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EVALUATION OF *IN VITRO* PROTOCOLS FOR ELIMINATION OF BANANA STREAK VIRUS FROM TISSUE CULTURED EXPLANTS IN BANANA SEEDLING PRODUCTION

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ABSTRACT

The banana industry in Kenya is threatened by the presence of Banana streak virus (BSV). The Jomo Kenyatta University of Agriculture and Technology (JKUAT) commercial banana laboratory uses tissue culture (TC) technique for mass propagation of plantlets which are free from most disease causing organisms for commercial purposes. To evaluate in vitro protocols for production of Banana Streak Virus-free TC banana planting materials for farmers, leaf samples were collected from Thika, Kisii, and JKUAT orchards for indexing. The corms were taken through the TC procedure up to the 2nd subculture stage after which they were subjected to three virus elimination techniques; chemotherapy, meristem tip culture and thermotherapy for evaluation. Indexing for BSV using PCR BSV indicated 90, 80 and 40% infection levels for Kisii, JKUAT and Thika orchards, respectively. For chemotherapy evaluation, concentrations of between 10 and 40 mg/l were used resulting in 0 to 90% virus elimination. For thermotherapy, 27°C (control), 32°C, 34°C, 36°C and 38°C for 10 days, resulted in 0 and 90% virus elimination. Meristem tip culture at 1, 2, 3, 4 and 5mm (control) gave between 0 and 90% virus elimination, respectively. The study indicates that BSV can be eliminated using chemotherapy, thermotherapy and meristem tip culture. Chemotherapy using salicylic acid at 20mg/l can be used to eliminate BSV up 90%. It is also easy to implement since it is incorporated into the medium.

Keywords: *Banana streak virus*, Chemotherapy, Thermotherapy, Meristem tip

Contribution/ Originality

This study contributes to the existing literature on virus elimination from banana planting materials globally and the first logical analysis in the fight against banana streak virus in Kenya. If adopted, the findings of the study can help banana farmers increase the yield translating to higher income and poverty eradication.

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1. INTRODUCTION

In Kenya, banana is grown for home consumption and for the national market [1]. It is also perceived as a major avenue to alleviate food insecurity in the region [2]. Despite this, optimum banana yields are lowered by banana streak disease (BSD) caused by *Banana streak virus* (BSV). BSV is prevalent in all banana growing regions in Kenya and all popular cultivars grown by farmers are susceptible [3]. The JKUAT banana tissue culture (TC) laboratory is one of the biggest in Kenya among others like Mimea limited and KARI Thika, producing over two million plantlets per year [4]. The propagation method in use at the JKUAT commercial laboratory is micro propagation of banana corms through tissue culture. This method ensures production of sufficient planting materials which are free from most disease causing organisms but are not free from banana viruses. The JKUAT TC laboratory supplies planting materials all over the country and the East African region including Ethiopia, Southern Sudan and Somalia [4].

Initiation of up to 400, 100, and 100 pieces from JKUAT, Kisii and Thika orchards, respectively are used in the TC laboratory every month. It is therefore important to screen for the presence of viruses in initiation materials before using for massive TC plantlets production. *Banana streak virus*-infected banana plants frequently express broken or continuous chlorotic or necrotic streaks on the leaves, stunting of diseased plants and occasionally heart-rot of the pseudostem and plant death. However, the disease symptoms may vary depending on virus strain, host genotype, level of management and environmental conditions Harper, et al. [5], Dahal, et al. [6], and Lockhart and Jones [7]. *Banana streak virus* causes yield loss of up to 90% [8], [9].

There are many *BSV* elimination methods in use today which include chemotherapy (the use chemicals such as Ribavirin and salicylic acid), thermotherapy and meristem tip culture which involves the use of the apical dome or shoot tip with a few leaf primordia of the size less than 2mm in length as explant, cryotherapy and electrotherapy [10]. In this study, three virus elimination methods namely chemotherapy, thermotherapy and meristem tip culture were evaluated to determine their effectiveness in eliminating BSV from infected banana explants.

2. MATERIALS AND METHODS

2.1. Sampling Sites

Three orchards were selected from four different banana orchards, namely Kisii, Thika and JKUAT for leaf and sucker samples. Nusu Ng'ombe, Kampala and Fhia 18 varieties were randomly sampled (five symptomatic and five asymptomatic) from Kisii, JKUAT and Thika orchards respectively.

2.2. Nucleic Acid Extraction, PCR and Gel Electrophoresis

Total genomic DNA extraction from the leaf samples was done using the CTAB method as described by Doyle and Doyle [11]. Nucleic acid quantification was done by observing the bands on 0.8% agarose gel (0.8 g agarose gel dissolved in 100ml of Tris boric EDTA (1 X TBE) to confirm presence and quality of nucleic acid. The purity and nucleic acid concentration was determined by measurement of the absorbance at 260 and 280 nm in a spectrophotometer.

