Virus titre determines the efficiency of *Pentalonia nigronervosa* (Aphididae: Hemiptera) to transmit banana bunchy top virus

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Abstract Banana bunchy top disease (BBTD) caused by banana bunchy top virus (BBTV) is one of the most serious viral diseases of banana and plantains. BBTV is transmitted by *Pentalonia nigronervosa* (Hemiptera, Aphididae) in a persistent circulative manner. Better knowledge of vector–virus–host relationship and the mechanism of transmission is essential for developing an effective control strategy. In this study, the viral copies in single to group of aphids with different acquisition access period (AAP) were quantified using SYBR green-based quantitative polymerase chain reaction (qPCR). The result indicated that a single aphid was able to acquire 861.04 copies of the virus after 24 h of AAP from the infected banana plant and transmitted the virus to 16.6% tissue culture plants, whereas 50 viruliferous aphids (15,066.94 viral copies) were necessary to achieve 100% transmission in a shortest time of 21.6 days. The number of viral copies acquired by the aphids were gradually increased with increased AAP. Hundred percent transmission was observed with 20 aphids in 48 h of AAP or 30–50 aphids in 24 h of AAP. The inoculated plants expressed typical bunchy top symptoms quickly when higher number of aphids (30 and above) were used with 24 h of AAP. Further, we report that the tissue culture banana plants are highly prone or vulnerable to BBTV infection compared to sucker grown plants. We conclude that higher the number of viral copies in the vector, higher the percent transmission and quicker the symptom

expression and the results will contribute to a better understanding of vector–BBTV interactions and useful for epidemiological studies.

Keywords Aphids · Banana bunchy top virus · qPCR assay · Viral titre

Introduction

Banana cultivation continues to face several pests and disease problems which affect the production and productivity constantly. Banana bunchy top disease (BBTD) caused by banana bunchy top virus (BBTV; Genus *Babuvirus*; Family *Nanoviridae*) is one of the major disease threatening the production in one quarter of the world's banana growing areas [8]. In 2008, 17.16 million plants of Cavendish banana were found affected with BBTV in Jalgaon district of Maharashtra and caused an economic loss of around US\$ 51 million [13]. Most of the plant viruses depend on biological vectors for dissemination, BBTV-a multipartite virus is dependent on banana black aphid *Pentalonia nigronervosa* (Family *Aphididae*; Order *Hemiptera*) for banana to-banana spread [4]. The viral load in the vector and the host is expected to play a crucial role in the spread of the persistently transmitted viruses like BBTV. Till date, studies on the transmission biology of BBTD in banana have been restricted to temperature, life stage of vector and inoculation feeding period [1, 5, 9, 15, 16]. Though weather factors might intervene in the vector survival and their fecundity [1, 9], the virus titre in the vector is expected to influence the rate of infection and quickness of disease establishment. The viral titre reported to influence the rate of transmission in case of *Lettuce mosaic virus*, *Plum pox potyvirus* and *Tobacco*

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mosaic virus [7, 10, 18]. However, there are no reports to expatiate the influence of BBTv titre in the vector on the transmission. So, it is pertinent to study about the viral load in the vector during transmission as these findings shall be helpful for understanding the host–virus interaction. In this present investigation, we have quantified the BBTv in the vector, *P. nigronervosa* with the aid of SYBR green based quantitative polymerase chain reaction (qPCR) assay and studied the influence of virus titre with transmission. We also report that the tissue culture (TC) plants are more susceptible than the suckers with the support of transmission and qPCR assay data.

Materials and methods

Source of virus inoculum, aphids and banana plants

The BBTv infected banana field samples were obtained from Chinnamanoor, Theni district, Tamilnadu, India during the growing season in 2015 and confirmed by polymerase chain reaction (PCR) as described by Selvarajan et al. [14]. Adult aphids of *P. nigronervosa* were collected as a single colony from a healthy field in the lower Pulney hill region, Tamilnadu, India and were transferred to virus free TC banana plants of cv. Grand Naine kept in an insect proof cage. A new progeny from this colony was used for further multiplication in TC banana plants. The aphid culture was maintained in insect-proof cages at 25 ± 2 °C and 80% relative humidity under a 16-h light/8-h dark photoperiod. The transmission studies were conducted in pot cultured plants kept in insect proof glass house. Sixty days old virus-free certified, hardened TC plants of cv. Grande Naine obtained from M/s Jain Irrigation Systems Limited, Jalgaon were used throughout the study.

