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DETECTION OF BANANA BUNCHY TOP VIRUS IN VIRUS-INFECTED PLANTS USING POLYMERASE CHAIN REACTION

Sadik, A.S.[1,2,3], El-Afifi Sohair I.[1], Harding, R.M.[2], Dale, J.L.[2] and Allam, E.K.[1]

[1]Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt, [2]Centre for Molecular Biotechnology, School of life Science, Queensland University of Technology, Brisbane, QLD, Australia, [3]Department of Biology, Faculty of Science, Taif University, P.O. Box 888, Taif, Kingdom of Saudi Arabia

ABSTRACT

Polymerase chain reaction (PCR) was developed for the detection of *Banana bunchy top virus* (BBTV) at maximum after 210 min and at minimum after 90 min using Pc-1 and Pc-2, respectively. PCR detection of BBTV in crude sap indicated that the freezing of banana tissue in liquid nitrogen (LN_2) before extraction was more effective than using sand as the extraction technique. BBTV was also detected using PCR assay in 69 healthy and diseased plants using Na-PO_4 buffer containing 1 % SDS. PCR detection of BBTV in nucleic acid extracts using seven different extraction buffers to adapt the use of PCR in routine detection in the field was studied. Results proved that BBTV was detected with high sensitivity in nucleic acid extracts more than in infectious sap. The results also suggested the common aetiology for the BBTV by the PCR reactions of BBTV in nucleic acid extracts from Australia, Burundi, Egypt, France, Gabon, Philippines and Taiwan. Results also proved a positive relation between the Egyptian-BBTV isolate and abaca bunchy top isolate from the Philippines, but there no relation was found with the *Cucumber mosaic cucumovirus* (CMV) isolates from Egypt and Philippines and *Banana bract mosaic virus* (BBMV) were found.

Key words: BBTV, Polymerase chain reaction, Infectious sap, Cucumber mosaic cucumovirus, Banana bract mosaic virus (BBMV).

INTRODUCTION

The banana is one of the most important crops in Egypt and all over the world. It is grown in all types of tropical agricultural systems from small, mixed, subsistence gardens to very large company owned monoculture, and for export trade in banana (Dale, 1987, Othman *et al*., 1996; Harish *et al*., 2008, Oben *et al*., 2009 and Lokossou *et al*., 2011).

BBTV is the most serious virus affecting bananas (*Musa* sp.). The disease was first reported in Fiji Island as early as 1889 and by 1895 was widespread and almost destroyed the banana industry of the island. Since then the disease has been described in Australia, Asia, India, the Pacific Islands and a few countries in Africa: Egypt, Gabon, and Congo. But it has not been found in the tropics of America. The disease is caused by systemic infection of the plant by virus-like pathogen and transmitted by the banana aphid (*Pentalonia nigronervosa* Coq) (Sadik and Gad El-Karim, 1997, Abdelsalam *et al*., 2012). BBTV was originally classified as a member of a Luteovirus group based on the biological properties of the disease (Dale, 1987). On the other hand, recently, some investigators purified 18-20 nm isometric virus-like particles (VLPs) from infected banana plants and contained ssDNA (Harding *et al*., 1991; Burns *et al*., 1995; Hafner *et al*.,

1997; Sadik *et al*., 1999, Beetham *et al*., 1999, Nour El-Din *et al*., 2005, Khalil *et al*., 2007 and Nour El-Din *et al*., 2011). Now, these new particles are assumed to be the particles of BBTV.

Polymerase chain reaction (PCR) was described which allows the amplification of very low amounts of target nucleic acids (Saiki *et al.*, 1985). This technique has been used successfully to detect very low amounts of nucleic acids (Larzul *et al*., 1988, Dangler *et al.*, 1990, Wetzel *et al*., 1991, Harding *et al*., 2000, Mansoor et al., 2005, Lokossou *et al*., 2011).

In the light of the above findings, this study was proposed for developing and applying of PCR for BBTV detection. The relationship between the Egyptian isolate of BBTV and other strains and/or viruses affecting banana was also aimed.

MATERIALS AND METHODS

Virus purification: The method used by Wu and Su (1990) with further adaptations by Thomas and Dietzgen (1991), with even further adaptation by Harding *et al.* (1991) was used for purification of BBTV from BBTD-infected one kilogram of banana midrib tissue collected from Nambour region of south-east Queensland.

