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# Comparative detection of banana bunchy top virus in banana cv. Safed velchi utilizing DNA/RNA by PCR technique

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### Abstract

The present study was carried out to describe the ideal PCR protocol to find viral infection in plantlets of banana. 50 plantlets selected from Plant Biotechnology Center, Dapoli from which DNA (50 samples bulked into 10) as well as RNA (20 samples) were extracted along with BBTB infected samples. The previously standard rapid DNA isolation protocol was used. A protocol using 1.80 g ml<sup>-1</sup> Tris-base, 3.75 g ml<sup>-1</sup> EDTA, 1.5 g ml<sup>-1</sup> SDS and 100 μl ml<sup>-1</sup> βMercaptoethanol produced the highest yields and best quantity RNA. cDNA synthesis (M-MuLV kit) containing Oligo-dT, dNTP's, Molecular Biology Grade Water and isolated RNA template were used to prepare reaction mixture. The optimum PCR procedure utilizes to synthesized cDNA from template RNA. 21 samples get assayed at RNA level by preparing cDNA. Strong reactions were obtained from the sample no 10, 11, 22 and 23 of BBTB infected samples and a positive control plant at no 12 and 24 confirms the presence of BBTB with amplicon size of 389 bp with primer BBTB F & R. All 50 DNA samples were tested for BBTB gene specific primers (CP, NSP and FPCR<sup>2</sup>4+30MER gene) all the tested regenerated plantlets were found to be negative and the lot no 11 of BBTB infected samples and a positive control plant at no 12 confirms the presence of BBTB with amplicon size of 511bp, 349 bp and 1,349 bp respectively. But, RNA level mechanism was effective in which the actual coding genome was transcribed. So it is easy and useful to detect the genome at RNA level than DNA which contains large genome size.

**Keywords:** Banana bunchy top virus, micropropagation, cDNA, RT-PCR, gene specific

### Introduction

Banana (*Musa paradisiaca* L.) is worldwide an important food and cash crop. Banana and plantains are grown in about 120 countries. Banana is a globally important fruit crop with 97.5 million tones of production. In India it supports livelihood of million of people. With total annual production of 16.91 million tones from 490.70 thousand ha., with national average of 33.5 T/ha. Maharashtra ranks first in production with 60 T/ha.

Commercially, bananas are classified as dessert types and culinary types. The culinary types have starchy fruits and are used in the mature unripe form as vegetables. Important cultivars include Dwarf Cavendish, Robusta, Monthan, Poovan, Nendran, Red banana, Nyali, Safed Velchi, Basrai, Ardhapuri, Rasthali, Karpurvalli, Karthali and Grand Naine etc. Bananas are one of the most consumed and cheapest fruits worldwide: they are the most traded fruit and the fifth most traded agricultural product. The global export value of the banana trade was estimated to be US \$11.8 billion in 2017-18, with a retail value between \$20 and 25 billion. India produces 30% of global banana production. Currently, some 150 containers stacked with bananas are shipped from India per month to markets in the West Asia. With 1,800 containers per year, the market is around 150- 200 crore.

Micropropagation is preferred over conventional method of propagation sowing to its faster multiplication rate, uniformity in planting materials and production of disease free materials. ELISA is convenient but use of this technique is limited in detection sensitivity with very low concentrations of BBTB (Harding *et al*, 2000; Wetzel *et al*, 1992) [8 2]. Polymerase chain reaction (PCR) is very reliable and economically important for testing banana plantlets for the detection of virus. PCR tests are more sensitive than ELISA tests (Shelake *et al*, 2013) [11]. (Source: Database of National Horticulture Board, Ministry of Agriculture, Govt. of India.)

