Production of Polyclonal Antiserum and Development of a Detection Technique by Enzyme Linked Immunosorbant Assay for Banana Streak Virus

R.M.C.P. RATHNAYAKE[1], W.G.S. PERERA[2] and E.M. DASSANAYAKE[2]

[1]*Department of biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila(NWP), Sri Lanka.*
[2]*Plant Virus Indexing Centre, Department of Agriculture, Gabadawatta, Homagama, Sri Lanka.*

ABSTRACT

Banana Streak Virus (BSV) is considered to be the most frequently occurring virus in banana cultivation and associated with yield loss. It is genomically and serologically heterogeneous world wide, and there has been need to have a locally produced antiserum to detect the virus. Therefore this research was conducted to raise antibodies against partially purified BSV at laboratory of Plant Virus Indexing Centre and to develop a low cost, effective detection technique by indirect ELISA as a routing diagnostic test. For the production of antibodies, the first injection was given intraveinally with equal volume of physiological saline (0.85%NaCl) and other injections were given intramuscularly with equal volumes of fruend's incomplete adjuent at weekly intervals. First bleed was taken two weeks after the first injection to check the development of antibodies against BSV. The final bleed was taken five weeks after the first injection and the serum was separated and preserved by adding equal volumes of glycerol and 0.025% Sodium azide. The prepared antiserum was absorbed by partially purified healthy sap to increase the effectiveness and indirect ELISA tests were done to obtain best antigen extraction buffer, best tissue dilution and best antiserum dilution. This study revealed that the locally produced antiserum can be used to detect BSV by indirect ELISA after it was absorbed by partially purified healthy sap, using tissue dilution at 1:5(w/v) in extraction buffer PBST + 1%Na_2SO_3 and antiserum dilution at 1:200(v/v) in PBS-TPO buffer.

KEY WORDS: Banana Streak Virus (BSV), Enzyme Linked Immunosorbant Assay (ELISA), Polyclonal antiserum

INTRODUCTION

Banana (*Musa spp.*) is one of the most popular fruit crops with a year round production in Sri Lanka, and it is grown both as a staple fruit as well as a cash crop mainly for the local market. In 2003 the extent and production of banana were 49,225 ha and 393,384 Mt respectively (Anon, 2004).

At present banana cultivation in Sri Lanka is affected by four major viral diseases including Banana Bunchy-top Virus (BBTV), Cucumber Mosaic Virus (CMV), Banana Streak Virus (BSV) and Banana Bract Mosaic virus (BBrMV). BSV, the causal agent of viral leaf streak, is considered to be the most frequently occurring virus of *Musa* world wide, and associated with yield loss (Agindotan *et al.*, 2003). David Jones reported BSV from Sri Lanka in 1995 and its presence was first confirmed by Thomas *et al* in 1997. BSV is a member of family *Caulimoviridae*, and genus *Badnavirus*, which contains a circular double stranded DNA genome of 7.4kb in size.

BSV is primarily disseminated in vegetatively propagated planting material such as suckers, corms and tissue cultured plantlets which are derived from infected sources (Ndowora *et al.*, 2002). Transmission is also possible via mealy bugs (*Planococcus citri* and *Saccharicoccus sacchari*) in a semipersistant manner and through true seed, which is an important consideration in breeding programs (Geering *et al.*, 2002).

Chlorotic streaks and flecks are the major symptoms of BSV and they become progressively darker as the leaf edges eventually becoming necrotic. The streak expression is periodic and plants may not show symptoms on all leaves. Large ranges of plant and bunch abnormalities can also be identified in BSV infected plants (Geering *et al.*, 2000).These symptoms can not be detected at young stages of banana. Hence, identification of the disease through symptoms is difficult. In addition this disease is more prevalent and spreads through micro propagated banana, especially in local variety Ambul, which has the highest demand for cultivation and micro propagated widely in Sri Lanka. Spreading of the disease is also enhanced by the integration of segments of viral genome in to B genome of certain *Musa spp.* (LaFleur *et al.*, 1996). It is then essential to release BSV free planting materials to the farmer to prevent the spread of the disease.

Therefore sensitive, reliable and a convenient method is needed to detect BSV. Molecular detection methods are more sensitive than serological methods, but their use is limited due to high cost of reagents and tedious sample preparation techniques. In addition they are strain specific and failed in use as routing diagnostic tests. Hence the banana plants must be detected for BSV by serological methods such as Enzyme Linked Immunosorbant Assay (ELISA).

