DETECTION OF *Banana bunchy top virus* (BBTV) THROUGH COLORIMETRIC CLOSED TUBE LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

By

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(2019-11-201)

THESIS

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DECLARATION

I, Nahla Binth T. (2019-11-201) hereby declare that the thesis entitled **"Detection of *Banana bunchy top virus* (BBTV) through colorimetric closed tube Loop Mediated Isothermal Amplification (LAMP)"** 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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All that I am, I owe to you
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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
CTAB	Cetyl Trimethyl Ammonium Bromide
DIBA	Dot Immuno Binding Assay
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribo Nucleoside Triphospate
ELISA	Enzyme Linked Immuno Sorbent Assay
EDTA	Ethylene Diamine Tetra Acetic acid
KAU	Kerala Agricultural University
Kb	Kilo base pairs
L	Litre
LAMP	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	*Thermusaquaticus*
TAE	Tris Acetate EDTA
U	Unit
V	Volts
µg	Microgram
µl	Microlitre

INTRODUCTION

1. INTRODUCTION

Banana (*Musa spp.*) is an important food crop worldwide. Often mentioned as a giant herb, the banana is a monocot that belongs to the family Musaceae and order Zingiberales. Banana is the second largest fruit crop of the world and is cultivated predominantly in the tropical and subtropical regions of Asia. It is native to South-East Asia (Janssens *et al.*, 2016).

Banana has a high export potential also. Among fruits, banana is the most consumed one and is highly nutritious fruit crop with high dietary fibers, vitamins and minerals. Its low price and high nutritive value makes it a popular fruit. India is the largest producer of banana with an annual output of about 31.5 million tons from 0.9 million ha area (NHB, 2020).

Despite the higher economic importance, biotic and abiotic stresses are the major threats in achieving higher production and productivity from banana cultivation. Among biotic stresses, viral diseases are considered as a significant barrier in banana cultivation causing up to complete yield loss (Amin *et al.,* 2008). There are four major viral diseases, namely, Banana Bunchy Top Disease (BBTD) caused by *Banana bunchy top virus* (BBTV), Banana Bract Mosaic Disease caused by *Banana bract mosaic virus* (BBrMV), banana mosaic or infectious chlorosis caused by *Cucumber mosaic virus* (CMV) and Banana Streak Disease caused by *Banana streak virus* (BSV) occurring in most of the banana growing areas. Among them, BBTD is considered to be most economically destructive and can contribute up to 100 % yield reduction (Dale, 1987).

The BBTV belongs to genus *Babuvirus* and family Nanoviridae. The vector of this virus is the banana aphid, *Pentalonia nigronervosa*. The primary transmission of the disease is through infected planting material. Banana is mainly propagated by suckers. Due to high demand for suckers during planting season the interstate sale of banana suckers on road side is a common site and there is no quality control for these planting materials. Nowadays, the demand for tissue culture raised plants is increasing in order to obtain virus free planting materials. Viral pathogens are also a cause of concern to banana micropropagation because they are not completely eliminated

through shoot tip culture, a technique commonly adopted by the industries for mass propagation (Selvarajan *et al.,* 2011). They can remain in latent form in the host for a period of time, causing no visible symptoms. The BBTV infected plants raised through tissue culture may remain asymptomatic for few months when planted in the field (Drew *et al.,* 1989).

In addition, symptoms of viral infection are often confused with nutrient deficiency symptoms. Early and accurate disease diagnosis is necessary for timely management of the disease and for restricting spread of the pathogen to healthy plants. In the absence of resistant varieties, a sensitive and rapid virus indexing is mandatory for disease management (Dale, 1987).

Different diagnostic techniques based on serological detection and molecular detection are used worldwide. Serological detection methods include different types of Enzyme Linked Immuno Sorbent Assay (ELISA), which have less sensitivity and are time consuming. The common molecular detection method used for BBTV is Polymerase Chain Reaction (PCR) which requires instrument for thermal cycling and post amplification sample handling which may predispose to cross contamination of the samples.

The nucleic acid amplification method called Loop Mediated Isothermal Amplification (LAMP) enables rapid, sensitive and highly specific amplification of template DNA under isothermal condition (Notomi *et al.,*2000). There are different routes for monitoring the positive LAMP reaction, of which the colorimetric detection based on Hydroxy Naphthol Sky blue (HNB) enables closed tube detection with naked eye (Goto *et al.,* 2009). The HNB dye is added prior to the reaction and the positive reaction shows colour change from violet to sky blue, thus enabling simple visual detection. The LAMP assay for molecular detection of BBTV based on SYBR Green fluorescence detection has been developed in China (Peng *et al.,* 2012). In a study on the comparison of five different dyes to accurately detect LAMP products for *Tomato yellow leaf curl virus* (TYLCV), it was observed that HNB produced long and stable color change (Almasi *et al.,* 2013).

Development of colorimetric LAMP assay for rapid detection of the BBTV would help to detect virus even before symptom development and would enable virus screening of tissue culture raised plants and suckers of banana. The present work on “Detection of *Banana bunchy top virus* (BBTV) through colorimetric closed tube Loop Mediated Isothermal Amplification (LAMP)” was carried out at the Center for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agriculture University, Thrissur, from 2019 to 2021 with the objective to develop colorimetric closed tube LAMP assay for the diagnosis of BBTV in banana.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Introduction

Banana (*Musa sp.*) is one of the most popular fruits worldwide. It is botanically a berry produced by several kinds of giant herbaceous monocots of the genus *Musa*, belonging to the family Musaceae and order Zingiberales. Banana is cultivated mainly in the tropical and subtropical areas around the world. It is native to South East Asia (Janssens *et al.,* 2016). There are more than thousand varieties of banana in the world (Israyeli and Lahav, 2017).

Banana is the most produced fruit crop in India. It is also the most consumed fruit (APAARI, 2019). Its low price, year-round availability, range of flavours, medicinal value and high nutritive value with high dietary fibers, vitamins and minerals make it the favorite fruit among consumers. India holds the title of largest producer of banana in the world with an annual production of 31.5 million tons from 0.9 million ha area (NHB, 2020). Banana contributed to about 341 billion Indian rupees in the Indian economy in the fiscal year 2018 (Statista, 2020). It has a high export potential also. India exported 1.95 lakh metric ton banana valued at Rs 660 crore in 2019-20 (India times, 2020).

Banana is a rich source of carbohydrate and is rich in potassium, calcium, phosphorus, magnesium and vitamins particularly vitamin B (Thompson, 2011). The fruit is fat free and easy to digest. Plantains are rich in starch and banana starch can be used as baby food. A variety of processed products can be made from the fruit, such as chips, puree, jam, jelly, juice and wine.

2.2. Diseases affecting banana

Banana production and productivity world wideare limited by a number of biotic and abiotic stresses. Different pests and fungal, bacterial and viral pathogens are causing threat to its cultivation.

The fungal diseases like Panama wilt or Fusarium wilt (*Fusarium oxysporum),* Sigatoka (*Mycosphaerella musicola* and *Mycosphaerella fijiensis*), Anthracnose (*Gloeosporium musae*), Cigar end tip rot (*Gloeosporium musarum, Trachsphaera fructigena* and *Verticillium theobromae*) Crown rot *(Verticillium theobromae, Colletotrichum musae, Nigrospora sphaericu, Botryodiploidia theobromae* and *Fusarium sp*.), Stem end rot (Thielaviopsis paradoxa) and Pseudostem heart rot (Fusarium sp., Botrydiplodia sp. and *Gloeosporium sp*.) affect the crop .

Bacterial diseases of banana include Moko or Bugtok disease (*Ralstonia solanacearum*), bacterial rhizome and pseudostem wet rot (*Dickeya paradisiaca*), Xanthomonas wilt (*Xanthomonas campestris* pv. *musacearum*), blood disease (*R. syzygii* subsp. *celebesensis*) and bacterial head rot or tip over disease (*Erwinia carotovora* ssp. *carotovora* and *E. chrysanthemi*) (Blomme *et al.*, 2017).

2.2.1. Viral diseases

Viral diseases are considered as a significant threat to banana cultivation causing up to complete yield loss (Amin *et al.*, 2008). Of the viruses infecting banana, the four main viruses causing severe economic losses, namely, BBTV, BBrMV, BSV and CMV are seen in most of the banana cultivated regions. Among this, the BBTV causes devastative yield reduction and thus it is considered to be the most economically destructive one (Dale, 1987).

2.3. Banana bunchy top disease

2.3.1. Occurrence and transmission

The first written report about the disease incidence was from Fiji during 1890 where it caused heavy destruction and then from Australia during 1913 (Magee, 1927). The disease was devastating in many countries such as Fiji (1927), Vietnam (1961), Tongo and Samoa (1967) and Guam (1982) (Dale, 1987). The BBTD is described from many countries like Hawaii (Conant, 1992), Australia (Harding *et al.*,1993), Vietnam (Wanitchakorn, 2000), China (Tien *et al.*, 2004), Pakistan (Amin *et al.*, 2008) and in many African countries (Kumar *et al.*, 2009 and Blomme *et al.*, 2013).

The BBTD was introduced to India from Sri Lanka in 1940s (Magee, 1953). The disease was confirmed in India in the Travancore state in 1943 (Manickam, 2002). The BBTD has been reported from Kerala, Tamil Nadu Karnataka, Maharashtra, Gujarat, Assam, West Bengal, Arunachal Pradesh, Andhra Pradesh, Meghalaya, Delhi, Andaman (Selvarajan *et al.*, 2010) and Lucknow (Vishoni *et al.*, 2009).

The viral nature of the disease was revealed by Magee (1927) who stated that it is spread by insect vector, black banana aphid. An 18- 20 nm isometric virus like particle associated with the disease was purified from infected plants (Wu and Su, 1990a). Harding *et al.* (1991) isolated circular ssDNA of nearly 1 kb from these virus particles, cloned and sequenced one ssDNA component, called as BBTV component 1.

The host range of BBTV include edible banana, plantain, ornamental and abaca plants in the two genera of Musaceae family. Besides *Musa* species, BBTV also infects its close relative *Ensete ventricosum* (Wardlaw, 1961). About 45 banana cultivars in India are found to be susceptible to BBTV (Capoor, 1967). Mohan and Lakshmanan (1987) reported that AA and AAA varieties were affected by BBTV more seriously.

Primary infection of the disease is through infected suckers, corms or bits which are used as planting materials commonly. Banana is mainly propagated by suckers. The long distance spread of disease worldwide occurs through infected suckers and corms

(Magee, 1927). However, BBTV is not transmitted through agriculture implements or by contact (Hafner *et al.*, 1995).

Secondary infection of virus from plant to plant is through banana aphid *Pentalonia nigronervosa* in a persistent circulative and non-replicative manner (Magee, 1940). After feeding infected plants a latent period of about 24 hours is required for the transmission of the virus by aphids (Bresen and Watanabe, 2010). Hafner *et al.* (1995) reported that BBTV does not replicate within the aphid vector. Wu and Su (1990a) used groups of aphids to compare BBTV acquisition efficiency at different temperature and demonstrated that temperature impacts efficiency as no transmission was observed at 16 °C and maximum transmission at 27 °C.

2.3.2. Economic impact

The BBTV has caused some disastrous epidemics including the one in Australia during 1913 to 1926 that destroyed a large fraction of the country's banana crop (Dale, 1987). Another epidemic in Pakistan during 1992 had sever disease intensity with incidence up to 100 % and destroyed about half of the banana cultivation (Khalid *et al.*, 1993). In India, one of the most significant BBTD epidemics was reported in Tamil Nadu which resulted in huge loss (Kesavamoorthy, 1980).

The famous special flavored dessert banana variety Virupakshi found in the hills of Pulney, Tamil Nadu, was massively destroyed by the BBTD (Kesavamoorthy 1980). In Kerala alone, a loss of 8.5 million dollars has been reported due to BBTV (Selvarajan and Balasubramanian, 2008). A casualty of ₹40 million yearly has been reported in Kerala state due to this malady (Selvarajan and Balasubramanium, 2014).

2.3.3. Symptoms

The main symptom of the disease is bunched appearance of leaves on top of infected plants which contributed to the name of the disease. The first visible symptom includes dark green streaks on the leaf veins, midribs and petioles, forming a dot dash appearance referred as "Morse code" pattern (Thomas and Caruana, 2000). Affected plant shows marginal chlorosis or yellowing, which resembles that of iron deficiency.

Leaves become gradually dwarfed, appear more upright, brittle, narrow and bunched at the apex of the plant. Infection at young stage results in stunted growth and no fruit production (Dale, 1987). Symptoms depend on the time and severity of infection. If infection occurs in later stage of plant growth, fruits may be produced, but distorted or stunted (Nelson, 2004).

The primary infected plants don't grow more than 9 inches to 2 feet. The plant growth is so slow that it would not die for 2 years (Magee, 1927). There are also indications of vein flecking in the lamina. Dark green streaks are seen at the bract tip of the male flower bud if infection develops late in the season. The male bud's bract tip is sometimes transformed into a green leaf like structure (Thomas *et al.,* 1994). Leaf exudates of BBTV infected plants contain lower concentrations of glucose and sucrose than those of the healthy banana plants (Nair, 1969).

2.3.4. Molecular biology of BBTV

Wu and Su in 1990(a) purified and characterized the BBTV for the first time. The BBTV is an isometric particle with a diameter between 18 to 22 nm having a single coat protein (Harding *et.al.,* 1991; Thomas and Dietzgen, 1991). The BBTV is a multi-component circular single stranded DNA virus of family Nanoviridae, genus *Babuvirus*. Initially, BBTV was the only virus in the genus *Babuvirus* (Harding *et al.,* 1991). But *Abaca bunchy top virus* and *Cardamom bushy dwarf virus* were added under this genus later (Mandal *et al.,* 2004; Sharman *et al.,* 2008).

The BBTV isolates from all over the world have relatively low sequence diversity (Banerjee *et al.,* 2014). The BBTV genome consists of six portions, each roughly 1.0 kb, namely DNA-C encoding the cell cycle linked protein, DNA-M encoding for movement protein, DNA-N for shuttle protein, DNA-R encoding replication initiator protein, DNA-S containing gene encoding coat protein and DNA-U which is undetermined (Burns *et al.,* 1995; Wanitchakorn *et al.,* 2000). The DNA-R contains an internal second ORF named as DNA-U5 (Beetham *et al.,* 1997).

2.3.5. Management strategies for BBTV

The disease once established is very difficult to eradicate. The strategies for controlling BBTV include roughing infected plants as early as possible, replacing with virus free planting materials and insecticide application to control aphid vector (Dale, 1987). The use of micro propagated banana plants is not only the most reliable source of infection free planting material, but it is also a better management approach (Kumar *et al.,* 2015). Efforts have also been made to generate transgenic banana resistant to BBTV (Borth *et al.,* 2011; Shekavath *et al.,* 2013). As no resistant varieties are currently available, a sensitive and rapid indexing method is inevitable component of disease management (Dale, 1987).

2.4. Diagnosis of plant viruses

2.4.1. Serology

Wu and Su (1990b**)** produced monoclonal antibodies against BBTV for use in ELISA and detected BBTV by plate trapped antigen (PTA) ELISA. The double antibody sandwich (DAS) ELISA and triple antibody sandwich (TAS) ELISA based detection of BBTV has been optimized for routine indexing of the virus (Othman *et al.,* 1996; Geering and Thomas,1996).

Wanitchakorn *et al*. (1997) expressed BBTV coat protein and produced a polyclonal antiserum which could successfully detect the virus even in asymptomatic plants. A polyclonal antiserum for BBTV 'Hill Banana' isolate was produced by Selvarajan *et al.* (2002). They also reported that the direct antigen coating (DAC)-ELISA method was more sensitive than dot immuno binding assay (DIBA) for detection of BBTV. Impedance spectroscopy has been developed for indexing BBTV (Mujumdar *et al.,* 2013). An improved gold nano particle-based DIBA was developed for BBTV detection which is rapid, sensitive and simpler than ELISA (Mujumdar and Johari, 2018).

2.4.2. Molecular detection

Nucleic acid-based detection approaches are more sensitive than serological methods and are better suited to plant tissues that have low titer of pathogens (Sadik *et al.,* 1997). The BBTV is a phloem limited virus occuring in small concentration in diseased plants (Harding *et al.,* 2000). Nucleic acid-based detection methods are thus better choice for BBTD diagnosis.