Five specific primer pairs (Table 1.0) for detecting each *BSV* strain were used for detecting the presence of the various BSV strains in the banana leaves. PCR mixes (20 µL) containing 10 µl 2x GoTaq Green Master Mix (Promega Corp, Madison, WI), 5 ρmol of each primer, 1 µl of nucleic acid extract and water to final volume. Polymerase chain reaction (PCR) cycling conditions included an initial denaturation of 94°C for 2 min followed by 35 cycles at 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 2 min. Reaction products were analyzed by agarose gel electrophoresis and amplicons visualized in 1.5% agarose gel.

Table-1. Primer pairs used in the amplification of various BSV strains

Primer name	Primer sequence	Gene bank Accession No[1]	Virus strain[2]	Fragment length (in base pairs)
RD-F1	5'-ATCTGAAGGTGTGTTGATCAATGC-3'	AF215816	BSV-RD	522
RD-R1	5'-GCTCACTCCGCATCTTATCAGTC-3'	AF215816	BSV-RD	522
Cav-F1	5'-AGGATTGGATGTGAAGTTTGAGC-3'	AF215815	BSV-Cav	782
Cav-R1	5'-ACCAATAATGCAAGGGACGC-3'	AF215815	BSV-Cav	782
GF-F1	5'ACGAACTATCACGACTTGTTGTTCAAGC-3'	AF215814	BSV-GfV	476
GF-R1	5'-TCGGTGGAATAGTCCTGAGTCTTC-3'	AF215815	BSV-GfV	476
BSV4673-F1	5'-GGAATGAAAGAGCAGGCC-3'	AJ002234	BSOEV	644
BSV5317-R1	5'-GGAATGAAAGAGCAGGCC-3' 5'-AGTCATTGGGTCAACCTCTGTC-3'	AJ002234	BSVOEV	644
1A-F1	5'-CTNTAYGARTGGYTNATGCCNTTYGG-3'	AY189378	Badna	597
4'-R1	5'-TCCAYTTRCANAYNSCNCCCCANCC-3'	AY189383	Badna	597

Adopted from Geering, et al. [12], Harper, et al. [13] BSV-RD = BSV Red Dacca, BSV- Cav= BSV Cavendish, BSV- GfV= BSV Gold Finger,
BSV- BSOEV= BSV – Mysore, Badna = degenerate primer
[1]Represents the accession number of the sequence used for primer designing
[2]Represents the name of the virus strain from which primers was designed

2.3. Multiplication of BSV Infected Plantlets

Suckers were uprooted from the three mother orchards supplying JKUAT banana commercial laboratory with planting materials and taken in the laboratory. They were surface sterilized using 70% ethanol for about 8 to 10 seconds after which they were transferred for 20 minutes in 70% sodium hypochlorite where three drops of tween 20 (wetting agent) had been added, the explants were then rinsed twice using double distilled water in the clean bench.

The scorched outer coverings caused by the sodium hypochlorite were removed using sterile surgical blades, leaving a cube of about 2.5 cm^3. Using sterile forceps, the explants were placed in 500ml^3 jam jars containing semisolid MS medium. Cultures were incubated at 26 ± 1°C under photoperiod cycles of 16 hour under light and 8 hours dark. Light intensity of 25000 lux, from white fluorescent tubes was used. The explants were sub cultured after every 4 weeks to fresh MS multiplication medium until they reached the 2nd subculture while those explants that were BSV infected were subjected to virus elimination methods namely chemotherapy, thermotherapy, and meristematic tip culture. The infected banana explants were cultured in Murashige and Skoog medium with specification [14] for multiplication.

2.4. Chemotherapy, Thermotherapy and Meristem Tip Culture of BSV-Infected Plantlets

The plantlets were transferred to fresh MS medium after every 4 weeks to obtain enough TC material for the purposes of eliminating the virus. After the second subculture, the plantlets were