Use of imported ELISA test kits was limited due to its high cost and high genetic and serological diversity of BSV worldwide. Therefore polyclonal antiserum for BSV was produced using locally collected diseased samples in the laboratory at Plant

Virus Indexing Center (PVIC) in 2002. However another batch of BSV polyclonal antiserum is essential for the frequent indexing purposes and to establish and maintain BSV free banana foundation stock. In this study, BSV which was partially purified at the laboratory of PVIC, according to minor modification to Mini prep method was used for the production of antibodies against BSV. Thereafter it is necessary to develop an effective detection method using the locally produced antiserum by indirect ELISA.

MATERIALS AND METHODS

This study was carried out at the laboratory of Plant Virus Indexing Center, Department of Agriculture, Gabadawatta, Homagama and antiserum production was done at Medical Research Institute, Boralla from January to June 2006.

1 Immunization of the Rabbit and Antiserum Preparation

Purified BSV pellet (according to the Mini prep method with minor modifications) was mixed with resuspension buffer (10mM Potassium phosphate buffer, pH 7) and diluted up to 1ml.Then it was mixed with an equal volume of physiological saline (0.85% NaCl) and the rabbit was injected intraveinally(IV) through marginal ear vein. After first IV injection four intramuscular (IM) injections were given to thigh muscles of the rabbit at weekly intervals. IM injections were prepared using 1ml of virus suspension in resuspension buffer and mixing with equal volume of Fruend's incomplete adjuent.

First bleed was taken from the ear vein of the rabbit, 2 weeks after the IV injection and final bleed was taken after the series of IM injections.

The collected blood was incubated at room temperature for one hour and it was again incubated at 4°C for overnight to clot blood cells. The separated serum was then decanted to centrifuge tubes and centrifuged at 5000 rpm for 10 minutes. The separated supernatant was mixed thoroughly with equal volume of glycerol and 0.025% w/v sodium azide was added to avoid microbial contaminations. Then the processed antiserum was stored at -20°C.

The Protocol of BSV detection in banana by indirect ELISA with locally produced antiserum in 1999 at PVIC was used to prepare the antiserum for the detection of BSV. In this research, healthy banana samples (H) were taken from PVIC net house and field which are proven as healthy, and plants with BSV symptoms and plants which are indexed for BSV by commercially available test kits (Agdia) as disease samples (D).

2 Preparation of Antiserum for the Detection of BSV by Indirect ELISA.

2.1 Absorption of the Antiserum by Healthy Crude sap.

The direct sap was extracted using healthy banana leaves and it was filtered through two layers of muslin cloth. Then it was mixed with equal volume of raw antiserum. The mixture was then vortexed well and incubated at room temperature for 3 hours. It was then centrifuged at 10,000rpm for 10 minutes and the supernatant was separated. An indirect ELISA test was done to check the effectiveness of antiserum.

2.2 Absorption of the Antiserum by Partially Purified Healthy sap.

Hundred grams of healthy banana leaves were blended with 3 volumes (w/v) of 0.2M potassium phosphate buffer with 15mM Ethylene diamine tetraacetic acid (EDTA), 2% Polyvinylpyrrolidone (PVP), 2% Polyethylene glycol (PEG) and 0.4% Sodium sulphite (pH 7), using a kitchen blender, and filtered through 2 layers of muslin cloth. The filtered sap was centrifuged at 10,000rpm for 20 minutes. The supernatant was decanted and 2% Triton x 100 was added while stirring for 5 minutes at 4°C. Then 30% sucrose solution which is prepared by using the same buffer used to blend healthy leaves was applied to the bottom of the supernatant to create a sucrose cushion and centrifuged at 40,000rpm for 60 minutes. After the supernatant was removed, the pellet was washed slightly with distilled water and dissolved in 600µl of raw antiserum. Then the suspension was vortexed well and incubated for 3 1/2 hours at room temperature. It was then centrifuged at 10,000rpm for 10 minutes and the decanted supernatant was stored at -20°C, as the absorbed antiserum.

An indirect ELISA test was done to detect the effectiveness of absorbed antiserum by partially purified healthy sap.

