Annals of the Sri Lanka Department of Agriculture, 2002. 4: 255-267

DEVELOPMENT OF VIRUS FREE BANANA FOUNDATION STOCK

E.M. DASSANAYAKE and B.M. RATHNABHARATHI
Plant Virus Indexing Centre, Gabadawatte, Homagama

ABSTRACT

Bananas in Sri Lanka are infected with banana bunchy top virus (BBTV), banana bract mosaic virus (BBrMV), banana streak virus (BSV) and cucumber mosaic virus (CMV). These viruses are usually spread from plant to plant by specific insect vectors. However the primary source of viruses are the infected vegetative planting material. Production of virus free planting material is therefore of vital importance to ensure a healthy crop. Collection of apparently virus free, high yielding banana plant was done from home gardens, promising varieties from researchers and export quality bananas from the private sector. These were maintained under aphid protected greenhouse conditions in cement pots containing a 1:1:1 potting mixture of top soil: cattle manure: coir dust. Initial virus indexing was done one month after collection and subsequently after 3-6 months in the aphid protected greenhouse. Protocols for virus indexing technologies such as enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) were perfected for each virus. These methodologies form a most essential component for a virus free foundation stock development programme. Timely pruning and heading enabled the banana plants to be kept in pots till they were ready to be issued for rapid propagation scheme. Samples of *Embul, Alukesel, Binkesel, Seenikesel, Embon, Kolikuttu*, IC_2 banana, *Ratahondarawalu, William* and *Grand Naine* HS3640 and FHIA 3 were identified as virus free. Of about 1000 plants subjected in this programme, only 16 % were found to be free of all 4 viruses.

KEY WORDS: Foundation Stock, Virus Indexing, Enzyme Linked Immunosorbent Assay, Polymerase Chain reaction, Tissue Culture

INTRODUCTION

The Banana *(Musa spp.)* is a widely adapted crop in Sri Lanka. This crop could be grown in the wet, dry and intermediate zones. In Sri Lanka bananas are found to be infected with banana bunchy top virus (BBTV) (Wardlaw, 1972)-(Figure 1), banana bract mosaic virus (BBrMV) (Thomas *et al.*, 1997)-(Figure 2), banana streak virus (BSV) (Thomas *et al.*, 1997)-(Figure 3), and cucumber mosaic virus (CMV) (Wardlaw, 1972)-(Figure 4). Furthermore, the presence of Banana mild mosaic virus (BanMMV)-(Figure 5), in Sri Lanka was recently confirmed (Dr. John Thomas Personal communication). These systemic virus diseases have been causing considerable damage to the banana industry by way of low fruit yields, poor quality fruits.

Viruses are usually spread from plant to plant by insect vectors. Banana bunchy top virus, banana bract mosaic virus and cucumber mosaic virus are transmitted by aphid vectors while banana streak virus is transmitted by the citrus mealy bug. However, primary sources of viruses are the vegetative planting material obtained from infected mother plants. At present sucker production of banana is mainly done through conventional methods. However due to virus incidence, it is very difficult to find a banana sucker without any virus infection. Even though there is a great demand for banana suckers, an organized program to supply virus free planting material yet to be initiated. Only a few private sector organizations have tissue-culture produced

banana planting material. Even these are not virus free as proper indexing programs have not been followed.

Due to the susceptibility of bananas to various virus infections the reported work was initiated with the objective of producing virus free banana foundation stock.

MATERIALS AND METHODS

Virus detection

These experiments were conducted at the Plant Virus Indexing Centre, Gabadawatte.

Serological detection: Indirect Enzyme Linked Immunosorbent Assay (ELISA) was done according to Mowat and Dawson (1987). Polyclonal antisera for some of the banana viruses produced locally are listed below. A series of tests were conducted to evaluate the optimum conditions to differentiate disease and healthy samples.

Antiserum	*Reference*
Banana bunchy top virus	Dassanayake (1997)
Banana bract mosaic virus	Dassanayake (2001)
Banana streak virus	Dassanayake (2000)

(Method of virus purification according to Lockhart, 1986 with minor modifications)

Cucumber mosaic virus was detected using an antiserum gifted by Professor Su of Taiwan University according to his protocol, using direct ELISA method. For virus indexing purposes, diseased samples from infected plants were taken from the following parts indicated below.