Development and application of PCR: Primer pairs: Four different primer pairs varying from 17 to 30 nucleotides in length were designed and synthesized in the Centre for Molecular Biotechnology and used for PCR (Table-1)

Table-1: Oligonucleotide primers used for PCR detection of BBTV from purified virus particles.

Primer pairs	Primers used	Nucleotide sequences (5' ----------3')	AT (^oC)
Pc-1	P1 (R)	GGA AGA AGC CTC TCA TCT GCT TCA GAG AGC	
	P2 (F)	GAA CAA GTA ATG ACT TT	40
Pc-2	P3 (R)	CGC CAT GAT ATT CTC CAC CTC TGA TGT CCA	
	P4 (F)	CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA	60
Pc-3	P5 (F)	TAC AGG ATA TGC GTG AA	
	P6 (R)	AGC AAG AAA CCA ACT TT	40
Pc-4	P4 (F)	CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA	
	P6 (R)	AGC AAG AAA CCA ACT TT	60

F: Forward. R: Reverse. AT: Annealing Temperature.

PCR of purified BBTV: The virus was purified using the procedure described by Harding *et al.* (1991) as mentioned before. For each primer pair, four different programs were used (A, B, C and D). The original program according to Harding *et al.* (1993), that was called A included denaturation at 94^{o}C/4 minutes for one cycle, followed by denaturation at 94^{o}C/1 minute, annealing at 40^{o}C for Pc-1 or 60^{o}C for Pc-2/1 minute and extension at 72^{o}C/1 minute for 30 cycles and finally extension at 72^{o}C/10 minutes for one cycle. Those programs B, C, and D were different in the reduction of the time of the 30 cycles from 1 minute for each temperature to 30, 15 and 10 seconds, respectively and the final extension cycle from 10 minutes to 2 minutes. The reaction was conducted in a volume of 50 µl PCR reaction as follows: 27 µl d.H_2O, 4 µl Primer (R) (5 pmol/5 µl), 4 µl Primer (F) (5 pmol/5 µl), 5 µl 10X PCR buffer, 8 µl dNTPs (1.25 mM), 1 µl template (10^{-1} purified virus), 1 µl *Taq*

DNA polymerase (1 unit) and then covered with equal volume of PCR oil.

The PCR product was checked by loading 5 µl from each reaction plus 1 µl dye in 1% agarose gel in TAE and running the gel at 70-100 V for 1 hour following by staining with ethidium bromide for 10-15 minutes and distinguishing the bands by visualising the gel under the UV light illuminator. All these reactions were replicated twice.

PCR of BBTV in crude sap: The aim was to develop and apply the use of PCR system for the detection of BBTV in Egypt. Two different extraction techniques based on using the sand and liquid nitrogen (LN_2) for grinding the banana tissues were applied.

For preparing the diluted crude sap using sand, 1 g of the healthy and diseased banana midrib tissue was ground with sand in the presence of 2 ml extraction buffer (EB) e.g. d.H_2O; EB-1; EB-2 and EB-4. Then 500 µl were transferred to a microfuge tube (1.5 ml eppendrof tube) and centrifuged at 4^oC for 10 minutes. The supernatant was stored in a new tube at 4^oC. For extracting the diluted crude sap using LN_2, only 0.2 g banana tissue were cut into fine pieces, then placed into a sterile 1.5 ml eppendrof tube. The tissue was then frozen using LN_2 and then ground into a fine powder. It was then allowed to thaw and 500 µl extraction buffer (EB) e.g. d.H_2O; EB-1; EB-2 and EB-4 was then added. The tube was then vortexed for 1 minute followed by centrifugation at 4^oC for 10 minutes. The supernatant was stored in a fresh tube at 4^oC. For PCR detection, 1 µl from the diluted crude sap, 10^{-1}, 10^{-2} and 10^{-3} dilutions were used as template and PCR reaction was achieved as described before, using Pc-4 and program B.

The PCR detection of BBTV in the diluted crude sap from health and diseased banana plants was applied using two sets of health (37 plants) and BBTV-infected (32 plants) using the four different primer pairs (Pc-1, Pc-2, Pc-3 and Pc-4). The diluted crude saps were prepared using the LN_2 and Na-PO_4 buffer containing 1% SDS. 1 µl of 10^{-2} was used as template and the PCR reaction using program B was achieved as mentioned before.

