Indian Phytopath. 63 (3) : 333-336 (2010)

Evaluation of immunological and molecular techniques for the detection of different isolates of *Banana bunchy top virus* in India

R. SELVARAJAN[1]*, V. BALASUBRAMANIAN[1], S. DAYAKAR[1], S. SATHAIMOORTHY[1] and Y.S. AHLAWAT[2]
[1]*Molecular Virology Lab, National Research Centre for Banana, Thogamalai Road, Thayanur Post, Tiruchiraplalli 620 102*
[2]*Advanced Centre for Plant Virology, Indian Agricultural Research Institute, New Delhi 110 012*

ABSTRACT: In this study, *Banana bunchy top virus* (BBTV) infecting hill banana cv. Virupakshi (AAB) was purified and raised polyclonal antiserum which had a titre of 1:500. Direct Antigen Coating (DAC)-ELISA, PCR and Nucleic Acid Spot Hybridization (NASH) with non-radioactive probe techniques were optimized and compared for detection of BBTV. In PCR, the virus could be detected at a dilution of 1:1000 whereas DAC-ELISA and NASH detected the virus at 50 and 500 dilutions respectively. Five BBTV isolates collected from various parts of India were tested by three methods, only PCR detected all the isolates. Primers specific to coat protein gene of BBTV was used for PCR and NASH tests. As part of validation, 417 samples collected from different banana growing regions of Tamil Nadu tested, DAC-ELISA detected the virus from 336 samples whereas 364 (87.29%) and 404 (96.8%) samples were positive in NASH and PCR respectively. When NASH test was performed for PCR products, three PCR negative samples tested positive. This is the first report that the coat protein gene specific primers have been used for detection of BBTV. The PCR and PCR-NASH combined techniques can be used for identifying virus-free plants in germplasm and routine indexing of BBTV in certification of tissue culture plants in India.

Key words: *Banana bunchy top virus*, detection, DAC-ELISA, PCR, NASH

Banana bunchy top disease (BBTD) is one of the most serious viral disease affecting banana world wide and cause great threat to banana cultivation in India (Dale, 1987). In lower pulney hills of Tamil Nadu state, a famous, unique flavoured elite dessert banana cultivar Virupakshi (Pome group AAB) has been almost destroyed by the BBTD and the area under this banana has been reduced from 18,000 ha to 2,000 ha (Kesavamoorthy, 1980). In Jalgaon district of Maharastra state, 17.16 million plants were affected with BBTV in 2008 and caused an economic loss of around US$ 51 million (unpublished).

The disease has spread to all banana growing states of India including North Eastern Hill States. BBTD is caused by *Banana bunchy top virus* (BBTV) belongs to the genus, *Babuvirus* and the family, *Nanoviridae*. BBTV is an isometric virus, 18-20 nm in diameter, with a genome comprising at least six circular, single-stranded DNA components (BBTV DNA 1-6), each with a size of approximately 1 Kb (Burns *et al*., 1995). BBTV is transmitted primarily through infected planting materials and secondary transmission by aphid vector (*Pentalonia nigronervosa*) in a persistent manner (Magee, 1927). Dale (1987) recommended a strategy for controlling BBTV based on identifying virus infected plants as early as possible followed by eradication of infected clumps and replanting virus free healthy planting material. In order to ensure virus-free suckers and tissue culture plants, there is a need to have sensitive detection methods. Many techniques such as ELISA (Wu and Su, 1990; Thomas and Dietzgen, 1991; Selvarajan *et al*., 2002) and PCR for detection of BBTV and other viruses (Xie and Hu, 1995; Shamloul *et al*., 1999; Selvarajan *et al*., 2008). Various nucleic acid based assays have been reported the detection of BBTV in plant tissue and viruliferous aphids including DNA and RNA probes labeled with nonradioactive or ^{32}P from other parts of the world (Xie and Hu, 1995; Harding *et al*., 2000). Though different detection techniques for BBTV have been reported, they were not validated for large scale virus indexing purpose. India, being the largest banana growing country, uses more than 50 million tissue culture banana plants every year to ensure those tissue culture plants free of BBTV, we need sensitive and reliable detection methods.

In this study, an attempt was made to purify the BBTV (Hill banana isolate) to raise polyclonal antiserum and develop serology based detection. The coat protein gene of BBTV has been cloned and sequenced previously by us has been used to develop PCR and NASH (non-radioactive) based detection. Further we have compared and simultaneously validated the techniques with field samples.