Virus	*Part of diseased plant*
BBTV	youngest 1-3 leaves, (mid rib)
BSV, BBrMV, CMV	young leaves with symptoms

Molecular based detection methods: Standard polymerase chain reaction (PCR) and reverse transcriptase PCR was done at the final stage due to the cumbersome sample preparation required and the high cost of reagents. Modifications such as leaf soak method and immunocapture steps can overcome some of these problems. Protocols for detection of BBTV, BBrMV, BSV and CMV by modified PCR methodologies were done according to Dassanayake 1999. Selected primers for detection of local strains of viruses and amplified DNA base pair numbers are given in Table 2. Names of Researchers who designed these primers are given below:

Virus	*Primer Pair*	*Researchers*
BBTV	FCPR4 ; 30 merF3	Burns, 1994
BBTV	BBT_1 : BBT_2	Harding *et al.*, 1993
BBrMV	Bract1; Bract2	Bateson & Dale, 1995
BBrMV	F_1/R	B.C.Rodonis' sequence (AF 071586)
BSV	BADNA 3; BADNA T	Lockhart & Olszewsk, 1993
BSV	Primer F_1 ; Primer F_2	John Thomas' sequence
CMV	CMV 3'; CMV 5'	Bariana *et al.*, 1994
CMV	U 93 –309; D 93-359	Hu *et al.*, 1995

Scheme for mother plant selection

In this programme, banana suckers were collected from 3 main areas. 1. General collection from home gardens, 2. Plants with proven agronomical characteristics from researchers, 3. Selected plants from private sector plantations

General collection from home gardens

Due to limitation of facilities the collections were done mainly from the two main districts of Colombo and Kalutara. Only the apparently healthy plants were collected during the survey. The following criteria were used in sample collection, 1. Selection to be done from home gardens only, 2. Vicinity of banana plants should be free of viral diseases, 3. Mother sucker should be vigorous and a higher yielder, 4. suckers of 90-120 cm to be selected.

Plants with proven agronomical characteristics from researchers

This collection was limited to three research Institutes. Suckers from promising cultivars were selected from the Horticultural Crops Research & Development Institute, Gannoruwa, Field Crops Research & Development Institute, Maha Illuppallama and Oil Crops Research & Development Centre, Angunukolapelessa.

Collection from private sector cultivations

This was done from three locations. Export quality bananas were collected from CIC farm at Hingurakgoda and Mahaweli Coconut plantation at Aralaganwila. Most of the *Kolikuttu* suckers were collected from a private farmer at Makewita.

Williams and Grand Naine were collected from CIC farm at Hingurakgoda. Fifty plants from each variety were collected from high yielding mother plants. Few plants were seen to be infected with banana bunchy top virus. Another 25 *Embul* plants were also collected from a different block of the same cultivation. In this block some plants were observed to be infected with banana streak virus.

Maintenance of selected suckers

Two aphid protected houses at the Fruit Crop Research & Development Centre, Horana and Plant Virus Indexing Centre, Gabadawatte Homagama were utilized. Collected suckers were cleaned and treated for weevil infestation. They were then planted in 45 cm cement pots containing a proven potting mixture consisting of top soil: cattle manure: coir dust at a 1: 1: 1 ratio. Stock plants in the greenhouses were regularly checked for any insect vectors and sprayed with insecticides whenever necessary. Banana fertilizer mixture 11: 10: 25 of N: P_2O_5: K_2O at the rate of 25 g per plant was applied at 3 month intervals. Once the plants become too tall they were cut at ground level. Meristem tissue was damaged to induce sucker production.

Scheme for Virus Indexing

Initial Stage: Selection of suitable cultivars and culling of plants on visual symptoms.

1st stage: Indexing of apparently healthy samples by ELISA for the 4 important viruses. (1 month after establishment)

2nd stage: ELISA negative samples for the 4 viruses were once again indexed by ELISA 3-6 months after collection. PCR tests were then carried out for the final ELISA negative samples

3rd stage: Multiplication of healthy banana through conventional method under aphid proof greenhouse methods.

