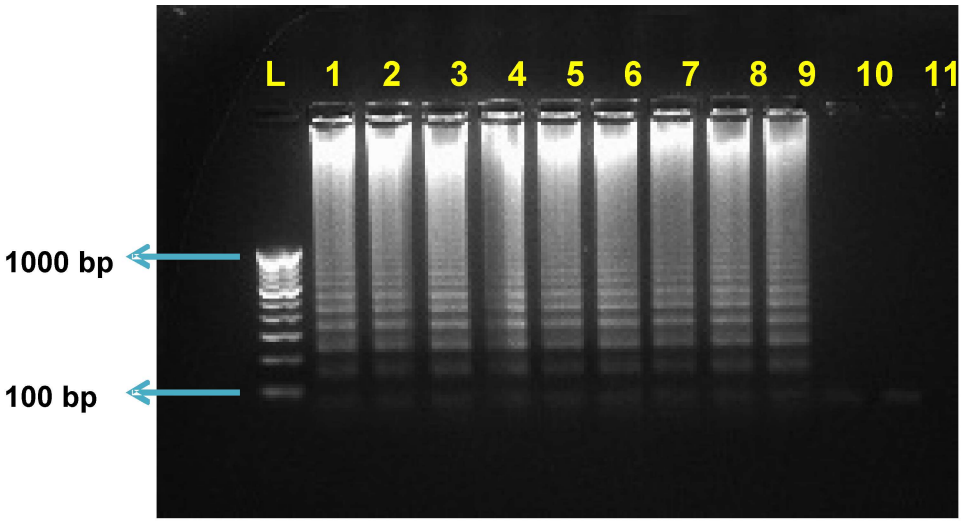
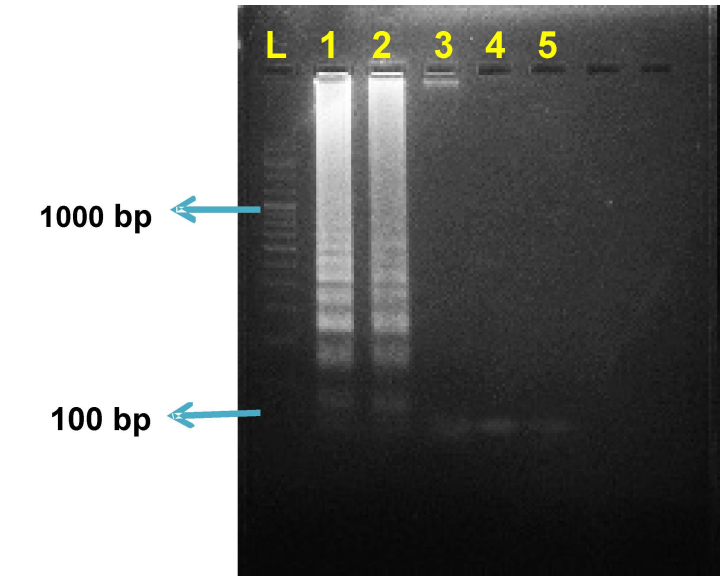
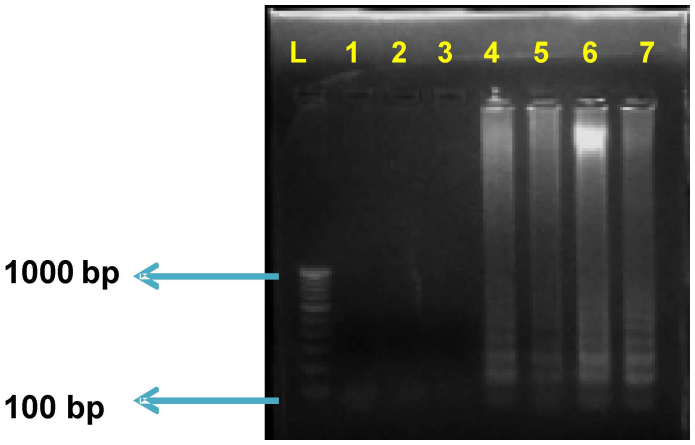


Plate 4.5. Detection of two step RT-LAMP amplicons using AGE

L- 1 kb DNA Ladder; 1-9- BBrMV symptomatic samples Kannara 1-9; 10- Healthy sample; 11- No template control



(a)

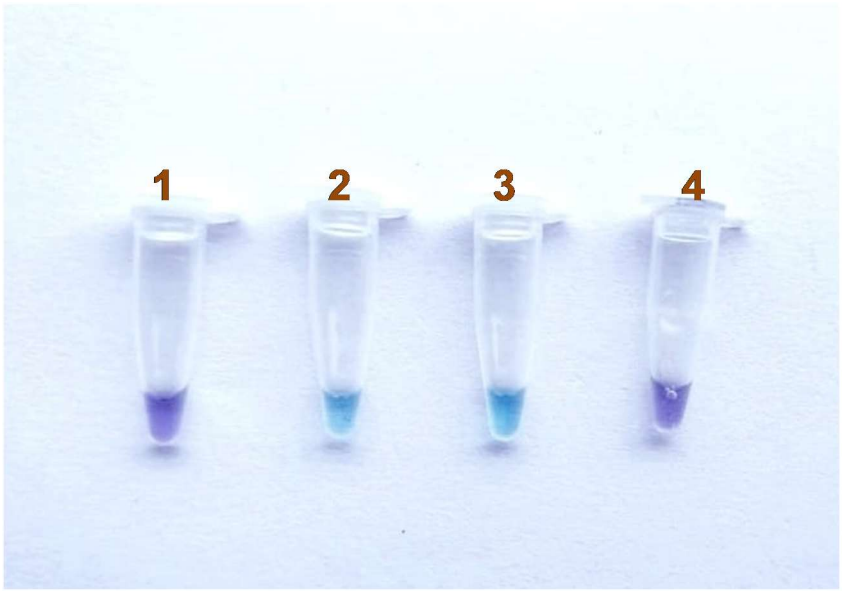


(b)

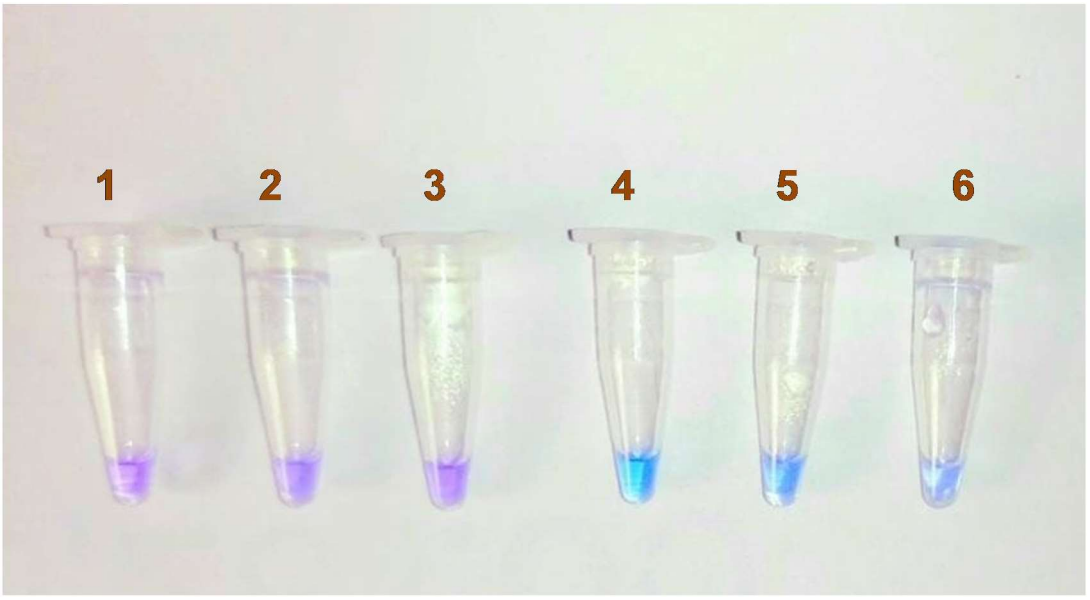
Plate 4.6. Agarose gel electrophoresis of one step RT-LAMP amplicons