Influence of different numbers of aphids on the transmission efficiency of BBTv

Same sized, 8–10 days old aphids (apterae) were transferred to BBTv-infected plant and kept in an insect proof chamber for a period of 24 h to acquire the virus. Then, one set of aphids were withdrawn with selective numbers viz., 1, 5, 10, 20, 30, 40 and 50 from BBTv infected plants and the total DNA from aphid/s were isolated based on a modified protocol of Selvarajan et al. [14]. Briefly, selective number of aphids were collected and frozen using liquid nitrogen and then macerated and powdered using sterile pestle and mortar. To this powder, 500 µl of CTAB extraction buffer (2% CTAB, 100 mM Tris.HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.1% (v/v) Mercaptoethanol) was added, mixed thoroughly and transferred the

homogenate to 1.5 ml tube. The sample was incubated at 55 °C in water bath, then 5 µl proteinase K was added to the sample and mixed by shaking in vortex. To this solution equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 5 min and the upper phase was collected. Finally, the precipitation was done by centrifugation with equal volume of isopropanol. The final pellet was washed with 70% ethanol and dried. The DNA pellet was dissolved in Tris EDTA buffer, quantified and tested for the presence of the virus using real time PCR [11]. The other set of viruliferous aphids with selective numbers viz., 1, 5, 10, 20, 30, 40 and 50 were allowed to feed on the healthy virus-free TC banana plants of cv. Grand Naine for a period of 48 h to transmit the virus at 28 ± 2 °C. After 48 h of transmission, the banana plants were sprayed with 0.02% imidacloprid to kill the aphids. One hundred milligram of tissues from each of the newly emerged leaves of all inoculated plants were collected at different point of time, stored at -80 °C and tested by standard PCR [14].

Determination of viral titre in aphids after different AAPs and transmission of BBTv with different AAPs

To find out the effects of viral titre in aphids with different acquisition access periods (AAP), same sized, 8–10 days old (apterae) adult aphids were allowed to feed on BBTv infected banana plant in a closed insect proof vector chamber and then 40 aphids (2 sets) were withdrawn each time at different time intervals viz, 0.15, 0.5, 0.75, 1, 2, 3, 4, 5, 24, and 48 h and the total DNA from one set of aphids was isolated. The viral titre in the aphids after allowing them for different AAP was analysed using qPCR and titre was calculated based on the absolute standard curve method [11]. Subsequently, another set of aphids with different AAP were transferred to 2 months old virus free TC banana plants of cv. Grand Naine with a fine tip brush for BBTv inoculation and the percent disease incidence was recorded at weekly intervals.

Polymerase chain reaction

Total DNA of banana leaf tissues was isolated using a method as described by Selvarajan et al. [14]. The positive control consisted of total DNA extracted from BBTv infected banana leaf tissue and for the negative control, total DNA was extracted from virus free tissue culture plants. PCR was conducted as described previously with a BBTv capsid protein (CP) gene primer pair that directs the amplification of a 513-bp DNA fragment [14].