Most of the banana viruses reside in the host in latent form, i.e. without exhibiting any visual symptoms in the host for some period (sometimes for up to 2 years) BBTV infected plants multiplied through tissue culture can be symptomless for over some months after planting out (Drew *et al.*, 1989). The virion consists of at least six components of circular ssDNA, each about 1kb (Burns *et al.* 1995), transmitted by *Pentalonia nigronervosa* (Hu *et al.*, 1996), and a phloem limited virus (Dale, 1987; Sadik *et al.* 1999). In addition, symptoms of viral diseases often get confused with nutrient deficiency. In order to make proper management decisions, early and correct diagnosis of viruses is important. This will help in checking further spread of pathogens, especially to a new area. Early detection by means of sensitive diagnostics methods is the main way to control them. If non indexed plants are used as mother plants, viruses are spread, with huge effect on yield. Tissue culture is a widely adopted technology in horticultural industry. In order to sustain and reap the benefits of tissue culture technology in banana, we have to ensure the growers have access to quality mother plants that have undergone virus indexing for producing healthy and high yielding planting material. Different molecular diagnostic techniques that are used worldwide by the tissue culture industry to detect banana viruses' pathogens developed by National Research Centre for Banana (NRCB) are also discussed.

## Materials and methods

### A) Plant material

Fresh green leaves were collected from Safed Velchi variety of banana Plant Biotechnology Center, College of Agriculture, Dr. B. S. K. K. V., Dapoli (50 plantlet) and S. P. College of

Horticulture, Kharawate Dahiwal (infected samples), for the present experimental study.

### B) RNA extraction

RNA was isolated following the protocol of (Beetham *et al.* 1997) with slight modifications (Table 1). Leaf samples (0.5 g each) were collected from tissue cultured plantlets of banana and sterilized with 75% ethanol prepared in DEPC to avoid contamination and used to extract genomic RNA. RNA was isolated from 20 plantlets while, one sample is of BBTV infected plant and used for further analysis. The size range and concentration of RNA sample was determined after electrophoresis using a standard DNA ladder in 1.2% (w/v) agarose gels in 1X TBE buffer and by comparing the intensity of staining with 10 mg ml<sup>-1</sup> ethidium bromide.

**Table 1:** Solution for RNA extraction buffer preparation

| Sr. No. | Chemicals        | T1     | T2     | T3     |
|---------|------------------|--------|--------|--------|
| 1       | Tris-base        | 1.60 g | 1.80 g | 2.80 g |
| 2       | EDTA             | 3.50 g | 3.75 g | 4.00 g |
| 3       | SDS              | 1.0 g  | 1.5 g  | 2.0 g  |
| 4       | βMercaptoethanol | 80 μl  | 100μl  | 120μl  |

### C) cDNA Synthesis

The components for construction of cDNA synthesis like Oligo-dT (1μl), RNA template (5μl) and Molecular Biology Grade Water (4μl) were added. Then the mixture was incubated in water bath for 5 min at 65 °C, and then cooled immediately on ice. The reaction mixture in total volume 20μl was prepared (table 2). For preparation of cDNA, the complete mixture was incubated at 42 °C for 5min followed by 70 °C for 60min. The cDNA was further used to perform PCR assay.

**Table 2:** Reaction mixture for cDNA synthesis

| Sr. No. | Ingredients                             | Volume (μl) |
|---------|---|-------------|
| 1       | Template RNA Primer Mixture             | 10          |
| 2       | RT Buffer for MmuLV                     | 4           |
| 3       | 10X Solution for MmuLV                  | 2           |
| 4       | M-MuLV Reverse Transcriptase (RNase H-) | 1           |
| 5       | 10mM dNTP mix                           | 2           |
| 6       | Molecular Biology Grade Water for PCR   | 1           |
|         | Total                                   | 20          |

### C) DNA extraction

DNA was isolated following the protocol of (Doyle and Doyle 1987) with slight modifications. Previously standardized solutions containing 0.095 g ml<sup>-1</sup> glucose, 0.020 g ml<sup>-1</sup> PVP, 0.0040 g ml<sup>-1</sup> sodium bisulphite, 0.0050 g ml<sup>-1</sup> SDS, and 50 μl ml<sup>-1</sup> sarcosine (Kadam *et al.*, 2018) were used to extract tissue cultured banana leaf DNA. Fresh leaf samples (0.1 g each) were collected from the tissue culture developed plantlets and used to extract genomic DNA. RNA as well as proteins were removed by treatment with 50 μg ml<sup>-1</sup> RNase and 120 μg ml<sup>-1</sup> ProteinaseK (Merck Pvt. Ltd., Mumbai, India) and incubated at 37°C for 1 h. DNA was isolated from

50 plantlets and bulked into ten lots while two sample is of BBTV infected plants as a eleventh lot and used for further analysis. Electrophoresis is done using a standard DNA ladder in 1% (w/v) agarose gels to determine the molecular weight and quality of DNA.