(a) L -1 kb DNA Ladder; 1-2- BBrMV symptomatic samples Kannara 10-11; 3- Healthy tissue culture sample; 4- Healthy field collected sample; 5- No template control

(b) L- 1 kb DNA Ladder ; 1- Healthy tissue culture sample; 2-Healthy field collected sample; 3- No template control; 4-7 – BBrMV symptomatic samples Kannara 10-12



(a)



(b)

Plate 4.7(a) and (b). Colourimetric detection of RT-LAMP amplicons

(a)1-Healthy sample; 2,3- BBrMV symptomatic samples Kannara 7,8; 4- No template control

(b)1-Healthy tissue culture sample; 2-Healthy field collected sample; 3-No template control; 4,5,6- BBrMV symptomatic samples Kannara 1-3

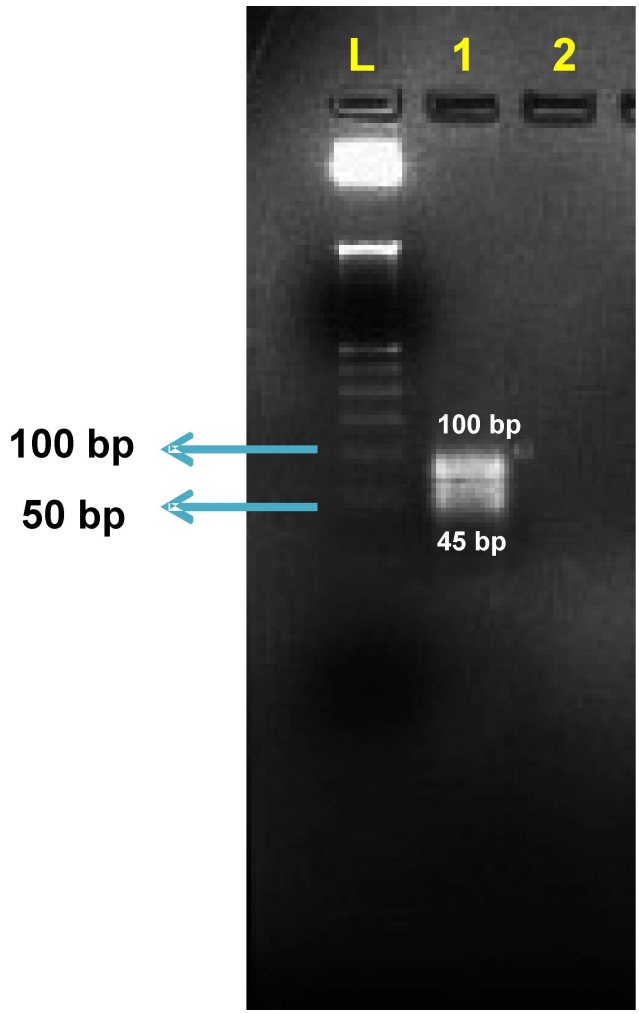


Plate 4.8. Restriction analysis of RT-LAMP product

L- 50 bp DNA Ladder 1- RT-LAMP Product after restriction digestion 2- NTC

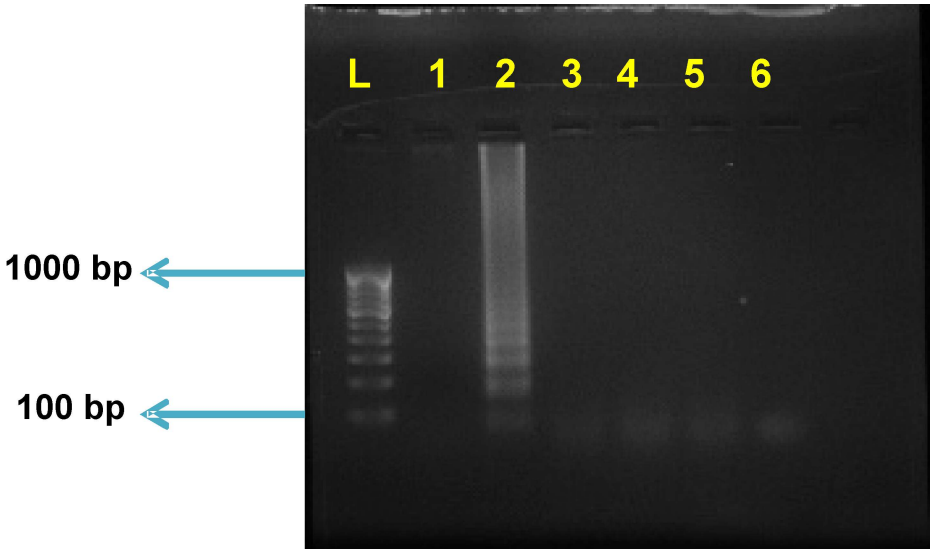
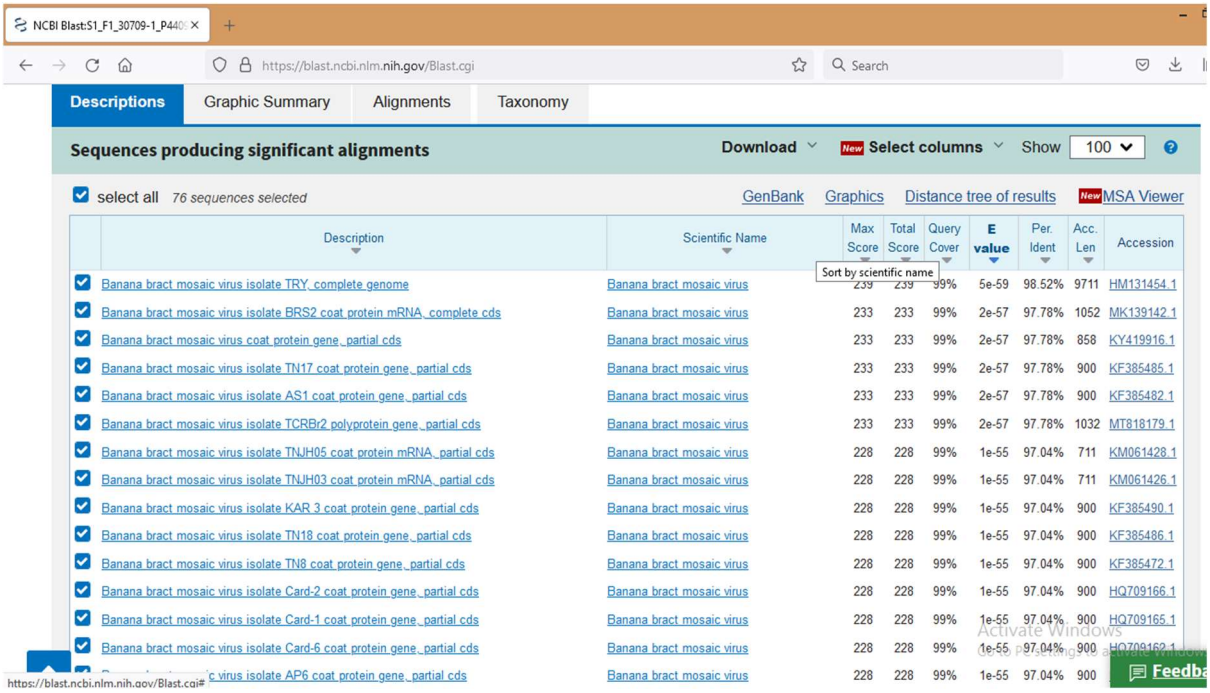
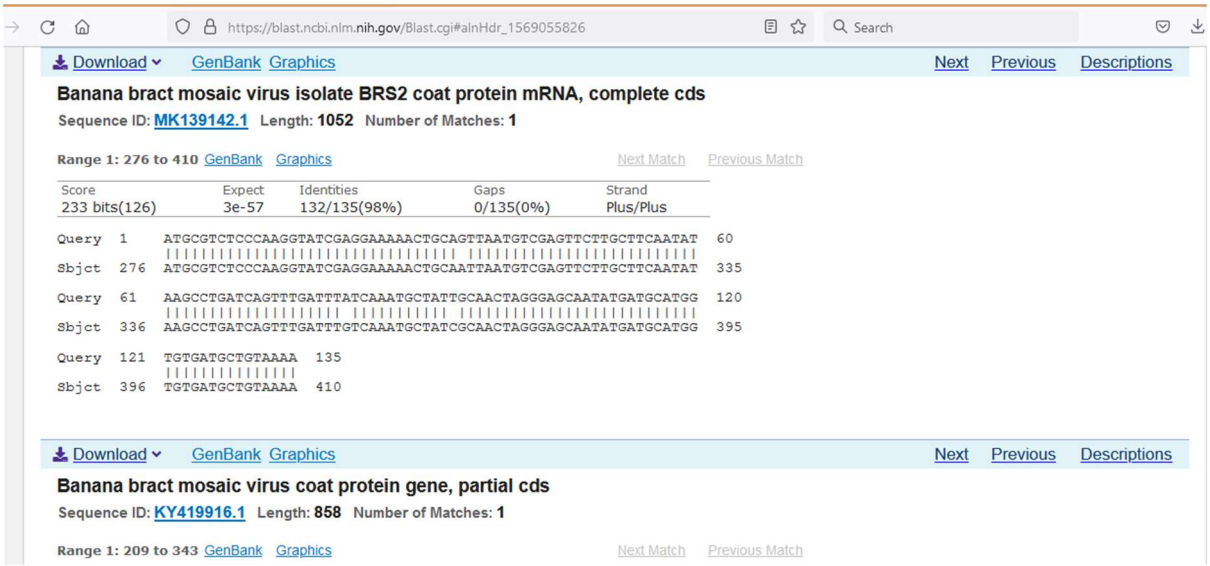