Absolute quantification of BBTV titre using SYBR green based qPCR assay

The quantity and quality of the DNA was determined by microvolume spectrophotometer (Colibri; Berthold Technologies GmbH & Co. Germany). Absolute quantification of BBTV in aphid and plant leaf samples were analyzed by qPCR using SYBR Green chemistry as described by Bernardouma [11]. and reactions were carried out in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA). The BBTV-specific primers RSF01 (5' TATGGCAGCAAGGCGGCAAC 3') and RSR02 (5' TCCGGGCTTCACTTTGCACA 3') were used to amplify part of coat protein (CP) gene of BBTV with 164 bp size. The standard amplification profile was 94 °C for 4 min and 40 cycles of 94 °C for 15 s and 60 °C for 1 min and followed by a melt curve analysis was performed by allowing the reactions at 94 °C for 15 s and 60 °C for 1 min. The amplification product was quantified using the absolute method, with a standard curve prepared using a cloned 513-bp fragment of the BBTV-CP gene into the pGEM-T Easy vector (Promega Corp, USA) as a template in qPCR using SYBR green chemistry.

Assessing the rate of transmission of BBTV in TC and sucker grown plants

Banana suckers of cv. Grand Naine (AAA) weighing ~ 1.5 kg, were obtained from the research farm of ICAR-NRCB and the suckers were planted in plastic pots (43 cm * 35 cm * 23 cm) having red soil, sand, farm yard manure at 1:1:1 ratio. The experimental design was completely randomized block design with 12 replications with three suckers per replication. Simultaneously, 60 days old virus-free certified, TC plants of cv. Grande Naine were planted in plastic pots (43 cm * 35 cm * 23 cm) with same manure composition used for suckers. BBTV was transmitted using 30 viruliferous aphids after they were allowed to feed on BBTV infected plants for 24 h. After 48 h of inoculation feeding by aphids on the midrib/petioles of youngest leaf, the banana plants were sprayed with 0.02% imidacloprid to kill aphids. Non-viruliferous aphids fed plants were used as a control. The banana leaf samples were collected and stored at - 80 °C every 2 weeks after the transmission, DNA was isolated from the pooled samples of all individual replications and the viral copy number were quantified through qPCR assay as mentioned earlier. The number of days taken for the expression of typical BBTD symptom was recorded. The data obtained were statistically analysed using SPSS statistics tool version 20 [3].

Results

Efficiency of transmission with variable number of aphids

The efficiency of *P. nigronervosa* to transmit BBTV was evaluated using variable number of adult aphids in TC banana cv. Grand Naine. The percent BBTD incidence was increased in plants with increasing number of viruliferous aphids (Table 1). The disease incidence ranged from 16.6 to 100%. The disease incidence of 83.3% was observed in plants inoculated with 20 viruliferous aphids per plant. The highest rate of 100% BBTD incidence was observed in plants inoculated with 30, 40 and 50 viruliferous aphids with 24 h AAP. The time taken to express typical BBTD symptoms varied with number of aphids used for transmission. Single aphid transmitted the virus up to 16.6%, however, the plants expressed the symptoms 102 days after inoculation (DAI). The least time taken to express the symptoms was 21.6 days when 50 viruliferous aphids per plant was used for transmission (Table 1). The BBTD symptom expression was observed after 49.8 and 23.3 days in plants inoculated with 30 and 40 viruliferous aphids respectively. In qPCR, the lowest average Ct value was obtained from fifty aphids (24.71), followed by forty (25.97), thirty (26.41), twenty (26.56), ten (26.79) and five (27.01) aphids. The highest average Ct value of 28.76 was obtained from single aphid and aphids fed on healthy plants showed no viral amplification. The melt curve analysis performed after the run showed a single peak (data not shown). BBTV titre in aphids used in these experiments ranged from 861.04 to 15,066.94 copies per ng (Table 1). Single aphid could acquire 861.04 viral copies, whereas, a group of 30 and 40 aphids acquired 6922.45 and 7378.40 viral copies respectively. The maximum viral copies 15,066.94 were recorded in DNA isolated from 50 aphids. In general, time taken to express BBTV symptoms was longer in banana plants inoculated with less number of aphids.

