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# Real Time PCR Based Quantification of Banana Bunchy Top Virus (BBTV) Titre in Banana cv. Grand Naine (*Musa acuminata*)

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author NT performed the experiment and wrote the manuscript. Authors AR and KKK designed the study. Authors SV, EKD and LA analysed the real-time PCR data. Authors KS, SV and DS corrected the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

Banana Bunchy Top Disease (BBTD) is one of the most severe viral diseases affecting major banana growing belts in India. Banana Bunchy Top Virus (BBTV) is transmitted by a black banana aphid (*Pentalonia nigronervosa* L Coquerel) in a persistent manner. BBTV virions are limited to the phloem tissue of banana resulting in low titre in banana. A reliable method to quantify the BBTV in the banana will be useful for monitoring the insect vector mediated BBTV transmission in banana,

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an essential requirement for characterizing the transgenic banana transformed for BBTV resistance. A protocol for real time PCR based absolute quantification of BBTV is reported in the present study. The partial BBTV coat protein gene (459 bp) was isolated, cloned into a plasmid vector and used to construct a standard curve using an SYBR green-based assay with known copies of BBTV coat protein gene. Using the standard curve, BBTV viral load was estimated in BBTV infected symptomatic and asymptomatic leaf samples of banana cultivar Grand Naine through SYBR green-based quantitative Polymerase Chain Reaction (qPCR). The study demonstrated that a higher viral tire was associated with BBTV disease symptoms appearance, whereas the low titre resulted in asymptomatic plants.

*Keywords: Grand Naine; BBTV; coat protein; PCR; real time quantification.*

## 1. INTRODUCTION

Banana bunchy top disease caused by banana bunchy top virus (BBTV, family *Nanoviridae*, genus *Babuvirus*) is one of the viruses affecting banana plants leading to a 100% loss in yield and has been recorded in all countries cultivating banana. BBTD is transmitted by the aphid *Pentalonia nigronervosa* [1] in a persistent, circulative, non-propagative manner; the virus enters the haemocoel and the aphids retain the virus throughout their life cycle without passing it on to their offspring [2]. The BBTV is a single stranded DNA virus comprising of six genome components and are termed as DNA-R, DNAU3, DNA-S, DNA-M, DNA-C, and DNA-N [3]. Based on the DNA-R sequences, the BBTV isolates were grouped into two: Asian and South Pacific groups [4]. The Asian group comprises of the isolates from the Philippines, Taiwan, China, Japan, Indonesia, and Vietnam. The South Pacific group includes India, Pakistan, Egypt, Australia, Burundi, Tonga, Myanmar, Fiji, and USA-Hawaii and isolates from Africa [5].

Earlier Polymerase Chain Reaction (PCR)-based method was employed for the detection of BBTV infection in banana [6,7]. Later methods for the quantification of BBTV through real-time PCR using SYBR Green or TaqMan chemistry were developed [5,8,9,10]. Aphid-mediated transmission is a standard method for the BBTV virus infection in banana. However, the BBTV transmission experiments require a suitable tool to monitor virus accumulation in banana plants. Real time PCR is a sensitive method to quantify the viral load during the insect mediated transmission study in banana. The real time PCR based methods will be useful for quantifying the BBTV viral load in transmission study and also to characterize the transgenic banana plants engineered for BBTV resistance by quantifying the viral load.

## 2. MATERIALS AND METHODS

### 2.1 Source of Plant Material and Virus Infection

BBTV infected samples of banana cv. Grand Naine were collected from the Orchard of Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu and confirmed the presence of BBTV infection through PCR. The tissue culture derived plants of banana cultivar, Grand Naine which were free from BBTV infection were used throughout the study. Healthy banana adult aphids of *P. nigronervosa* were obtained from NRCB (National Research Centre for Banana), Trichy and multiplied on the tissue culture derived banana plants of cv. Grand Naine. The new aphid population was continuously obtained by culturing in new disease- free tissue culture derived plants.

The transmission studies were conducted by releasing the virus-free, same-sized, 8-10 days old aphids (apterae) on BBTV- infected plant for a period of 24 h to acquire the virus in an insect-proof chamber. After the acquisition of the virus by the aphids, 20 viruliferous aphids were allowed to feed on the virus- free tissue culture banana plants of cv. Grand Naine for a period of 48 h to transmit the virus. Then, the infected banana plants were sprayed with 0.02% Imidacloprid to kill the aphids.