Plate 4.9. Validation of RT-LAMP results with samples showing symptoms of other viruses – Agarose gel profile of RT-LAMP products

L- 1 kb DNA Ladder; 1- No template control; 2- BBrMV symptomatic sample Kannara 5; 3-BBTV symptomatic sample; 4- BSV symptomatic sample; 5-CMV symptomatic sample; 6- Healthy sample



(a)



(b)

Fig 4.5. BLASTN analysis of PCR product after sequencing

Discussion

5. DISCUSSION

The study entitled "Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) for diagnosis of *Banana bract mosaic virus* in banana (*Musa* spp.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur, during 2019-2021 to develop an efficient virus indexing platform for screening of tissue culture plants and banana planting materials for BBrMV. The results of the study are discussed in this chapter.

For plant viral detection, most of the laboratories use serological and molecular assay. However, detection methods based on molecular techniques are found to have more sensitivity compared to serological method like ELISA, but they need expensive and sophisticated instruments (Saiki *et al.*, 1988). In the present study, we developed a user-friendly RT-LAMP assay for rapid and sensitive detection of BBrMV. The method is simple, highly sensitive, cost-effective, specific and facilitates simple visual detection.

Viral diseases are of great concern in banana cultivation due to their effects on quality and yield as well as their spread through planting materials. The BBrMV is a major virus affecting growth and yield of banana and the disease causes about 40 per cent yield loss in banana. The disease was first detected in 1979 in the Philippines (Magnaye and Espino, 1990). The BBrMV is a single stranded RNA virus, of the genus *Potyvirus*. The banana bract mosaic disease is characterised by spindle-shaped, reddish streaks on bracts, fruits, peduncles and even pseudostem (Rodoni *et al.*, 1997).

In this study, total RNA was isolated from the leaves of the *Banana bract mosaic virus* symptomatic and healthy banana plants for developing RT-LAMP assay. Different methods were tried to obtain good quality RNA from banana leaf samples. Initially RNA isolation was done using TRI reagent. But this method did not give bands corresponding to RNA in 1 per cent agarose gel. The TRI reagent is basically a monophasic solution of guanidinium isothiocyanate and phenol that solubilizes

biological material. Following solubilization, adding chloroform induces separation of the components into various phases, with protein sedimenting to the organic phase, DNA resolving at the interface, and RNA remaining in the aqueous phase. However, RNA pellets purified using TRI reagent are often difficult to resuspend (Rio *et al.*, 2010).

The isolation of RNA was also tried using Purelink plant RNA reagent. Similar to the previous results, good quality RNA was not obtained. The PureLink® Plant RNA Reagent contains 2-mercaptoethanol and sodium azide. Plant RNA Reagent is optimized to have less sample DNA contamination. Even though there were no DNA contamination, RNA obtained was degraded. The RNA isolated using Purelink Plant RNA reagent did not show distinct bands on agarose gel. Because of the high mucilaginous and polyphenolic content of banana, preparation of good quality RNA is difficult. Using The PureLink protocol it is difficult to isolate RNA from damaged or very mucilaginous samples (Yang *et al.*, 2017).

Good quality RNA was isolated using RNeasy plant mini kit (Qiagen). The kit contains different buffers. Buffer RLT contains guanidine thiocyanate, Buffer RW1 contains small amount of guanidine thiocyanate and Buffer RLC contains guanidine hydrochloride. First, biological samples are homogenized in a denaturing buffer. The samples are then applied to the column where other contaminants are removed. The RNA is then eluted in 30–100 µl nuclease free water. The isolation of RNA using the kit was found highly successful in mature leaves of blueberry (Kalinowska *et al.*, 2012). For all of the banana leaf samples, agarose gel electrophoresis of the isolated RNA revealed good quality RNA with intact bands. There were intact bands corresponding to 28S and 18S rRNA (Plate 4.4). The OD_{260}/OD_{280} ratio was used to verify the purity of isolated RNA using a NanoDrop spectrophotometer, and the ratio was between 1.80 and 1.99, indicating pure RNA.

The LAMP is an isothermal amplification platform developed by Notomi *et al.* (2000). The RT-LAMP is a nucleic acid amplification method used to diagnose infectious diseases caused by RNA viruses. In this method, LAMP DNA-detection is performed in conjunction with reverse transcription, which makes cDNA from RNA

before running the LAMP reaction. The LAMP does not require thermal cycling (unlike PCR) and is performed at temperatures ranging from 60 to 65 °C. Designing of primers is an important factor for development of LAMP assay to avoid non-specific detection (Boubourakas *et al.,* 2009). The LAMP uses two sets of primers and additional loop primers for specific amplification. Six primers named internal primers FIP and BIP, external primers F3 and B3, and loop primers LF and LB were designed for RT-LAMP assay. The external and internal primers bind to six distinct regions on the target DNA sequence. Forward and reverse internal primers bind at end region of the target sequence which in turn produces a intermediate stem-loop structure and this acts as template for further rounds of amplification. With the inclusion of loop primers, the reaction sensitivity is enhanced even more, and the incubation time required could be reduced to 30-60 minutes (Li and Ling, 2014). In our study, six primers were developed targeting the coat protein sequence of BBrMV. The RT-LAMP primers were designed using the software Primer Explorer version 5.0 (primerexplorer.jp/lampv5e/index.html). The sequences of selected targets for primer designing were retrieved from NCBI GenBank database. Initially three targets were selected for picking LAMP primers including coat protein gene, movement protein gene and replicase gene of BBrMV. Due to unavailability of loop primers for movement protein gene and replicase gene, primers were synthesised targeting coat protein gene.