### D) PCR amplification

The standard Gene specific primers were effectively used for RNA as well as for DNA were given in table 3(A&B) for detection of BBTV infection. These molecular markers were effectively used to screening of the infected samples of banana by isolated RNA and DNA.

**Table 3(A):** List of Gene specific primers with their sequence and GC content (For RNA).

| Sr. No. | Primer   | Primer Sequence (5'-3')      | GC Content (%) | Tm value(°C) |
|---------|----------|------------------------------|----------------|--------------|
| 1       | BBTV – F | 5'ATGGCGCGATATGTGGTATGC 3'   | 52.3           | 53°C         |
|         | BBTV – R | 5'TCAGGTTTTATTCATCTCTGCTTG3' | 37.5           | 53°C         |

**Table 3(B):** List of Gene specific primers with their sequence and GC content (For DNA).

| Sr. No. | Primer | Primer Sequence (5' – 3')             | GC content (%) | Tm Value(°C) |
|---------|--------|---------------------------------------|----------------|--------------|
| 1       | CP-F   | 5'ATGGCTAGGTATCCGAAG 3'               | 50             | 50.8         |
|         | CP-R   | 5'CCAGAACTACAATAGAATGCC 3'            | 42.85          | 50.8         |
| 2       | NSP-F  | 5'CCTCGCAAGGTACTTCTTAG 3'             | 50             | 52.4         |
|         | NSP-R  | 5'CCATGTCTCTGCTCCAATCT 3'             | 50             | 54.3         |
| 3       | FPCR4  | 5'TTCCCAGGCGCACAC CTTGAGAAAACGAAAG 3' | 53.33          | 65.4         |
|         | 30mer  | 5'GGAAGAAGCCTCTC ATCTGCTTCAGAGAGC 3'  | 53.33          | 63.1         |

A PCR protocol was standardised for all gene specific markers. Each 20 µl PCR contained 20 ng template DNA, 2.5 µl of 10× PCR buffer, 0.5 µl of 15 mM MgCl<sub>2</sub>, 1 µl of 10 mM dNTPs (HiMedia biosciences, Mumbai), 10 pmol of each gene specific primers (Bioresource Biotech Pvt. Ltd., Pune, India) and 3.0 units of *Taq* polymerase (HiMedia biosciences, Mumbai). Thermal profiles were standardised for each primer (i.e. marker) based on its melting temperature and the standard annealing temperatures of all gene specific markers

are given in Table 4. The amplified products of reaction were separated by electrophoresis on 2 percent agarose gel (Marck, India), containing ethidium bromide in 1X TAE Buffer (pH 8.0) and separations was carried-out by applying constant voltage of 70 Volts for 1 hour. The standard molecular marker and 1 Kb DNA ladder was used. PCR and gel electrophoresis were carried-out twice and only reproducible patterns were used for data analysis.

**Table 4:** Thermo profile of PCR used in detection of *BBTV*

| Sr. No. | PCR steps            | RNA Primer                         | DNA Primer                |                            |                                      |
|---------|----------------------|------------------------------------|---------------------------|----------------------------|--------------------------------------|
|         |                      | Reaction condition for <i>BBTV</i> | Reaction condition for CP | Reaction condition for NSP | Reaction condition for FPCR4 & 30mer |
| 1       | Initial Denaturation | 94 °C for 5 min.                   | 94 °C for 5 min.          | 94 °C for 5 min.           | 94 °C for 5 min.                     |
| 2       | Denaturation         | 94 °C for 20 sec.                  | 94 °C for 20 sec.         | 94 °C for 20 sec.          | 94 °C for 20 sec.                    |
| 3       | Annealing            | 53.1 °C for 45sec.                 | 57.1 °C for 30sec.        | 51.0 °C for 45sec.         | 35.2 °C for 45sec.                   |
| 4       | Primer extension     | 72 °C for 45sec                    | 72 °C for 45sec.          | 72 °C for 45sec.           | 72 °C for 45sec.                     |
| 5       | Final extension      | 72 °C for 7 min                    | 72 °C for 7 min           | 72 °C for 7 min            | 72 °C for 7 min                      |
| 6       | Hold                 | 4 °C for 10 min                    | 4 °C for 10 min           | 4 °C for 10 min            | 4 °C for 10 min                      |