PCR of BBTV-DNA in nucleic acid extracts: The nucleic acid of purified virus was extracted using 2 µl of purified virus and 18 µl of Na-PO_4 buffer (0.07 M, pH 7.2 and containing 1% SDS). Seven different EBs, *i.e.*, d.H_2O; 1% SDS; Tris-HCl (pH 8.3); Tris-HCl containing 1% SDS; Tris-HCl, 1%SDS, 0.1% PVP; Na-PO_4 (pH 7.2) and Na-PO_4, 1% SDS were suggested by Dale (1987) to adapt the PCR technique for the detection of BBTV under routine detection in the field. Extracts were prepared according to Harding *et al.*, (1991). For PCR detection, 1 µl of crude, 10^{-1}, 10^{-2}, and 10^{-3} of these extracts were used as template. The PCR reaction was carried out using Pc-2 and program B as mentioned before.

The PCR detection of BBTV-DNA in nucleic acid extracts was successfully applied for routine detection of BBTV-DNA in the field. Extracts of nucleic acid from 13 health and 36 banana plants infected with BBTV were prepared using Na-PO_4 containing 1% SDS based on the method of Salama *et al.*, (2007) and PCR reactions of crude, 10^{-1}, 10^{-2} and 10^{-3} (for each plant), were conducted using Pc-2 and program B as described above.

Detection of Egyptian BBTV isolate and its relation to overseas isolates: Nucleic acid extracts from 13 overseas isolates from Australia (Q1); Burundi (B1, B2 and B3); Egypt (E1); France (F1 and F2); Gabon (G1); the Philippines (A1 and P1) and finally from Taiwan (T1 and T2) were collected from the glasshouse to study the

relationship between the Egyptian isolate and other overseas isolates using the PCR. The crude sap and only two dilutions (10^{-1} and 10^{-2}) were used as template for the PCR reactions as described before using Pc-3 and program B. It must be mentioned that the isolates had been provided to us as nucleic acid extracts.

The relationship between Egyptian-BBTV and other bananas viruses: Extracts of nucleic acid of banana-CMV isolate and banana bract mosaic virus (BBMV) isolate occurring in the Philippines were provided by glasshouse culture. But Egyptian BBTV and CMV-infected plants and abaca bunchy top virus (AbBTV)-infected plants were extracted using Na-PO_4 (0.07 M, pH 7.2 containing 1% SDS) to obtain the nucleic acid as described before. For PCR detection, crude, 10^{-1}, and 10^{-2} dilutions were prepared and used as template for PCR assay that was performed using Pc-3 and program B as explained before.

RESULTS AND DISCUSSION

PCR as an enzymatic procedure was used successfully to detect very low amounts of nucleic acid belonging to several plant viruses with high sensitivity and specificity (Nicolas and Laliberte, 1991 and Harding *et al.*, 1993, Harding *et al*., 2000, Mansoor *et al*., 2005).

PCR of purified BBTV: BBTV purified according to Harding *et al.*, (1991) and occurred in Na-PO_4 buffer (0.07 M, pH 7.2) was used. Isometric virus particles with a diameter of 20 nm and a density of 1.28 to 1.30 g/ml in caesium sulphate gradients were purified from banana infected with BBTV (Figure-1).

Two different primer combinations (Pc-1 and Pc-2) and four different programs (A, B, C and D) as described in materials and methods were used. Results in Figure-2 showed that BBTV could be detected using four primer combinations (Pc-1, Pc-2, Pc-3 and Pc-4) producing 4 different sizes of PCR product are 437, 320, 537 and 774 bp respectively.

Results in Table-2 show that BBTV was detected at maximum after 210 minutes (Pc-1) and 170 minutes (Pc-2) and at minimum after 110 minutes (Pc-1) and 90 minutes (Pc-2). The difference in reaction time between Pc-1 and Pc-2 was due to the annealing temperature, where, 60°C for Pc-2 was too close to both extension temperature (72°C) and denaturation temperature (94°C) than 40°C for Pc-1. Results also showed that 1 unit of *Taq* DNA polymerase was found to be able to extend about 437 bp for Pc-1 in 10 seconds using program D.

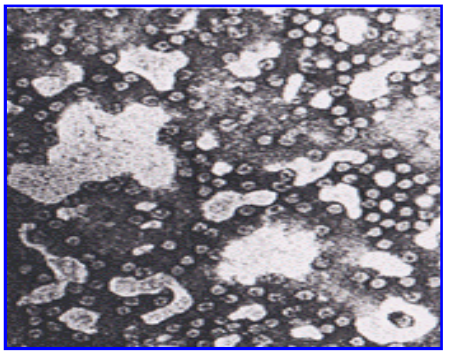

Figure-1: Isometric virus particles with a diameter of 20 nm with a density of 1.28 to 1.30g/ml in caesium sulphate gradients purified from banana infected leaves.