Effect of AAPs and viral titre on the BBTV incidence

The absolute quantification (number of viral copies) of BBTV was quantified by real time PCR from a group of aphids (20 number) allowed to feed on a BBTV infected plant for different time periods are furnished in Table 2. After the shorter AAPs of 0.15 h and 0.30 h on the infected plant, the BBTV was not successfully transmitted to banana as evidenced from the non-expression of BBTV symptoms and the virus titre were 7.29 and 12.13 copies respectively. After a longer AAP of 48 h, 100% disease

Table 1 Absolute quantification of BBTV titre in distinct number of viruliferous aphids based on SYBR green real-time PCR and transmission rate

Number of aphids	Virus titer (viral copies $\pm$ SD)	C _T (mean $\pm$ SD)	BBTD incidence (%)	Time taken for BBTD symptom expression (days) (mean $\pm$ SD)
1	861.04 $\pm$ 207.04	28.76 $\pm$ 0.32	16.6 (5/30)	102.8 $\pm$ 20.06
5	2384.24 $\pm$ 164.89	27.01 $\pm$ 0.10	26.6 (8/30)	80.6 $\pm$ 16.07
10	3868.48 $\pm$ 937.75	26.79 $\pm$ 0.38	36.6 (11/30)	67.5 $\pm$ 7.01
20	4897.98 $\pm$ 736.48	26.56 $\pm$ 0.22	83.3 (25/30)	54.2 $\pm$ 6.76
30	6922.45 $\pm$ 1042.62	26.41 $\pm$ 1.03	100 (30/30)	49.8 $\pm$ 6.45
40	7378.40 $\pm$ 3839.18	25.97 $\pm$ 1.92	100 (30/30)	23.3 $\pm$ 3.62
50	15,066.94 $\pm$ 2280.54	24.71 $\pm$ 0.24	100 (30/30)	21.6 $\pm$ 3.87

For each transmission, 01–50 aphids (*Pentalonia nigronervosa*) were used, with a 24-h virus acquisition access period

Table 2 Absolute quantification of BBTV through SYBR green based qPCR assay for various acquisition access periods of aphids and transmission rate

AAP (h)	Virus titer (viral copies $\pm$ SD)	C _T (mean $\pm$ SD)	BBTD incidence (%)	Time taken for BBTD symptom expression (days) (mean $\pm$ SD)
0.15	7.29 $\pm$ 4.99	26.35 $\pm$ 1.46	0 (0/30)	0
0.30	12.13 $\pm$ 2.50	25.28 $\pm$ 0.29	0(0/30)	0
0.45	15.72 $\pm$ 2.51	24.91 $\pm$ 0.23	3(1/30)	112.0
1	110.53 $\pm$ 5.91	22.24 $\pm$ 0.07	6(2/30)	109.5 $\pm$ 9.19
2	937.74 $\pm$ 254.22	19.35 $\pm$ 0.36	13(4/30)	102.5 $\pm$ 10.66
3	1741.10 $\pm$ 669.60	18.54 $\pm$ 0.54	23 (7/30)	93.8 $\pm$ 9.09
4	2171.50 $\pm$ 1531.22	18.34 $\pm$ 1.38	26(8/30)	88.2 $\pm$ 7.55
5	2361.22 $\pm$ 468.36	18.08 $\pm$ 0.29	36(11/30)	73.5 $\pm$ 8.07
10	2867.74 $\pm$ 115.84	17.79 $\pm$ 0.05	63(19/30)	61.8 $\pm$ 5.51
24	4868.72 $\pm$ 2160.71	17.50 $\pm$ 0.96	86 (25/30)	54.6 $\pm$ 5.59
48	37,866.60 $\pm$ 18,182.94	14.36 $\pm$ 0.61	100 (30/30)	46.1 $\pm$ 8.73

For each transmission, 20 aphids (*Pentalonia nigronervosa*) were used

incidence was recorded, and the days taken to express the BBTD symptoms was 46. For 24 h AAP, 54 days were taken to express the BBTD symptoms in 86% of plants inoculated. Forty-five minutes (0.45 h) of AAP provided only 3% transmission among the inoculated plants and the time taken for symptom expression was 112 days. Differences in infection rates between different AAPs were also reflected in the quantitation analysis (Table 2). At the shortest period, the mean titre value was estimated to be 7.29 copies of BBTV whereas at longer periods the titre was 4868.72 copies (24 h AAP) and 37,866 copies (48 h of AAP) (Fig. 1a). Non-viruliferous aphids showed no amplification. The melt curve analysis performed after the run, showed a single peak with a melting temperature of 78.43 °C (Fig. 1b). When the CP gene of BBTV from the same samples were amplified by standard PCR [14], the virus could be detected only from 2 h of AAP (Fig. 2).