2.4.2.1. Nucleic acid hybridization

Xie and Hu (1995) developed hybridization based detection of BBTV using ^{32}P and digoxigenin (DIG) labelled probes. The dot blot hybridization technique has been used to detect BBTV from infected plant samples (Hu *et al.,* 1996; Hafner *et al.,* 1997). Nucleic Acid Spot Hybridization (NASH) technique has been established for diagnosis of BBTV which yielded satisfactory results as that of PCR in tissue culture samples (Selvarajan *et al.,* 2011).

2.4.2.2. Polymerase Chain Reaction

The use of PCR for detecting BBTV in symptomatic as well as in asymptomatic banana samples paved way for virus control in plants produced via tissue culture (Galal, 2007). Xie and Hu (1995) detected the Hawaiian isolates of BBTV using PCR and found the method to be 1000 times more sensitive than ELISA or dot blot methods using DNA probe. A single step plant DNA extraction protocol to reduce plant inhibitory factors interfering with PCR for the detection of BBTV in corm, leaf and root tissues has been developed (Thomson and Dietzgen, 1995). Mansoor *et al.* (2005) detected a Pakistan isolate and Selvarajan *et al.* (2007) detected Indian isolates of BBTV by PCR.

The PCR reaction is often inhibited by contaminants present in banana plant DNA extracts (Das *et al.,* 2009). Moreover, PCR requires thermal cycling instrument and post amplification sample handling which often predisposes to sample cross contamination. High throughput detection of BBTV was developed using real time TaqMan PCR (Chen and Hu, 2013). All these methods are cumbersome, tedious and

require sophisticated well-equipped laboratories and highly trained and skilled personnel.

2.4.2.3. Isothermal amplification methods

Isothermal amplification methods do not require thermal cycling. The amplification takes place at a constant temperature and thus a water bath or heat block is sufficient to carry out the reaction. Usually, an enzyme having strand displacement activity is used for polymerization. The isothermal amplification techniques available are Recombinase Polymerase Amplification (RPA), Loop Mediated Isothermal Amplification (LAMP) and Helicase Dependent Amplification (HDA).

2.4.2.3.1. Recombinase polymerase amplification

An RPA assay has been developed for BBTV detection targeting replicase initiator protein gene of BBTV and the result obtained was reported to be superior to the PCR based detection (Kapoor *et al.,* 2017). In RPA, the isothermal amplification of target DNA fragments is achieved by the combination of enzymes and proteins, *viz.* the recombinase, strand displacing polymerase and single stranded binding proteins (SSB) reacting at a constant low temperature. This technique produces result in 10-15 min and there is no need for initial denaturation of DNA target. The RPA products can be visualized on gel after purification. Hybridization based and fluorescence based methods for detection of amplified products have also been reported. The RPA assay has also been established for detection of other viruses such as *Bean golden yellow mosaic virus, Tomato mottle virus* and *Tomato yellow leaf curl virus* (Londono *et al.,* 2016).

2.4.2.3.2. Helicase dependent amplification

The HDA is an alternative isothermal amplification technique developed by Vincent *et al*. (2004) for New England Biolabs. The HDA uses DNA helicase to generate ssDNA for primer annealing followed by primer extension at isothermal condition. The SSB and MutL endonuclease are added to the reaction to prevent hybridization of complementary ssDNA. Detectable amount of amplicons are produced

within 60 min in this method (Vincent *et al.,* 2004). It has been employed to identify *Tobacco mosaic virus* and *Citrus leprosis virus* (Corona *et al.,* 2010).

2.3.3.3. Loop Mediated Isothermal Amplification

The LAMP technique has been developed by Notomi *et al.* (2000). It is a rapid and simple amplification method, using only a water bath or heating block. The LAMP assay can amplify DNA under constant temperature using a strand displacing polymerase like *Bst* DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences in target DNA (Notomi *et al.,* 2000). The amplification utilises the strand displacement activity of *Bst* DNA polymerase, which is a polymerase I enzyme from *Bacillus stearothermophilus,* to amplify the target (Aliota *et al.,* 1996). So, no denaturation of template is needed and we can amplify target sequence at a constant temperature of 60 to 65 ^{o}C. It is a highly exponential amplification method that accounts for up to 10^9 fold amplification of the target DNA (Notomi *et al.,* 2000).

2.5. LAMP assay for molecular diagnosis

The LAMP technique has been successfully employed for disease diagnosis due to its high specificity, quickness, sensitivity and simplicity.

2.5.1. Principle of LAMP

The LAMP is a one-step amplification assay that amplifies the target DNA or RNA sequence under isothermal condition. Two pairs of primer are used in polymerization reaction detecting six distinct regions in the target sequence. These four primers are labelled as F3 (Forward external), B3 (Backward external), FIP (Forward internal) and BIP (Backward internal). The FIP and BIP are combination of two distinct regions. The FIP contains F1c and F2, while B1c and B2 for BIP, in which F1c and F2c are complementary to the F1 and B1 regions in the target sequence (Plate 2.1).

The LAMP process can be explained using three steps: initial steps, loop structure production and the cycling amplification. Initially, the internal primer (FIP) binds to the specific complement sequence (F2c) and begins the DNA synthesis. Then

the external primer (F3) anneals to the F3c region and starts the strand displacement DNA elongation, releasing first synthesized FIP linked strand, which later forms loop structure. This single stranded DNA acts as template for BIP initiated DNA synthesis and B3 primed strand displacement DNA synthesis. Thus, a dumbbell shaped DNA is produced with loops at both ends. This is quickly converted to stem loop DNA which serves as a single strand template in the cycling amplification step and amplification proceeds similar to that of the initial and loop structure producing steps.

Two amplified products are formed through each cycle for each ssDNA template, in which, one is identical to the template and the other is double the length of the template. The external primers are needed in the initial steps, while, as reaction proceeds only internal primers are needed. The initial use of four primers that enables the recognition of six distinct regions ensures the high selectivity for target amplification.

Additional pair of primers called as loop primers, namely LF (Loop forward) and LB (Loop backward), can be applied in the reaction which further accelerate the amplification (Nagamine *et al.*, 2002). Loop primers hybridize to stem loops except for loops that hybridize to inner primers. To accelerate the reaction, loop primers act from elongation step. As the sensitivity of the reaction is further increased by addition of loop primers, time required for amplification can be shortened to 30–60 minutes. The region between F1 and F2 or B1 and B2 is hybridized by loop primer. The final products are stem loop DNA with continuous inverted repeats of the target DNA and multiple looped cauliflower like structure (Nagamine *et al.*, 2002).

2.5.2. LAMP primer designing

The LAMP primers are designed specific to the DNA sequence of each pathogen. Primer Explorer, a specialized free online software available, is commonly used to pick primers for LAMP assay (https://primerexplorer.jp/e/). Several key factors to be considered for designing primers include length, GC content, stability at 3’ and 5’ end of primers, primer secondary structures and melting temperature (Tm). The primers are designed so that the distance between F2 and B2, the region amplified by the LAMP method, is between 120 and 160 bp.

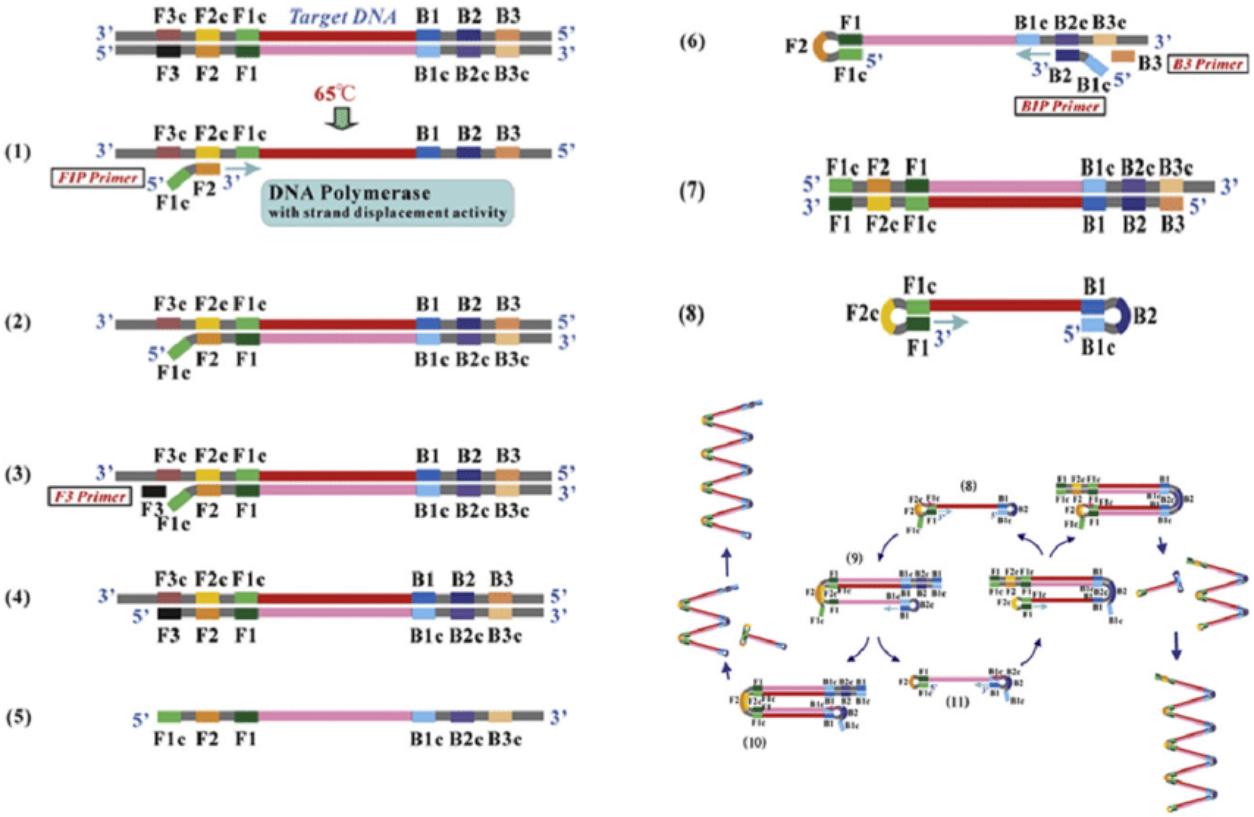


Fig.2.1 Schematic representation of LAMP (Notomi *et al.*, 2000)

PREMIER Biosoft (https://premierbiosoft.com) developed a commercial tool called LAMP Designer for LAMP primer designing. In addition, opensource tools like Electronic LAMP (eLAMP) and LAMP Assay Versatile Analysis (LAVA) have been developed with similar objectives (Torres *et al.,* 2011; Salinas and Little, 2012).

2.5.3. Detection of LAMP products

The amplified DNA can be visualized under UV using gel electrophoresis and staining in ethidium bromide solution that entails opening the reaction tubes, increasing the possibility of carryover contamination dramatically (Zanoli and Spoto, 2013). Closed tube reactions decrease this danger and maximize the applicability of the assay for field experiments. Several methods suitable for such closed tube detection that enable both end point and real time detection of amplified DNA are known today.

2.5.3.1. Detection base on turbidity

The production of pyrophosphate during polymerization after the integration of deoxynucleotide triphosphates (dNTPs) into the DNA synthesized allows for indirect detection of amplification. A large amount of pyrophosphate is generated during LAMP and it combine with calcium, magnesium and manganese bivalent metal ions forming a stable complex (Mori *et al.,* 2001). The magnesium pyrophosphate thus formed precipitates at concentrations above 0.5 mM and can be measured quantitatively using a turbidimeter in terms of turbidity or observed as a pellet after centrifugation (Mori *et al.,* 2004).

2.5.3.2. Fluorescence detection

EvaGreen, Pico Green and SYBR Green are used as indicators for the direct analysis of the LAMP amplicons (Peng *et al.,* 2012). Their use depends on the fluorescence signal collecting time to positive (Tp) values throughout each amplification session (Tomlinson *et al.,* 2007). SYBR Green I has negative impact on DNA amplification when present at higher concentration. Thus, it is used for end point detection either by adding it to the reaction mixture after the reaction only or by depositing the dye into the lid of the tube without mixing with reaction mixture and then mixing after reaction (Zangh *et al.,* 2013). Peng *et al.* (2012) developed the LAMP

closed tube detection method for BBTV adding SYBR Green into the lid of tube before reaction and mixing it with product after the reaction, which enables visualization of colour change of positive products with naked eye.

2.5.3.3. Closed tube detection

The LAMP amplicons can be identified after reaction by adding indicators into the reaction before the reaction like HNB, Genefinder^{TM} or calcein (Tomita *et al.,* 2008 and Goto *et al.,* 2009). Among these, HNB and calcein are metal ion indicators which are usually preferred for closed tube reactions because of their safety. These closed tube detection methods avoid post reaction tube opening, thus avoiding chance of sample cross contamination and enables easy detection.

Calcine is used along with manganese ions, which have a quenching effect on chelating dyes like calcine. Manganese has strong affinity to pyrophosphate. So, as reaction progress manganese bind to pyrophosphate and calcine thus released forms complex with magnesium, leading to an intense green fluorescence following excitation with UV light or visible light. Since the signal recognition is so sensitive, this technology allows for visual result discrimination (Tomita *et al.,* 2008). But manganese has been reported with inhibitory impact on the reaction, reducing the sensitivity of the LAMP assay (Goto *et al.,* 2009).

2.5.3.3.1. Colorimetric detection using HNB

The HNB dye acts as an indicator by variation in the reaction mixture color of the positive LAMP product (Goto *et al.,* 2009). The HNB was reported as a colorimetric indicator for the titration of Mg^{2+} ions at pH 10.0 (Ito and Ueno, 1970). The large fragment of *Bst* DNA polymerase synthesizes DNA under alkaline conditions. Goto *et al.* (2009) reported that when the solution contained 8 mM Mg^{2+} ions and no dNTPs, its color was magenta at pH 8.6–9.0 and violet at pH 8.4. Whereas in presence of dNTPs 1.4 mM and above, the color of HNB becomes violet irrespective of the pH due to the chelation of Mg^{2+} ions by dNTPs. The HNB changes color from violet to sky blue when free Mg^{2+} ions are depleted as amplification progresses because Mg^{2+} binds with pyrophosphates formed.

In a study on the comparison of five different visual dyes to detect LAMP products for *Tomato yellow leaf curl virus*, it was observed that both HNB and GeneFinder^{TM} produced long and stable color change (Almasi *et al.,* 2013). The colour brightness and stability of HNB in positive and negative reaction tubes remained constant after two to three weeks when kept under ambient light (Almasi *et al.,* 2013). The one step RT LAMP for *Potato leaf roll virus* detection (Ahmadi *et al.,* 2012) and LAMP assay for coconut root wilt disease and arecanut yellow leaf disease diagnosis (Nair *et al.,* 2016) were also developed based on colorimetric detection using HNB dye.

2.5.3.4. Real time LAMP

The real time detection of LAMP can be done by observing the time taken for amplification and the annealing peak formed while using a fluorescent intercalating dye. The real-time LAMP has been developed successfully detecting *Botrytis cinerea* causing root rot of flowers, fruits and vegetables (Tomilson *et al.,* 2010) and for quantitative detection of *Fusarium oxysporum f. sp. cubense*, which causes Fusarium wilt in banana (Zeng *et al.,* 2014). The real time LAMP assays for detection of phytoplasma causing coconut root wilt disease and arecanut yellow leaf disease generated positive amplification signals within 30 min of reaction (Nair *et al.,* 2016).

2.5.4. LAMP assay for plant disease detection

Detection platform for plant viruses based on LAMP has been developed for *Tobacco mosaic virus* (Tsutsumi *et al.,* 2010), viral spots on coffee and potatoes (Liu *et al.,* 2010), *Yellow leaf virus* in wheat and tomato (Fukuta *et al.,* 2003), *Yellow dwarf virus* in rice (Le *et al.,* 2010) as well as viruses affecting flowers like chrysanthemum (Fukuta *et al.,* 2004). A LAMP assay developed for identifying wheat mosaic virus showed a sensitivity 100 times greater than PCR (Zang *et al.,* 2011).

The LAMP assays have been standardized to identify *Fusarium oxysporum f. sp. lycopersici* (Almasi *et al.,* 2013) which causes vascular wilt disease in tomato, *Pythium aphanidermatum*, causing tomato root rot disease (Fukuta *et al.,* 2013) and *Bursaphelenchus xylophilus*, causing pine wilt disease (Kikuchi *et al.,* 2009). The LAMP has proven faster and less costly than real time PCR in accurately detecting

Leptosphaeria which causes phoma leaf spotting and stem canker of rape (Je *et al.,* 2013). They used the enzyme *Gsp*SSD DNA polymerase instead of *Bst* polymerase, which is having more amplification efficiency than conventional LAMP.

