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Original Research Article

Real time PCR based quantification of Banana Bunchy Top Virus (BBTV) titre in banana cv. Grand Naine (*Musa acuminata*)

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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, 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 CP gene. Using the standard curve, BBTV viral loads 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 proves that a higher viral titre is associated with BBTV disease symptoms appearance, whereas the low titre results in asymptomatic plants.

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Keywords: [Grand Naine, BBTV, Coat protein, PCR, Real time quantification]

1. INTRODUCTION

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Banana bunchy top disease caused by a banana bunchy top virus (BBTV, family *Nanoviridae*, genus *Babuvirus*) is one of the significant viruses affecting banana plants leading to a 100 % loss in yield and has been recorded in all countries cultivating banana across the globe. BBTD is transmitted by the aphid *Pentalonia nigronervosa*[11] in a persistent, circulative, non-propagative manner; the virus enters the haemocoel and the aphids retain the virus throughout their lives without passing it on to their offspring [7].

26 *Pentalonia nigronervosa* was first described by [3] from banana. The BBTV is an ssDNA virus
27 and comprises of six genome components and are termed as DNA-R, DNAU3, DNA-S,
28 DNA-M, DNA-C, and DNA-N [10]. Based on the DNA-R sequences, the BBTV isolates were
29 grouped into two: Asian and South Pacific groups [9]. The Asian group comprises of the
30 isolates from the Philippines, Taiwan, China, Japan, Indonesia, and Vietnam. The South
31 Pacific group includes India, Pakistan, Egypt, Australia, Burundi, Tonga, Myanmar, Fiji, and
32 USA-Hawaii and isolates from Africa [15].

Comment [ST1]: BY WHO? Include the name of the author

33 Earlier Polymerase Chain Reaction (PCR)-based method was employed for the detection of
34 BBTV infection in banana [6, 12]. Recent methods demonstrate the quantification of BBTV
35 through real-time PCR using SYBR Green or TaqMan chemistry [2, 5, 8 and 15]. Aphid
36 mediated transmission is a standard method for the BBTV virus infection in banana.
37 However, the BBTV transmission experiments require a suitable tool to monitor the virus
38 accumulation in banana plants. Real time PCR is a sensitive method to quantify the viral
39 load during the insect mediated transmission study in banana. [The real time PCR based
40 developed will be useful for the BBTV transmission study and also to characterize the
41 transgenic banana plants engineered for BBTV resistance.]

Comment [ST2]: Since the study had been done there is need to modify this statement

42 43 **2. MATERIAL AND METHODS**

44 45 **2.1 Source of plant material and virus inoculum**

46 BBTV infected samples of banana cv. Grand Naine were collected from the orchard of Tamil
47 Nadu Agricultural University, Coimbatore, Tamil Nadu and confirmed the presence of BBTV
48 infection through PCR. The tissue culture plants of banana cultivar Grand Naine obtained
49 from Sunglow Biotech, Coimbatore were used throughout the study. Healthy banana adult
50 aphids of *P. nigronervosa* were obtained from Dr. R. Selvarajan, NRCB, Trichy and
51 multiplied in the tissue culture banana plants of cv. Grand Naine. The new aphid population
52 was continuously obtained by culturing in new tissue culture plants.

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

59 **2.2 Isolation of BBTV coat protein gene**

60 The total DNA was extracted from BBTV infected leaf tissues of Grand Naine using the
61 CTAB method [4]. One hundred milligram of leaf tissues was homogenised with one ml of
62 CTAB buffer ([10 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2 % CTAB, 0.1 % (v/v) β -
63 Mercaptoethanol and 2 (v/w) %PVP] to isolate the total DNA. Then DNA was precipitated
64 with ice cold iso-propanol and washed with 70 % ethanol to remove the salts. The pellet
65 obtained was air-dried and dissolved with 100 μ l of sterile water and stored at -20 $^{\circ}$ C for
66 further analysis. The DNA samples were quantified using Nanodrop spectrophotometer ND-
67 1000. The DNA samples were resolved on a 1.0 % agarose gel.