Both one-step and two-step RT-LAMP assays were optimized for detection of the virus. The two-step RT-LAMP process uses cDNA as a template. In this step, total RNA is reverse transcribed to cDNA. When there is a potential that more than one virus will show up in the sample, two-step RT-LAMP is preferred. Also for initial standardisation procedures, two-step method is ideal. The cDNA was synthesized from the total RNA using Revert Aid First Strand cDNA Synthesis Kit (Thermoscientific). The kit uses RevertAid reverse transcriptase, which has a decreased RNase H activity and works best at 42 °C to 50 °C. This is suitable for cDNA synthesis up to 13 kb in length. At 55 °C, the Ribolock RNase inhibitor protects RNA from destruction (Rubio *et al*., 2003). The poly (A) tail of mRNA was annealed to the oligo (dT) primer. The synthesised first strand cDNA was used as the

template for isothermal amplification. Initially, optimisation of RT-LAMP method was done by varying the concentrations of reagents such as betaine, dNTPs, $MgSO_4$. Several set of reactions were carried out to optimise the conditions for RT-LAMP. Different concentrations of $MgSO_4$ (4-8 mM), dNTP (1.4-1.6 mM each), betaine (0.8-1 M) and *Bst* polymerase (4-8 U) were tried for optimisation.

A major component of the LAMP reaction is *Bst* polymerase enzyme. The *Bst* DNA polymerase permits rapid sequence analysis from nanogram amounts of template (Mead *et al.*, 1991). Primer annealing and enzyme activity also depends on Mg^{2+} concentration in the reaction. In this assay 6-8 mM $MgSO_4$ concentration amplified target virus. However, for colourimetric detection 6 mM $MgSO_4$ was used because of the distinct colour change. For the amplification of different plant RNA viruses, the use of, 4–8 mM $MgSO_4$ concentration is reported to be optimum (Nie, 2005). It has been suggested that betaine improves amplification of GC-rich DNA sequences by reducing formation of secondary structure and helps in improving the amplification of DNA (Henke et al., 1997).

One molar betaine was used in the reaction mixture for both one-step and two-step RT-LAMP assay. In our investigation, we optimized the two-step assay with the reaction mixture containing 40 ng cDNA, 1.6 mM each dNTP, 0.2 µM each primers F3 and B3, 0.8 µM each primers BrFIP and BrBIP and 0.4 µM each primers BrLF and BrLB, 1 M betaine, 4 mM $MgSO_4$, 1x Thermopol buffer with 2 mM $MgSO_4$ and 4 U *Bst* polymerase large fragment in 25 µl reaction volume for two-step RT-LAMP assay.

The optimum temperature for LAMP amplification mainly depends on the primers used. Literature suggests that the temperature for amplification ranges from 59° to 69 °C (Siljo and Bhat, 2014). Increase or decrease in reaction temperature may result in decreased yields due to inactivation of enzymes or instability of reaction (Fukuta et al., 2004; Wei et al., 2012).

However, we found 65°C as the optimum temperature. A simple heat block or a water bath can be used to perform the RT-LAMP reaction instead of a thermal

cycler for PCR (Sui et al., 2018). The RT-LAMP assay when performed with a thermal cycler and using a heat block produced comparable results.

Under UV light, isothermally amplified DNA can be visualised using gel electrophoresis and ethidium bromide staining, and it can also be detected using other post-amplification detection methods which may increase the risk of carry-over contamination by opening the tubes (Zanoli and Spoto, 2013). For closed tube detection, colourimetric detection method is generally used. Hydroxy Naphthol Blue dye was used for colourimetric detection of RT-LAMP amplicons where a change in colour from violet to sky blue can be observed in positive samples (Ahmadi *et al.,* 2013; Banerjee *et al.,* 2016). The change in colour with HNB after the reaction is generated by the pH shift and this indicates successful amplification (Goto *et al.*, 2009). The colour of the reaction mixture turned violet after addition of HNB dye. Positive amplicons showed a colour change from violet to sky blue after the RT-LAMP reaction while negative samples remained violet. The colour change was observed when the reaction was optimised with 120 µM HNB dye. When 150 µM HNB was added, no colour change observed in amplified samples. Similar findings were recorded by Liu *et al.* (2015) in diagnosis of *Maize chlorotic mottle virus*.

We also developed a one-step RT-LAMP assay to make the test simpler and to reduce the time involved. This assay requires only one step of master mix preparation and isothermal amplification for one hour to detect BBrMV effectively. When RNA template is directly used in case of one-step RT-LAMP assay, a process of reverse transcription is also involved in the same tube. One-step RT-LAMP assay could be completed within an hour for the detection of plant RNA viruses (Fukuta *et al.*, 2004). One-step reaction utilized the RNA as template in the reaction along with M-MLV reverse transcriptase (Promega) and RNasin® plus RNase inhibitor (Promega). The RNase inhibitor contains 20 mM HEPES-KOH (pH 7.60 at 4°C), 50 mM KCl, 8mM DTT and 50 per cent (v/v) glycerol. The RNasin® Plus RNase Inhibitor prevents ribonucleases action during reaction assembly. One-step RT-LAMP is generally used for routine detection of viral samples. One-step RT-LAMP could easily detect virus from total RNA and had hundreds of times the sensitivity of conventional RT-PCR for detection of *Cucumber green mottle virus* (Li *et al.*, 2013). The one-step RT-

LAMP assay was optimised with 2 µg RNA, 1.6 mM each dNTP, 0.2 µM each primers BrF3 and BrB3, 0.8 µM each primers BrFIP and BrBIP and 0.4 µM each primers BrLF and BrLB, 1 M betaine, 4 mM $MgSO_4$, 1x Thermopol buffer with 2 mM $MgSO_4$, 8 U *Bst* polymerase large fragment, 100 U M-MLV reverse transcriptase and 20 U RNasin® plus RNase inhibitor in 25 µl reaction volume.

In this study, BBrMV symptomatic samples were tested with both one-step and two-step procedure and results obtained were comparable. One-step RT-LAMP assay was optimized with 8 U *Bst* and two-step with 4 U *Bst* enzyme. Ladder like bands were observed in both the type of reactions when observed on agarose gel electrophoresis. Ladder like bands are the result of stem loop structure formed as a result of LAMP amplification (Notomi *et al.* 2000). The specificity of primers is an important aspect in achieving efficient amplification of a particular target by molecular diagnostic assays, including RT-LAMP. As a result, understanding the specificity or cross-reactivity of RT-LAMP primers, as well as non-specific amplification, is critical. We had validated all our primers using BLASTN analysis. We also tested different targets in our RT-LAMP assay. We observed a positive reaction to BBrMV but a negative reaction to other viruses such as BBTV, CMV and BSV indicating that the LAMP primers are highly specific to BBrMV. The LAMP assay has 10 to 1000 fold more sensitivity than RT-PCR or nested PCR assays due to its increased amplification efficiency (Zhang *et al.*, 2011).

In our research study, we did the restriction profiling of the RT-LAMP amplicons with *Sau*3AI restriction enzyme having internal cut site in the BrF2-BrB2 flanking region. The enzyme has single cut site generating fragments of size 100 bp and 45 bp. Expected results were produced after agarose gel electrophoresis indicating the accuracy and specificity of the developed RT-LAMP platform. Restriction digestion has been used to confirm the accuracy of LAMP assay for the detection of phytoplasma 16S rRNA gene (Nair *et al.,* 2016). We further confirmed the fidelity of our assay by RT-PCR with external primers and sequencing. Sequence after BLASTN analysis showed 97.78 % identity with BBrMV isolate BRS2 coat protein mRNA, complete cds (GenBank Acc no: MK139142.1).

The LAMP has been successfully used for detection of various viruses (Liu *et al.*, 2010). To avoid false-positive results, careful and strict conditions are necessary owing to its higher sensitivity (Tomita *et al.*, 2008). We maintained a sterile area for RT-LAMP reaction with UV attached PCR work station. Sample preparation was done in a separate space to reduce contamination. The developed RT-LAMP assay can be performed in a dry bath or a water bath with a constant temperature.