MATERIALS AND METHODS

Source of plant materials and vectors

Suckers of banana plants showing characteristic bunchy top symptom were collected from lower Puleny hills, Western Ghats, Tamil Nadu and different banana growing regions of India (Table 2) were planted in pots and maintained in insect proof glass house at National Research Centre for Banana, Tiruchirapalli or the symptomatic leaf samples collected were frozen and stored at -86 °C. Healthy tissue cultured

*Corresponding author: selvarajanr@gmail.com

banana plantlets with two to three leaves were inoculated with viruliferous aphids and the plants exhibited typical symptoms were used for virus purification.

Virus purification and antiseraum production

BBTV infected young leaf tissues along with midrib was processed as per the method described by Thomas and Dietzgen (1991). Purified virus (200µg) was emulsified with an equal volume of Freund's incomplete adjuvant and three intramuscular injections administered at weekly intervals into the rear flank of a 10 week old New Zealand white rabbit. Blood taken 10 days after the last injection was used throughout. The antiserum titre was estimated in DAC-ELISA.

Direct antigen coating enzyme linked immunosorbant assay (DAC-ELISA)

DAC-ELISA was performed in 96 well polystyrene microtitre plate (Tarson, India) as per the protocol described by Clark and Bar-Joseph (1984). Infected and healthy leaf tissues were triturated at 1:10 dilution in carbonate buffer (pH 9.6). The extract were centrifuged at 5000 rpm for 5 min and the resulting supernatant (200 µl) was added into three wells per sample and incubated for 1 h at 37 °C. Polyclonal rabbit antiserum produced in this study was used at 1:500 dilutions. Cross absorption with healthy tissue was done to remove background. Antirabbit immunoglobulin- alkaline phosphatase conjugate (Sigma, USA) was used as secondary antibody. Absorption values at 405 nm were recorded using a micro plate reader (Asys Hitech, Austria), 1h after adding the *P*- nitro phenyl phosphate (0.5 mgl^{-1}, of substrate buffer). ELISA readings were considered positive when the absorbance of sample wells was at least three times greater than the mean absorbance reading of healthy controls.

PCR amplification

Total DNA was isolated from infected samples following the protocol (Selvarajan *et al*., 2008). This DNA preparation was used as template for PCR and NASH. The primers were designed from the previously cloned coat protein gene sequence of BBTV- Hill banana isolate (Accession no. AY534140). The PCR reaction mixture in a volume of 50 µl contained 5.0 µl of 10X PCR buffer with 15mM $MgCl_2$, 4.0 µl of 10mM dNTP's, 1.0 µl (100ng/µl) each of BBTV forward (5'ATGGCTAGGTATCCGAAGAAATCC3') and reverse primer (5'TCAAACATGATATGTAATTCTGTTC3'), 1.5 units of *Taq* DNA polymerase (Genei, Bangalore, India) and 2.0 µl of total DNA. The PCR was performed in Master Cycler gradient PCR machine (Eppendorf, Germany) with initial denaturation at 94°C for 4 min was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 49-53 °C for 1 min and extension at 72°C for 2 min with a final extension at 72°C for 10 min. 10 µl of amplified products were loaded in 1% agarose gel and electrophoresed. The gels were visualized and photographed in Alpha Imager (Alpha Inotech Corp, USA).

Nucleic acid spot hybridization

Digoxigenin-labelled DNA probe for BBTV coat protein gene was generated using PCR-DIG probe synthesis kit (or) DIG high prime labeling kit (Roche Diagnostic, Germany) as per manufacturer instructions. The probe was prepared using BBTV DNA which was obtained by PCR of DNA fragment cloned previously (database accession no: AY534140). For NASH, approximately 2.0 µl of total DNA extracted from samples and PCR amplified products were spotted on positively charged nylon membrane (Sigma, USA) using either hand or dot blot apparatus (Bio-Rad). After spotting, the membranes were dried and either baked at 80°C for 2 h or UV cross linked for 3 min (Constart, USA**)** and pre hybridization and hybridization was done at 68°C for 1 h and 18 h respectively. Hybridized blots were washed in 2X SSC containing 0.1% SDS twice for 5 min each time and then 0.5X SSC twice at 68°C for 15 min each time. Hybridization and detection were carried out using DIG luminescent detection kit for nucleic acids (Roche Diagnostic, Germany) according to manufacture's instructions.