68 BBTV coat protein specific forward primer (5' TCCGAAGAAATCCATCAAGA 3') and reverse
69 primer (5'CCAGAACTA CAATAGAATGCCAAA 3') were used for amplifying the coat protein
70 gene. PCR condition for amplification of Coat protein gene of BBTV was as follows: Initial
71 denaturation at 94 °C for 5 min followed by 35 cycles of Denaturation at 94 °C for 1 min, 60
72 °C annealing for 30 sec, extension at 72 °C for 1 min and final extension was given at 72 °C
73 for 5 min. PCR reactions were performed in Thermal Cycler (Eppendorf, Germany) in a final
74 volume of 20 µl. The PCR reaction mixture contained 1 µl of banana total DNA (50 ng/µl),
75 2.0 µl of 10X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl and 1.5 mM MgCl₂), 0.5 µl of
76 100 mM dNTPs, 1.0 µl each of 10 µM respective forward and reverse primers, 0.3 µl of 1
77 unit Taq DNA polymerase (TaKaRa Bio USA, Inc.) and 14.2 µl of sterile distilled water. The
78 PCR amplified product was resolved on 1% Agarose gel. The amplified products were
79 visualized on a UV transilluminator and documented in a gel documentation system
80 (Syngene, UK).

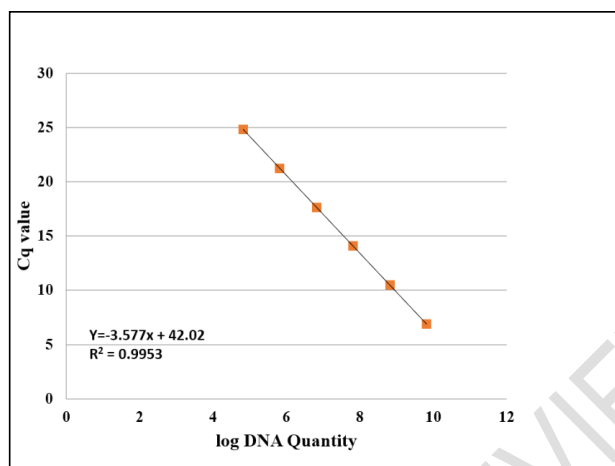
81 2.3 Cloning of partial BBTV Coat protein gene

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

89 2.4 Standard curve for absolute quantification BBTV DNA through qPCR assay

90 The purified 2.9 kb plasmid DNA containing a partial coat protein gene was used to prepare
91 a standard curve for absolute quantification of the BBTV DNA-S component as described by
92 [16]. The plasmid was serially diluted to obtain a standard series up to 10⁷ with each step
93 differing by 10- fold. The copy number of the DNA in each dilution was calculated with the
94 following formula $Y \text{ molecules} = X \text{ g}/\mu\text{l DNA} \times 6.022 \times 10^{23} / (\text{Base pair of recombinant plasmid} \times$
95 $660)$. qPCR was performed using these diluted DNA with three replicates. After the assay,
96 the values of threshold cycles (Cq) were obtained. A standard curve was established by
97 plotting the Cq value on the Y-axis and natural log of concentration (copies/µl) on the X-axis,
98 and a regression equation was obtained and coefficient of determination (R²) were achieved
99 (Fig. 2).

Comment [ST3]: Place the name of the author before the reference



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101 **Fig. 2. The standard curve obtained for BBTV DNA-S coat protein quantification. The**
 102 **X-axis represents the number of DNA copies, while the Y-axis represents the Cq**
 103 **value.**

104 **2.5 Quantification of viral load through qPCR assay in BBTV infected plants**

105 Banana plant DNA isolated from three symptomatic and non-symptomatic Grand Naine
 106 infected plants (120 dpi) and each sample was tested in triplicate using qPCR. DNA isolated
 107 from leaf tissue samples from wild-type virus-free Grand Naine plants were used as negative
 108 control and the cloned CP plasmid was used as a positive control. Equal quantity of banana
 109 genomic DNA (25 ng) was taken for the absolute quantification of BBTV by qPCR using
 110 SYBR green chemistry as described by [1] and reactions were carried out in a Biorad® CFX
 111 connect Real time PCR system (Biorad, USA). The designed Real-time PCR primers, BBTV-
 112 RT-CP-F(5'TCAACCAGCCGACAACCTGT3') and RT-CP-R
 113 (5'TGTCCCTGTTGCGACTCCTG3') were used to amplify 116 bp of coat protein gene of
 114 BBTV. The standard amplification profile was 94 °C for 4 min and 40 cycles of 94 °C for 15 s
 115 and 60 °C for 1 min and followed by a melt curve analysis was performed by allowing the
 116 reactions at 94 °C for 15 s and 60 °C for 1 min. The qPCR cycle provided the melt curve for
 117 each sample, for assessing the specificity of amplification. For each PCR reaction, the
 118 samples were taken in three replicates.

119 **3. RESULTS AND DISCUSSION**

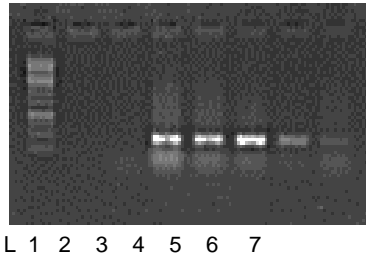
120 **3.1 Isolation of BBTV coat protein gene from infected banana samples**

121 Genomic DNA samples were isolated from BBTV infected Grand Naine banana collected
 122 from Coimbatore, Tamil Nadu. PCR amplification of partial length BBTV DNA-S was
 123 observed in all five samples of Grand Naine (Fig. 1).