The LAMP is considered a useful technique to identify bacterial pathogens with the sensitivity about 10 pg target DNA for *Phytophthora sojae* and 1 ng total DNA for *Candidatus liberobacterasiaticum* and *C. liberobacterafricanum* from citrus samples having citrus greening (Dai *et al.,* 2012 and Okuda *et al.,* 2005). The LAMP has proven to be an economic, efficient, rapid and reliable method for identifying *C.liberobacter solanacearum* in potatoes (Ravindran *et al.,* 2012).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The research work entitled "Detection of *Banana bunchy top virus* (BBTV) through colorimetric closed tube Loop Mediated Isothermal Amplification (LAMP)" was carried out at Center for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agriculture University, Thrissur, from 2019 to 2021. The details of the materials and methods used in the study are described below.

3.1. Materials

3.1.1. Plant materials

Leaf samples from 15 BBTV symptomatic plants and two asymptomatic plants were collected from Banana Research Station, Kannara, Thrissur. Leaf samples of healthy banana plants were also collected from tissue culture raised plants at Center for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agriculture University, Thrissur. Leaf samples were also collected from banana plants showing symptoms of other viruses like BSV, CMV and BBrMV from Banana Research Station, Kannara, Thrissur. The leaf samples were collected from different banana varieties like Nendran, Kadali, Poovan, Palayankodan, Njali Poovan and Robusta (Plate 4.1)

3.1.2. Laboratory chemicals and glass wares

Pure and certified chemicals obtained from firms like Merck India Ltd., HIMEDIA and Sigma Aldrich Pvt. Ltd. were used in the present study. The PCR primers and LAMP primers specific for coat protein gene of BBTV were synthesized with Sigma Aldrich Pvt. Ltd. The components for PCR *viz., Taq* DNA polymerase, dNTPs, 10X reaction buffer, $MgCl_2$ and DNA ladder were obtained from GeNei Pvt. Ltd. The components of LAMP *viz., Bst* DNA polymerase, Thermopol buffer and $MgSO_4$ were bought from New England Biolabs Pvt. Ltd. Other components of LAMP such as betaine, dNTPs and HNB dye were procured from Sigma Aldrich Pvt. Ltd. The

plastic wares were procured from Tarson India Ltd. and glass wares used were that of Borosil and Vensil.

3.1.3. Laboratory equipment and facilities

The study was carried out using molecular biology laboratory facilities available at CPBMB, College of Agriculture, Vellanikkara. The instruments such as electronic balance (Shimadzu), pH meter (EUTech instrument pH tutor), micropipettes (Eppendorf), icematic (F100 Compact), water bath (Rotek), high speed refrigerated centrifuge (KUBOTA 6500, HITACHI CR22GIII), Thermocycler (Agilent and BioRad) and heat block (NeuationiTherm D150-2) were used for research work. Nanodrop spectrophotometer (NanoDrop ND- 1000) was used for quality and quantity analysis of DNA. The quality of DNA and amplicons of PCR and LAMP were analyzed using gel electrophoresis unit (BioRad) and agarose gel were visualized using gel documentation unit (BioRad Gel DocTM XR+).

3.2. Methods

3.2.1. Isolation of DNA from diseased sample

Total genomic DNA was isolated from 3.0 g banana leaf samples as per Doyle and Doyle (1987) protocol with some modifications.

Reagents

- Extraction buffer – CTAB buffer (2X)
  2.0% CTAB (w/v)
  100 mM Tris (pH 8)
  20 mM EDTA (pH 8)
  1.4 M NaCl
- β-mercaptoethanol
- Poly Vinyl Pyrrolidine (PVP)
- Chloroform isoamyl alcohol (24:1)
- Isopropanol
- 70 % ethanol

- Nuclease free water

Procedure

1. The extraction buffer was preheated to 60 °C in water bath with β-mercaptoethanol.
2. Approximately 3.0 g of leaf tissue was chopped into small pieces using sterile scissors wiped with 70 % alcohol and ground into fine powder using liquid nitrogen using prechilled mortar and pestle.
3. The tissue powder was transferred immediately into an Oakridge tube containing 7.5 ml warm CTAB extraction buffer.
4. A pinch of PVP was added to the tube.
5. The content was mixed by gentle inversion and then incubated at 60 °C on water bath for 30 minutes with intermittent shaking.
6. Equal volume of chloroform isoamyl alcohol (24:1) was added to the homogenate and incubation was done at 4 °C for 5 minutes.
7. The contents were centrifuged at 10,000 rpm for 15 minutes at 4 °C. The supernatant was transferred to another sterile Oakridge tube using sterile pipette.
8. Two third volume of chilled isopropanol was added to the supernatant and mixed by inversions.
9. The mixture was incubated at -20 °C overnight to precipitate the nucleic acid.
10. After incubation, precipitated DNA was pelleted down by centrifugation at 12,000 rpm for 10 minutes at 4 °C.
11. The supernatant was decanted and the pellet was washed in 70 % ethanol. The pellet was then air dried and dissolved in 120 µl of sterile nuclease free water.

3.2.2. Quantification and quantity analysis of isolated total DNA using NanoDrop spectrophotometer

The quantity and quality of isolated total DNA was analyzed using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Blank was set using 1 µl of nuclease free water to zero before the sample readings were taken. For taking the sample reading, 1 µl of sample was pipetted in to the sample holder and the readings

were measured in ng/µl. The absorbance of nucleic acid was measured at wavelength of 260 nm for quantification. The purity of DNA was checked based on A_{260}/A_{280} value.

3.2.3. Quality analysis of total DNA by agarose gel electrophoresis

The quality of the isolated total DNA was analyzed by agarose gel electrophoresis.

Materials used

- Agarose (HIMEDIA)
- 50X TAE buffer (pH 8.0) (Tris buffer (1 M) 100 ml, 0.5 M EDTA (pH 8.0)100 ml and glacial acetic acid 57.1 ml, Total volume made up to 1 L)
- Sterile distilled water
- Loading dye (Bromophenol blue and glycerol)
- Ethidium bromide solution
- Electrophoresis unit (BioRad)
- Power pack (BioRad)
- Casting tray and comb (BioRad)
- Gel documentation and analysis system (BioRad/ UV transilluminator)

Procedure

1. One liter of 1X TAE buffer was prepared by diluting 50X TAE stock solution with sterile distilled water. The TAE buffer was used to fill the electrophoresis tank and to prepare the gel.
2. The casting dam, casting tray and comb were cleaned with 70 % ethanol. The casting tray was mounted horizontally over the casting dam such that the free ends were tightened and the comb was placed on the gel tray at one end of the tray leaving 1 cm from one end of the tray.
3. Agarose gel of 0.8 % (0.8 g agarose in 100 ml 1X TAE buffer) was prepared in a conical flask by boiling in microwave oven to dissolve the agarose completely.
4. The solution was then cooled to hand bearable temperature, 0.5 µg/ml Ethidium bromide was added as the intercalating dye and the solution was mixed thoroughly.
5. The solution was then poured on gel casting tray. The gel was left undisturbed to solidify.

6. After the gel was solidified, comb was removed gently.
7. The gel was then placed in electrophoresis tank containing 1X TAE buffer (with the wells at the cathode end) such that the wells are completely submerged in the buffer.
8. From DNA samples 3 µl were mixed with 1 µl loading dye and loaded into the wells using micropipettes.
9. The cathode and anode ends were connected to the power pack and the gel was run at voltage of 80 V.
10. The power was turned off when loading dye reached 2/3rd of the gel.
11. The gel was then visualized in gel documentation unit.

3.2.4. RNase treatment

The RNase treatment was done to remove RNA contamination in the DNA samples isolated.

Procedure:

- In an Eppendorf tube with 50 µl DNA, 2 µl of RNase (10 mg/ml) was added and incubated at 37 °C in water bath.
- Chloroform isoamyl alcohol (24:1) 50 µl was added and centrifuged at 10,000 rpm for 15 minutes at 4 °C.
- The supernatant formed after centrifugation was transferred into new tube and 2/3 volume chilled isopropanol was added.
- That tubes were incubated overnight at -20 °C.
- The precipitated DNA was pelleted down by centrifugation at 12,000 rpm for 10 minutes at 4 °C.
- The supernatant was decanted and the pellet was washed in 70 % ethanol.
- The pellet was then air dried and dissolved in 30 µl of sterile nuclease free water and stored in -20 °C for long term use.

The DNA obtained was electrophoresed in 0.8 % agarose gel as mentioned in 3.2.3 and gel was visualized in gel documentation unit.

3.2.5. LAMP primer designing

The LAMP primers were designed using Primer Explorer 5.0. **(**http://primerexplorer.jp/lampv5e/index.html). Three different targets in the viral genome were initially selected. The BBTV coat protein gene (GenBank Accession number GU125413.1), movement protein gene (GenBank accession number MF540875.1) and BBTV replicase gene (GenBank Acc. No. AY222303.1) were used. A pair of designed external primers (BBTVF3 and BBTVB3) and internal primers (BBTVFIP and BBTVBIP) were selected initially and its sequence information file was saved. This file was used to design a pair of loop primer (BBTVLF and BBTVLB). One set of designed primers were further validated using BLASTN analysis and synthesized with Sigma Aldrich Pvt. Ltd.

3.2.6. LAMP primer mix preparation

A primer mix was made using external, internal and loop primers. Primer mix was made to 50 µl stock containing 20 µM of FIP and BIP each, 10 µM of LF and LB each, 5.0 µM each of the primers BBTVF3 and BBTVB3. From this, 1 µl primer was taken for each LAMP reaction giving a final concentration of 0.2 µM each of BBTVF3 and B3 0.8 µM each of BBTVFIP and BBTVBIP and 0.4 µM each of the primers BBTVLF and BBTVLB.

3.2.7. Establishment of LAMP assay

Different concentrations of various reagents were tried to optimize the LAMP assay. The dNTPs at 1.4 mM and 1.6 mM each were tried. The LAMP reaction was set with $MgSO_4$ concentration ranging from 4mM to 8mM. Reactions were set with betaine concentration 0.8 M and 1 M and *Bst* DNA polymerase 8U and 4U. The reaction was incubated at different temperatures *viz* 55 °C, 60 °C, 65 °C and 68 °C for 60 minutes. The LAMP reaction was set in 0.2 ml tubes.

The final optimized reaction mixture contained 50 ng DNA template, 1.6 mM each dNTP, 0.2 µM each of BBTVF3 and BBTVB3, 0.8 µM each of BBTVFIP and BBTVBIP and 0.4 µM each of the primers BBTVLF and BBTVLB, 1.0 M betaine

(Sigma), 6 mM $MgSO_4$ (New England BioLabs), 4U *Bst* polymerase large fragment (New England BioLabs) and 1X Thermopol buffer with 2.0 mM $MgSO_4$. The HNB dye (Sigma) 150 µM and 120 µM where titrated against total $MgSO_4$ concentration at 6mM to 8 mM. Finally, 120 µM HNB was added in 25 µl reaction volume with total $MgSO_4$ concentration 8 mM. Molecular biology grade water (Sigma) was used to make up the volume to 25 µl. The reaction was incubated at 65°C for 60 minutes followed by enzyme inactivation at 80 °C for 20 minutes on a dry bath (NeuationiTherm D150-2). Along with each assay, a no template control (NTC) was also run.

Table 3.1. Reaction cocktail for LAMP

Components	Final concentration	Volume (µl)
DNA template	50 ng	2.0
Primer mix	BBTVF3/BBTVB3 - 0.2 µM, BBTVFIP/BBTVBIP- 0.8 µM, BBTVLF/BBTVLB - 0.4 µM	1.0
Bst polymerase	4 U	0.5
Thermopol Buffer (with 2 mM $MgSO_4$)	1X	2.5
Betaine	1 M	5.0
dNTP mix 10 mM each	1.6 mM each	4.5
$MgSO_4$	6 mM	1.5
HNB	120 µM	0.3
Molecular grade water		9.0
Total		25.0

3.2.8. Detection of LAMP products

The LAMP products were observed by electrophoresis on 2.0 % agarose gel stained with Ethidium bromide. Colorimetric detection of LAMP positives was done by adding HNB. The HNB dye (Sigma) was added at a concentration of 120 µM to the LAMP reaction mixture prior to the amplification. Then colour change was observed after the reaction.

3.2.9. Confirmation of specificity of LAMP reaction

3.2.9.1. Sequencing confirmation

A PCR reaction was carried out using LAMP external primers, BBTVF3 and BBTVB3.

Materials used

- DNA sample
- Forward primer BBTVF3
- Reverse primer BBTVB3
- *Taq* DNA polymerase
- 10 X reaction buffer A (with $MgCl_2$)
- 2.5 mM each dNTP mix
- PCR grade water

Procedure

The reaction mixture for PCR reaction was prepared as mentioned below. The reagents were properly mixed. The DNA samples were added separately and aliquots of PCR reaction mixture were added to 0.2 ml PCR tubes and reaction mix was gently spun for proper mixing. Along with each assay a NTC was also run.

Table 3.2. Reaction cocktail for PCR

Components	Volume (µl)
DNA template (50 ng)	2.0
Forward primer (BBTVF3)	1.0
Reverse primer (BBTVB3)	1.0
dNTP mix (2.5 mM each)	1.0
Taq polymerase	0.5
10 X Reaction buffer A (with 1.5 Mm $MgCl_2$)	1.5
Molecular grade water	8.0
Total	15.0

The PCR reaction was carried out in thermocycler along with a no template control under the thermal profile given in Table 3.3.

Table 3.3. Thermal profile for PCR amplification

Steps	Temperature	Time	No. of cycles
Initial denaturation	95 °C	1 minute	1
Denaturation	95 °C	30 seconds	35
Annealing	55 °C	30 seconds	35
Elongation	72 °C	1 minute	35
Final elongation	72 °C	8 minutes	1

The PCR product was observed on 1 % agarose gel. The amplified band of expected size was cut out from gel and eluted using Nucleospin gel elution kit (Macherey Nagel).

3.2.9.1.2. Gel elution

Gel elution was done using Nucleospin gel and PCR clean up kit of Macherey Nagel.

Materials required

- NT1 buffer (provided with the kit)
- NT3 solution with ethanol (provided with the kit)
- Nuclease free water

Procedure

1. Gel slice was solubilized by adding 200 µl of NT1 for each 100 mg of agarose gel and incubated for 10 minutes at 50 °C. Sample was vortexed briefly every 2 minutes until gel slice was completely dissolved.
2. Sample was loaded in to a gel clean up column placed on a collection tube of 2 ml.
3. The sample was then centrifuged for 30 seconds at 11000 g.
4. The flow through in collection tube was discarded and the column was placed back into collection tube.
5. The NT3 buffer 700µl was added to column and centrifuged for 30 seconds at 11000 g. The flow through was discarded. This step was repeated to minimize salt carry over.
6. The tube was spun at 11000 g for 1 minute to remove NT3 completely.
7. The column was incubated for 5 minutes at 70 °C on heat block.
8. The column was then placed in a 1.5 ml tube and 15 µl nuclease free water was added to it.
9. This was centrifuged for 1 minute at 11000 g. Again, 10 µl nuclease free water was added centrifuged for 1 minute at 11000 g.
10. The eluted DNA sample was stored at -20 °C.

3.2.9.1.3. Sequencing and sequence analysis

The sequencing of eluted DNA sample using BBTVF3 primer was carried out at Agri Genome Labs Pvt. Ltd., Kochi. The sequences obtained were analyzed by similarity search using the local alignment search algorithm, BLASTN (Altschul *et al*., 1997).

3.2.9.2. Restriction digestion

The LAMP products were cut with a restriction enzyme having cut site in the particular LAMP product. Enzyme specific to the sequence between F2 – B2 region was identified using NEBcutter version 2.0.**(**http://nc2.neb.com/NEBcutter2/) software. The LAMP product was digested with restriction enzyme *Sau*3AI (Thermo Fischer Scientific). The restriction digestion reaction was set as follows in a 0.2 ml tube:

- *Sau*3AI restriction enzyme - 2 µl
- 10 x buffer – 5 µl
- LAMP product - 10 µl
- Water – 33 µl

The reaction tubes containing final volume 50 µl were incubated at 37 °C on a heat block (NeuationiTherm D150-2) for 8 hours followed by enzyme inactivation at 65 °C for 20 minutes. Restriction fragments were analyzed by electrophoresis on 2.5 % agarose gel.