The RT-LAMP is a simple method which could be used for screening of large number of samples as well as for field level diagnostics, if simple sample preparation procedures are available. It could be used where sophisticated instruments are not accessible and also where reagents might not be readily available (Liu *et al.*, 2015). The RT-LAMP reactions can be carried out under field conditions in a simple heating block using tissue extracts and by using colourimetric detection with HNB, direct visualization is possible. Such rapid and low cost detection technique will facilitate the implementation of virus indexing facility in tissue culture units to screen plants. The RT-LAMP assay developed in this study is an efficient tool for monitoring of BBrMV disease.

Summary

6. SUMMARY

The research work entitled "Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) for diagnosis of *Banana bract mosaic virus* in banana (*Musa* spp.)" was carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Agriculture, Kerala Agricultural University, Thrissur, from 2019-2021 with the objective to develop a rapid and efficient molecular diagnostic method for BBrMV.

The salient findings of the study are summarised below:-

- Twelve BBrMV symptomatic banana samples and samples showing symptoms of other viruses like BBTV, BSV and CMV were collected from Banana Research Station, Kannara
- Healthy banana samples were collected from CPBMB, CoA, Vellanikkara
- Total RNA was isolated from these samples using RNeasy plant mini kit, Qiagen
- Agarose gel profile of isolated RNA indicated distinct bands corresponding to 28S and 18S rRNA
- Purity of total RNA isolated was checked using NanoDrop spectrophotometer and OD_{260}/OD_{280} ratio was between 1.80 and 1.99 indicating pure RNA
- Three targets were tried for designing of RT-LAMP primers using software Primer Explorer version 5.0 (primerexplorer.jp/lampv5e/index.html)
- Three targets include BBrMV coat protein gene, replicase gene and movement protein gene
- Due to unavailability of loop primers for replicase gene and movement protein gene, primers were designed for coat protein gene and synthesised with Sigma-Aldrich

- Six RT-LAMP primers namely external primers (BrF3 and BrB3), internal primers (BrFIP and BrBIP) and loop primers (BrLF and BrLB) were synthesised
- Initially, the two-step RT-LAMP assay was standardised. The RevertAid^{TM} first strand cDNA synthesis kit by ThermoFischer Scientific^{TM} was used for first strand cDNA synthesis
- Several sets of reactions were carried out to optimise the conditions for RT-LAMP
- Different concentrations of $MgSO_4$ (4-8 mM), dNTP (1.4-1.6 mM each), betaine (0.8-1 M) and *Bst* polymerase (4-8 U) were tried for optimisation
- One-step RT-LAMP assay was optimised using 100 U M-MLV reverse transcriptase and 20 U RNasin® plus RNase inhibitor directly in the RT-LAMP reaction and RNA as template
- Positive amplicons showed ladder like bands on agarose gel electrophoresis whereas no template control and healthy samples did not show any amplification
- Positive samples showed a colour change from violet to sky blue when 120 µM HNB was added to the reaction mixture prior to amplification while the negative samples remained violet using HNB
- PCR amplification was done using primers F3 and B3, this gave amplicons of the expected size (196 bp) and sequences when subjected to BLASTN, showed 97.78 % identity with query coverage of 99 % with BBrMV isolate BRS2 coat protein mRNA, complete cds (GenBank Acc no: MK139142.1)
- A representative sequence was deposited in the GenBank database (GenBank Acc no: OL757512)

- Restriction digestion of RT-LAMP products were done using *in silico* identified enzyme *Sau3*AI. Restriction digestion produced fragments of expected size (100 bp and 45 bp) when run on 2.5 per cent agarose gel

- The RT-LAMP assay was validated using 12 BBrMV symptomatic samples, healthy samples, samples with symptoms of BBTV, CMV and BSV

- Only the positive samples of BBrMV showed ladder like bands in agarose gel as well as colour change. All other samples did not show any amplification indicating the specificity of RT-LAMP primers.

References

REFERENCES

Ahmadi, S., Almasi, M. A., Fatehi, F., Struik, P. C. and Moradi, A. 2013. Visual detection of Potato Leaf roll Virus by one-step reverse transcription loop-mediated isothermal amplification of DNA with hydroxyl naphthol blue dye. *J. Phytopathol. 161*(2):120-124.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W and Lipman, D. J.1997.Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids Res. 25*(17):3389-3402.

Atreya, P. L., Atreya, C. D., Pirone, T. P. 1991. Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. *Proceedings of the National Academy of Sciences, USA.* 88(7): 887–7 891.

Balamuralikrishnan, M., Doraisamy, S., Ganapathy, T., and Viswanathan, R. 2002. Serological specificity and titre of Sugarcane mosaic virus polyclonal antisera raised under varying immunization procedures and bleeding time. *J. Plant Dis. Prot.* 109(6):646-654.

Balasubramanian, V. and Selvarajan, R. 2014. Genetic diversity and recombination analysis in the coat protein gene of Banana bract mosaic virus. *Virus Genes*. 48(3):509-517.

Banerjee, A., Roy, S., Sharma, S. K., Dutta, S. K., Chandra, S. and Ngachan, S.V. 2016. Reverse transcription loop-mediated amplification (RT-LAMP) assay for rapid diagnosis of chilli veinal mottle virus. *Adv. Virol*. 161:1957–1961.

Bateson, M. F. and Dale, J. L. 1995. Banana bract mosaic virus: characterisation using potyvirus specific degenerate PCR primers. *Arch.Virol.* 140: 515–527 https://doi.org/10.1007/BF01718428.

Bhat, A. I. and Maheshwari, Y. 2017. Application of Immuno-Diagnosis for plant viruses occurring in India. In *A century of Plant Virology in India*. pp. 583-619.

Blomme, G., Dita, M., Jacobsen, K. S., Pérez Vicente, L., Molina, A., Ocimati, W., Poussier, S. and Prior, P. 2017. Bacterial diseases of bananas and enset: current state of knowledge and integrated approaches toward sustainable management. *Front. Plant Sci*. 8:1290.

Boubourakas, I. N, Fukuta, S. and 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.

Budziszewska, M., Wieczorek, P. and Steplowska, A. O. 2016. One-step reverse transcription loop- mediated isothermal amplification (RT-LAMP) for detection of *Tomato torrado virus*. *Arch. Virol.* 161: 1359-1364.

Chen, Z., Mao, S., Zhang, W., Fan, X., Wu, W., Liu, C., Zhao, K. and Lu, R. 2020. Rapid visual detection method for barley yellow mosaic virus using reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Plant Dis*. 12: 550-556.

Cherian, K. A., Menon, R., Suma, A., Nair, S. and Sudeesh, M. V. 2002. Effect of banana bract mosaic disease on yield of commercial varieties in Kerala (Abstr.) In: Global conference on banana and plantain, Bangalore, pp.155.

Clark, M. F. and Adams, A. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34: 475–483. doi: 10.1099/0022-1317-34-3-475.

Dassanayake, E. M. 2001. Detection of banana bract mosaic potyvirus by immunocapture polymerase chain reaction (IC·PCR). *Ann. Sri Lanka Dept. Agric.* 3 : 19-25.

Demertzis, M. A. 1988. Fluorimetric determination of calcium in serum with calcein: complexation of calcein with calcium and alkali metals. *Anal. Chim. Acta*. 209: 303–308.

Diehl, H. and Ellingboe, J. L.1956. Indicator for titration of calcium in presence of magnesium using disodium dihydrogen ethylenediamine tetraacetate. *Anal. Chem.* 28, 882–884.

Drew, R. A., Moisander, J. A. and Smith, M. K. 1989. The transmission of banana bunchytop virus in micropropagated bananas. *Plant Cell, Tissue and Organ Culture.* 16: 187-93.

Edwardson, J. R. and Christie, R. G. 1978. Use of virus-induced inclusions in classification and diagnosis. *Ann. Rev. Phytopathol*. 16(1): 31-55.