RESULTS AND DISCUSSION

In this study first we evaluated the sensitivity of DAC-ELISA, PCR and NASH using young leaf samples of BBTV infected banana cv. Virupakshi (AAB, Pome). Serial dilutions of BBTV infected leaf sap was made with healthy banana sap extract and tested using each assay. In case of DAC-ELISA, the virus was detected with 50 time dilution whereas NASH and PCR technique detected the virus at 500 and 1000 dilutions respectively (Table 1). The result is in harmony with that of other investigators who reported that the PCR was 1000 times more sensitive than dot blot and ELISA assays for detection of BBTV in banana (Xie and Hu, 1995). In another experiment, seven BBTV isolates collected from five different states of India were grouped into five strains based on symptoms and tested by three methods *viz*., DAC-ELISA, PCR and NASH. The results are presented in

Table 1 Comparison of the sensitivity limits of DAC-ELISA, NASH and PCR for the detection of BBTV

	Healthy [a]	Undiluted	Dilution[b]						
			1/10	1/20	1/50	1/100	1/500[b]	1/1000	1/2000
ELISA	-	++	++	+	+	-	-	-	-
NASH	-	++	++	+	+	+	+	-	-
PCR	-	++	++	++	++	+	+	+	-

[a]Healthy
[b]serial dilutions were prepared by mixing BBTV infected sap with healthy sap
-Negative; +, Positive; + +, strong positive reaction.

Table 2. Comparison of DAC-ELISA, NASH and PCR techniques for detection of BBTV isolates collected from different states of India

Isolates[1]	Variety with sub group and ploidy	Symptoms[2]	ELISA* reading at 405 nm	NASH	PCR
PB	Alpon (Mysore, AAB)	VC,GS,BT,SST	+(1.079)	+	+
THTN	Virupakshi (Pome, AAB)	VC,GS,BT,SST	+(1.158)	+	+
KA	Amrithpani(Silk, AAB)	VC,GS,BT	-(0.155)	+	+
KHTN	Namaran (Pome, AAB)	VC,GS,BT	-(0.129)	+	+
BO	Monthan(ABB)	Mild vein clearing	-(0.104)	-	+
JM	Grand Naine (Cavendish,AAA)	Yellowing of emerging leaf	-(0.098)	-	+
TTN	*Musa laterita*	VC,BT,ST	+(1.054)	+	+
	Negative control		-(0.086)	-	-
	Positive control		+(1.546)	+	+

[1]PB: Pusa, Bihar; THTN: Thadiankudisai, Tamil Nadu; KA: Kovur, Andhra Pradesh; KHTN: Kolli Hills, Tamil Nadu; BO: Bhuvaneswar, Orissa; JM: Jalagon, Maharastra; TTN; Trichy, Tamil Nadu.

[2]VC- Vein Clearing; GC- Green Streak; BT- Bunchy Top; ST- Stunting; SST- Severe Stunting.

*Negative ELISA values ranged from 0.086 to 0.155 whereas positive values ranged from 1.054-1.546. Figures in the parenthesis are absorbance values of ELISA results. Average of three reading at 405 nm, 1h after substrate addition.-, Negative: + positive. Minimum of three replicates were used in PCR and NASH.

Table 2. DAC-ELISA, detected the virus only from three isolates having severe symptoms and in NASH test, two isolates were negative whereas PCR detected the virus from all the isolates with an expected product of 513 bp (Fig. 1). Two of the samples exhibiting vein clearing (Orissa isolate) and yellowing of newly emerging leaf (Maharastra isolate) were also positive in PCR but not in DAC-ELISA and NASH. The healthy banana sample tested negative in all three assays. Su *et al*. (2003) have also grouped six Asian BBTV isolates into five strains based on the symptoms induced upon BBTV inoculation and by PCR with three designed primer pairs. Detection of BBTV by PCR is reliable and sensitive than probe based detection using replicase gene of DNA component –R (Harding *et al*., 2000; Hu *et al*., 1996).

As part of validation, all three techniques were used to test the field samples collected during survey from 15 banana growing regions of Tamil Nadu. The results are furnished in Table 3. DAC-ELISA technique detected the virus in 336 samples of 417 tested whereas 87.29 and 96.8 percent samples were positive in NASH and PCR respectively. The result showed that PCR technique was more sensitive and reliable for detection of BBTV than DAC-ELISA and NASH. PCR based detection is reportedly several orders of magnitude more sensitive than immunological based tests (Randles *et al*., 1996). DAC-ELISA is convenient but limited in detection sensitivity, especially for BBTV which normally occur at low concentration (Harding *et al*., 2000). In the present study the PCR amplification was done for *cp* gene of BBTV which is part of DNA component- S, a highly conserved sequence in both South Pacific and Asian group of isolates (Wanitchakorn *et al*., 2000). This is the first report that the cp gene primers have been used for detection of geographically different isolates of BBTV. Replicase gene targeted "PCR" based detection of BBTV has been used by several workers (Hu *et al*., 1996; Harding *et al*., 2000; Lavakumar *et al*., 2008). However none of the tests were validated with field samples for routine indexing of BBTV. We have also tested the *rep* gene primers which were not giving consistent results in detection (data not shown).