3 Optimization of Conditions in ELISA Procedure to Detect BSV.

3.1 Determination of Best Antigen Extraction Buffer

Three healthy samples and three disease samples were extracted separately using 3 different extraction buffers, Phosphate buffered saline plus Tween 20 (PBS-T), 1% Na_2SO_3 ,pH 7.4 (EB1), 0.5M Potassium phosphate buffer with 0.01M EDTA and 0.1% Na_2SO_3 ,pH 7.5 (EB2), and 0.2M potassium phosphate buffer with 15mM EDTA, 2%PVP, 2% PEG and 0.4% Sodium sulphite, pH 7(EB3). An indirect ELISA test was done using antiserum absorbed by partially purified healthy sap, diluted 1:200(v/v) in PBS-TPO (PBS-T, 2% PVP, Ovalbumin) buffer and keeping other conditions constant. Absorbance values were taken at 405nm for 1/2hr, 11/2hr at 37°C and 18hr at 4°C incubation periods, after substrate addition. The buffer which was given highest mean value of disease to healthy ratio was selected.

3.2 Determination of Best Tissue Dilution and Best Antiserum Dilution

Two healthy samples and two disease samples were ground and 3 different tissue dilutions were

made as 1:5(w/v), 1:10(w/v), and 1:15(w/v) in PBST+ 1% Na_2SO_3 (pH 7.4) buffer, for each sample. An indirect ELISA test was done using 2 different antiserum dilutions as 1:100(v/v) and 1:200(v/v) in PBS-TPO buffer, and keeping other conditions constant. Readings were taken at 405nm for 1/2hr, 1 1/2hr, and 2 1/2hr at 37°C and 18hr at 4°C incubation periods, after substrate addition. The tissue dilution and antiserum dilution which were given highest mean values of disease to healthy ratios were selected.

3.3 Analysis of Disease to Healthy Ratios

Disease to healthy ratios was obtained by dividing total averages of absorbance values of disease samples by total averages of absorbance values of healthy samples. They were statistically analyzed using the Statistical Analysis Software (SAS) package. Analysis of variance (ANOVA) procedure was applied to analyze the effect of antigen extraction buffer by using the 3 incubation periods (1/2hr and 11/2hr at 37°C, 18hr at 4°C) as replicates. Two ANOVA procedures were done separately for two different antiserum dilutions to detect the effect of tissue dilution on disease to healthy ratio and three ANOVA procedures were done separately for the 3 different tissue dilutions to detect the effect of antiserum dilution on disease to healthy ratio. Four incubation periods (1/2hr, 11/2hr and 2 1/2hr at 37°C, 18hr at 4°C) were used as replicates for each analysis.

RESULTS AND DISCUSSION

1 Immunization of Rabbit

An indirect ELISA test was done using antiserum prepared from the first bleed with 1:200(v/v) in PBST-PO buffer to check the development of antibodies specific for BSV. In this test 4 healthy samples and 4 disease samples were used.

THV2 was calculated with respect to the absorbance value of H2. When compared with THV2, all samples gave negative values except D4. It indicates the development of more antibodies against plant proteins other than for BSV coat proteins.

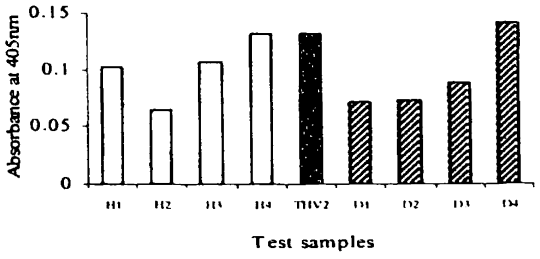

Figure 1 - Testing of antiserum of first bleed to check the development of antibodies against BSV:

H1=H3, H2=PV1, H3=72D, H4=35D, TVH2= Threshold Value, D1=A6, D2=A9, D3=A4, D4=A10

Then the antiserums of first and final bleed were compared to check the increment of antibodies in final bleed, by using 3 healthy samples and 2 disease samples.

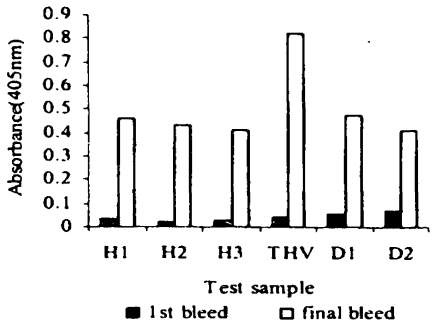

Figure 2 - Comparison of antiserums of first and final bleeds:

H1=34, H2=T, H3=V, THV=Threshold value, D1=A9, D2=A10

All absorbance values of antiserum of final bleed gave significantly higher values than the first bleed, though it couldn't differentiate disease samples from healthy samples. It indicates the presence of large amount of non specific antibodies in the antiserum of final bleed as well.