Differential reaction of TC and sucker grown plants to BBTV

Based on the results obtained on AAP and number of aphids together with virus titre, a transmission study was conducted to find if there are variation occur in the rate of transmission when different planting material viz., suckers and TC plants were used. The results of pot culture experiment are furnished with Table 3. In this experiment, we used 30 viruliferous aphids with 24 h of AAP for the transmission to obtain an expected 100% incidence of BBTV. As expected, we observed 100% incidence in TC plants, whereas in suckers the per cent infection was 44.4. The days taken for expression of typical BBTV symptoms were 33.16 $\pm$ 4.46 and 97.31 $\pm$ 15.61 in TC and sucker grown banana plants respectively. Sucker grown plants took longer time to express the disease symptoms compared to TC plants. The absolute number viral copies of

Fig. 1 **a** Standard curve based on virus copies from plasmid BBTVCP-pGEMT versus the number of cycles that was required to reach CT. The regression equation is $CT = -3.473 \times \log(\text{number of virus copy}) + 39.388$; red square in the graph indicates standard dilution's of BBTVCP-pGEMT and blue square represents virus titre

in 20 aphids with various AAPs (refer Table 2). **b** Melt peak chart showing the melt profile for qPCR amplicons of the coat protein gene of BBTV from all test samples (various AAP) with replications. Flat line indicates the negative control that failed to amplify (colour figure online)

Fig. 2 Agarose gel electrophoresis of conventional PCR products generated by BBTV-CP gene specific primers. Lanes-1, 1 kb marker DNA; 2–12, PCR products amplified using DNA from viruliferous aphids with different acquisition access periods (AAP); 13, virus free aphid DNA; 14, negative control; 15, positive control

Table 3 Differential reaction of TC and sucker grown plants to BBTV

Type of plants	No of plants infected	Percent incidence (mean $\pm$ SD)	Time taken for BBTD symptom expression (days) (mean $\pm$ SD)	Viral titre [(mean $\pm$ SD) $\times 10^5$ copies]
Tissue culture	36/36	100	33.16 $\pm$ 4.46	4.02 $\pm$ 0.22
Sucker	16/36	44.44 $\pm$ 16.41	97.31 $\pm$ 15.61	5.23 $\pm$ 0.31

SD standard deviation

For each transmission, 30 aphids (*Pentalonia nigronervosa*) were used

BBTV quantified by real time PCR at different weeks after the BBTV inoculation by transmission in TC and sucker grown plants are furnished in Fig. 3. The viral titre in TC plants 2 weeks after transmission was 4887.9 ± 1840.26 whereas, the viral titre after 4 weeks was $402,714.09 \pm 29,053.98$. In sucker grown plants, the virus could not be detected till the 6th week after transmission.

The viral titres in sucker grown plants recorded at 6th, 8th, 10th and 12th weeks after transmission were 1004.89 ± 131.02 , 5396.38 ± 1292.5 , 7143.98 ± 2112.44 , $13,178.58 \pm 2878.85$ copies respectively. The viral copies reached $523,091.72 \pm 31,842.32$ at 14 weeks after transmission coinciding expression of typical BBTV symptoms. The

Fig. 3 Comparison of viral titre in both tissue culture and sucker grown plants at different number of weeks after transmission with banana bunchy top virus by SYBR green based qPCR assay. In X-

axis, the different number of weeks after the transmission and in Y-axis, the viral titre values are plotted as copy number

melt curve analysis showed a single peak, which proved the presence of a single amplicon (data not shown).