4th stage: Issue of healthy banana material for rapid multiplication to tissue culture laboratories.

5th stage: Random indexing after tissue culture propagation

RESULTS AND DISCUSSION

Detection of viruses

Serological Method: Results show that for the detection of each virus, it is necessary to extract the virus sample using different buffer solutions. This causes practical difficulties in sample preparation. However for BBTV, BBrMV and BSV antiserum and conjugate dilution conditions are similar (Table 1).

CMV was detected using a protocol provided by Prof. Su, Taiwan University, Taiwan. Locally produced antisera effectively detected these viruses with or without symptoms.

Table 1. Optimum conditions for ELISA

Name of Virus	*ELISA Method*	*Gamma Globulin Dilution*	*Extraction Buffer*	*Sample / Buffer*	*Ant. Dilution*	*te Dilution*
BBTV	Indirect ELISA	-	PBST + .13% Na_2SO_3 + 2% PVP + 0.2% Albumin	1 g / 5ml	1: 500 PBS-TPO	1:2000 Protein A conjugate in PBS-TPO
BBrMV	- do -	-	.05M Carbonate buffer pH 9.6 + 10 mM Na DIECA	1g / 10 ml	- do -	- do -
BSV	- do -	-	PBST	1g / 5 ml	- do -	- do
CMV (Prof.Su)	Direct ELISA	1:2000 µl in .05M carbonate buffer pH 9.6	0.2M K phosphate pH 7.4 + .5 % Na_2SO_3	1g / 10 ml	-	1:500 Alkaline Phosphate conjugate in PBS-TPO

Abbreviations: PBST - Phosphate buffered saline saline + 0.5 ml/ L Tween 20, PVP - Polyvinyl pyrrolidone (MW - 40,000), NaDIECA - Sodium di-ethyl dithio carbamic acid, Ant - Antiserum

Table 2. Suitable conditions for PCR Methodology

Virus	*Method of DNA/RNA Extraction*	*Selected Method for DNA/RNA Extraction*	*Primers Tested.*	*Selected Primer Pair*	*PCR Method*	*Amplified Product Size (bp)*
BBTV	Phenol extrn Leaf soak mtd	Leaf soak	FPCR4 } 30merF3 } BBT_1 } BBT_2 }	BBT_1 BBT_2	PCR	350
BBrMV	CTAB extrn Coating virus Sample in .05M Carbonate buffer PH 9.6	Immuno capture	$Bract_1$ } $Bract_2$ } F1/R	F1/R	IC/PCR	324
BSV	Phenol extrn Leaf soak mtd	Phenol extrn	BADNA T/3 Thomas sequence	Thomas sequence	PCR	220
CMV	Phenol extrn Leaf soak mtd	Phenol extrn	93-309 } 93-359 } CMV3 } CMV5 }	CMV3 CMV5	RT/PCR	500

Abbreviations: PCR-Polymerase chain reaction, IC/PCR -Immuno capture polymerase chain reaction, RT/PCR-Reverse transcriptase polymerase chain reaction

In PCR, nucleic acid extraction was time consuming. This problem was overcome up to a certain extent by the use of leaf soak method and IC-PCR method. However leaf soak extraction method could be only worked with

BBTV. Similarly immuno-capture method could be applied only to BBrMV. For BSV and CMV extraction takes 1 day and therefore amplification takes two days. For BBTV amplification takes 1 day and for BBrMV 2 days (Table 2).