## E) Data analysis

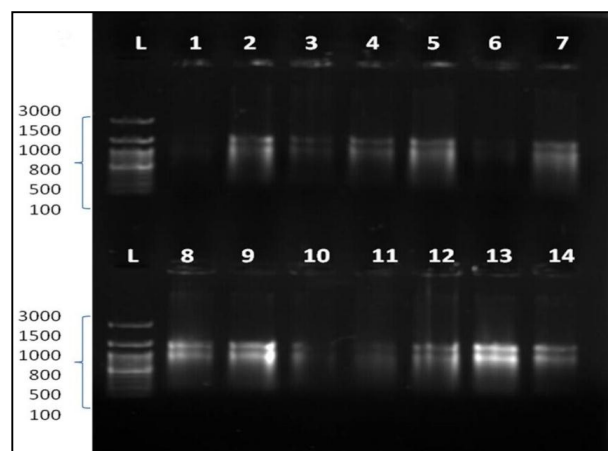
The images of gel was taken by the documentation systems (Uvi-Tech. Fire reader, Cambridge, UK) and saved in computer for further analysis. The standard Gene specific primers across the 51 samples of DNA and 21 samples of RNA were scored for their absence (0) and one lot was scored for their presence (1) of bands for each primer. By comparing the banding patterns of those samples for a specific primer specific bands were identified. The data so generated was used to estimate the presence or absence of viral gene/ bands.

## Results and discussion

### A) RNA isolation

The isolation of good quality RNA is important for further analysis like cDNA synthesis. Because of contaminants such as DNA, proteins and other metabolites can interfere with cDNA synthesis. RNA was isolated from fresh leaves of each banana plantlets. The minor modifications in buffer composition are done to extract good quality of RNA. Different concentrations of stock components were tested which given in table 1. A protocol using 1.80 g ml<sup>-1</sup> Tris-base, 3.75 g ml<sup>-1</sup> EDTA, 1.5 g ml<sup>-1</sup> SDS and 100µl ml<sup>-1</sup> βMercaptoethanol produced the highest yields and best quantity RNA and also minimizes contamination by metabolites (Fig.1). PCR requires an appropriate, low quantity of DNA (10–30 ng). DNA concentrations above or below the optimum can result in incorrect amplification. By using the

formula, the isolated RNA samples were quantified and shown in table 5.



**Fig 1:** RNA bands of banana (Safed velchi) plantlets. Lanes M, 1.0 kbp DNA ladder; lanes 1-11, RNA of banana test plants; lane 12-13, BBTv infected plant samples; lane 14, Positive control.

### RNA Quantification

Formula for calculating RNA quantity:  $A_{260} \times 30 \times 200 = \text{µg/ml}$   
 $A_{260}$ : Optical density at 260 nm  
 30: Quantity of DEPC water to dissolve RNA  
 200: Dilution factor

**Table 5:** RNA Quantification

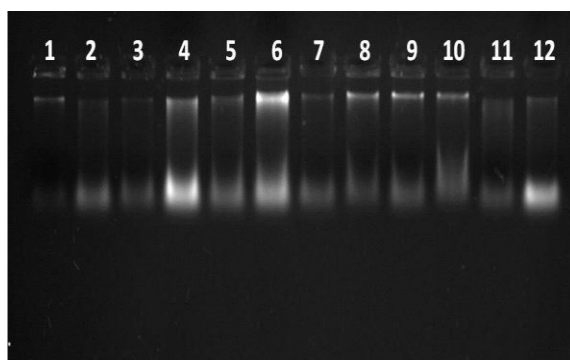
| Sr. No | A <sub>260</sub> | A <sub>280</sub> | Quantity(µg/ml) |
|--------|------------------|------------------|-----------------|
| 1      | 0.086            | 0.090            | 516             |
| 2      | 0.040            | 0.030            | 240             |
| 3      | 0.014            | 0.021            | 84              |
| 4      | 0.024            | 0.008            | 144             |
| 5      | 0.030            | 0.033            | 180             |
| 6      | 0.029            | 0.039            | 174             |
| 7      | 0.017            | 0.016            | 102             |
| 8      | 0.028            | 0.024            | 168             |
| 9      | 0.006            | 0.004            | 36              |
| 10     | 0.040            | 0.038            | 240             |
| 11     | 0.012            | 0.029            | 72              |
| 12     | 0.103            | 0.120            | 618             |
| 13     | 0.115            | 0.112            | 690             |
| 14     | 0.093            | 0.102            | 558             |