3.2.10. Validation of LAMP assay

The developed LAMP assay was tested with 15 BBTD symptomatic field collected banana samples. The LAMP assay was also validated with DNA samples from banana plants showing symptoms of BSV, CMV and BBrMV. Healthy tissue culture raised banana plants and field collected symptomless banana plants were also tested using the developed assay. The colour change in each reaction was observed. The amplified products were visualized on 2 % agarose gel.

3.2.11. Comparison of sensitivity of LAMP assay with conventional PCR in the detection of BBTV

The total DNA from all the 15 symptomatic leaf samples was used for PCR based detection using coat protein gene sequence specific primers.

3.2.11.1. Primer designing

The BBTV Kerala isolate coat protein gene sequence was retrieved from NCBI (GenBank accession number GU125413.1). This sequence was used as input in the Primer 3 software, version 0.4.0 (https://bioinfo.ut.ee/primer3-0.4.0/) for PCR primer designing. The primers designed were validated using BLASTN and then synthesized with Sigma Aldrich Pvt. Ltd.

Table 3.4. Primers for PCR amplification of BBTV coat protein gene

Orientation	Name of primer	sequence (5' to 3')	No. of bases	Product size
Forward	BBTVF1	GGTATCCGAAGAAATCCATCAA	22	416 bp
Reverse	BBTVR1	ATTCTTCCTCAACACGGTTGTC	22	

3.2.11.2. PCR reaction

Materials required

- DNA sample
- Forward primer
- Reverse primer
- *Taq* DNA polymerase
- 10 X reaction buffer A (with $MgCl_2$)
- 2.5 mM each dNTP mix
- PCR grade water

Procedure

The reaction mixture for PCR reaction was prepared as mentioned in table 3.5. The reagents were properly mixed. The DNA samples were added separately and aliquots of PCR reaction mix were added to 0.2 ml PCR tubes and reaction mix was gently spun for proper mixing. All the fifteen BBTV symptomatic banana samples were tested using PCR along with healthy sample and NTC. The PCR product was electrophoresis on 1 % agarose gel and documented.

Table 3.5. Preparation of reaction mixture for PCR

Components	Volume (µl)
DNA template (50 ng)	2.0
Forward primer (BBTVF1)	1.0
Reverse primer (BBTVR1)	1.0
dNTP mix (2.5 mM each)	1.0
Taq DNA polymerase	0.5
10 X Reaction buffer A (with 1.5 Mm $MgCl_2$)	1.5
Molecular grade water	8.0
Total	15.0

The PCR reaction was carried out with the thermal profile as mentioned in Table 3.3. The PCR product was observed on 1 % agarose gel. A 100 bp ladder was also run to monitor the amplification. Electrophoresis was done at 80 V till the loading dye reached more than half of the gel. The gel was documented using gel documentation unit of BioRad.

3.2.11.3. Confirmation of the sequence

The amplified bands of expected size were eluted as mentioned in 3.2.9.1.2. for a representative sample. Sequencing was done using BBTVF1 and BBTVR1 primers at AgriGenome Labs Pvt. Ltd. The forward and reverse sequences obtained after sequencing were combined to contig using Cap3 software. The sequence was analyzed using NCBI BLASTN tool.

RESULTS

4. RESULTS

The research work entitled “Detection of *Banana bunchy top virus* (BBTV) through colorimetric closed tube Loop Mediated Isothermal Amplification (LAMP) assay” was carried out at the Center for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agriculture University, Thrissur, from 2019 to 2021. The results of the study are presented in this chapter.

4.1. Total genomic DNA isolation

The leaf samples were collected from 15 banana plants showing BBTV symptoms (Kannara 1 to 15), two field collected healthy banana plants (H1, H2) and one tissue culture raised healthy banana plant (H3) (Plate 4.1). The total genomic DNA was isolated from leaf samples as mentioned in 3.2.1. The genomic DNA isolated was analyzed for quality and quantity.

4.1.1. Quality and quantity analysis of isolated total DNA

The total genomic DNA upon electrophoresis on 0.8 % agarose gel in 1X TAE buffer showed intact bands of DNA (Plate 4.2). However, RNA contamination was observed. So, RNase treatment was done as mentioned in 3.2.2 and samples after treatment had no RNA contamination (Plate 4.3). The total DNA was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA) by determining absorbance at 260 nm and the purity of DNA was checked by measuring the absorbance at 260 nm and 280 nm wavelength. The quality of isolated DNA was good with $OD_{260/280}$ ranging between 1.8 to 2.26. The yield of DNA was high, between 290 to 1250 ng/µl (Table 4.1).

4.2. LAMP primer designing

The coat protein gene, movement protein gene and replicase gene were used for primer designing, but loop primers were not obtained for movement protein gene and replicase gene sequences (Fig. 4.1.,4.2.,4.3.,4.4.,4.5.,4.6.). Hence, a set of six

LAMP primers compliment to eight regions on the coat protein gene of BBTV were designed (Fig. 4.7., Table 4.2.).

Table 4.1. Quantitative analysis of total DNA isolated

Sample	Variety	A 260/280	Conc. ng/µl
Kannara1	Robusta	1.90	286.29
Kannara 2	Robusta	1.94	361.40
Kannara 3	Nendran	1.91	965.64
Kannara 4	Nendran	2.20	940.0
Kannara 5	Kadali	2.19	513.0
Kannara 6	Kadali	2.26	1064.0
Kannara 7	Palayankodan	2.00	309.0
Kannara 8	Poovan	1.80	894.0
Kannara 9	NjaliPoovan	2.10	723.42
Kannara 10	Nendran	1.96	1256.0
Kannara 11	Nendran	1.93	925.84
Kannara 12	Nendran	2.22	847.5
Kannara 13	Nendran	2.00	737.0
Kannara 14	Kadali	2.20	1000.0
Kannara 15	Kadali	1.92	546.0
H1	Nendran	1.81	390.0
H2	Nendran	1.89	679.0
H3	Nendran	1.80	600.0
CMV	Nendran	2.00	806.0
BSV	Nendran	1.90	700.87
BBrMV	Nendran	1.93	760.0

(a)

(b)

(c)

(d)

(e)

Plate 4.1. Banana plants showing symptoms of BBTD

(f)

(g)

(h)

(i)

Plate 4.1. Banana plants showing symptoms of BBTD

a to d – Banana plants showing bunchy top appearance

e – Banana leaf showing Morse code pattern

f, g – plants with marginal necrosis

h, i – plants with yellowing of leaf

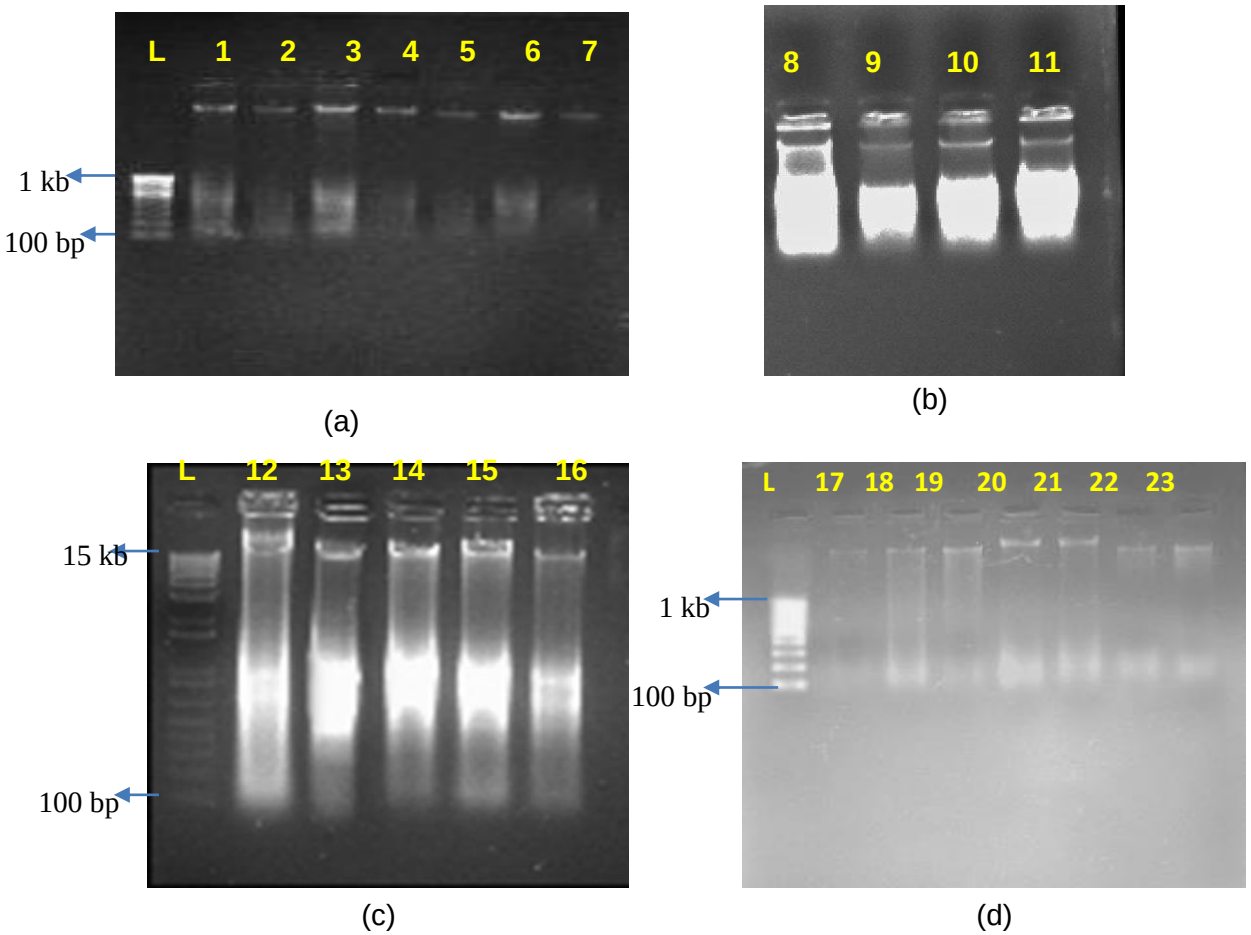


Plate 4.2. Quality analysis of DNA isolation

L- Ladder

1 to 16 – Kannara 1 to 15

17– BSV sysmptomatic banana

18– CMV sysmptomatic banana

19–BBrMV sysmptomatic banana

20,21 – H3

22, 23 – H1, H2

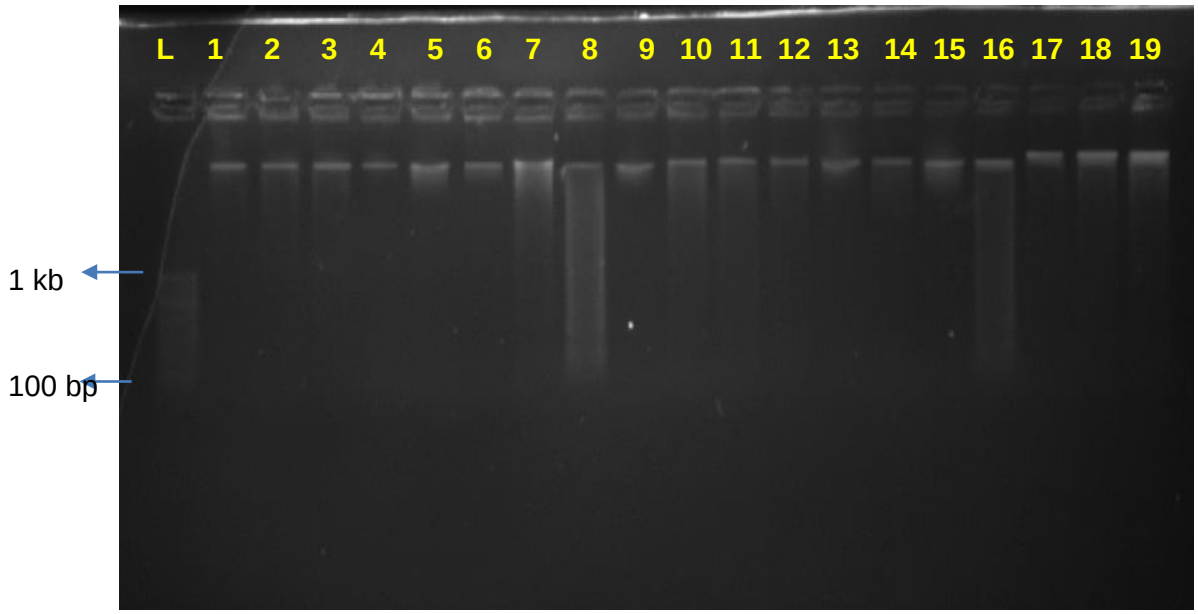


Plate 4.3. Agarose gel profile of DNA after RNase treatment

L – Ladder (Invitrogen 100 bp step up)

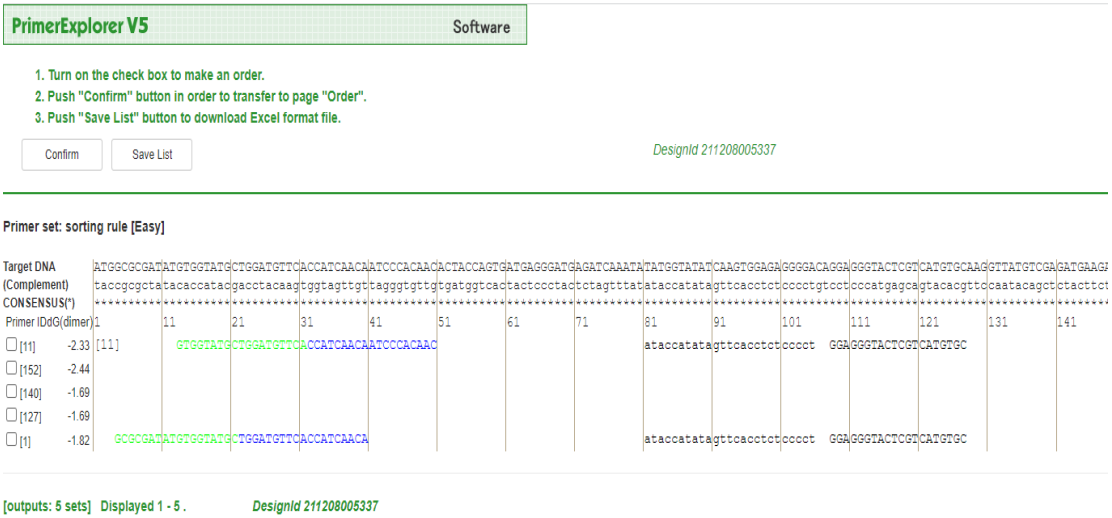
Lanes1 to 15 – Kannara 1 to 15

Lane 16 - BSV symptomatic sample

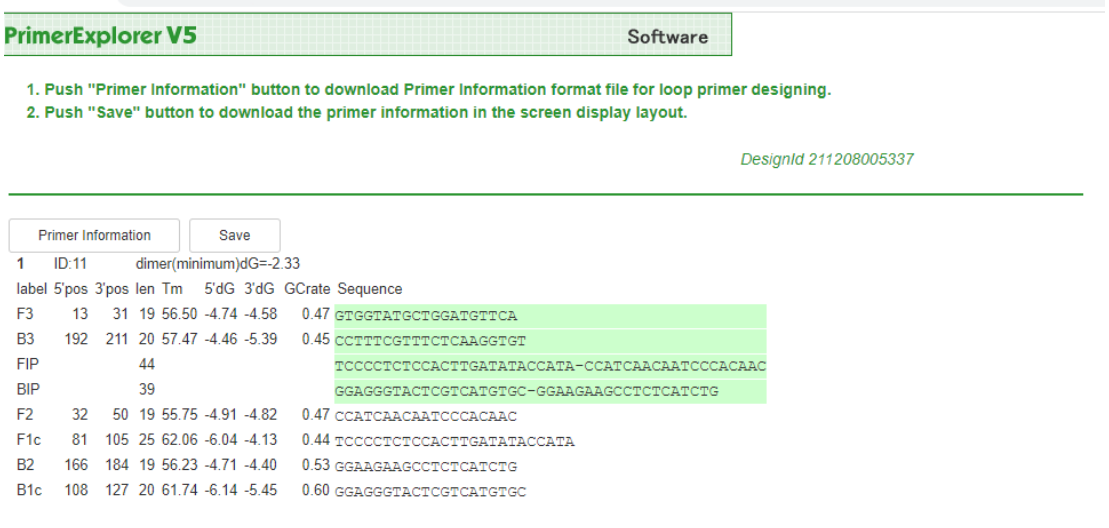
Lane 17 – CMV symptomatic sample

Lane 18 – BBrMV symptomatic sample

Lane 19 – H1



(a)