In this study partially purified BSV preparation was used to produce polyclonal antiserum. Therefore it may consist of a lot of antibodies against plant proteins, other than for antibodies against BSV coat proteins. The 260/280nm ratio of this preparation was 0.9 (according to the PVIC literature), whereas the expected ratio was 1.25(Brunt *et al.*, 1990). It confirms the impurity of the virus preparation. Moreover it is difficult to obtain BSV specific polyclonal antibodies due to periodicity of symptom expression, absence of propagational hosts, low titer of the virus in infected host and difficulty in purifying the virus (Agindotan *et al.*, 2003).

Therefore the final bleed was absorbed by crude healthy sap to remove non specific antibodies for BSV.

2.1 Testing of final bleed absorbed by healthy crude sap

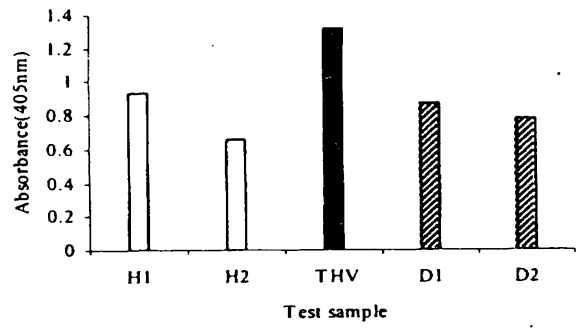

Figure 3 – Testing of final bleed absorbed by healthy crude sap:

H1=V, H2=T, THV= Threshold value, D1=BSV1, D2=BSV2

Even though the antiserum of the final bleed was absorbed by healthy crude sap, the absorbance values of disease samples gave negative values, indicating the presence of further nonspecific antibodies. Therefore the antiserum was absorbed by partial purification of the healthy sap.

2.2 Absorption of Final bleed by Partially Purified Healthy sap.

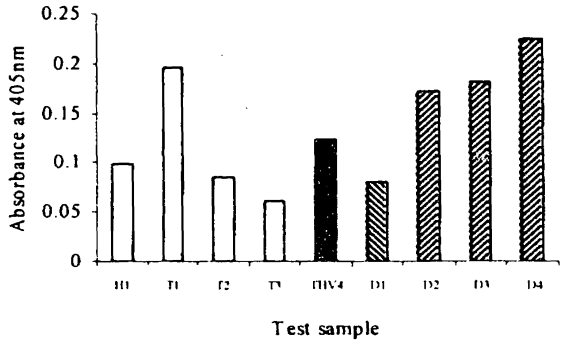

Figure 4 - Testing of the antiserum of final bleed absorbed by partially purified healthy sap:

H1=DTCq, T1=DTCf1, T2=DTCf2, T3=DTCf3, THV=Threshold value, D1=Lotus T3, D2=BSV2, D3=V3, D4=BSV4.

According to this ELISA results, D2, D3 and D4 disease samples gave positive values with respect to the THV, indicating the increment of BSV specific antibodies in the antiserum after it had absorbed by partially purified healthy sap.

But still some healthy samples gave positive values compare to THV. It may be due to the presence of BSV in healthy looking samples as well since the periodicity of foliar symptom expression and ability of infected banana plants to remain symptom less. One diseases sample also gave negative value indicating the absence of BSV. The reason may be due to absence of antibodies specific for that BSV strain in the antiserum. However this purified antiserum was used to optimize the conditions of the ELISA procedure.

3. Analysis of Conditions in ELISA Procedure to Detect BSV.

3.1 Determination of Best Antigen Extraction Buffer

Mean values of disease to healthy ratios of different extraction buffers were significantly different at P=0.06. Effect of EB1 was significantly different from EB2 and EB3. Buffer EB1 (PBST+ 1% Na_2SO_3, pH 7.4), was the best antigen extraction buffer as it was given highest mean value of disease to healthy ratio (Table 1).

Table 1 - Mean values of disease to healthy ratios in different antigen extraction buffers:

Antigen extraction buffer	Mean values of disease to healthy ratio
EB1	1.277^a
EB2	1.077^b
EB3	1.056^c
LSD	0.195

Mean values of disease to healthy ratios with same superscript are not significantly different.
Effects of antigen extraction buffers are significantly different at P=0.06

3.2 Determination of Best Tissue Dilution and Best Antiserum Dilution.

Mean values of disease to healthy ratios of tissue dilution 1:5, 1:10 and 1:15 were significantly different at P=0.05 for two antiserum dilutions and the best tissue dilution was 1:5(w/v) in PBST+ 1% Na_2SO_3, as it was given the highest mean value of disease to healthy ratio(Table 2).