FAOSTAT, 2017. Production and area (on-Line). Available: http://www.fao.org/economic/est/est- commodities/bananas/bananafacts/en.[20 Jan 2020]

Francki, R. I. B., Fauquet, C. M., Knudson, D. L., Brown, F. 1991. Classification and Nomenclature of Viruses. Fifth Report of the International Committee on Taxonomy of Viruses. *Arch. Virol*. 2(1).

Fukuta, S., Lida, T., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M. and Ishimoto, Y. 2003. Detection of Japanese yam mosaic virus by RT-LAMP. *Arch. Virol*. 148(9): 1713-1720.

Fukuta, S., Nimi, Y., Ohishi, K., Yoshimura, Y., Anai, N., Hotta, M., Fukaya, M., Kato, T., Oya, T. and Kanbe, M. 2005. Development of reverse transcription loop-mediated isothermal amplification (RT-LAMP) method for detection of two viruses and chrysanthemum stunt viroid. *Ann. Report Kansai Plant Prot. Society*. 47: 31-36.

Fukuta, S., Ohishi, K., Yoshida, K., Mizukami, Y., Ishida, A. and 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(1): 49-55.

Galvez, L. C., Koh, R.B.L., Barbosa, C.F.C. and Aquino, V.M. 2020. Multiplex reverse transcription-polymerase chain reaction for simultaneous detection of banana bract mosaic virus (BBrMV) and sugarcane mosaic virus (SCMV) in abaca. *Canadian J. Plant Pathol*. 42(4): 572-583.

Gawande, S. P., Raghavendra, K. P., Monga, D., Nagrale, D. T. and Kranthi, S. 2019. Rapid detection of Tobacco streak virus (TSV) in cotton (Gossypium hirsutum) based on Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP). *J. Virol. Methods*. 270: 21-25.

Gerhold, D., Rushmore, T. and Caskey C.T. 1999. DNA chips, promising toys have become powerful tools Trends. *Biochem. Sci*., 24: 168-173.

Gibbs, A. J. and Ohshima, K. 2010. Potyviruses and digital revolution. *Ann. Rev. Phytopathol*. 48:205–223.https://doi.org/10.1146/annurev-phyto-073009-114404.

Goto, M., Honda, E., Ogura, A., Nomoto, A. and Hanaki, K. I. 2009. Colorimetric detection of Loop Mediated Isothermal Amplification reaction by using hydroxy napthol blue. *Biotechniques.* 46: 167-172.

Henke, W., Herdel, K., Jung, K., Schnorr, D. and. Loening, S. A. 1997. Betaine Improves the PCR Amplification of GC-Rich DNA Sequences, *Nucleic Acids Res*. 25(19): 3957–3958, https://doi.org/10.1093/nar/25.19.3957.

Herranz, M. C., Sanchez-Navarro, J. A., Aparicio, F. and Pallás, V. 2007. Simultaneous detection of six stone fruit viruses by non-isotopic molecular hybridization using a unique riboprobe or 'polyprobe'. *J. Virol. Methods*. 124(1-2): 49-55.

Huang, C. H., Lai, G. H., Lee, M. S., Lin, W. H., Lien, Y. Y., Hsueh, S. C., Kao, J. Y., Chang, W.T., Lu, T.C., Lin., et al. 2010. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of chicken anaemia virus. *J. Appl. Microbiol*. 108: 917–924.

Hull, R. and Al-Hakim, A. 1988. Nucleic acid hybridization in plant virus diagnosis and characterization. *Trends Biotechnol.* 6: 213–218. doi: 10.1016/0167-7799(88)90076-5

Iskra-Caruana, M. L., Galzi, S., and Laboureau, N. 2005. A reliable IC One-step RT-PCRmethod for the detection of BBrMV to ensure safe exchange of Musa germplasm. *J. Virol. Methods*. 153: 223–231.

Ivars, P., Alonso, M., Borja, M., and Hernandez, C. 2004. Development of a non-radioactive dot-blot hybridisation assay for the detection of *Pelargonium flower break virus* and *Pelargonium line pattern virus*. *European J. Plant Pathol*. 110: 275–283.

Jones, D. R. 2002. Risk of spread of banana diseases in international trade and germplasm exchange. In: Proceedings XV ACORBAT meeting 2002, Colombia: Cartagena de Indias.

Joshi, S. and Haenni, A. L. 1984. Plant RNA viruses: strategies of expression and regulation of viral genes. *FEBS letters*. 177(2): 163-174.

Jyotsna, P., Parayil, C., Shanmugasundaram, B. and Shankar, M. 2019. Production and Coping Strategies in Banana due to Climate Variability in Kerala. *Indian Hortic. J*. *9*(3and4): 29-35.

Kalinowska, E., Chodorska, M., Paduch-Cichal, E. and Mroczkowska, K. 2012. An improved method for RNA isolation from plants using commercial extraction kits. *Acta Biochimica Polonica*. 59(3).

Karwitha, M., Feng, Z., Shen, Y., Essendi, W., Zhang, W., Li, J. and Tao, X. 2014. Rapid detection of *Tomato chlorosis virus* from infected plant and whitefly by one-step reverse transcription loop mediated isothermal amplification. *J. Phytopathol.* 164(4): 255-263.

Kiranmani, G., Kumar, P. L., Hema, M., Venkatramana, M., Kirshna-Prasadji, Mandheva-Rao, J., et al. 2005. Partial characterization of a potyvirus causing bract mosaic of banana in Andhra Pradesh. *Indian J. Virol*. 16: 7–11.

Landicho, D. M. 2014. Virus detection in tissue cultured abaca (Musa textilis Nee) by serological and molecular methods.Th: Bachelor of Science in Agriculture. College of Agriculture and Food Science (CAFS).

Lenarčič, R., Morisset, D., Mehle, N. and Ravnikar, M. 2013. Fast real-time detection of Potato spindle tuber viroid by RT-LAMP. *Plant Pathol*. 62(5): 1147-1156.

Li, R. and Ling, K. S. 2014. Pepino mosaic virus genotype shift in North Americaand development of a loop-mediated isothermal amplification for rapid genotype identification. *Virol. J.* 10: 117.

Li, X. H., Valdez, P., Olvera, R. E. and Carrington, J. C. 1997. Functions of the tobacco etch virus RNA polymerase (NIb): subcellular transport and protein-protein interaction with VPg/Pro (NIa). *J. Virol*. 71: 1 598–1 607.

Li, Y., Liu, R., Zhou, T. and Fan, Z. 2013. Genetic diversity and population structure of Sugarcane mosaic virus. *Virus Res*. 171(1): 242-246.

Liu, Y., Wang, Z., Qian, Y., Mu, J., Shen, L., Wang, F. and Yang, J. 2010. Rapid detection of tobacco mosaic virus using the reverse transcription loop mediated isothermal amplification method. *Arch. Virol*. 155:1681.

Liu, Z., Xia, X., Yang, C. and Huang, J. 2015. Colorimetric detection of *Maize chlorotic mottle virus* by reverse transcription loop- mediated isothermal amplification (RT-LAMP) with hydroxynapthol blue dye. Royal Society of Chemistry. *RSC Adv*. 6(1):73-78.

Lu, Y., Yao, B., Wang, G. and Hong, N. 2018. The detection of ACLSV and ASPV in pear plants by RT-LAMP assays. *J. Virol. methods*. 252: 80-85.

Magnaye, L. V. and Espino, R. R. C. 1990. Banana bract mosaic, a new disease of banana. I. Symptomatology. *Philipp. Agric.* 73: 55-59.

Magnaye, L. V. and Valmayor, R. V. 1995. BBTV, CMV and other viruses affecting banana in asia and the pacific. Food and fertilizer technology centre Ext. Bull. No. 418. 7p.