(b)

Fig 4.1. Primer explorer version 5.0. window displaying output for LAMP primers targeting replicase gene of BBTV

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

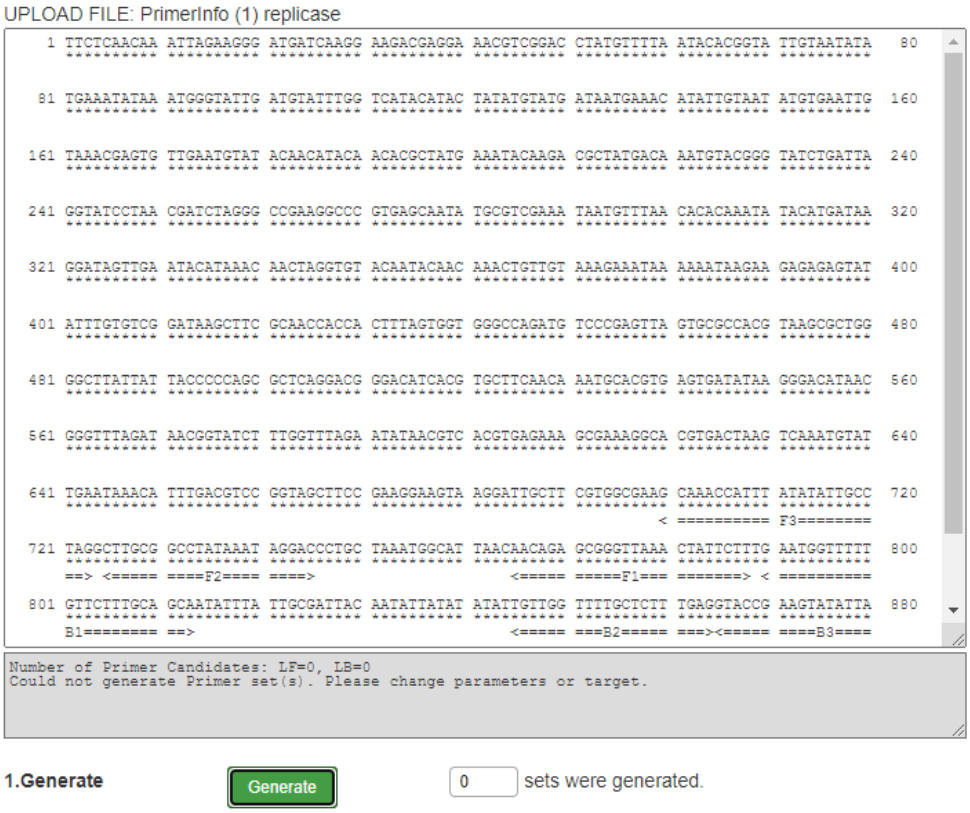
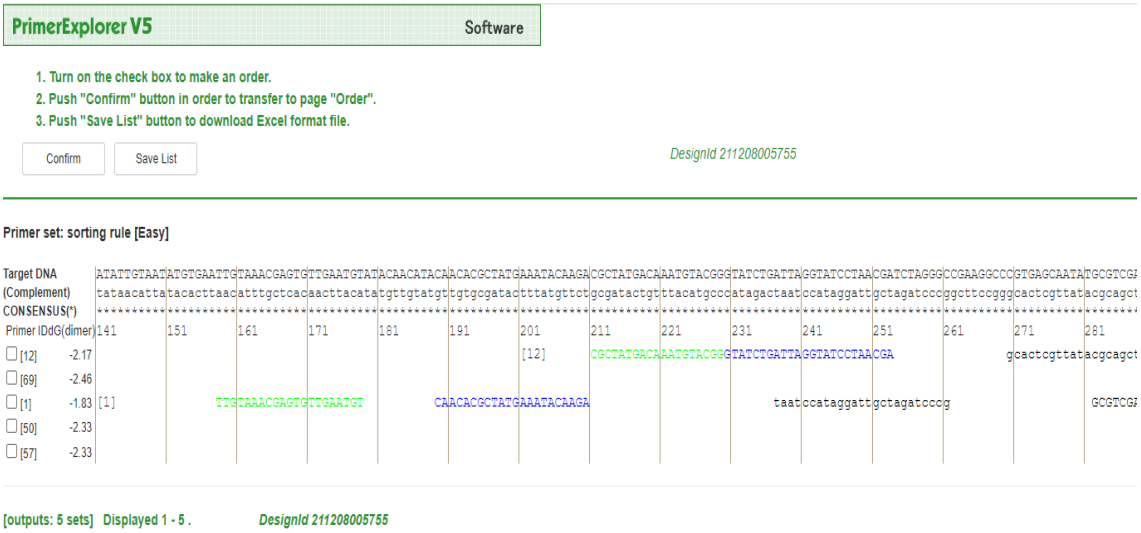
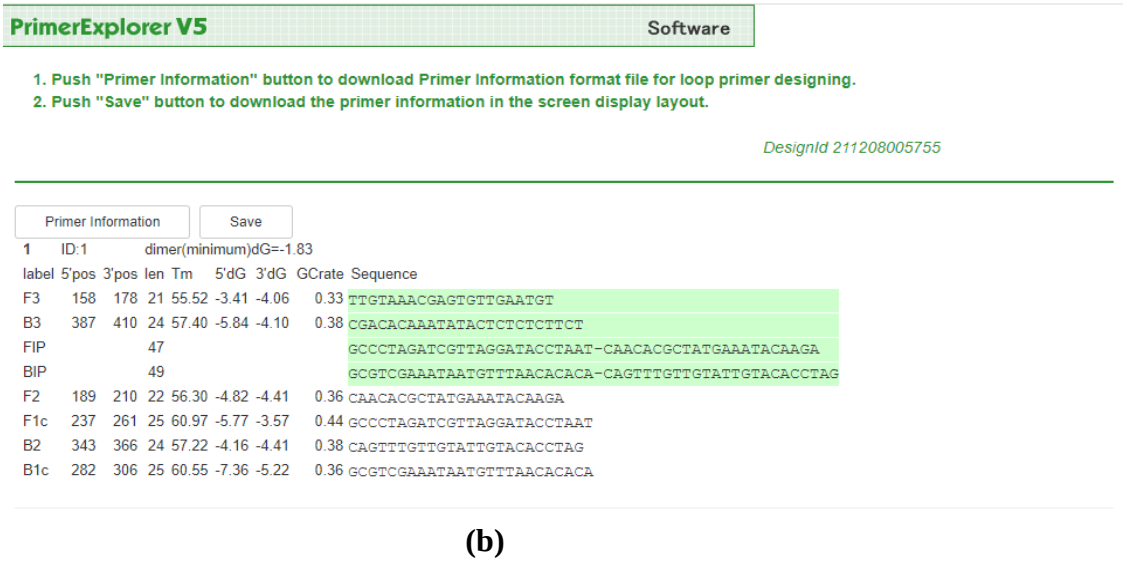


Fig. 4.2. Primer explorer version 5.0. window displaying output for loop primers targeting replicase gene of BBTV

No loop primers obtained for replicase gene



(a)



(b)

Fig 4.3. Primer explorer version 5.0. window displaying output for LAMP primers targeting movement protein gene of BBTV

(a) Regular primers specific to movement protein gene

(b) Regular primer set selected

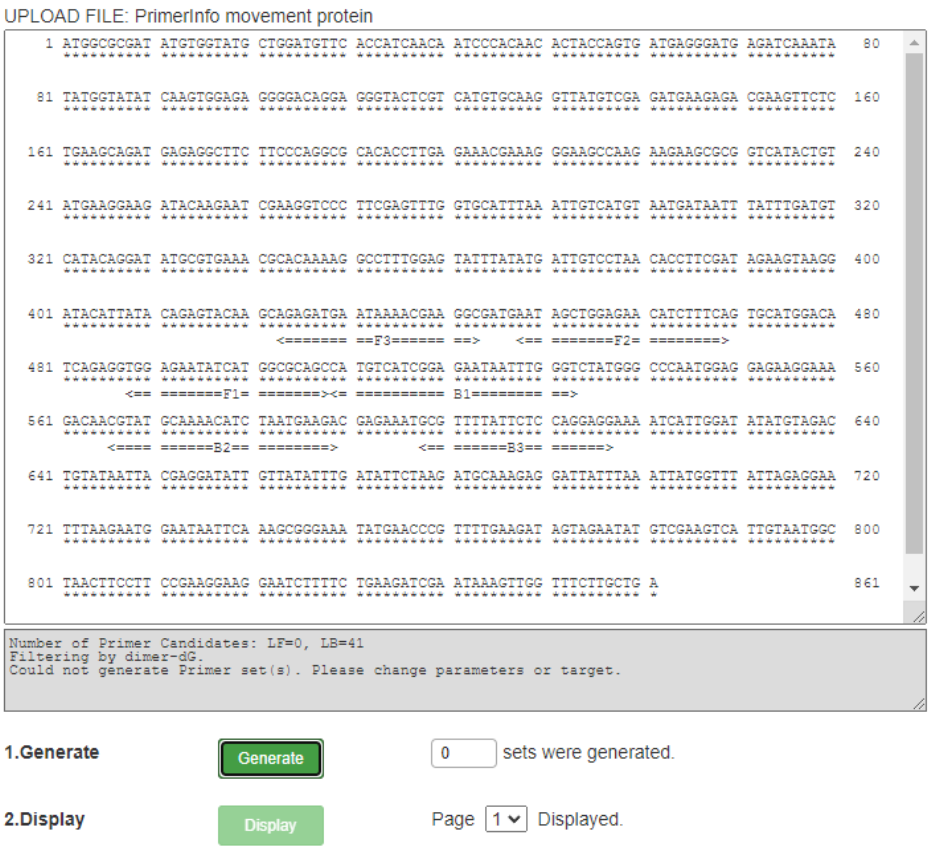
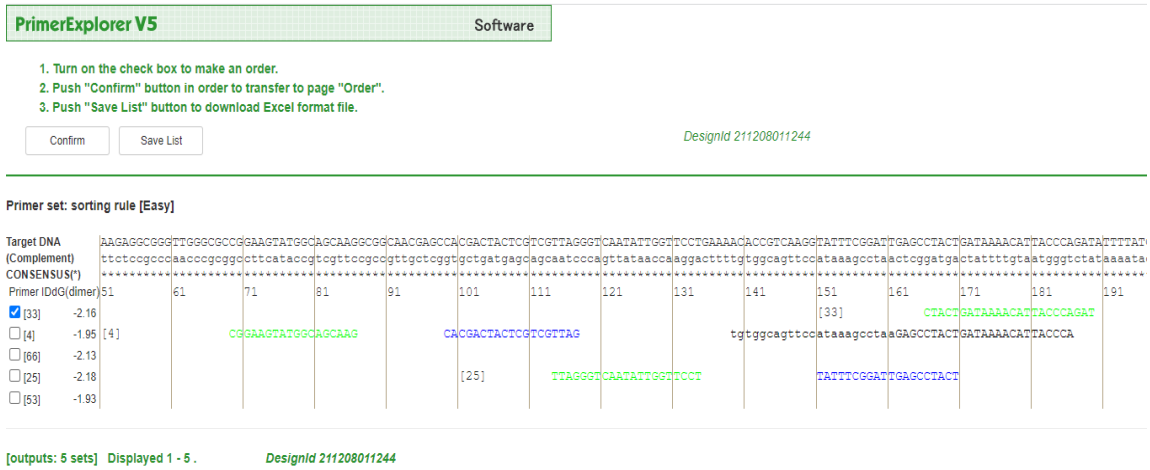
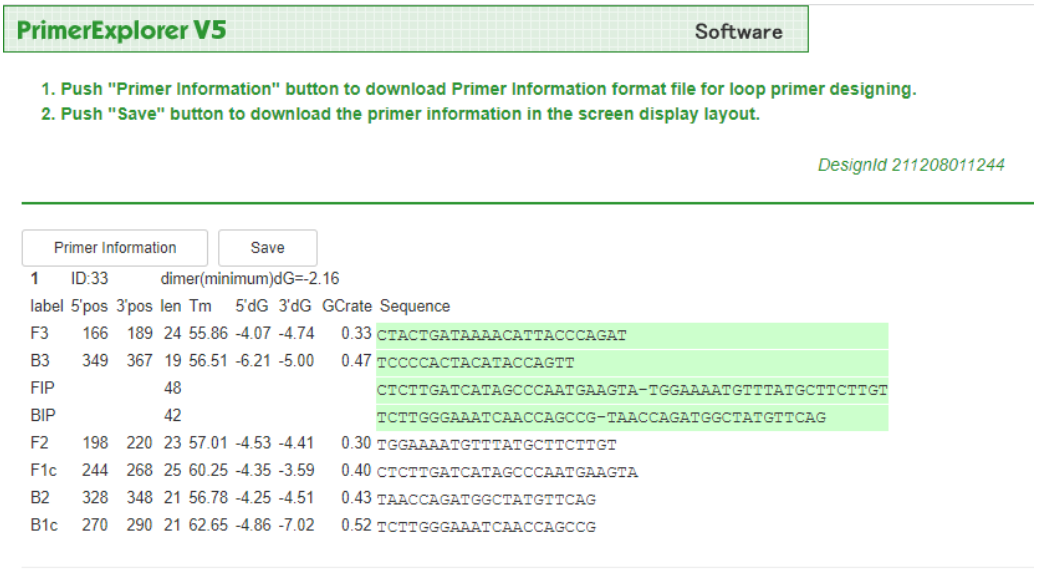


Fig 4.4. Primer explorer version 5.0. window displaying output for LAMP loop primers targeting movement protein gene of BBTV

No loop primers obtained for movement protein gene



(a)



(b)

Fig 4.5. Primer explorer version 5.0. window displaying output for LAMP primers targeting coat protein gene of BBTV

(a) Regular primers specific to coat protein gene

(b) Regular primer set selected

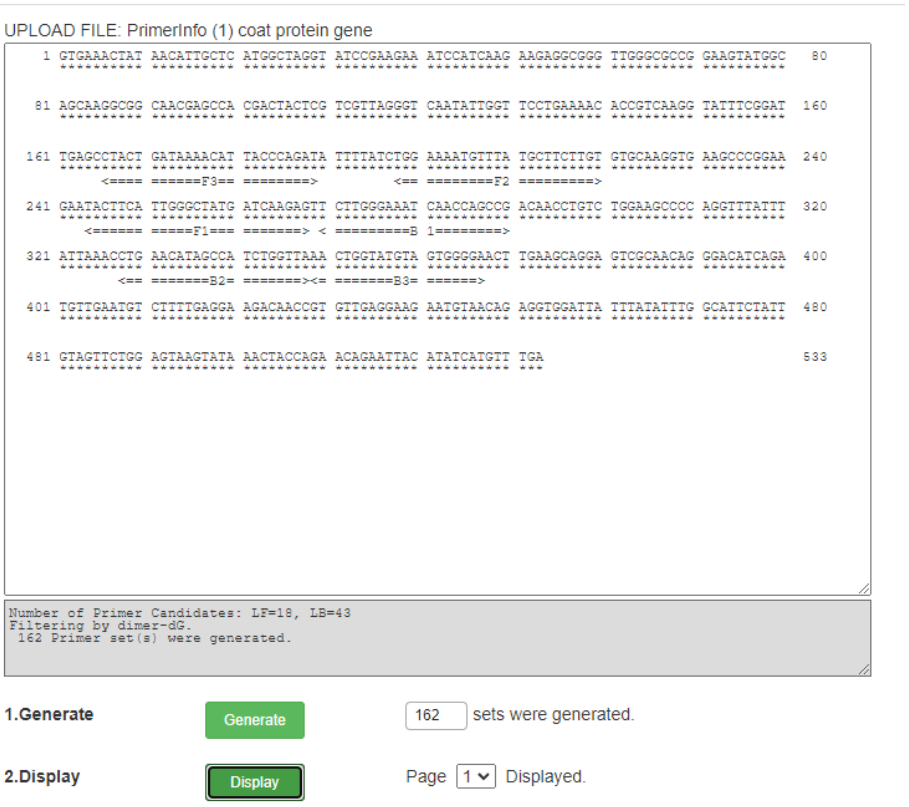


Fig. 4.6. Primer explorer version 5.0. window displaying output for LAMP loop primers targeting coat protein gene of BBTV