Mean values of disease to healthy ratios of antiserum dilution 1:100 and 1:200 were significantly different at P=0.05 for all tissue dilutions and the best antiserum dilution was 1:200(v/v) in PBS-TPO as it was given the highest mean value of disease to healthy ratio (Table 3).

Table 2 - Mean values of disease to healthy ratios:

Tissue dilution(w/v)	Mean values of disease to healthy ratio	
	Antiserum dilution 1:100	Antiserum dilution 1:200
1: 5	1.572^a	1.862^a
1: 10	1.415^b	1.727^b
1: 15	0.857^c	0.912^c
LSD	0.0788	0.0886

Mean values of disease to healthy ratios with same superscript are not significantly different.
Effects of tissue dilutions are significantly different at P=0.05

Table 3 - Mean values of disease to healthy ratios for tissue dilutions:

Antiserum dilution(v/v)	Mean values of disease to healthy ratio		
	Tissue dilution 1:5	Tissue dilution 1:10	Tissue dilution 1:15
1:100	1.572^a	1.415^a	0.857^a
1:200	1.862^b	1.727^b	0.912^b
LSD	0.126	0.08	0.048

Mean values of disease to healthy ratios with same letters are not significantly different.
Effects of antiserum dilutions are significantly different at P=0.05

CONCLUSIONS

The locally prepared antiserum against partially purified BSV could be use to detect BSV infected plants by indirect ELISA after it was absorbed by partially purified healthy sap. The ELISA protocol developed in 1999 at PVIC can be used to detect the virus using antigen extraction buffer as PBST + 1% Na_2SO_3 , tissue dilution as 1:5(w/v) in PBST + 1% Na_2SO_3 buffer and antiserum dilution as 1:200(v/v) in PBS-TPO buffer.

ACKNOWLADGEMENT

The authors wish to express their gratitude to all the staff members of Plant Virus Indexing Centre of the Department of agriculture and to the technical staff at Animal House of Medical Research institute. Prof. D.P.S.T.G Attanayaka, Head of the Biotechnology department, Faculty of Agriculture and Plantation Management is gratefully acknowledged for his guidance throughout this study. They are also grateful to Ms. W.M.R. Kumari, Programming assistant at Plant Virus Indexing Centre of the Department of agriculture for the assistance in statistical analysis.

REFERENCES

Anon., (2004). Agricultural and Livestock Data, Economic and Data Management Division, HORDI, Gannoruwa, Sri Lanka.

Agindotan, B.O., G. Thottappilly, A. Uwaifo and S. Winter,(2003). Production of monoclonal and polyclonal antibodies against a Nigerian isolate of banana streak virus. African Journal of Biotechnology, 2:171-178.

Ariyarathne and T. Liyanage, (2002). Survey on incidences and severity of virus disease of banana in Sri Lanka. Annals of the Sri Lanka department of Agriculture, 4:245-254.

Brunt, A.,K., Crabtree and A.Gibbs, (1990). Viruses of tropical plants.Mellesham, Redwood press Ltd.,Wiltshire, UK, 93-94.

Dassanayake, E.M. and Rathnabharathi, B.M, (2002). Development of virus free banana foundation stock. Annals of the Sri Lanka department of Agriculture.

Geering, A.D.W., L.A.McMichael, R.G. Dietzge and J.E. Thomas,(2000). Genatic diversity among banana streak virus isolates from Australia. Phytopathology, 90:921-927

Gimhani, D.R., B.M.V.S. Basnayake and E.M. Dassanayake, (2005). Proceedings of 5th agricultural Research symposium. In faculty of Agriculture and Plantation Management, Makandura, Gonawila.

LaFleur, D.A., B.E.L. Lockhart and N.E. Olszewski, (1996). Portions of the banana streak badnavirus genome are integrated in to the genome of its host Musa. Phytopathology, 86:100-102.

Lockhart, B.E.L.,(1986). Purification and serology of a bacilliform virus associate with banana streak disease. Phytopathology, 76:995-999.

Ndowora, T.C.R. and B.E.L. Lockheart, (2002). Development of a serological assay for detecting serologically diverse BSV isolates. Archives of Virology, 147:2393 – 2404