In order to improve the detection, we combined PCR and NASH for nineteen BBTV infection suspected samples collected from Yercadu, Shevroy hills, Tamil Nadu and compared with the result of PCR and NASH alone. In PCR, 13 samples were positive and only 10 were positive with NASH (DNA extract from infected samples were spotted) whereas 16 samples were positive when PCR products

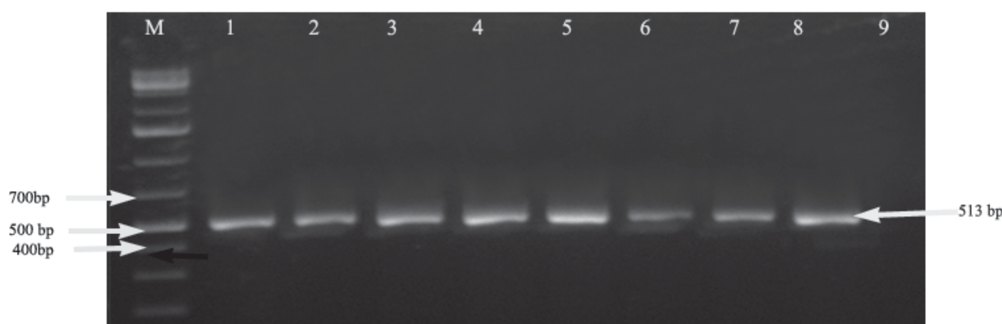

Fig. 1. Agarose gel electrophoresis of PCR amplified products from banana samples collected from different places tested with BBTV coat protein gene specific primers. Lanes : 1,Alpon (Pusa, Bihar); 2,Amrithpani (Kovur, Andhra Pradesh); 3,Monthan (Bhuvaneshwar, Orissa); 4, Grand Naine (Jalgaon, Maharastra); 5,Namaran (Kolli hills, Tamil Nadu(TN)); 6, *Musa laterita* (Trichy, TN); 7,Virupakshi (Thadiankudisai, TN);8, Positive control; 9, Negative control; M, Marker (1kb ladder)

Table 3. Detection of BBTV from surveyed samples using DAC-ELISA, PCR and NASH

Name of the locations*	No. of samples tested	No. of positive samples		
		DAC-ELISA	NASH	PCR
Thadiankudisai	37	28	30	34
Kolli hills	25	20	22	25
Sirumalai	33	28	30	32
Thirukattupalli	18	15	16	17
Perumparai	22	16	18	22
KC patti	35	29	32	34
Thandikudi	29	23	24	27
Puthoor	32	27	29	32
Agasthiar Puram	26	22	23	26
Nadukauveri	38	32	34	35
Kandamangalam	24	20	21	24
Mahadhanapuram	28	23	25	28
Krishnarayapuram	21	15	17	21
Thirukampliyur	17	13	15	16
Yercadu	32	25	28	31
Total number of samples	417	336	364	404
Percentage		80.57	87.29	96.8

*All the areas are located in Tamil Nadu state of India, known for more BBTV incidence

were spotted onto membrane and detected using DIG labeled probe, the samples negative in PCR or NASH became positive in NASH test when PCR products were used instead of DNA extracted from samples (Fig. 2A, B). The result showed that combination of PCR and NASH was better than either PCR or NASH alone. Our results corroborated with finding of Harding *et al.* (2000), however they used radioactive probe for detecting PCR products of BBTV. The high sensitivity of dot blot in detection of PCR product may be due to trapping most of the amplified nucleic acid in the spots onto the nylon membrane. Non radio active probes are more economical, safer, consumes short time and multiple reuse of hybridization probe make this method very cheaper than the nested PCR technique.

The results obtained in this study suggest that the PCR and combination of PCR and NASH are more reliable and useful for production of virus free tissue cultured plantlets, epidemiological studies, quarantine departments, germplasm screening and certification programmes.