Loop primers obtained for coat protein gene

Table 4.1. Primers for LAMP reaction

Orientation	Name of primer	Sequence (5' to 3')	Position
Forward	BBTVF3	CTACTGATAAAACATTACCCAG AT	24
Reverse	BBTVB3	TCCCCACTACATACCAGTT	19
Forward	BBTVFIP (F1c -F2)	CTCTTGATCATAGCCCAATGAA GTA-TGGAAAATGTTTATGCTTCTTGT	48
Reverse	BBTVBIP (B1c -B2)	TCTTGGGAAATCAACCAGCCG-TAACCAGATGGCTATGTTCAG	42
Forward	BBTVLF	CGGGCTTCACCTTGCAC	17
Reverse	BBTVLB	TCTGGAAGCCCCAGGTTT	18

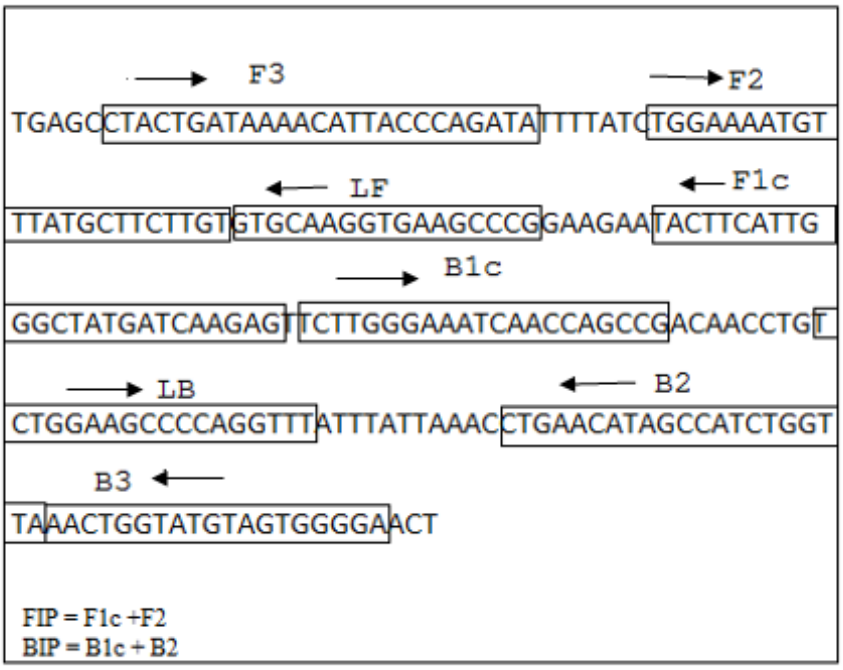


Fig. 4.7. Position of LAMP primers on the BBTV coat protein gene sequence (GenBank Acc. No. GU125413.1)

4.3. Establishment of LAMP assay

The LAMP primers amplified the coat protein gene from BBTD positive samples. The final optimized reaction mixture containing 50 ng DNA template, 1.6 mM each dNTP, 0.2 µM each of BBTVF3 and BBTVB3, 0.8 µM each BBTVFIP and BBTVBIP and 0.4 µM each of the primers BBTVLF and BBTVLB, 6 mM $MgSO_4$ (New England BioLabs), 1X Thermopol buffer with 2 mM $MgSO_4$, 4U *Bst* polymerase large fragment (New England BioLabs) and 1M betaine (Sigma) in 25 µl reaction volume gave positive amplification in symptomatic samples. No amplification was observed in the tissue culture raised healthy banana sample and NTC. Also, no amplification was observed in DNA samples of symptomless plants collected from the same field. The positive LAMP amplicons upon electrophoresis on 2 % agarose gel produced a series of ladder like bands which represents the stem-loop structure of DNA with inverted repeats of the target sequence formed after LAMP amplification (Plate 4.4.).

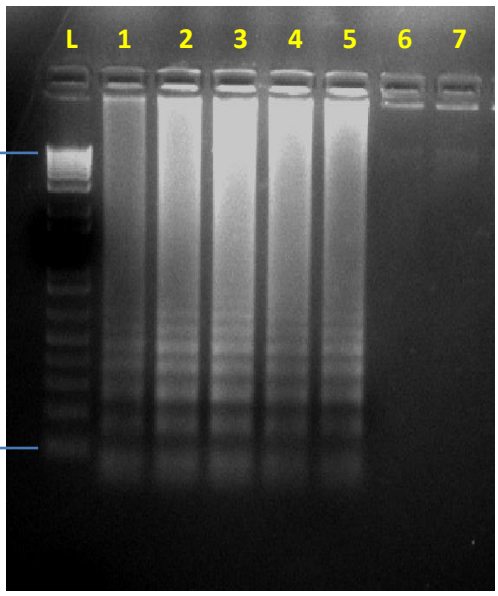
4.4. Colorimetric detection of LAMP positives

The LAMP reaction mix upon addition of 120 µM HNB appeared violet in colour prior to amplification with 8mM total $MgSO_4$ concentration. The positive LAMP products after reaction showed colour change from violet to sky blue (Plate 4.5.). The NTC and asymptomatic samples remained violet itself. When 150 µM HNB was added, no colour change observed in amplified samples.

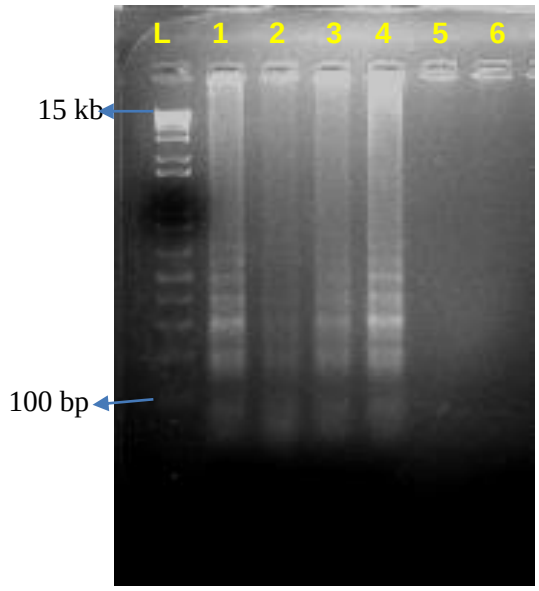
4.5. Molecular identification of LAMP products

4.5.1. Sequencing

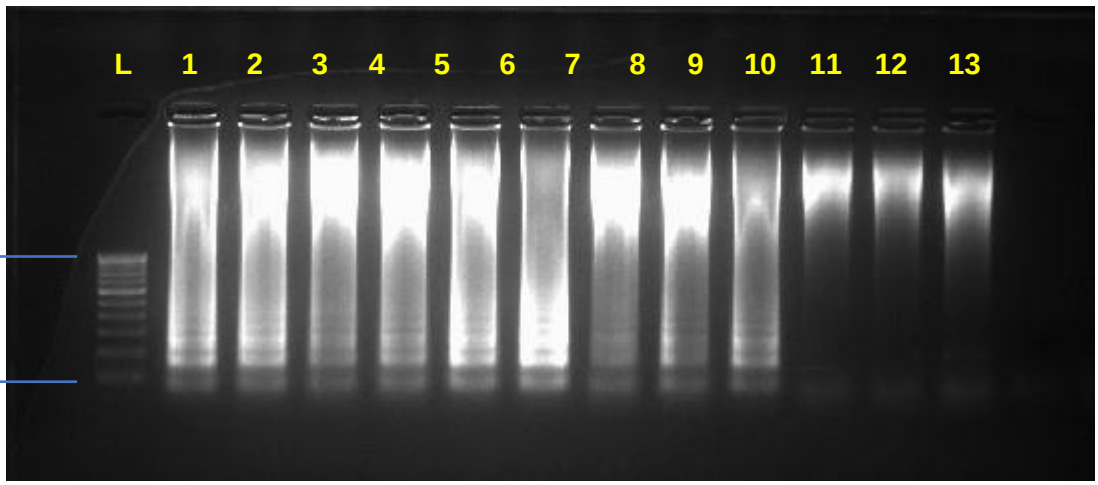
The amplification of diseased samples through conventional PCR using the LAMP external primers, BBTVF3 and BBTVB3, produced bands of expected size (202 bp) upon electrophoresis in 1 % agarose gel (Plate 4.6.). The healthy sample and NTC produced no amplification. Sequencing of amplicon and its BLASTN analysis showed that the sequences had 100 % identity with BBTV isolate KOL1 coat protein gene (GenBank Accession number MT174330.1) (Fig. 4.8.). Representative sequence was deposited in GenBank database (GenBank Accession No. OL757511).



(a)



(b)



(c)

Plate 4.4. Agarose gel profile of LAMP amplicons

(a) L – 1 kb plus ladder (Invitrogen);

Lanes 1 to 5 – Kannara 1 to 5; Lane 6 H3; 7 – NTC

(b) L- 1 kb plus DNA Ladder (Invitrogen);

Lanes 1 to 4 – Kannara 1,6,7,8; Lane 5 – H3; 6- NTC

(c) L- 100 bp Ladder (Genei); 1 to 9 – Kannara 7 to 15;

Lanes 10, 11, 12- H1, H2, H3; Lane 13 – NTC

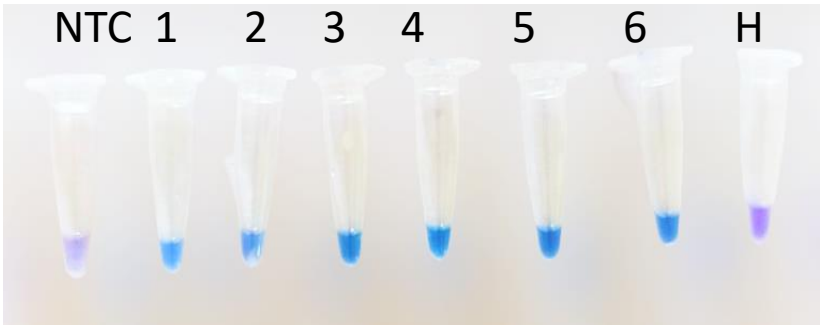
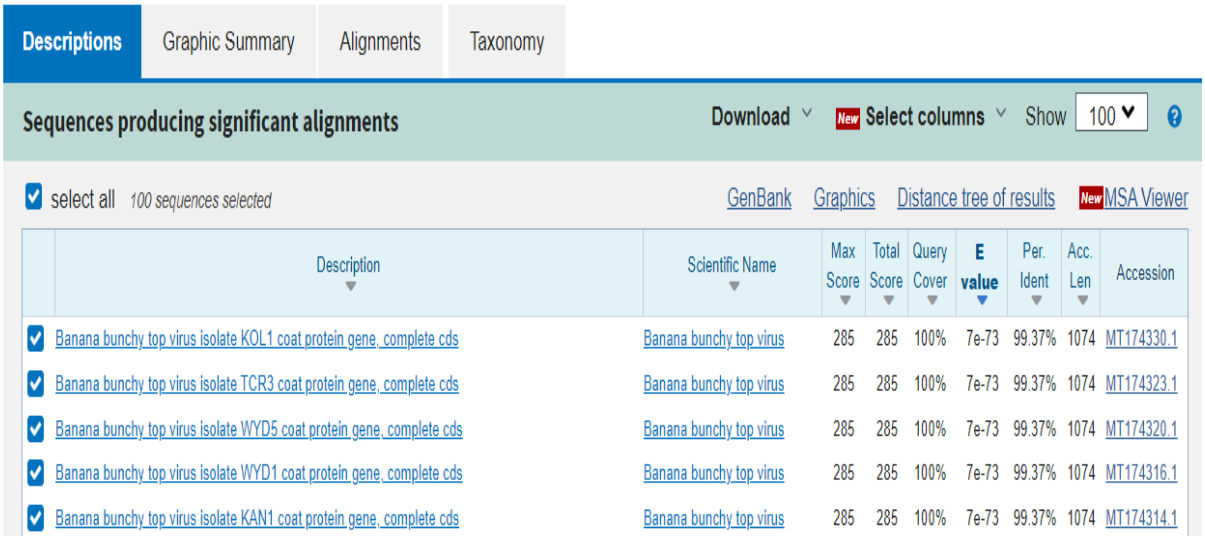


Plate 4.5. Colorimetric detection of LAMP products

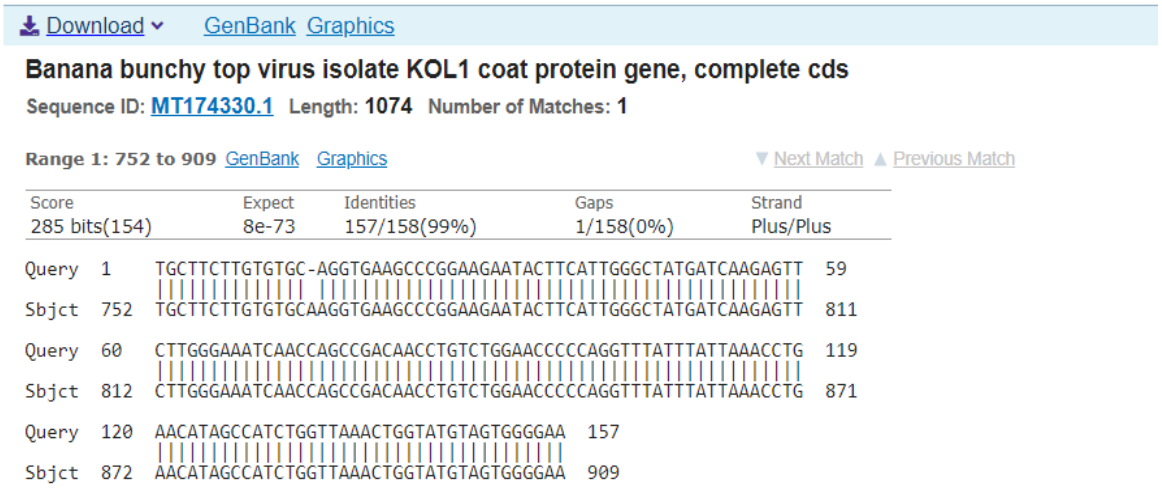
1 to 6 –Kannara 1 to 6

H – H1

NTC – No template control



(a)



(b)