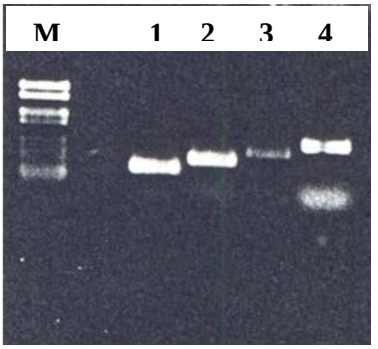

Figure-2: Agarose gel 1% in TAE buffer stained with ethidium bromide showing the amplified fragments from BBTV in purified virus preparation. Lane 1, amplified fragment (AF) using Pc-2 (320 bp); lane 2, AF using Pc-1 (437 bp); lane 3, AF using Pc-3 (537 bp) and lane 4, AF using Pc-4 (774 bp).

Table-2: PCR of purified BBTV using four PCR programs and different primer pairs.

Primer pairs	Programs	Time of reaction (min)	Size of PCR product (bp)
	A	210	
Pc-1	B	145	437
	C	125	
	D	110	
	A	170	
Pc-2	B	125	320
	C	101	
	D	90	

PCR of BBTV in crude sap: Two extraction techniques using sand and liquid nitrogen (LN_2) were used with four different extraction buffers to prepare the crude sap from 0.2 g midrib of cv. Cavendish BBTV-infected banana plants.

Results in Table-3 showed that, BBTV was not detecting directly in the infected crude sap in 10^{-1} dilutions. But all 10^{-2} dilutions showed exhibited positive reactions. Similar results were observed by Wetzel *et al.*, (1991), where he found that the amplification of PPV directly in plant extracts decreased the efficiency of synthesis of the amplification product. It was also found that the LN_2 extraction technique was more sensitive than the sand extraction technique. This may be due to obtaining a large number of viral particles in the crude sap using LN_2. This was also confirmed by the ability to detect BBTV in 10^{-3} dilutions using LN_2 as extraction technique. Results also showed that both Na-PO_4 (0.07 M, pH 7.2, 1% SDS) and 1% SDS containing 0.5% Na_2-SO_3 buffers appeared highly sensitive using LN_2 as extraction buffers.

Dale (1987) reported that, pulverisation of banana tissues frozen in LN_2 before extraction greatly reduced the interference between latex and phenolic compounds contained in banana and the virus extraction. LN_2 also appeared to be one of the main factors contributing to the successful extraction and purification of BBTV (Su and Wu, 1989).

Table-3: PCR detection of BBTV in crude sap prepared using different extraction buffers and different extractions tools.

Extraction buffers	Sand				LN_2			
	Crude	10^{-1}	10^{-2}	10^{-3}	Crude	10^{-1}	10^{-2}	10^{-3}
d.H_2O	-	-	+	-	-	-	+	±
Na-PO_4 (0.07 M, pH7.2)	-	-	+	±	-	-	+	±
Na-PO_4 (0.07 M, pH7.2)+ 1% SDS)	-	-	+	-	-	-	+	+
1% SDS+ 0.5% Na_2SO_3	-	-	+	±	-	-	+	+

(-): Negative reaction. (+): Positive reaction. (±): In doubt.

Results represented in Table-4 demonstrated that, PCR was one of the appeared to react negatively, but 29 of 32 BBTV-infected banana plants collected from the field appeared to have positive amplification with 10^{-2} dilution of crude sap. On the other side, 3 plants were found to need more dilution up to 10^{-3} to allow the amplification of BBTV in the crude sap. This may be due to the presence of high amounts of inhibitors in banana crude sap that affect the activity of *Taq* DNA polymerase.

Table-4: Application of PCR in detection of BBTV in crude saps from healthy and infected plants.

Primer pairs	Healthy looking plants		BBTV-infected plants		Size of PCR product (bp)
	No. of tested plants	No. of +ve plants	No. of tested plants	No. of +ve plants	
Pc-1	22	0	16	14	437
Pc-2	2	0	2	2	320
Pc-3	11	0	12	11	537
Pc-4	2	0	2	2	774

Results illustrated in Figure-3 proved the use of the PCR for the detection of BBTV under routine detection in the field to be more sensitive than either molecular hybridization using ^{32}P-labelled DNA or ELISA. Similar results have been reported by Dr. J. Thomas (Pers. comm) who found that positive reactions of health banana plants were obtained using ELISA for detection of BBTV.