**B) cDNA synthesis**

The components for construction of cDNA synthesis (M-MuLV kit) containing Oligo-dT, dNTP's, Molecular Biology Grade Water and isolated RNA template were used to prepare reaction mixture. The optimum PCR procedure utilizes to synthesized cDNA from template RNA.

**C) DNA isolation**

The genomic DNA extracted from a 50 samples of safed velchi variety of banana by using standardized rapid protocol of DNA isolation. The protocol is modified which ultimately control good quality of DNA, means removes proteins, polysaccharides and phenolic contaminants. Five no of DNA samples were bulked into ten lots and one is of infected sample (Fig. 2).



**Fig 2:** DNA bands of banana (Safed velchi) plantlets. Lanes 1-10, lots of tissue culture banana plants; lane 11, lot of BBTV infected plant samples; lane 12, Positive control.

**D) Marker analysis**

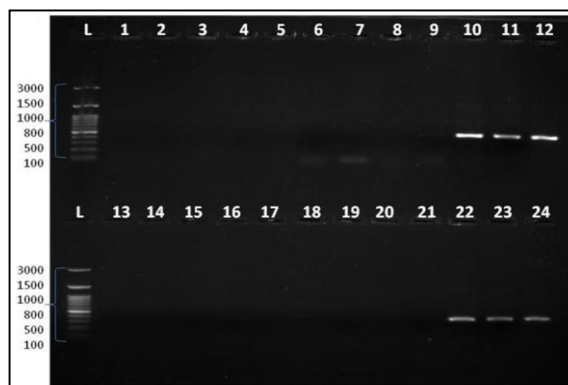
The standard annealing temperature range for gene specific primer was 35.2°C for FPCR 4 & 30MER, 57.1°C for CP (F & R), 51°C for NSP (F & R) and 53.1°C for BBTV (F & R). The standardized annealing temperatures for Gene Specific Primer is given in table 6.

**Table 6:** Standardize annealing temperatures of Gene Specific Primer

| Sr. No. | Primer | T <sub>m</sub> value(°C) | Stand. Annealling Temp.°C |
|---------|--------|--------------------------|---------------------------|
| 1.      | BBTV-F | 52.3                     | 53.1                      |
|         | BBTV-R | 37.5                     |                           |
| 2.      | CP-F   | 50.7                     | 57.1                      |
|         | CP-R   | 50.8                     |                           |
| 3.      | NSP-F  | 52.4                     | 51.0                      |
|         | NSP-R  | 54.3                     |                           |
| 4.      | FPCR   | 65.4                     | 35.2                      |
|         | 30MER  | 63.1                     |                           |

**Gene Specific Primer Analysis (RNA)**

cDNA synthesis want to optimize by virus specific antisense primers about 50nm of total RNA was mixed with these antisense primer. 21 samples get assayed at RNA level by preparing cDNA. After cDNA synthesis the template strand of cDNA was amplified with those gene specific primers with PCR and there was amplification observed (table 6). Strong reactions were obtained from the sample no 10, 11, 22 and 23 of BBTV infected samples and a positive control plant at no 12 and 24 confirms the presence of BBTV with amplicon size of 389 bp with primer BBTV F & R (Fig. 3).