### 2.2 Isolation of BBTV Coat Protein Gene

The total DNA was extracted from BBTV infected leaf tissues of Grand Naine using the CTAB method [11]. One hundred milligram of leaf tissues was homogenised with one ml of CTAB buffer ([10 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB,0.1% (v/v) β-Mercaptoethanol and 2 (v/w) %PVP] to isolate the total DNA. Then DNA was precipitated with ice cold iso-propanol followed by washing with

70% ethanol to remove the salts. The pellet obtained was air-dried and dissolved with 100 µl of sterile water and stored at -20ºC for further analysis. The DNA samples were quantified using Nanodrop spectrophotometer ND-1000. The DNA samples were resolved on a 1.0% agarose gel.

BBTV coat protein gene specific forward primer (5' TCCGAAGAAATCCATCAAGA 3') and reverse primer (5'CCAGAACTA CAATAGAATGCCAAA 3') were used for amplifying the coat protein gene. PCR condition for amplification of coat protein gene of BBTV was as follows: initial denaturation at 94ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 1 min, 60ºC annealing for 30 sec, extension at 72ºC for 1 min and final extension at 72ºC for 5 min. PCR reactions were performed in Thermal Cycler (Eppendorf, Germany) in a final volume of 20 µl. The PCR reaction mixture (20 µl) contained 1 µl of banana DNA (50 ng/µl), 2.0 µl of 10X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl and1.5 mM $MgCl_2$), 0.5 µl of 100 mM dNTPs, 1.0 µl each of 10 µM respective forward and reverse primers, 0.3 µl Taq DNA polymerase (TaKaRa Bio USA, Inc.) and 14.2 µl of sterile distilled water. The PCR amplified product was resolved in 1% agarose gel and visualized on a UV transilluminator and documented in a gel documentation system (Syngene, UK).

### 2.3 Cloning of Partial BBTV Coat Protein Gene

A 459 bp PCR amplified CP gene of BBTV was purified using PCR clean up kit (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instruction. The purified product was ligated and cloned using pJET1.2/blunt PCR Cloning Kit (Thermo Scientific Inc., USA) and transformed using *E. coli* DH5α competent cells and plated on Luria and Bertini agar media containing ampicillin (100 mg/L). Plates were incubated at 37ºC overnight. The transformed clones were analysed for the presence of recombinant clones through PCR followed by DNA sequencing (Agrigenomes Pvt. Ltd., Cochin, India).

### 2.4 Standard Curve for Absolute Quantification BBTV DNA through qPCR Assay

The purified 2.9 kb plasmid DNA containing a partial coat protein gene was used to prepare a standard curve for absolute quantification of the BBTV DNA-S component as described by Xue et al. [12]. The plasmid was serially diluted to obtain a standard series up to $10^7$ with each step differing by 10- fold. The copy number of the DNA in each dilution was calculated with the following formula Y molecules=X g/µl DNA x $6.022\times10^{23}$/(Base pair of recombinant plasmid x 660). qPCR was performed using these diluted DNA with three replicates. After the assay, the values of threshold cycles (Cq) were obtained. A standard curve was established by plotting the Cq value on the Y-axis and natural log of concentration (copies/µl) on the X-axis, and a regression equation was obtained and coefficient of determination ($R^2$) were achieved (Fig. 1).

### 2.5 Quantification of Viral Load through qPCR Assay in BBTV Infected Plants

Banana plant DNA isolated from three symptomatic and non-symptomatic Grand Naine infected plants (120 dpi) and each sample was tested in triplicate using qPCR. DNA isolated from leaf tissue samples from wild-type virus-free Grand Naine plants were used as negative control and the cloned CP plasmid was used as a positive control. Equal quantity of banana genomic DNA (25 ng) was taken for the absolute quantification of BBTV by qPCR using SYBR green chemistry as described by [13] and reactions were carried out in a Biorad® CFX connect Real time PCR system (Biorad, USA). The designed Real-time PCR primers, BBTV-RT-CP-F (5'TCAACCAGCCGACAACCTGT3') and RT-CP-R (5'TGTCCCTGTTGCGACTCCTG3') were used to amplify 116 bp of a part of coat protein gene of BBTV. The standard amplification profile was 94ºC for 4 min and 40 cycles of 94ºC for 15 s and 60ºC for 1 min and followed by a melt curve analysis by allowing the reactions at 94ºC for 15 s and 60ºC for 1 min. The qPCR cycle provided the melt curve for each sample, for assessing the specificity of amplification. For each PCR reaction, the samples were taken in three replicates.