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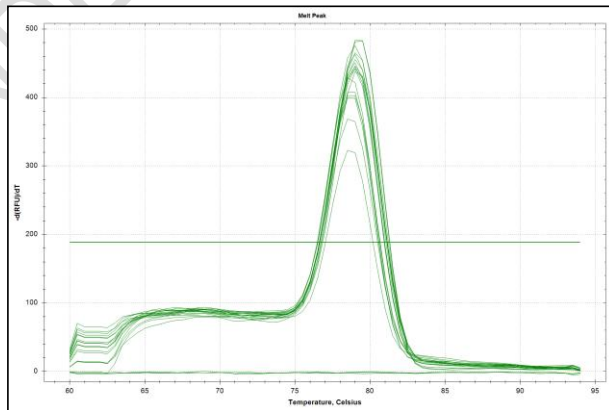
131 **Fig. 1. PCR detection of BBTV in Grand Naine banana leaf samples**
132 Lane L- 1 Kb ladder; 1 and 2- healthy Grand Naine samples; 3 to 7- BBTV infected Grand
133 Naine samples
134 **3.2 Cloning of BBTV coat protein gene**

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

Comment [ST4]: This should be under methodology

139 **3.3 Standard curve for quantification of BBTV DNA in the infected banana samples**

140 Absolute quantification of viral DNA is done using the standard curve made with the cloned
141 viral gene with known copy number. Melt curve analysis confirmed the presence of specific
142 real time PCR amplified product (116 bp). All the PCR products melt between 75 °C and
143 83.5 °C which indicates the breakdown of only one PCR product (Fig. 3). The Cq values
144 obtained for each dilution of the plasmid were used for regression analysis taking copy
145 number and Cq value. The mean squares of the coefficient of determination (R^2) value was
146 above 0.995 which indicates good linearity between the initial copy numbers and the
147 fluorescence values (Cq values) for obtaining a high-quality standard curve for absolute
148 quantification [16]. BBTV coat protein gene is an ideal target gene for the establishment of
149 standard curve. Earlier studies for BBTV DNA quantification also used the CP gene for
150 quantification [8].



151

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

155 **3.4 Viral titre in asymptomatic and symptomatic BBTV infected plants of cv. Grand**
 156 **Naine**

157 The results revealed that viral titre was higher in the symptomatic plant compared to non-
 158 symptomatic plants (Table 1). Equal quantity (25 ng/μl) of the total DNA of the banana
 159 samples were taken for real time PCR analysis to ensure that BBTV DNA. This was
 160 achieved by the quantification of DNA concentration in Nanodrop and diluted to get a DNA
 161 concentration of 25 ng per μl, followed by further confirmation in Nanodrop. Based on qPCR
 162 results, the viral load in symptomatic plants was higher compared to non- symptomatic. In
 163 the symptomatic plants, the viral load ranges from 1.4×10^8 to 7.7×10^7 , whereas in the
 164 non-symptomatic plants it ranges from 1.1×10^5 to 2.3×10^5 . Even though good correlation
 165 exists among the analyzed plants and disease symptoms, one plant in each show's
 166 deviation, which may be associated with an experimental error. Previous reports in BBTV
 167 DNA quantification also show real time PCR is an effective and simple method for
 168 quantification of viral load [2, 15]. It clearly proves that the symptomatic plants harbour more
 169 viral load which is the reason for symptom expression. The viral titre plays a major role in
 170 symptom expression [8] and the delay in symptom expression in non-symptomatic plants
 171 may be due to unequal transmission of aphids during the incidence [13]. The absolute
 172 amount of each BBTV DNA component were measured by real- time PCR [1,17]. qPCR
 173 results will invoke the importance of latency of BBTV in non symptomatic plants. For various
 174 studies on viral accumulation in plants, real time PCR provides a confirmation protocol to
 175 study the quantity of virus for transmission studies.

Comment [ST5]: No result to to prove this. Kindly show the result that back it up

Comment [ST6]: Not clear

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

Target gene	Sample type	Cq value	Cq SEM	Copy number of BBTV protein* coat
CP	Positive control	18.97	0.01790	2.7×10^6
CP	Negative control	25.81	0.06283	4.9×10^4
CP	Non- symptomatic -H1	24.03	0.81199	1.1×10^5
CP	Non- symptomatic -H2	23.66	0.21572	1.3×10^5
CP	Non- symptomatic -H3	22.79	0.35354	2.3×10^5
CP	Symptomatic - I1	13.79	0.08139	7.7×10^7
CP	Symptomatic - I2	15.33	0.14517	2.8×10^7

Comment [ST7]: A negative contro supposed not to have viral coat protein
 What do you mean by Positive and Negative control.