Table 3. No. of Plants collected from each location

Location	*Cultivar*	*Number (total)*
Hanwella	Seenikesel	86
	Alukesel	
	Anamalu	
	Ratakolikuttu	
Kosgama	Anamalu	14
	Embul	
	Seenikesel	
	Ratahondarawalu	
	Embon	
Horana	Embon	127
	Embul	
	Rathkesel	
	Seenikesel	
	Ranel	
	Puwalu	
	Alukesel	
	Suwadel	
Madurawala	Wanduru Anamalu	22
	Seenikesel	
	Embul	
	Embon	
	Rathkesel	
	Alukesel	
	Anamalu	
Gabadawatte	Embon	120
	Seenikesel	
	Rathkesel	
	Ratahondarawalu	
	Embul	
	Suwandel	
	Anamalu	
	Puwalu	
Pitipana	Embon	57
	Seeni	
	Anamalu	
	Embul	
	Kolikuttu	
	Sudukochchi	
	Suwandel	
	Rathkesel	
Kananwila	Rathkesel	76
	Embon	
	Alukesel	
	Kolikuttu	
	Embul	

	Seenikesel	
	Anamalu	
	Puwalu	
FCRDI	Seenikesel	16
Maha Illuppallama	Alukesel	15
	Embul	22
	Discarded	17
OCRDI A'Pellessa	Embul	25
	Ash Plantain	2
	FHIA	4
	HS 3640	4
HORDI, Gannoruwa	Rathkesel	2
	Embon	18
CIC, Hingurakgoda	Embul	25
	Williams	50
	Grand Naine	50
	Kolikuttu	55
Makewita	Kolikuttu	19
	Embon	1
Weligatte	Embul (Nadee)	10
Mahaweli Coconut plantation, Girandurukotte	Dwarf Cavendish (Israel banana)	10

Collection from home gardens

In this survey it was found that BBTV was common in home gardens. However a few banana suckers were found with banana streak virus in Horana and Kirindiwela areas. Banana bract mosaic virus was very common in *Embul* cultivars. It was not possible to find any CMV infected plants. When collections were made diseased areas were left out.

Collections from researchers

Promising varieties from Researchers were brought for indexing. They were apparently free of viruses but had to be discarded as most of the samples brought were severely infested with weevil attack. Bigger plants were pruned at ground level and their subsequent new growth was indexed for viruses.

Collection from private sector

It was not possible to select a single *Embul* plant from this collection as all were infected with the virus. However 80% of *Williams* and *Grand Naine* were selected as they were free of all 4 viruses.Similarly Dwarf Cavendish plants from Mahaweli Plantation were also selected. Some *Kolikuttu* plants from the well managed 5 acres at Makewita were also selected for foundation stock.

Table 4. Characteristics of selected virus free cultivars

Cultivar	*No.*	*Location*	*Yield of Mother Plant*
Alukesel	03	Hanwella	12 kg
	58	Madurawela	12 kg
	71	Gabadawatte	10 kg
	135	Pitipane	10 kg
Alukesel	Ak1:3:4	Maha Illuppallama	10 kg
	Ak1:1:2	- do –	12 kg
	Ash(1p)	A'Pelessa	15 kg
	Ash(2p)	- do –	15 kg
Embul	15	Kosgama	18 kg
	73	Gabadawatte	12 kg
	82	Gabadawatte	20 kg
	111	Pitipane	12 kg
	37	Horana	na
Embul (Nadee)	P1	Weligatte	20 kg
	P3		20 kg
	P4		20 kg
	P5		20 kg
	P9		20 kg
	P10		20 kg
Rata-Hondarawalu	21	Kosgama	Na
Seenikesel	36	Horana	20 kg
	77	Gabadawatte	18 kg
	SK 1:1:2	M'Illuppallama	15 kg
Embon	86	Pitipane	Na
	121	Pitipane	Na
	128	Pitipane	Na
	164	Hanwella	Na
	GN16	HORDI	}
	GN34	HORDI	}Varieties are
	GN51	HORDI	}Under
	GN53	HORDI	}evaluation
	GN167	HORDI	}
	GN168	HORDI	18-20 kg
IC2	2P	PVIC, H'gama	18-20 kg
	5P	- do -	- do -
Kolikuttu	106	Pitipane	10 kg
	147	Kananwila	12 kg
	152	Kananwila	12 kg
	154	Kananwila	15 kg
Kolikuttu	M5, M6, M7	Makawita	10 – 15 kg
	M8, M9, M14	- do -	- do-
Kolikuttu	1,2,7,8,11,12,13	CIC	-do-
	16,21,23,24,31,	Hingurakgoda	-do-
	33,40,42,45	- do -	-do-
Binkesel	71	Kirindiwela	20 kg
Dwarf Cavendish (Israel)	1p-10p	Maweli c'nut Plantation Aralaganwila	Na
	1,2,3,4,8,9, }	CIC	20-25 kg