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation of BBTV Coat Protein Gene from Infected Banana Samples

Genomic DNA samples were isolated from BBTV infected Grand Naine banana collected from Coimbatore, Tamil Nadu. PCR amplification of partial length BBTV DNA-S was observed in all five samples of Grand Naine [14] (Fig. 2).

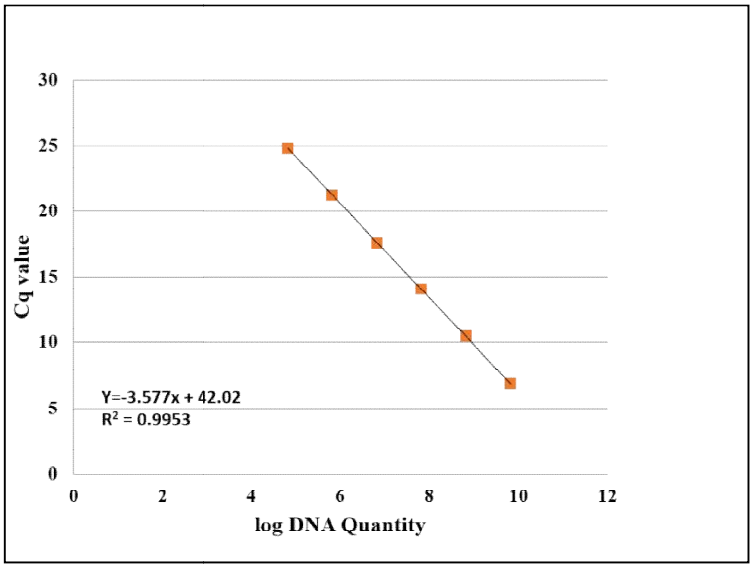


**Fig. 1. The standard curve obtained for BBTV DNA-S coat protein quantification. the X-axis represents the number of DNA copies, while the Y-axis represents the Cq value**

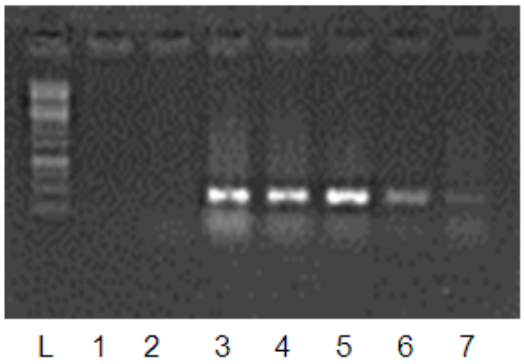


**Fig. 2. PCR detection of BBTV in Grand Naine banana leaf samples**
*Lane L- 1 Kb ladder; 1 and 2- healthy Grand Naine samples; 3 to 7- BBTV infected Grand Naine samples*

### 3.2 Cloning of BBTV Coat Protein Gene

The BBTV coat protein gene was amplified by PCR using gene specific primer BBTV-CPF and BBTV-CPR. The PCR amplified BBTV coat protein gene (459 bp) was cloned into pJET1.2/ blunt vector. The transformed clones were confirmed by DNA sequencing (Agrigenome, Kerala, India).

### 3.3 Standard Curve for Quantification of BBTV DNA in the Infected Banana Samples

Absolute quantification of viral DNA was done using the standard curve made with the cloned viral gene with known copy number. Melt curve analysis confirmed the presence of specific real time PCR amplified product (116 bp). All the PCR products melted between 75ºC and 83.5ºC which indicated the breakdown of only one PCR product (Fig. 3). The Cq values obtained for each dilution of the plasmid were used for regression analysis taking copy number and Cq value. The mean squares of the coefficient of determination ($R^2$) value was above 0.995 which indicated good linearity between the initial copy numbers and the fluorescence values (Cq values) for obtaining a high-quality standard curve for absolute quantification [12]. BBTV coat protein gene is an ideal target gene for the establishment of standard curve. Earlier studies for BBTV DNA quantification also used the CP gene for quantification [10].