CP	Symptomatic - I3	12.78	0.07265	1.4 x10 ⁸
CP	NTC	0.00	0.00	0.00

178 *Copy numbers have been calculated using the formula.

179 Copy No, 1µl of the sample= $X \text{ g/}\mu\text{l DNA} \times 6.022 \times 10^{23}$

180 (base pair of recombinant plasmid x 660)

181 4. CONCLUSION

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

Comment [ST8]: Real time What?
 Kindly make it complete

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191 REFERENCES

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1. Bernard Ouma. Development of Banana bunchy top virus resistance in bananas: RNAi approach. Ph. D. thesis. Queensland University of Technology. 2016.
2. ChenY, Hu X. High-throughput detection of banana bunchy top virus in banana plants and aphids using real-time TaqMan® PCR. Journal of virological methods. 2013;193 (1):177-183.
3. Coquerel C. Observations entomologiques sur divers insectes recueillis a Madagascar. Annales de la Societe entomologiques de France. 1859; 3(7), 239-260.
4. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus. 1990; 12:13.
5. Fu HC, Hu JM, Hung TH, Su HJ, Yeh HH. Unusual events involved in Banana bunchy top virus strain evolution. Phytopathology. 2009;99 (7):812-822.
6. Furuya N, Somowiyarjo S, Natsuaki KT. Virus detection from local banana cultivars and the first molecular characterization of Banana bunchy top virus in Indonesia. J Agri Sci Tokyo. 2004; 49(3):75-81.
7. HuJS, Wang M, Sether D, Xie W, Leonhardt KW. Use of polymerase chain reaction (PCR) to study transmission of banana bunchy top virus by the banana aphid (*Pentalonia nigronervosa*). Annals of Applied Biology. 1996; 128(1): 55-64.
8. Jebakumar RM, Balasubramanian V, Selvarajan R. Virus titre determines the efficiency of *Pentalonia nigronervosa* (Aphididae: Hemiptera) to transmit banana bunchy top virus. Virus Disease. 2018; 29 (4):499-505.

- 214 9. Karan M, Harding RM, Dale JL. Evidence for two groups of banana bunchy top virus
215 isolates. *Journal of General Virology*. 1994; 75 (12):3541-3546.
- 216 10. King AMQ, Adams MJ, Lefkowitz EJ. Virus taxonomy: classification and nomenclature of
217 viruses: ninth report of the international committee on taxonomy of viruses. Springer, Wein.
218 2011.
- 219 11. Magee, C.J. Investigation on the bunchy top disease of bananas. *Bulletin of the Council*
220 *for Scientific and Industrial Research in Australia*. 1927; 30.
- 221 12. Mansoor S, Qazi J, Amin I, Khatri A, Khan IA, Raza S, et al. A PCR-based method, with
222 internal control, for the detection of Banana bunchy top virus in banana. *Molecular*
223 *biotechnology*. 2005; 30 (2):167-169.
- 224 13. Selvarajan R, Balasubramanian V, Anuradha C. Population Structure and Diversity of
225 Banana Bunchy Top Virus and Banana Bract Mosaic Virus. In *Plant Viruses*. 2018; 149-170.
226 CRC Press.
- 227 14. Stainton D, Martin DP, Muhire BM, Lolohea S, Halafihi MI, Lepoint P, et al. The global
228 distribution of Banana bunchy top virus reveals little evidence for frequent recent, human-
229 mediated long-distance dispersal events. *Virus evolution*. 2015;1 (1).
- 230 15. Watanabe S, Greenwell A, Bressan A. Localization, concentration, and transmission
231 efficiency of Banana bunchy top virus in four asexual lineages of *Pentalonia aphids*. *Viruses*
232 2013; 5 (2):758-776.
- 233 16. Xue B, Guo J, Que Y, Fu Z, Wu L, Xu L. Selection of suitable endogenous reference
234 genes for relative copy number detection in sugarcane. *International Journal of Molecular*
235 *Sciences*. 2014;15(5): 8846-8862.
- 236 17. Yu NT, Xie HM, Zhang YL, Wang JH, Xiong Z, Liu ZX. Independent modulation of
237 individual genomic component transcription and a cis-acting element related to high
238 transcriptional activity in a multipartite DNA virus. *BMC genomics*. 2019; 20: 573.