Dwarf Cavendish (Grand Naine)	10,13,16,17, 18,19,20,23, 29,30,34,36. 42.45,46,48, 50,51,53,56, 57,60,59,38, 15,25,47,58	Hingurakgoda	
	1,2,4,8,9 11,14,	CIC	20-25 kg
Dwarf Cavendish (Williams)	15w1,15w2 19,20,22,23, 25,26,27,29, 31,32,34,16, 37,38,39,17.	Hingurakgoda	
HS 3640	1P	A'Pelessa	22 kg
	2P	- do -	- do -
	4P	- do -	- do -
FHIA 3	1P	A'Pelessa	25 kg
	2P	- do -	- do -

Na= not available

Apparently virus free plants collected from various locations were indexed serologically. Final indexing was done by the use of molecular based methods. Indexing results showed that most of the plants were infected with banana bract mosaic virus, banana bunchy top virus and banana streak virus. Mixed infections were also found in some instances. Nearly 1000 plants were collected and were subjected to this program. However only 16% plants were found to be free of all 4 viruses. Cucumber mosaic virus was hardly found in the collected banana samples in the present investigation. When a mixture of viruses was present, it was found that some symptoms were suppressed. For instance when BBTV & BBrMV were found in a mixture, some BBTV symptoms were masked.

Maintenance and multiplication of selected suckers through conventional methods

Maintenance of suckers in cement pots under greenhouse conditions for banana is a very cumbersome process. It was a problem for the first 6-9 months after which the plants were cut back and new growth encouraged for further maintenance.

Scale insects were observed in some plants while aphids were seen to colonise a few banana suckers. Insecticidal sprays were given for their control. The source of these insects maybe from the collected suckers.

Suckering of bananas in pots under aphid protected house was poor. Only Williams and Grand Naine produced a few suckers. Cutting of mother plants at ground level resulted in the production of a few suckers from the meristem. Unsuitable micro-environment maybe a reason for the poor

suckering. Therefore multiplication will have to be by rapid propagation through tissue culture and preservation under aphid protected greenhouse conditions for the establishment of a virus free foundation stock.

CONCLUSIONS

Bananas are heavily contaminated with one or more viruses. Sensitive virus detection methods are essential to develop a virus free foundation stock. Antisera produced locally has been an important component in this exercise due to the exorbitant costs of the commercially available ones. Even though molecular detection methods are more sensitive than serological methods, their use is limited to the final stages as the reagents are costly and the sample preparation techniques are tedious and time consuming.

Maintenance of selected virus free foundation stock should be throughout under aphid protected greenhouse conditions. Due to the poor suckering habit in bananas grown in cement pots under aphid protected greenhouse conditions, rapid multiplication programs have to be implemented. In this context multiplication through tissue culture means will provide the necessary material to build up a foundation stock. Multiplication of good quality, virus free cultivars will result in disease free planting material for commercial cultivations. This is a continuous programme

ACKNOWLEDGEMENTS

The authors wish to thank Council for Agricultural Research Policy for funds made available under its research project No. 12/442/326 to conduct this investigation

REFERENCES

Bariana, H.S., A.L. Shannon, P.W.G. Chu and P.M. Waterhouse. 1994. Detection of five seed borne legume viruses in one sensitive multiplex polymerase chain reaction test. Phytopathology: 84: 1201-1205.

Bateson, M.F. and J.L. Dale. 1995. Banana bract mosaic virus: Characterization using potyvirus specific degenerate primers. Arch. Virol 140: 515-527.

Burns, T.M. 1994. Genome organization of Banana bunchy top virus. PhD Thesis, Queensland University of Technology.

Dassanayake, E.M. 1997. Production of polyclonal antibodies against banana bunchy top virus and their use in virus detection. The Sri Lanka J of Agri Sciences 1997 Vol 34: 119-125 pp.

Dassanayake, E.M. 1999. Terminal report CARP Project No: 12/371/288.