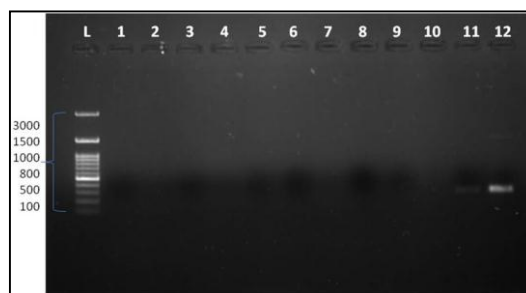
**Fig 3:** PCR assay of cDNA of banana plantlets by using primer BBTV (F&R). Lanes M, 1.0 kbp DNA ladder; lanes 1-9, 13-21, test banana samples (cDNA); lane 10-11, 22-23, BBTV infected plant samples; lane 12, 24, Positive control.

**Gene Specific Primer Analysis (DNA):**

All DNA samples were tested for BBTV gene specific primers (CP and NSP gene) with PCR. Interestingly, all the tested regenerated plantlets were found to be negative and the lot no 11 of BBTV infected samples and a positive control plant at no 12 confirms the presence of BBTV with amplicon size of 511bp and 349 bp (Fig. 4 & 5) for CP and NSP respectively.

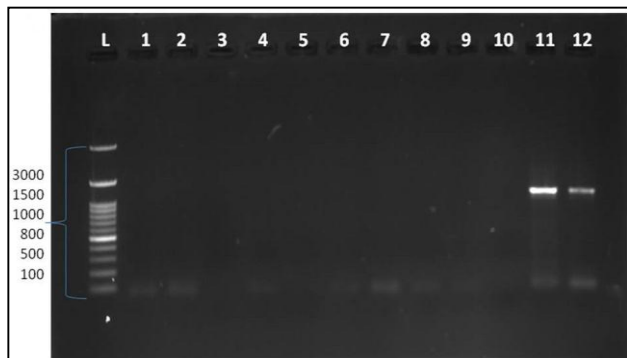


**Fig 4:** PCR assay of banana plantlets by using primer CP (F&R). Lanes M, 1.0 kbp DNA ladder; lanes 1-10, amplicons of banana lots; lane 11, lot of BBTV infected plant samples; lane 12, Positive control.



**Fig 5:** PCR assay of banana plantlets by using primer NSP (F&R). Lanes M, 1.0 kbp DNA ladder; lanes 1-10, amplicons of banana lots; lane 11, lot of BBTV infected plant samples; lane 12, Positive control.

Banana Bunchy top virus was detected in 12 lots assayed. Strong reactions were obtained from the lot no 11 of BBTV infected samples and a positive control plant at no 12 confirms the presence of BBTV with amplicon size of 1,349 bp with primer FPCR'4 and 30MER (Fig. 6). While all ten lots of collected from apparently healthy looking plants show negative result hence, there was completely absence of Banana Bunchy Top Virus in all lots of tissue culture banana plant samples.



**Fig 6:** PCR assay of banana plantlets by using primer F-PCR'4 & 30 MER. Lanes M, 1.0 kbp DNA ladder; lanes 1-10, amplicons of banana lots; lane 11, lot of BBTV infected plant samples; lane 12, Positive control.

### Conclusion

Micropropagation is an effective protocol for generation of disease-free, virus-free or any other contamination free planting material. Also the strategies of molecular biology techniques are basis on rapid detection. Nucleic acid based detection by using polymerase chain reaction for infected plants using their genome so that potential source of infection can be assayed and plants of BBTV can be destroyed promptly. The specific primers to coding sequences of coat protein and replicas' 21 and combination of FPCR4 and 30 MER sequence at DNA level and RNA transcript like BBTV F & R sequence at RNA level can be effectively analysed infectious planting materials. The major objective of micropropagation is to eliminate contaminants like fungal, bacterial or nematode pathogens or insect pests.

Here, we present investigation showing the comparison between RNA (BBTV F & R) and DNA (CP, NSP gene and FPCR+30MER) level determination of BBTV infected samples. From present data we can conclude both nucleic acid based mechanisms were effective for viral infection detection. But according to the central dogma of life, RNA level mechanism was effective in which the actual coding genome was transcribed. So it is easy and useful to detect the genome at RNA level than DNA which contains large genome size.

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