Fig. 4.8. BLASTN analysis of sequenced product

(a) BLASTN result window

(b)Alignment of the sequence with BBTV isolate KOL1 coat protein gene

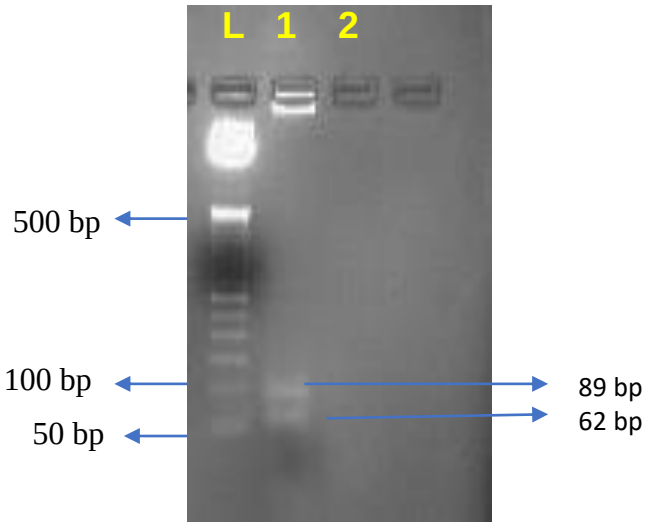


Plate 4.6. Restriction profile of LAMP amplicon

L – 50 bp Ladder (Genei); 1 –LAMP product; 2 – NTC

Bands of 62 bp and 89 bp were obtained

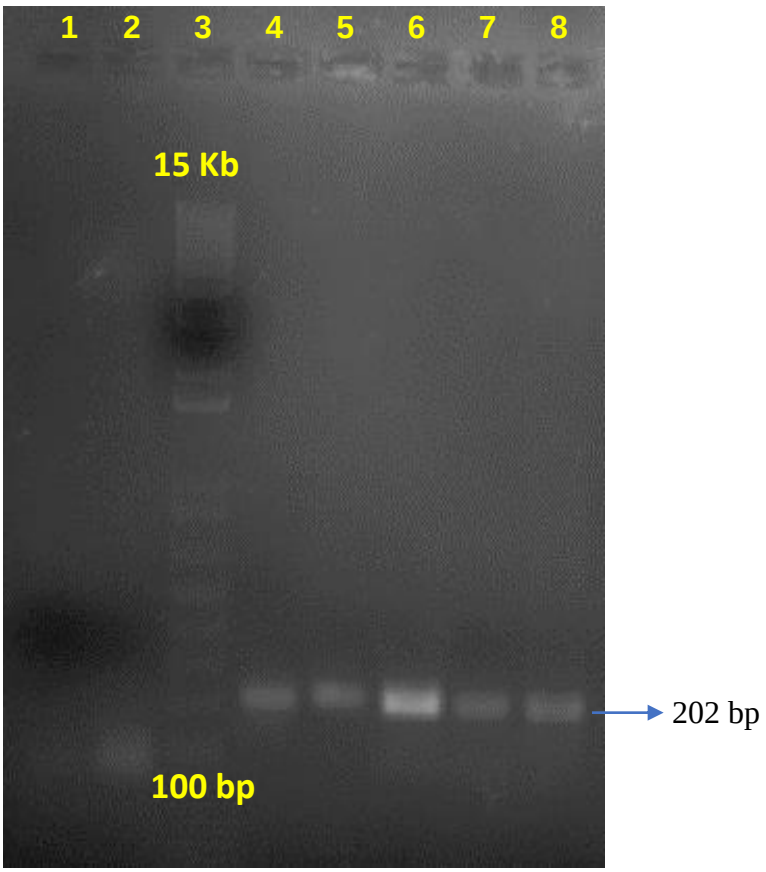


Plate 4.7. PCR amplified bands using LAMP external primers

Lane 1- NTC; Lane 2- H3; Lane 3- 1 kb plus Ladder (Invitrogen)

Lane 4 to 8 – Kannara 1 to 5

4.5.2. Restriction digestion

The restriction enzyme *Sau*3AI, specifically cutting LAMP amplicon between F2 and B2 region was identified using NEB cutter software. The enzyme has single cut site generating two fragments of size 62 bp and 89 bp theoretically. The restriction digestion of LAMP amplicons produced fragments of sizes as expected for the diseased samples as observed by electrophoresis on 2.5 % agarose gel (Plate 4.7.). Hence, we could confirm the accuracy of LAMP based detection of BBTD.

4.6. Validation of LAMP assay

The developed LAMP assay was validated with 15 BBTV symptomatic, 3 healthy banana simples and banana leaf samples plants showing BBrMV, BSV and CMV symptoms. All the 15 symptomatic samples showed positive amplification. No amplification was observed for these samples (Plate 4.8.). These samples did not show any colour change with HNB (Plate 4.9.). Thus, the specificity of LAMP method for detection of BBTV was confirmed.

4.7. Comparison of LAMP assay with PCR for BBTV detection

The PCR reaction was performed using forward BBTVF1 and reverse BBTVR1 primers. The designed PCR primers amplified 416 bp region of coat protein gene of BBTV (Plate 4.10.). Healthy sample and NTC did not shown amplification. Sequencing followed by BLASTN analysis gave 100 % identity with BBTV isolate KOL1 coat protein gene (GenBank Accession number MT174330) (Fig. 4.9.).

Among the 15 diseased samples tested, only 13 samples showed positive amplification in the PCR based detection, while all the symptomatic samples had amplified in the LAMP assay (Plate 4.9.). The reaction time for PCR was 1 hour 38 min for amplification and another 1 hour for gel electrophoresis; while LAMP assay took only 1 hour for amplification and detection. The colorimetric detection in LAMP reduced the time for detection and there was no need for post amplification sample handling.

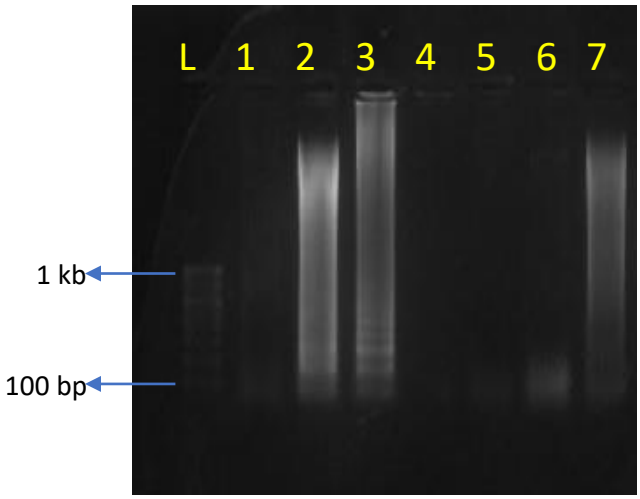


Plate 4. 8. Validation of LAMP assay- Agarose gel profile of amplicons

L – 100 bp plus ladder (GeNei); 1- NTC;
Lanes 2 and 3 – Kannara 1, 2; Lane 4 – BSV;
Lane 5 -CMV; Lane 6 – BBrMV; Lane 7 – H1

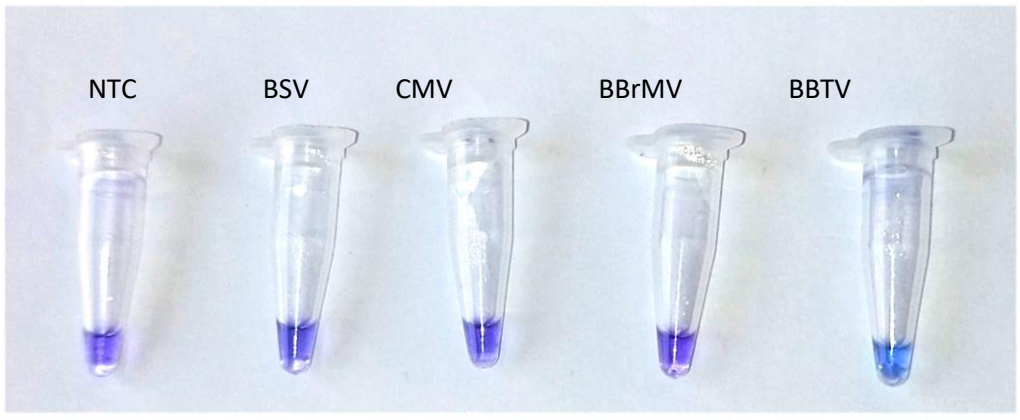


Plate 4.9. Validation of LAMP assay- Clorimetric detection with HNB
Positive sample shows colour change from violete to blue

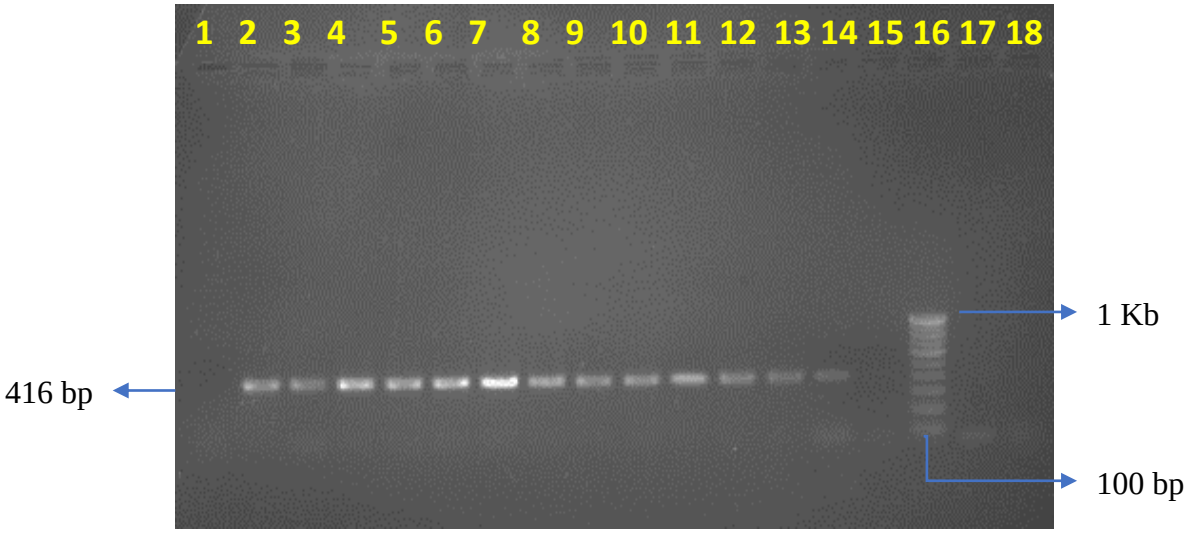
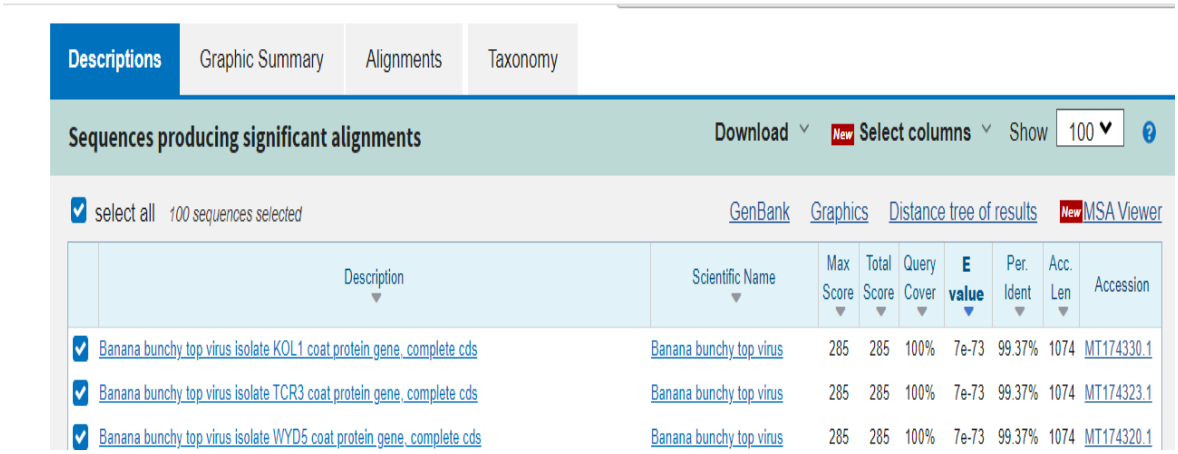


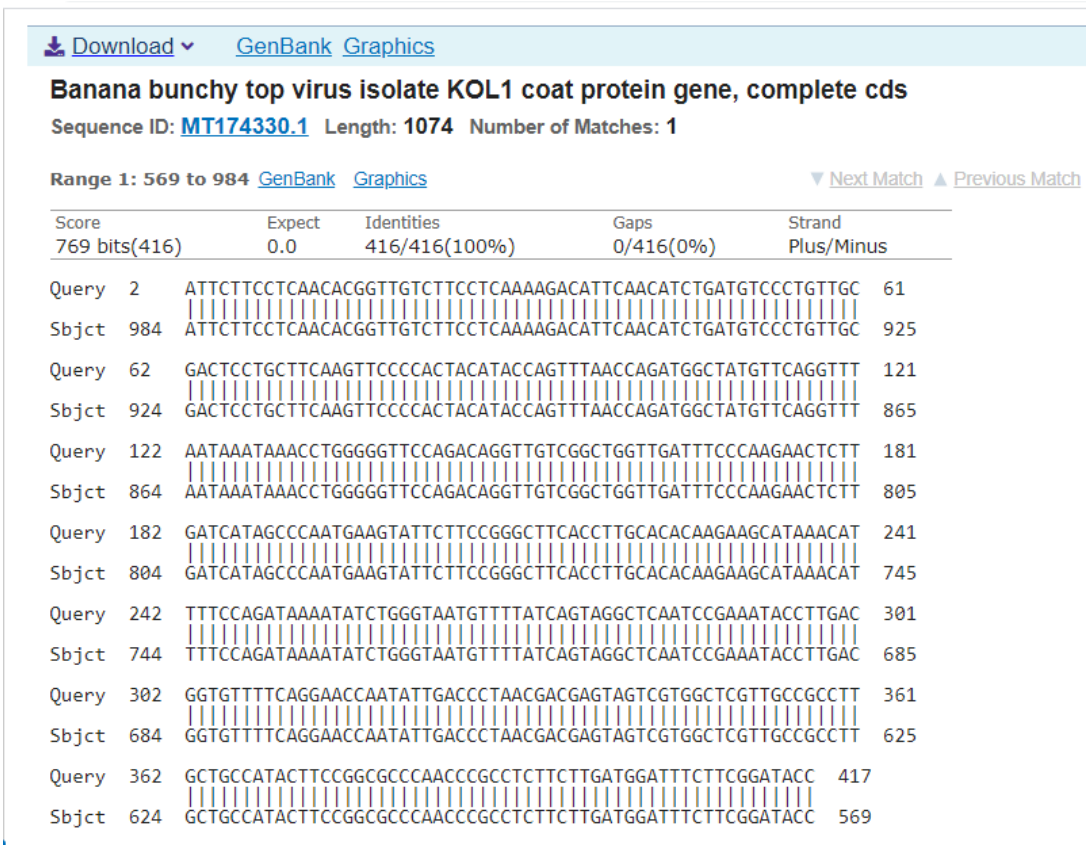
Plate 4.10. PCR amplification of coat protein gene fragment of BBTV

1 to 15 – PCR products of symptomatic samples (Kannara 1 to 5); 16 – 100 bp Ladder (Genie); 17- H3; 18 – NTC

Out of 15 tested, 13 samples shown positive amplification. Samples Kannara 1 and Kannara 15 did not amplify



(a)



(b)

Fig. 4.9. BLASTN analysis result of the PCR amplicon after sequencing

(a) BLASTN result window

(b) Alignment of the sequence with BBTV isolate KOL1 coat protein gene

DISCUSSION

5. DISCUSSION

The study entitled "Detection of *Banana bunchy top virus* through colorimetric closed tube Loop Mediated Isothermal Amplification (LAMP)" was carried out at Center for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agriculture University, Thrissur, from 2019 to 2021. The results obtained in the study are discussed in this chapter.

Banana is an important food and cash crop worldwide and it is the second largest fruit crop grown in India. Diseases are the major threat to banana production and viral diseases are considered to be most destructive as they can cause complete yield loss (Amin *et al.,* 2008*)*. The BBTV is considered to be the most economically destructive among the viruses infecting banana as it can contribute up to 100 % yield reduction (Dale, 1987). A loss of ₹40 million annually has been reported in Kerala alone due to BBTV (Selvarajan and Balasubrhamanium, 2014).

The first visible symptom of disease is the development of morse code pattern in the leaf veins, midribs, and petioles and marginal chlorosis (Thomas and Caruana, 2000). As the disease develops, the leaves become upright and bunched at the apex of the plant. Infection at young stage results in stunted growth and no fruit production (Dale, 1987).

The BBTV is a multi- component circular ssDNA virus belonging to the family Nanoviridae, genus *Babuvirus*. Primary transmission of disease is through infected planting material, and the vector for this virus is the banana aphid, *Pentalonia nigronervosa*. Banana is primarily propagated by suckers which does not have any quality check by farmers for large scale cultivation. Now tissue culture techniques are being used increasingly to get planting materials free of infections. Virus indexing of suckers and tissue culture plantlets is essential to assure that infection free planting materials reach farmers. In the absence of resistant varieties, a sensitive and rapid virus indexing is necessary for proper disease management (Dale, 1987; Harding *et al.,* 2000).

The ELISA being employed as common detection method, DAS-ELISA and TAS-ELISA have been developed for routine detection of BBTV (Othman *et al.*,1996; Geering and Thomas, 1996). The DAC-ELISA method using polyclonal antiserum is reported to be more sensitive than DIBA and satisfactorily detected BBTV in symptomless samples (Selvarajan *et al.,* 2002; Wanitchakorn *et al.,* 1997). However, ELISA is less sensitive than molecular detection methods and is time and labour consuming.