Discussions

The biology of BBTB transmission has been studied for many decades. Since then many studies have examined to find out epidemiological factors such the temperature, vector life stage, plant access period and age of the hosts [1, 4, 5, 12, 16, 19] but the effects of viral titre in vector–host interactions and effective transmission have not been examined in previous studies. Hence, in this study, we examined the influence of BBTB viral titre during the vector–host interactions by qPCR assay and in pot culture for the expression of symptoms. Our results clearly demonstrated that the viral titre in vector influence the transmission of BBTB. AAP also impacted virus acquisition and transmission of BBTB to banana plants. On force inoculation of the virus by aphids, we could not get 100% incidence in inoculated plants with 20 aphids. This is may be due to less viral load acquired by the aphids in 24 h of AAP. It is likely that the virus titre within infected source tissues is also known to affect the quantity of virions acquired by aphids [6]. We have followed the standardised weather factors for transmission assay described by Almeida and Anhalt [1]. Interestingly, our results showed

15 min of AAP acquired 7.29 copies of BBTB from a group of 20 aphids. Though aphids could acquire the virus with 15 min of AAP, the efficient transmission occurred only when they were allowed to feed 48 h for acquisition access. This result is in conformity with the findings of Almeida and Anhalt [1] that longer AAP elevates the BBTB incidence in plants. The viral copies acquired in 24 h of AAP was 4868.72, and in 48 h of AAP, the titre was 37,866.60 which is eightfold higher than 24 h of AAP, this increase might be due to the longer period of acquisition [2, 8, 16]. So, the qPCR assay clearly explains that the viral titre acquired and transmitted by aphids significantly influence the rate of transmission in terms of per cent incidence. In our transmission studies, the plants expressed typical BBTB symptoms between 20 and 112 DAI. The time taken to express the symptom in virus inoculated plants varied and this might be due to the latency of virus inside the host. Latency (delay between host infection and the onset of disease) of BBTB is an unknown mechanism [15, 16]. The cause of latency in BBTB inoculated plants is not clearly understood, but may include temperature, pre-acquisition feeding, BBTB source plants and the viral titre in aphids. Our quantitative study clearly indicates that the concentration of BBTB accumulated in vector during acquisition determines the higher per cent infection in inoculated plants and the duration of symptom expression in transmitted plants which was

quicker when a higher number of aphids were used with longer duration of AAP. After optimising the AAP and number of aphids for BBTV inoculation using TC plants, we compared the efficiency of transmission of BBTV once again with sucker grown plants with that of TC plants. Interestingly the per cent infection of BBTV in sucker grown plants was much lesser than TC plants. The time taken for the typical BBTVD symptom expression in TC plants was significantly less than sucker grown plants. This differential transmission of BBTV could be due to the age of the plants. Our findings corroborated with the reports of Almeida and Anhalt [1] and Wu et al. [17] who have shown that the per cent transmission varied with the age of TC banana plants. The suckers are conventionally used for plantation establishment and TC plants are used especially for a ruling cultivar, i.e. Grande Naine for harvesting higher yields. The factors are not known why the suckers exhibit resistance over TC plants despite the high number of viruliferous aphids with higher titre of BBTV were used in the transmission. Our quantitative results explain that the virus was not detected up to 4th weeks after the viral inoculation in sucker grown plants, but it could be detected even in the 2nd week after the viral transmission in TC plants. This clearly explains the phenomena of latency of BBTV in banana as the plants were not showing any symptoms, but it carried a countable copy number of BBTV. It is evident based on our transmission study the TC banana plants are highly prone or susceptible to BBTV compared to sucker grown plants. In case of TC plants, we opine that the time taken for the movement of BBTV from the point of entry to the growing point of the plant (meristem tip) is lesser compared to suckers, hence it can be assumed that the TC banana plants are more susceptible or quick to get infected with BBTV. This quantitative study will contribute to a better understanding of epidemiological implications and designing the strategies for managing BBTV. This finding shall be useful for screening the *Musa* germplasm against BBTV which is more important for *Musa* genetic improvement and germplasm exchange.

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