ACKNOWLEDGEMENTS

The authors wish to express sincere thanks to the Director, NRC for Banana for the facilities and encouragement. Financial support from ICAR, New Delhi in the form of NATP Project and Netwok project on Diagnostics of emerging plant viruses is greatfully acknowledged.

REFERENCES

Burns, T.M., Harding, R.M. and **Dale, J.L.** (1995). The genome organization of *Banana bunchy top virus*: analysis of six ssDNA components. *J. Gen. Virol.* **76**: 1471-1482.

Clark, M.F. and **Bar-Joseph, M.** (1984). Enzyme immunosorbent assays in plant virology. In *Methods in Virology* (Eds. Maramorosch, K. and Koprowski) Vol. VII, pp. 51-85. Academic Press, New York USA.

Dale, J.L. (1987). Banana bunchy top: an economically important tropical plant virus disease. *Adv. Virus Res.* **33**: 301-325.

Harding, R.M., Sadik, A.S., Bahieldin, A. and **Dale, J.L.** (2000). A sensitive detection of *Banana bunchy top nanovirus* using molecular genetic approaches. *Arabian J. Biotech.* **3**: 103-114.

Hu, J.S., Wang, M., Sether, D., Xie, W. and **Leonhardt, K.W.** (1996). Use of polymerase chain reaction (PCR) to study transmission of *Banana bunchy top virus* by the banana aphid (*Pentalonia nigronervosa*). *Annals Appl. Biol.* **128**: 55-64.

Kesavamoorthy, R.C. (1980). Radical changes in ecosystem in the Pulney hills. *Proc eedings of National Seminar on Banana production Technology*, TNAU, Coimbatore, P. 23-28.

Lava Kumar, P., Ayodele, M., Oben, T.T., Mahungu, N.M., Beed, F., Coyne, D., Londa, L., Mutunda, M.P., Kiala, D. and **Maruthi, M.N.** (2008). First report of *Banana bunchy top virus* in banana and plantain (*Musa* spp) in Angola. *New disease reports* 18.

Magee, C.J.P. (1927). Investigation on the bunchy top disease of the banana. Council for Scientific and Industrial Research, Bulletin, **30**: 1-64

Randles, J.W., Hodgson, R.A.J. and **Wefels, E.** (1996). The rapid and sensitive detection of plant pathogens by molecular methods. *Australasian Plant Pathology* **25**: 71-85.

Selvarajan, R., Sathiamoorthy, S., Dayakar, S., Balasubramanian, V., Viswanathan, R. and **Ahlawat, Y.S.** (2002). Purification and detection of an Indian isolate of *Banana bunchy top virus* (BBTV). Paper presented at the 54th *National symposium on "Crop protection and WTO-An Indian Perspective*". CPCRI, Kasaragod. P 30.

Selvarajan, R., Balasubramanian, V., Kavitha, K., Kavitha, K.S, Sathiamoorthy S, and **Ahlawat, Y.S.** (2008). Detection of *Banana bunchy top virus* and *Banana streak Mysore virus* by PCR: Impact of storing virus infected banana samples. *Indian J. Virol.* **19**: 28-33.

Shamloul, A.M., Hadidi, A., Madkour, M.A. and **Makkouk, K.M.** (1999). Sensitive detection of *Banana bunchy top virus* from infected leaves, tissue cultures and viruliferous aphids using polymerase chain reaction. *Can. J. Pl. Path.* **21**: 326-337.

Su, H.J., Tsao, L.Y., Wu, M.L. and **Hung, T.H.** (2003). Biological and molecular categorization of strains of *Banana bunchy top virus*. *J. Phytopath.* **151:** 290-296.

Thomas, J.E. and **Dietzgen, R.G.** (1991). Purification, characterization and serological detection of virus-like particles associated with *Banana bunchy top virus* in Australia. *J. Gen. Virol.* **72**: 217-224.

Wanitchakorn, R., Harding, R.M. and **Dale, J.L.** (2000). Sequence variability in the coat protein gene of two groups of *Banana bunchy* top isolates. *Arch. Virol.* **145**: 593-602.

Wu, R.Y. and **Su, H.J.** (1990). Production of monoclonal antibodies against *Banana bunchy top virus* and their use in enzyme-linked immunosorbent assay. *J. Phytopath.* **128**: 203-208.

Xie, W.S. and **Hu, J.S.** (1995). Molecular cloning, sequence analysis and detection of *Banana bunchy top virus* in Hawaii. *Phytopathology* **85**: 339-347.

Received for publication February 10, 2010