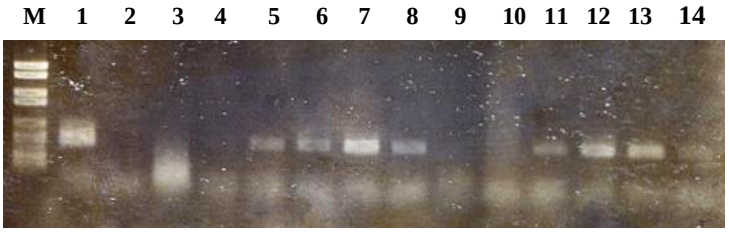

Figure-3: Ethidium bromide stained 1% agarose gel in TAE buffer showing the PCR detection of BBTV in the crude sap extracted from healthy lanes 3, 4, 9 and 10 and from BBTV-infected plants (lanes, 5, 6, 7, 8, 11, 12, 13 and 14). Lane 1, purified BBTV as positive control. Lane 2, PCR reaction without template as negative control.

Wetzel *et al.*, (1991) reported that, in a field indexing trial, the PCR assay proved to be more sensitive than molecular hybridization using ^{32}P-labelled RNA probes for PPV detection.

PCR of BBTV in nucleic acid extracts: To adapt the PCR technique for the detection of BBTV under routine detection in the field, seven different buffers were used for the extraction of BBTV-DNA from infected banana plants. Results in Table - 5 showed that BBTV was not detect absolutely in crude nucleic acid extracts, but was detected in most of 10^{-1} dilutions. These results agree with that found by Wetzel *et al.*, (1991). Results also revealed that, using d.H_2O as an extraction buffer was not recommended. The dilution 10^{-2} was found to be the best dilution for the amplification of BBTV-DNA in the PCR detection of BBTV.

The modifications in the contents of extraction buffers that were suggested by Dale (1997) proved the PCR detection technique to be more sensitive in the routine detection of BBTV in the field. Tris-HCl (containing 1% SDS), Tris-HCl (1% SDS; 0.1% PVP) and Na-PO_4 (containing 1% SDS) were recommended to be the most suitable buffers which could be used for the extraction of nucleic acid of BBTV to adapt the PCR detection of BBTV.

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Table -5: PCR of BBTV-nucleic acids using different extraction buffers.

Extraction buffers	Crude	10^{-1}	10^{-2}	10^{-3}
d.H_2O	-	+	-	-
1% SDS	-	-	+	+
Tris.Cl (10 mM, pH 8.3)	-	-	+	-
Tris.Cl (10 mM, pH 8.3, 1% SDS)	-	+	+	+
Tris.Cl (10 mM, pH 8.3, 1% SDS, 0.1% PVP)	-	+	+	+
Na-PO_4 (0.07 M, pH 7.2)	-	-	+	±
Na-PO_4 (0.07 M, pH 7.2, 1% SDS)	-	+	+	+

(-): Negative reaction. (+): Positive reaction. (±): In doubt.

Results represented in Table-6 and illustrated by Figures-4 and 5 revealed that the use of nucleic acid extracts increased the sensitivity of the PCR technique in the detection of BBTV in the field. No nucleic acids were synthesized in the amplification of nucleic acid extracts from 13 health looking banana plants. But all the 36 BBTV-infected plants appeared to have positive amplification. The high sensitivity

of nucleic acid extracts from purified virus and from BBTV-infected plants provides strong evidence that the use of PCR technique in the detection of BBTV was more sensitive than ^{32}P-labelled DNA probe Wetzel *et al.*, (1991) supported these results .

Table-6: Application of PCR in the detection of BBTV in nucleic acid extracts from purified BBTV, healthy and diseased plants.

Dilutions	Purified virus	Healthy plants		Diseased plants	
		Total	No. of +ve plants	Total	No. of +ve plants
Crude	+	13	0	36	0
10^{-1}	+	13	0	36	11
10^{-2}	+	13	0	36	27
10^{-3}	+	13	0	36	9

(+): Positive reaction.