Martin, R. R., James, D. and Lévesque, C. A. 2000. Impacts of molecular diagnostic technologies on plant disease management. *Annu. Rev. Phytopathol.* 38: 207-239.

Mead, D. A., McClary, J. A., Luckey, J. A., Kostichka, A. J., Witney, F. R. and Smith, L.M. 1991. Bst DNA polymerase permits rapid sequence analysis from nanogram amounts of template. *BioTechniques*. 11(1): 76-8.

Milne, R. G., 1992. Immunoelectron microscopy of plant viruses and mycoplasmas. *Adv. Dis. vector Res*. pp. 283-312

Mori, Y. and Notomi, T. 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *J. Infect. chemotherapy*. 15(2): 62-69.

Morton, J. 1987. Banana. Fruits of warm climates. *FEBS letters*. pp. 29–46.

Munez, A. R. 1992. Symptomatology transmission and purification of banana bract mosaic virus (BBMV) in giant cavendish banana. Th: Philippines Univ., Los Banos, College, Laguna (Philippines). 1 – 57

Nagamine, K., Hase, T. and Notomi T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol. Cell Probes.* 16(3):223-9. doi: 10.1006/mcpr.2002.0415. PMID: 12144774.

Nair, S., Manimekalai, R. and Gangaraj, P. 2016. Loop mediated isothermal amplification (LAMP) assay for detection of coconut root wilt and arecanut yellow leaf disease phytoplasma. *World J. Microbiol. Biotechnol*. 32: 108

NHB (National Horticulture Board) 2010. Indian Horticulture Data Base-2010. In: Kumar B (ed) National Horticulture Board, Gurgaon

NHB (National Horticulture Board) 2018. Area and Production estimates for horticultural crops for 2018-2019 (On-Line). Available: http://nhb.gov.in/statistics/State_Level/2017-18- (Final).pdf [20 Jan 2020]

Nie, X. 2005. Reverse transcription loop-mediated isothermal amplification of DNA for detection of Potato virus Y. *Plant Dis*. 89(6): 605-610.

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

Przewodowska, A., Zacharzewska, B. and Chołuj, J. 2015. A One-Step, Real-Time Reverse Transcription Loopmediated Isothermal Amplification Assay to Detect Potato Virus Y. *Ann. J. Potato Res.* 92: 303–311 https://doi.org/10.1007/s12230-015-9430-3.

Punyarani. and Kshetrimayum. 2013. In vitro production of genetically stable and virus free plantlets of Musa sp. var. Meitei Hei using male inflorescence as explant. *Sci. Hortic.* 164: 440-447.

Rio, D. C., Ares, M., Hannon, G. J. and Nilsen, T. W. 2010. Purification of RNA using TRIzol (TRI reagent). *Cold Spring Harbor Protoc*. 2010(6): 5439.

Rodoni, B. C., Ahlawat, Y. S., Varma, A., Dale, J. L. and Harding, R. M. 1997. Identification and characterization of *Banana bract mosaic virus* in India. *Plant Dis*. 81: 667-672.

Rubio, L., Galipienso, L. and Ferriol, I. 2020. Detection of plant viruses and disease management: relevance of genetic diversity and evolution. *Front. Plant Sci.* 11:1092. doi: 10.3389/fpls.2020.01092

Rubio, L., Janssen, D., Cuadrado, I. M., Moreno, P. and Guerri, J. 2003. Rapid detection of cucumber vein yellowing virus by tissue–print hybridisation with

digoxigenin–labelled cDNA probes. *J. Virol. Methods.* 114: 105–107. doi: 10.1016/j.jviromet.2003.08.008

Rubio, L., Soong, J., Kao, J. and Falk, B. W. 1999. Geographic distribution and molecular variation of isolates of three whitefly-borne closteroviruses of cucurbits: *Lettuce infectious yellows virus*, *Cucurbit yellow stunting disorder virus*, and *Beet pseudo–yellows virus*. *Phytopathology*. 89: 707–711. doi: 10.1094/PHYTO.1999.89.8.707.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science.* 239(4839): 487-491.

Saldarelli, P., Barbarossa, L., Grieco, F. and Gallitelli, D. 1996. Digoxigenin-labelled riboprobes applied to phytosanitary certification of tomato in Italy. *Plant Dis.* 80: 1343–1346.

Samraj, J., Menon, M. R., Christudas, S. P. and Satyarajan, P. K. 1966. Kokkan a new disease of banana (*Musa paradisiaca* Linn). *Agric. Res. J. Kerala.* 4: 116.

Sánchez-Navarro, J. A., Cañizares, M. C., Cano, E. A. and Pallás, V. 1999. Simultaneous detection of five carnation viruses by non-isotopic molecular hybridization. *J. Virol. Methods*. 82(2): 167-175.

Sankaralingam, A., Virendra Baranwal, K., Ahlawat, Y. S., Renuka Devi and Madan Ramiah. 2006. RT-PCR detection and molecular characterization of *Banana bract mosaic virus* from the pseudostem and bract of banana. *Arch. Phytopathol. Plant Prot*. 39:4:273-281. DOI: 10.1080/03235400500181709.

Selvarajan, R. and Jeyabaskaran, K. J. 2006. Effect of *Banana bract mosaic virus* (BBrMV) on growth and yield of cultivar Nendran (Plantain, AAB). *Indian Phytopathol*. 59(4): 496-500.

Selvarajan, R., Balasubramanian, V. And Sathiamoorthy, S. 2006. Vector transmission of banana bract mosaic and banana streak viruses in India. In:

Abstracts of XVI Annual convention and International symposium on "Management of vector – borne viruses, conducted by IVS, at ICRISAT, 7-10 February 2006. pp. 110.

Selvarajan, R., Balasubramanian, V., Sheeba, M. M., Raj Mohan, R. and Mustaffa, M. M. 2009. Virus-indexing technology for production of quality banana planting material: a boon to the tissue-culture industry and banana growers in India. In *V International Symposium on Banana: ISHS-ProMusa Symposium on Global Perspectives on Asian Challenges.* 897: 463-469.

Selvarajan, R., Kanichelvam, P.S., Balasubramanian, V. and Subramanian, S.S. 2020. A rapid and sensitive lateral flow immunoassay (LFIA) test for the on-site detection of banana bract mosaic virus in banana plants. *J. Virol. Methods*. *284:* 113929.

Sharman, M., Thomas, J. E. and Dietzgen, R. G. 2000. Development of a multiplex immunocapture PCR with colourimetric detection for viruses of banana. *J. Virol*. *Methods.* 89: 75–88.

Siljo, A. and Bhat, A. I. 2014. Reverse-transcription loop-mediated isothermal amplification assay for rapid and sensitive detection of banana bract mosaic virus in Cardamom (*Elettaria cardamomun*). *Eur J Plant Pathol*. 138:209–214.

Siljo, A., Alangar., Biju, C., and Venugopal. 2011. Occurrence of Banana bract mosaic virus on cardamom. *Phytoparasitica.* 40. 10.1007/s12600-011-0193-1.

Singh, H. P., Uma, S., Selvarajan, R. and Karihaloo, J. L. 2011. Micropropagation for production of quality banana planting material in Asia-Pacific. *Asia-Pacific Consortium Agric. Biotechnol. (APCoAB), New Delhi, India*, *92*.

Singh, S. J. and Selvarajan, R. 2000. In Banana-improvement, production and utilization, ed. by H.P. Singh, K.L. Chadha, pp. 381–383.

Sui, X., Zhang, S., Wu, Z. and Ling, K.S. 2018. Reverse transcription loop-mediated isothermal amplification for species-specific detection of *Tomato chlorotic spot orthotospovirus*. *J. Virol. Methods*. 253: 56-60.

Suresh., Byadgi, A. S., Hegde. and Mokashi, A. N. 2004. Survey and detection of banana bract mosaic virus. *Phtopathology*. 16(1).