Dassanayake, E.M. 2001. Detection of Banana bract mosaic virus (BBrMV) through immuno-capture polymerase chain reaction. Annals of the Sri Lanka Dept. of Agriculture, 2001. Vol.3: 19-25 pp.

Harding, R.M., T.M. Burns,. G. Hafner, R.G. Dietzgen and J.L. Dale. 1993. Nucleotide sequence of one component of the banana bunchy top virus genome contains a putative replicate gene. J.of General Virology. 74: 323-328.

Hu, J.S., H.P. Li, K. Barry and M. Wang. 1995. Comparison of Dot-Blot, ELISA and RT-PCR assays for detection of two cucumber mosaic virus isolates infecting banana in Hawaii. Plant Disease 7 (9):

Hwang, S.C. and H.J. Su. 1998 "Production and cultivation of virus free banana tissue cultured plantlets in Taiwan". *In* Managing banana and citrus diseases. Proceedings of a regional workshop on disease management of banana and citrus through the use of disease free planting material. Held in D.O.O. city Philippines. 14-16 Oct 1998.

Lockhart, B.E.L. 1986. Purification and serology of a bacilliform virus associated with banana streak disease. Phytopathology. 76 (10):

Lockhart, B.E.L. and N.E. Olszewski. 1993. Serological and genomic heterogeneity of banana streak badnavirus: Implications for virus detection in *Musa* germplasm. Pages 105-113 *In* Breeding Banana and Plantain for the resistance to Diseases and Pests. J. Ganry Ed. International network for the Improvement of Banana and Plantain. Montpellier, France.

Magnaye, L.V. 1998. "The impact of tissue cultured plants in the ongoing eradication and rehabilitation programme in the Philippines". *In* Managing Banana and Citrus diseases. Proceedings of a regional workshop on disease management of banana and citrus through the use of disease free planting material. Held in D.O.O. city Philippines. 14-16 Oct 1998.

Mowat, W.P. and S. Dawson. 1987 "Detection and identification of plant viruses by ELISA using crude sap extracts and unfractionated antisera". Journal of Virological Methods. 15: 233-247.

Ong Ching. and Lily Ong. 1998. "Virus and virus like diseases of banana and Citrus in Malaysia, Status and Control strategies". *In* Managing banana and citrus diseases. Proceedings of a regional workshop on disease management of banana and citrus through the use of disease free planting material. Held in D.O.O. city Philippines. 14-16 Oct 1998

Thomas, J.E., A.D.W. Geering, C.F. Gambley, A.F. Kessling and M. White 1997. "Purification, properties and diagnosis of Banana Bract Mosaic Potyvirus and its distinction from Abaca Mosaic Potyvirus". Phytopathology: Vol. 87, No.7. 698-705

Wardlaw, C.W. 1972. "The virus diseases. Banana diseases including plantation abaca. Williams Clows and Sons Ltd. London. Pp 115-139.

Figure 1

Figure 2

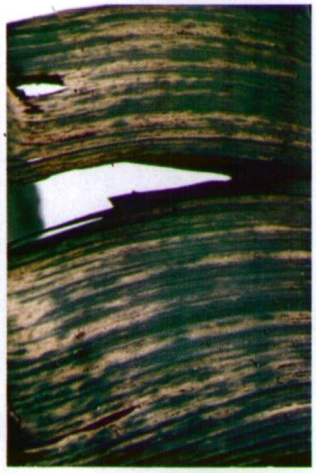

Figure 3

Figure 4

Figure 5

Figure 6

Figure 1. A young banana plant infected with banana bunchy top virus (BBTV), showing chlorosis, rosetting, narrow leaves and dwarfing symptoms

Figure 2. Banana infected with bract mosaic virus (BBrMV), showing yellow blotching symptom in the bract

Figure 3. Chloritic and necrotic streaking in banana leaf infected with banana streak virus (BSV)

Figure 4. Banana plant infected with cucumber mosaic virus (CMV) showing heart-rot symptom

Figure 5. Infected banana leaf showing mild mosaic virus (BanMMV) symptom

Figure 6. Virus free banana cultivars