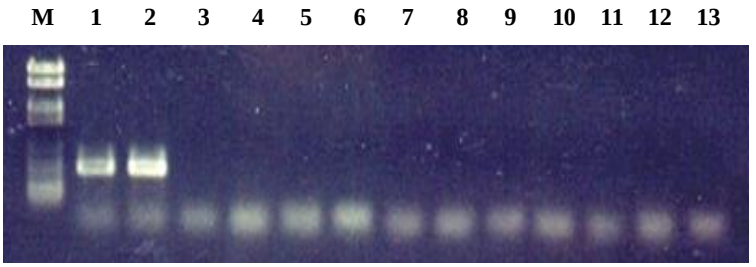

Figure-4: Ethidium bromide stained 1% agarose gel in TAE buffer showing the PCR detection of BBTV in healthy banana plants. Lane 1, AF using Pc-2 (320 bp) of purified virus (as positive control); lane 2, AF using Pc-2 (320 bp) of 10^{-2} of nucleic acid extracts from BBTV-infected plant; lane 3, PCR reaction without any template (as negative control) and lanes (4-13), PCR reactions of 10^{-2} of crude sap from 10 healthy banana plants.

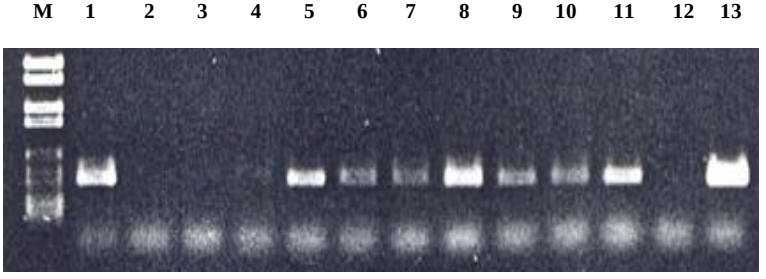

Figure-5: Ethidium bromide stained 1% agarose gel in TAE buffer showing the PCR detection of BBTV in nucleic acid extracts (10^{-2} dilution). Lane 1, positive control (purified virus); lane 2, negative control (tube reaction without template) and lanes 3-13, BBTV-infected plants.

Detection of Egyptian BBTV isolates and its relation with other overseas isolates: The detection of BBTV was achieved in nucleic acid extracts from Australia, Burundi, Egypt, France, Gabon, Philippines and Taiwan obtained from the glasshouse.

No specific reactions as band corresponding to the viral amplified fragment were observed with healthy extracts from France. The crude (undiluted) nucleic acid extracts appeared to have negative synthetic reactions in the amplification of BBTV in the PCR detection technique. The Egyptian-BBTV isolate appeared to have amplification reaction similar to that performed by other international isolates, which suggests a common aetiology for the BBTD. These results are represented in Table-7.

Table-7: Detection of Egyptian BBTV and its relation with other overseas isolates.

Overseas isolates		Crude	10^{-1}	10^{-2}
Australia	(Q1)	-	+	+
	(B1)	-	+	+
Burundi	(B2)	-	+	+
	(B3)	-	+	+
Egypt	(E1)	-	-	+
	(F1)	-	-	-
France	(F2)	-	-	-
Gabon	(G1)	-	+	-
Philippines	(A1)	-	+	+
	(T1)	-	-	±
Taiwan	(T2)	-	-	+
	(T3)	-	-	+

(-): Negative reaction. (+): Positive reaction (±): In doubt.

Thomas and Dietzgen (1991) reported that, BBTV isolates from Australia, Taiwan, People's Republic of China, Tonga, Western Samoa and Hawaii were found to be serologically related.

The relationship between Egyptian-BBTV and other banana viruses: The PCR detection system was used to detect the relationship between the Egyptian-BBTV isolate and other common viruses affecting banana. Nucleic acid extracts from banana-CMV infected plants from Egypt and Philippines; from AbBTV and from BBMV-infected plants from Philippines were tested.

Results illustrated in Table -8 indicate that, the specific band corresponding to Egyptian-BBTV amplified fragment was observed with Philippine-AbBTV, but not with the other banana viruses mentioned above. This result suggests that perhaps the Philippine-AbBTV isolate could be identified as a strain of BBTV.

Dale (1987) reported that AbBTV was very probably a strain of BBTV, based on the similarity of the biological properties of both viruses (found by Ocfemia, 1926, Magee, 1927, Ocfemia , 1930 and Ocfemia and Buhay , 1934).

Table -8: PCR of Egyptian BBTV and its relationship with other banana viruses.

Nucleic acid extracts	Crude	10^{-1}	10^{-2}
Egyptian-BBTV	-	-	+
Egyptian-banana-CMV	-	-	+
Philippine-AbBTV	-	-	+
Philippine-banana-CMV	-	-	+
Philippine-BBMV	-	-	+

(-): Negative reaction. (+): Positive reaction.

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