Suzuki, Y., Gojobori, T. and Nakagomi, O. 1998. Intragenic recombinations in rotaviruses. *FEBS letters*. 427(2): 183-187.

Thangavelu. R., Selvarajan, R. and Singh, H. P. 2000. Status of banana streak virus and banana bract mosaic virus diseases in India. Banana: Improvement, production and utilization. 364-376.

Thomas, J. E., Geering, A. D. W., Gambley, C. F., Kessling, A. F. and White, M. 1997. Purification, properties, and diagnosis of banana bract mosaic potyvirus and its distinction from abaca mosaic potyvirus. *Phytopathology*. 87(7): 698-705.

Tomita, N., Mori, Y., Kanda, H. and Notomi, T. 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature protoc*. 3(5): 877-882.

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

Varsani, A., van der Walt, E., Heath, L., Rybicki, E. P., Williamson, A.L. and Martin, D. P. 2006. Evidence of ancient papillomavirus recombination. *J. Gen. Virol.* 87(9):2527–2531.

Wang, I. C., Sether, D. M., Melzer, M. J., Borth, W. B. and Hu, J.S. 1997. Flowering Ginger in Hawaii. *Phytopathology*. 87(698).

Warghane, A., Misra, P., Bhose, S., Biswas, K. K., Sharma, A. K., Reddy, M. K. and Ghosh, D. K. 2017. Development of a simple and rapid reverse transcription

loop mediated isothermal amplification (RT-LAMP) assay for sensitive detection of *Citrus tristeza virus. J. Virol. Methods*. 250: 6-10.

Wei, Q. W., Yu, C., Zhang, S. Y., Yang, C. Y., Miriam, K., Zhang, W. N., Dou, D. L. and Tao, X. R. 2012. One-step detection of bean pod mottle virus in soybean seeds by the reverse-transcription loop-mediated isothermal amplification. *Virol J*. 9:187–192.

WeiGang, W., Zhong, T. and Ying, Z. 2009. Detection of *Banana bract mosaic virus* by real time fluorescent RT-PCR. *Acta Phytophylacica Sinica*.36:417–20.

Yang, Y., Moore, M. J., Brockington, S. F., Timoneda, A., Feng, T., Marx, H. E., Walker, J. F. and Smith, S. A. 2017. An efficient field and laboratory workflow for plant phylotranscriptomic projects. *Appl. Plant Sci*. 5(3): 1600128.

Zanoli, L. M. and Spoto, G. 2013. Isothermal amplification methods for the detection of nucleic acids in microfluidic devices. *Biosensors*. *3*(1):18-43.

Zhang, J., Borth, W. B., Lin, B., Dey, K. K., Melzer, M. J., Shen, H., Pu, X., Sun, D. and Hu, J. S. 2016. Deep sequencing of banana bract mosaic virus from flowering ginger (Alpinia purpurata) and development of an immunocapture RT-LAMP detection assay. *Arch. Virol.* 161(7): 1783-1795.

Zheng, L., Rodoni, B. Gibbs, A. And Gibbs, M. 2009. A novel pair of universal primers for the detection of potyviruses. *Plant. Pathol*. 18(6).

Abstract

REVERSE TRANSCRIPTION LOOP MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) FOR DIAGNOSIS OF *Banana bract mosaic virus* IN BANANA (*Musa* spp.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Banana (*Musa* spp.) is one of the most important and widely cultivated crops around the world. Banana is cultivated on 0.9 million hectares in India with a production of 30.8 million tonnes (NHB, 2020). Despite its importance, growers are not in a position to achieve high productivity due to the presence of various biotic and abiotic stresses. Viruses are a major concern to banana production causing up to 100 per cent yield reduction. The *Banana bract mosaic virus* (BBrMV), a ssRNA virus of the genus *Potyvirus* is reported to cause 40 per cent yield reduction in banana. The virus is transmitted through vegetative propagules and insect vectors like *Pentalonia nigronervosa*, *Aphis gossypi* and *Rhopalosiphum maidis.* Internal quarantine is necessary to prevent the spread of the virus. We need a platform for virus indexing, so that farmers can get good quality virus-free planting materials. The available detection methods for BBrMV are the Enzyme-Linked Immunosorbent Assay (ELISA) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The ELISA is time consuming while the RT- PCR needs post PCR sample handling predisposing to sample cross contamination.

Hence the study entitled "Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) for diagnosis of *Banana bract mosaic virus* in banana (*Musa* spp.)" was undertaken during the period from 2019 to 2021 at the Department of Plant Biotechnology, College of Agriculture (CoA), Vellanikkara, Thrissur, with an objective to develop a rapid and efficient molecular diagnostic method for BBrMV. The LAMP is an auto-cycling strand displacement isothermal DNA synthesis method using a strand displacement DNA polymerase. The RT-LAMP is a specific, highly sensitive and relatively faster diagnostic method for RNA viruses.

Initially, leaf samples of BBrMV symptomatic banana plants were collected from Banana Research station (BRS), Kannara, Kerala. Healthy leaf samples were collected from asymptomatic plants in the field and from tissue culture plants produced at CPBMB, CoA, Vellanikkara. Samples from banana plants showing symptoms of other viruses such *Banana bunchy top virus* (BBTV), *Banana streak virus* (BSV) and *Cucumber mosaic virus* (CMV) as were also collected from BRS,

Kannara, to validate the results of the study. Three different methods using TRI reagent, Purelink Plant RNA reagent and RNeasy plant mini kit were tried for isolation of total RNA. Good quality RNA with intact bands could be obtained with RNeasy plant mini kit (Qiagen).

For the RT-LAMP reaction, a set of six primers were designed using the software Primer Explorer version 5.0. Initially, three targets in the BBrMV genome namely, coat protein gene, replicase gene and movement protein gene were selected for picking LAMP primers. Due to unavailability of loop primers for BBrMV replicase gene and movement protein gene, six primers targeting the BBrMV coat protein gene were selected. All the primers were validated using BLASTN.

Both one-step and two-step RT-LAMP assay was optimized for diagnosis of BBrMV. For two-step RT-LAMP assay, RNA was converted to cDNA and was used as template for isothermal amplification. Optimisation of the assay was done by varying the concentration of the reagents like $MgSO_4$ (4-8 mM), dNTP (1.4-1.6 mM each), betaine (0.8-1 M) and *Bst* polymerase (4-8 U). The final optimised reaction mixture contained 40 ng cDNA, 1.6 mM each dNTP, 0.2 µM each primers F3 and B3, 0.8 µM each primers BrFIP and BrBIP and 0.4 µM each primers BrLF and BrLB, 1 M betaine, 4 mM $MgSO_4$, 1x Thermopol buffer with 2 mM $MgSO_4$, 4 U *Bst* polymerase large fragment and 120 µM HNB in 25 µl reaction volume. Incubation temperature at 65° C was found optimum. One step RT-LAMP is the method suited for routine diagnostics as the RNA sample can be directly used as the template for amplification. One step RT-LAMP reaction mixture contained reverse transcriptase and RNase inhibitor along with the other components of the LAMP reaction and RNA was used as the template instead of cDNA.

The positive amplicons showed ladder like bands on agarose gel and showed a colour change from violet to sky blue in the presence of HNB dye in the reaction mixture. Molecular typing of RT-LAMP products was done using sequence analysis and restriction digestion. For restriction profiling, the enzyme *Sau3*AI having internal cut site in the RT-LAMP internal primer flanking region was used. The enzyme having single cut site produced fragments of size 100 bp and 45 bp. The RT-LAMP assay was validated using 12 BBrMV symptomatic samples, healthy samples and samples showing symptoms of other banana viruses like BBTV, BSV and CMV. All

the 12 BBrMV symptomatic samples amplified in the RT-LAMP assay while no amplification was observed for healthy samples and samples showing symptoms of other viruses. Hence, in the current study, a rapid, sensitive and specific RT-LAMP based detection method for *Banana bract mosaic virus* was developed.