The BBTV is a phloem limited virus occurring in small concentration in infected plants and PCR for detecting BBTV in symptomatic banana samples paved way for virus indexing in plants produced through tissue culture (Galal, 2007). But the contaminants present in banana plant DNA preparations often interfere with PCR reaction affecting its sensitivity (Das *et al.,* 2009). The PCR requires thermal cycling instrument and post amplification sample handling which often predisposes to sample cross contamination. High throughput detection of BBTV has been developed using real time TaqMan PCR (Chen and Hu, 2013). All these techniques are costly, cumbersome, tedious and require well equipped laboratories and highly trained personnel.

The LAMP enables rapid, sensitive and specific amplification of template DNA under isothermal condition using a strand displacing polymerase like *Bst* DNA polymerase producing 10^9 copies of the target in less than 1 h (Notomi *et al.,* 2000). This technique is highly specific as four specially designed primers bind to six positions on template DNA. Two more primers called loop primers, can be applied in addition to accelerate the reaction making it more sensitive (Nagamine *et al*., 2002). There are different routes for monitoring the positive LAMP reaction, of which the colorimetric detection based on HNB enables closed tube detection with naked eye which avoids the chance for post amplification cross contamination of samples (Goto *et al.*, 2009).

In the present study, an attempt was done to develop colorimetric closed tube LAMP assay for the detection of BBTV using HNB dye. The total genomic DNA was isolated from banana leaves showing bunchy top appearance, leaf margin necrosis and morse code pattern. The leaf samples were collected from different varieties like

Nendran, Kadali, Poovan, Palayankodan, Njali Poovan and Robusta. The protocol followed was the Doyle and Doyle (1987) method with some modification as mentioned in 3.2.1. The DNA samples isolated had RNA contamination. (Plate 4.2). Further, RNase treatment was done to get rid of RNA contamination and to get pure DNA (Plate 4.3).

In the present study, the LAMP assay targeted BBTV coat protein gene. We used four primers in reaction binding to six regions in the target thus ensuring high specificity. Additionally, a pair of loop primers were used which further increases the amplification efficiency (Nagamine *et al.,* 2002). In total, six primers were designed targeting eight regions on coat protein gene of BBTV. Other genes like movement protein gene and replicase gene were also tried for primer designing. As no loop primers were obtained for these genes, the coat protein gene was used for our study. In the LAMP assay developed by Peng *et al.* (2012) developed LAMP assay for the detection of BBTV, replication initiator protein gene was used for picking primers and the loop primers were not included.

The concentration of different components in the LAMP reaction were optimized initially. In order to standardize the LAMP assay, reaction was set with dNTPs at 1.4 mM and 1.6 mM each. We got positive amplification with 1.4 mM dNTP while observed on agarose gel. However, colour change was not obtained with HNB. At 1.6 mM dNTP all the positive samples showed colour change from violet to sky sky blue. Goto *et al.* (2009) reported that colour of the HNB changes depending upon dNTP and pH of the solution. The LAMP reaction was set with $MgSO_4$ concentration ranging from 4 mM to 8 mM. Reaction was tried with betaine concentration 0.8 M and 1 M and *Bst* polymerase 8U and 4U. The reaction was incubated at different temperatures *viz* 55°C, 60°C, 65°C and 68°C for 60 minutes. Finally, the best result with visual detection was obtained with 1.6 mM each dNTP, 0.2 µM each of F3 and B3, FIP and BIP each with 0.8 µM and 0.4 µM each of the primers LF and LB, 6 mM $MgSO_4$ (New England BioLabs), 4U *Bst* polymerase large fragment (New England BioLabs), 1X Thermopol buffer with 2 mM $MgSO_4$, 1M betaine (Sigma) and 120 µM HNB. We got amplification with 4U and 8U concentration of *Bst* polymerase. So, we could reduce the cost by using

4U of enzymes. In the LAMP assay developed by Peng *et al.* (2012) and Almasi *et al.* (2013), reaction contain 8U *Bst* polymerase.

The incubation was also tried out in thermal cycler. The result was same as that from reaction incubated on a heat block. So, a simple heat block or water bath is sufficient to carry out the reaction. The LAMP assay for molecular detection of BBTV based on SYBR Green fluorescence has been developed in China, but the assay did not use loop primers (Peng *et al.,* 2012). The loop primers increase the amplification rate and hence our assay is more sensitive in the detection of BBTV. Moreover, the HNB enables closed tube simple visual detection by adding the dye in the reaction tube prior to amplification and observing color change at the end of the reaction (Goto *et al.,* 2009). The LAMP assay developed here is a better alternative to common molecular detection methods used for virus detection.

The LAMP assay for detection of Fuzarium wilt of chickpea (Gosh *et al.,* 2015) and coconut root wilt disease and arecanut yellow leaf disease (Nair *et al.,* 2016) has been developed based on colorimetric detection using 150 µM HNB dye. In the present study, HNB added at concentration 150 µM gave no colour change in the positive reaction. Thus, titration of the HNB against total $MgSO_4$ concentration 6 mM to 8 mM was done and we got optimum colour change with 120 µM HNB in the presence of 8 mM $MgSO_4$. In the LAMP assays developed for detection of tomato *yellow leaf curl virus* (Almasi *et al.,* 2013), phytoplasma associated cassava witches broom disease (Vu *et al.,* 2016) and wheat leaf rust (Manjunatha *et al.,* 2018), 120 µM HNB gave optimum colour change. The HNB changes colour from violet to sky blue upon decrease in the Mg^{2+} ion concentration as it combines with pyrophosphate produced during amplification (Goto *et al.,* 2009). Before the reaction Mg^{2+} ions chelated by dNTP make the solution appear violet (Goto *et al.,* 2009). In our study, the reaction mix appeared sky blue before reaction when 120 µM HNB added against $MgSO_4$ below 7 mM. Above 7 mM reaction mix had a violet colour but reaction colour change to sky blue was observed in reaction with 8 mM $MgSO_4$.The positive products could be differentiated from the negatives clearly by including 120 µM of HNB in the reaction at total concentration of 8 mM $MgSO_4$ and 1.6 mM each dNTP.

The molecular confirmation of LAMP amplicon was done by restriction digestion using *Sau*3AI enzyme which has single restriction site between F2 and B2 region producing fragments of size 65 bp and 89 bp. The restriction digestion produced bands of expected size. Thus confirmed the specificity of the developed LAMP assay. Notomi *et al.,* (2000) carried out restriction analysis to identify structure of LAMP products. Restriction analysis was employed for molecular typing of LAMP amplicons in the LAMP assay developed for detection of phytoplasma causing coconut root wilt disease and arecanut yellow leaf disease using *Hyp*CH4V enzyme (Nair *et al.,* 2016).

The PCR amplification of the diseased samples using LAMP external primers BBTVF3 and BBTVB3 followed by sequencing and BLASTN analysis also confirmed the specificity of our LAMP assay. The LAMP method has been successfully applied for virus detection from various hosts. A LAMP assay was employed to develop a simple and efficient system for the detection of *Squash leaf curl virus* in diseased plants of squash and melon (Kaun *et al.,* 2010). Zang *et al*. (2011) also developed a LAMP assay that can identify mosaic virus in wheat at a sensitivity 100 times greater than PCR. The LAMP assays using a Genelyzer™ III portable fluorometer combined with a toothpick method successfully detected begomoviruses, namely *Tomato leaf curl New Delhi virus*, *Pepper yellow leaf curl Indonesia virus* and *Tomato yellow leaf curl Kanchanaburi virus* infecting Cucurbitaceae and Solanaceae plants in infected melon, pepper, and eggplant samples (Wilisiani *et al.,* 2019). Walilullah *et al.* (2020) developed real time LAMP assay for detection of *Cucurbit leaf crumple virus.* A LAMP assay for the detection of *Dahlia mosaic caulimovirus* in Dahlia has been developed (Ifthikhar *et al.,* 2020). Most recently, colorimetric LAMP assay using phenol red dye has been optimized for the detection of *Cotton leaf curl virus* (Rafiq *et al.,* 2021).

The LAMP test always raises the risk of carryover contamination due to strong target amplification and sensitivity. Closed tube detection combined with proper sample handling, on the other hand, ensures contamination free reactions. We used a sterile, UV-enabled PCR work station for setting the reaction. A separate space was kept for setting up the LAMP reaction. A different laboratory room was used for DNA sample preparation. Hence, we ensured contamination free reaction. For routine detection, solely closed tube assays should be carried out.

The presence of phenolic chemicals and inhibitors in the DNA preparation often interfere with molecular detection. However, as compared to PCR, the LAMP assay identified a higher proportion of positives from the same tested samples. Among the fifteen symptomatic samples tested, only thirteen showed positive amplification in PCR, while all the tested symptomatic samples showed positive amplification in LAMP assay. The two samples showed negative result in PCR turned out BBTV positive in LAMP assay. The *Bst* polymerase used in LAMP is less sensitive to inhibitors in the DNA samples compared to *Taq* polymerase used in PCR (Dikinson, 2015).

As LAMP is an isothermal amplification method, it requires minimal laboratory condition with a heat block or water bath which can maintain a constant temperature. This replaces the need of a thermal cycler equipped laboratory. Moreover, LAMP took only one hour for disease detection while PCR requires an hour and 40 minutes for reaction only and the additional time needed for gel electrophoresis. Since a set of six primers target eight regions on the template, the specificity is higher in the LAMP amplification as compared to a pair of primers in PCR. Peng *et al.* (2012) reported that LAMP assay for BBTV detection could detect as low as 1 pg/µl DNA, while detection limit of PCR was about 100 pg/µl plasmid DNA.

In a comparative study of LAMP and PCR based assay for detection of *Alternaria solani* in tomato and potato, LAMP proved to be faster, simpler and sensitive test than PCR (Khan *et al.,* 2018). Zang *et al.* (2011) developed a LAMP assay to identify wheat mosaic virus at a sensitivity 100 times greater than PCR. Also, LAMP is highly sensitive due to production of multiple repeats of the target site. The cross-contamination chance in the post amplification handling for detection is also eliminated by closed tube detection method along with ease of naked eye detection. Thus, the LAMP assay developed here is a better alternative for routine screening of planting materials.

SUMMARY

SUMMARY

Banana is a widely cultivated crop and its production is threatened by many diseases. The BBTD caused by BBTV is such a disease, causing devastative yield reduction. It is transmitted through infected planting materials and insect vector, banana aphid. As a management strategy, a sensitive and rapid virus indexing method is needed to provide virus free planting materials to farmers.

The study entitled "Detection of *Banana bunchy top virus* (BBTV) through colorimetric closed tube Loop Mediated Isothermal Amplification (LAMP) was carried out in the Center for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agriculture University, Thrissur, from 2019 to 2021. The study was commenced with an objective to develop a colorimetric closed tube LAMP assay for detection of BBTV.

- Fifteen BBTV symptomatic banana leaf samples, three symptomless samples and leaf samples from plants showing symptoms of BSV, CMV and BBrMV were collected
- Total genomic DNA was isolated through Doyle and Doyle (1987) protocol followed by RNase treatment
- Good quality DNA was obtained with $O.D_{260/280}$ ranging between 1.8-2.2 and quantity ranging 286 to 1250 ng/µl
- A set of six LAMP primers were designed targeting the coat protein gene of BBTV using Primer Explorer v.5.0. software
- The LAMP assay for detection of BBTV was optimized
- Colorimetric detection of LAMP products was developed by adding 120 µM HNB
- Molecular typing of LAMP amplicon was done by sequencing and restriction analysis
- Sequencing of amplified bands after conventional PCR using LAMP external primers (BBTVF3 and BBTVB3) and similarity search using BLASTN confirmed the identity with BBTV coat protein gene

- Restriction analysis of the LAMP products using restriction enzyme *Sau*3AI, which has a single cut site in the internal primer binding region yielded bands of expected size (62 bp and 89 bp)
- Validation of the developed LAMP assay with 15 BBTV symptomatic samples, symptomless samples and BSV, CMV, BBrMV symptomatic samples was done
- All the BBTV symptomatic samples showed positive amplification and colour of the reaction changed from violet to sky blue after rection while healthy and BSV, CMV and BBrMV symptomatic samples showed no amplification and the colour remained violet
- All the 15 symptomatic samples were tested using conventional PCR also, and only 13 samples showed amplification
- The LAMP assay for detection of BBTV is more rapid, sensitive and easier than PCR based detection of BBTV
- Thus, the LAMP assay developed here is a better alternative for routine screening of planting materials

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DETECTION OF *Banana bunchy top virus* (BBTV) THROUGH COLORIMETRIC CLOSED TUBE LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

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ABSTRACT OF THE THESIS

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ABSTRACT

Banana is an important food and cash crop world wide. India leads in world banana production with 31.5 million tons from 0.9 million ha area. The Banana Bunchy Top Disease caused by *Banana bunchy top virus* (BBTV) is considered to be the most economically destructive of the viral diseases. Primary transmission of disease is through infected planting material. Virus indexing of suckers and tissue culture plantlets is essential to ensure the availability of virus free planting materials. The available detection methods for the BBTV are ELISA and PCR. The ELISA is time consuming while PCR requires thermal cycling and the post amplification sample handling predisposing to sample cross contamination. A nucleic acid amplification method termed Loop Mediated Isothermal Amplification (LAMP) enables rapid, sensitive and specific amplification of template DNA under isothermal condition using a strand displacing polymerase like *Bst* DNA polymerase and 4 to 6 primers producing 10^9 copies of the target in less than 1 h. Combined with simple visual detection of amplicons using the Hydroxy Naphthol Blue (HNB) dye, closed tube detection can be made hence avoiding sample cross contaminations.

Hence, the present study entitled "Detection of *Banana bunchy top virus* through colorimetric closed tube Loop Mediated Isothermal Amplification (LAMP)" was undertaken at the Center for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agriculture University, Thrissur, during the period from 2019 to 2021 with the objective to develop a colorimetric closed tube LAMP assay for rapid and sensitive detection of BBTV. As the initial step, samples were collected from 15 BBTV symptomatic banana plants, two symptomless plants and from plants showing symptoms of other viruses infecting banana such as *Banana bract mosaic virus* (BBrMV), *Banana streak virus* (BSV) and *Cucumber mosaic virus* (CMV). The samples were collected from Banana Research Station, Kannara. One sample was taken from healthy tissue culture raised plant at the Center for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agriculture University, Thrissur. Good quality DNA was obtained with Doyle and Doyle method followed by RNase treatment. For LAMP primer designing, coat protein gene, replicase gene and

movement protein gene sequences of BBTV were selected as the target. The LAMP external and internal primers could be picked from all the targets, but loop primers were not available for replicase gene and movement protein gene sequences. Hence, six LAMP primers were designed targeting BBTV coat protein gene using the software Primer Explorer version 5.0.

The concentration of different components in the LAMP reaction like dNTPs (0.8 to 1.8 mM), MgSO4 (4 to 8 mM), HNB (120 µM and 150µM) and betaine (0.8 to 1M) was optimized. The final reaction mixture contained 50 ng template DNA, 1.4 mM each dNTP, 0.8 µM each primer BBTVFIP and BBTVBIP, 0.2 µM each primer BBTVF3 and BBTVB3, 0.4 µM each primer BBTVLF and BBTVLB, 0.8 M betaine, 6 mM $MgSO_4$, 1X Thermopol buffer with 2 mM $MgSO_4$, 8U *Bst* polymerase large fragment and 120 µM HNB dye in 25 µl reaction volume. Isothermal amplification was set at 65 ^oC and end point detection made using 120 µM HNB dye in the reaction mixture where the BBTV positive samples showed colour change from violet to blue. The LAMP positive amplicons appeared as ladder like bands on agarose gel.

Molecular characterization of LAMP amplicon was made with sequencing and restriction analysis. A restriction enzyme having cut site in between the LAMP internal primer flanking region was identified. When the LAMP amplicons were digested with the restriction enzyme *Sau*3AI having single cut site, fragments of expected size (62 bp and 89 bp) were obtained. The optimized LAMP assay was validated using 15 BBTV symptomatic samples, three healthy samples and samples showing symptoms of BBrMV, CMV and BSV, as observed by the agarose gel profile and the colour change with HNB dye. All the 15 BBTV symptomatic samples showed amplification in the LAMP assay while the healthy samples and the samples showing symptoms of other viruses remained unamplified. Comparison of LAMP assay with conventional PCR in the detection of BBTV was also made. Only 13 samples out of the 15 BBTV symptomatic samples tested positive in the PCR assay, thus showing higher sensitivity of the LAMP method. Moreover, the closed tube visual detection in LAMP avoids the post amplification sample handling. Since LAMP reaction can be carried out on a dry bath or water bath, it is a rapid and simple alternative to the available PCR based detection method for BBTV.