subjected to the three virus elimination techniques namely chemotherapy, using two chemicals (ribavirin and salicylic acid) at 10, 20, 30 and 40 mg/l concentrations, thermotherapy at 32°C, 34°C, 36°C and 38°C and 27°C as control and meristematic tip culture using 1, 2, 3, 4, and 5mm size explants. In chemotherapy, *In vitro* meristems tips (5mm long) at the 2nd subculture were excised in a laminar flow hood and cultured in MS medium supplemented with 0 (control), 10, 20, 30 and 40 mg /l of ribavirin. Another set of cultures were supplemented with 0 (control), 10, 20, 30 and 40 mg/l of salicylic acid. After 4 weeks of incubation in the laboratory, the leaves were removed using sterile surgical blades in a laminar flow hood, and put in well labelled packing bags then stored in a freezer for virus indexing. In thermotherapy, explants at 2nd stage sub-culture with well-formed shoots were incubated at varying temperatures, 27°C (control), 32°C, 34°C, 36°C and 38°C for ten days. The leaves were then removed using sterile surgical blades in a laminar flow hood, and placed in well labelled packing bags and then stored in a freezer for PCR detection of BSV. In meristem tip culture, at the 2nd sub-culture, explants of 1mm, 2mm, 3mm, 4mm and 5mm (control) in size were excised using clean surgical blades in the clean bench, before being inoculated onto petri dishes containing MS medium. They were left for 2 to 3 days after which they were transferred to jars containing 500mls hormone free MS medium to enhance shoot elongation, for 4 weeks. The explants were then transferred to jars containing fresh MS medium supplemented with 10mg/l NAA to enhance root formation and the leaves were put in well labeled packing bags and stored at –20° C. DNA was extracted using CTAB method according to Doyle and Doyle [11] and DNA visualized in 0.8% agarose gel. Once the quantity of DNA was confirmed, PCR was done to determine whether the virus had been eliminated.

2.5. Data Collection and Analysis

The number of explants that survived and number of plantlets without the BSV was recorded after every four weeks in spread sheets and before the analysis, the collected data was converted into percentages then they were analyzed using the generalized linear model using the Poisson log linear model.

3. RESULTS

3.1. Detection BSV Infection in Banana Plantlets by PCR

All BSV- specific strains were detected in all the samples except BSV- Cavendish. Double infections of BSV strains were observed in three samples while one Sample had three BSV strains (Fig 1).

Only Red Dacca and Gold Finger strains were amplified when PCR was performed on Fhia 18 samples. Out of the ten samples amplified, five had at least one BSV strain infection (Table 2.0).

In Nusu Ng'ombe variety, PCR analysis using the five BSV specific primers, showed that out of the ten samples tested, nine had at least one or more BSV strain detected (Table 2.0).

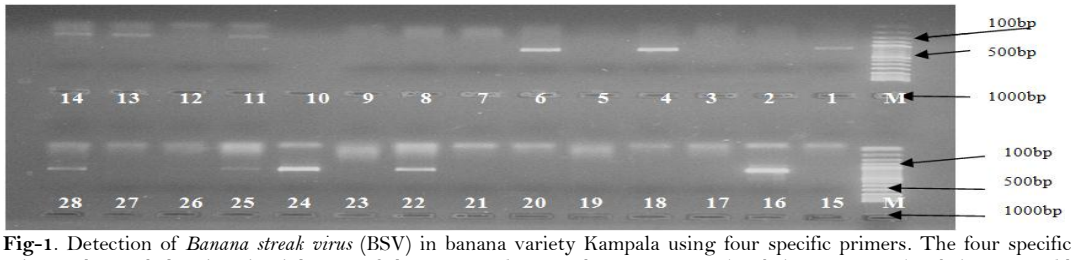

Fig-1. Detection of *Banana streak virus* (BSV) in banana variety Kampala using four specific primers. The four specific primers detected the virus in eight out of the ten samples tested, BSV Mysore (589bp), BSV –RD (522bp), BSV Gold Finger (476bp). Lane 1-10: BSV Mysore, lane 11-20: BSV Red Dacca, lane 21-29: BSV Gold Finger, lane M: molecular weight marker

No BSV– Cavendish strain was detected in this variety while the most prevalent BSV strain from Nusu Ng'ombe variety was Red Dacca (522bp), which was amplified in seven out of ten samples tested. BSV- Gold Finger (476bp) strain was amplified in three samples. Three samples had double infections while the other six had only one infection. When the degenerate primer (Badna-596bp) was used, BSV strains were detected in all samples except in 56K.

Table-2. Detection of BSV in mother orchards by PCR

Orchard	Variety	% BSV infection detection via PCR
JKUAT	Kampala	80
KISII	Nusu Ng'ombe	90
THIKA	Fhia 18	50

3.2. Virus Elimination

The BSV infected samples confirmed by PCR were subjected to the three virus elimination techniques and the virus eliminations recorded (Table 3, 4 and 5). For chemotherapy, the higher the concentration of the chemicals used (Ribavirin and Salicylic acid), the higher the percentage in virus elimination in all the three banana varieties (DF=1; $P < 0.05$). For thermotherapy, there was 100% survival in all the temperatures used except at 38°C in which all the explants were scorched. Higher virus elimination was recorded at 36°C (DF=2; $P<0.01$) (Table 4.0). For meristem tip culture, the smaller the size of the explants, the higher the percentage in virus elimination. However, the smaller the size of the explants the lower was the regeneration of the explants (DF= 4; $P < 0.001$), (Table 5.0)