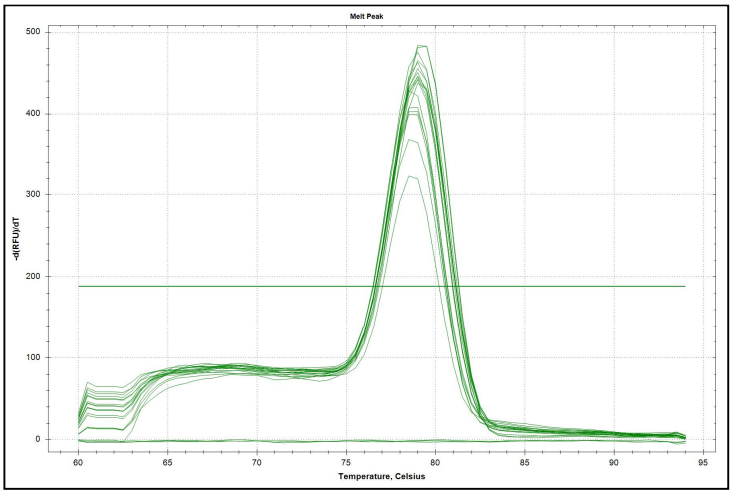


**Fig. 3. The melt curve of 116 bp BBTV CP gene product amplified in the real time PCR assay. The X-axis represents the temperature value and the Y-axis represents the value of the fluorescence**

**Table 1. qPCR analysis between symptomatic and non-symptomatic BBTV infected plants of banana cv. Grand Naine**

| Target gene | Sample type | Cq value | Cq SEM | Copy number of BBTV coat protein* |
|---|---|---|---|---|
| CP | Positive control | 18.97 | 0.01790 | $2.7 \times 10^6$ |
| CP | Negative control | 25.81 | 0.06283 | $4.9 \times 10^2$ |
| CP | Non- symptomatic -H1 | 24.03 | 0.81199 | $1.1 \times 10^5$ |
| CP | Non- symptomatic -H2 | 23.66 | 0.21572 | $1.3 \times 10^5$ |
| CP | Non- symptomatic -H3 | 22.79 | 0.35354 | $2.3 \times 10^5$ |
| CP | Symptomatic - I1 | 13.79 | 0.08139 | $7.7 \times 10^7$ |
| CP | Symptomatic - I2 | 15.33 | 0.14517 | $2.8 \times 10^7$ |
| CP | Symptomatic - I3 | 12.78 | 0.07265 | $1.4 \times 10^8$ |
| CP | NTC | 0.00 | 0.00 | 0.00 |

**Copy numbers have been calculated using the formula*

*Copy No, 1µl of the sample= $\frac{X \text{ g/µl DNA } X\ 6.022 \times 10^{23}}{\text{(base pair of recombinant plasmid x 660)}}$*

### 3.4 Viral Titre in Asymptomatic and Symptomatic BBTV Infected Plants of cv. Grand Naine

The results revealed that viral titre was higher in the symptomatic plant compared to non-symptomatic plants (Table 1). Equal quantity (25 ng/µl) of the total DNA of the banana samples were taken for real time PCR analysis to ensure that BBTV DNA. This was achieved by the quantification of DNA concentration in Nanodrop and diluted to get a DNA concentration of 25 ng per µl, followed by further confirmation in Nanodrop. Based on qPCR results, the viral load in symptomatic plants was higher compared to non- symptomatic. In the symptomatic plants, the viral load ranged from 1.4 X $10^8$ to 7.7 X $10^7$, whereas in the non-symptomatic plants, it ranged from 1.1 x $10^5$ to 2.3 x $10^5$. Even though good correlation existed among the analyzed plants and disease symptoms, one plant in each showed deviation, which may be due to experimental error. Previous reports in BBTV DNA quantification also showed real time PCR as an effective and simple method for quantification of viral load [5,8]. The results showed that the symptomatic plants harboured more viral load which was the reason for symptom expression. The viral titre plays a major role in symptom expression [10] and the delay in symptom expression in non-symptomatic plants may be due to unequal transmission of aphids during the incidence [15]. The absolute amount of each BBTV DNA component were measured

by real- time PCR [13,16]. qPCR results will invoke the importance of latency of BBTV in non-symptomatic plants. For various studies on viral accumulation in plants, real time PCR provides a confirmation protocol to study the quantity of virus for transmission studies.

## 4. CONCLUSION

Real time PCR is a rapid and sensitive method for the quantification of BBTV DNA in banana. During BBTV transmission through banana aphids, real time can be employed as a suitable tool for the accurate quantification of the BBTV DNA. The study confirms that BBTV disease symptoms occur in banana at a higher viral load and absence of symptom at lower viral load. There exists good correlation with the BBTV viral load and disease symptom in banana. The protocol described in this study can also be employed for BBTV DNA quantification of transgenic banana developed for BBTV resistance.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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