3.2.1. Chemotherapy

In chemotherapy, variety, chemical and chemical concentration had an effect on survival of the explants and virus elimination. (Table 3.0, 4.0, 5.0)

3.2.1.1. Effect of Variety on Survival of Explants and BSV Elimination

Variety used had an effect on virus elimination and survival (df=; $P<0.05$). Nusu Ng'ombe variety had the highest 80.9±2.35 BSV elimination against Kampala and Fhia 18 at 73.3±5.42 and 80.8±2.16 respectively using the same chemicals and same chemical concentrations. Variety Nusu

Ng'ombe had the highest mean of the surviving explants of 65.65 against Kampala and Fhia 18 at 62.6 ±6.01 and 57.92 ±7.57 respectively (Df=2; P<0.05) (Table 3.0)

Table-3. Effect of variety on survival of explants and BSV elimination

Variety	Survival of explants	Virus elimination
Fhia 18	57.92+7.566	80.83+2.163
Kampala	62.59+6.006	73.33+5.417
Nusu Ng'ombe	65.65+6.090	80.87+2.345

There was an effect of variety used on survival and BSV elimination (DF=2, P<0.05).

3.2.1.2. Effect of Chemical on Survival of Explants and Virus Elimination

Even though both chemicals eliminated the virus, elimination using salicylic acid was higher than that of ribavirin (DF=2; P<0.05) Salicylic acid had a higher survival mean of 62.1 ± 3.97 compared to ribavirin at 61.9 ± 5.18 salicylic acid had a higher BSV elimination of 81.1 ± 1.816^{b}% as compared to 75.26 ± 3.969^{a}% across the three varieties and chemical concentrations (Table 4.0).

Table 4. Effect of chemical on survival of explants and virus elimination

Chemical	survival	BSV elimination
Ribavirin	61.94 ± 5.177	75.26 ± 3.969
Salicylic acid	62.11 ± 3.969	81.11 ± 1.816

3.2.1.3. Effect of Chemical Concentration to Survival of Explants and BSV elimination

At 0mg/l, survival of explants was as higher as 96.8+3.33 with 0.0+0.0 BSV elimination. When concentration was increased, survival of explants reduced with increase in BSV elimination. At 10, 20, 30 and 40mg/l had 83, 78, 66 and 11explants survival and 76, 82, 84 and 88 BSV elimination respectively (Table 5.0) (DF=4; P<0.05)

Table 5. Effect of chemical concentration on survival of explants and BSV elimination

Chemical concentration (mg/l)	Explant Survival	BSV Elimination
0	96.67+3.333	0.00+0.00
10	83.33±3.052	76.67±2.567
20	78.33±3.052	82.78±1.948
30	66.11±5.248	84.44±2.318
40	11.76±2.461	88.76±0832

Chemical concentration had a significant effect on explant survival and BSV elimination at DF= 4; P<0.05)

3.2.2. Thermotherapy

3.2.2.1. Effect of Variety on BSV Elimination and Survival of Explants

The variety had a significant effect on both BSV elimination from the explants (df=2; P<0.001) Kampala variety had the highest BSV elimination (39.6 ±10.86 while Nusu Ng'ombe had the lowest virus elimination (37.9±10.93) at the same temperatures (Table 6.0).

Table 6. Effect of variety to BSV elimination and survival of explants

Variety	Virus elimination	Survival
Fhia 18	39.17±10.88[a]	100.00±0.00[a]
Kampala	39.58±10.86[a]	100.00±0.00[a]
Nusu Ng'ombe	37.92±10.93[a]	100.00±0.00[a]

Variety used had a significant effect on both BSV elimination from the explants (df=2; P<0.001)

3.2.2.2. Effect of Temperature on BSV Elimination and Survival of Explants

Temperature had a significant effect on BSV elimination as well as survival of explants (p<0.05). The higher temperature, contributed to higher BSV elimination. All temperature levels used had a 100.0 ± 0.00 explants survival except when the explants were placed at 38°C when all explants got scorched The lower the temperature, the higher the BSV elimination (Table 7.0).

Table 7. Effect of Temperature on BSV elimination and survival of explants

Temperature (°C)	Virus elimination	Survival
27	0.00±0.00	100.00±0.00
32	13.33± 6.47	100.00±0.00
34	57.78±5.54	100.00±0.00
36	84.89±1.54	100.00±0.00
38	n/a	died

Temperature had a significant effect on BSV elimination (Df =4; P<0.05)

3.2.3. Meristem Tip Culture

3.2.3.2. Effect of Meristem Tip Size on BSV Elimination and Explants Survival

The highest virus elimination was at 1mm sized explants. The smaller the explant used the lower the survival and the higher the BSV elimination and vice versa in all the three varieties. At 1mm, BSV elimination was 89.0±1.20 and 32.4±0.38 plants survival respectively, (DF = 4; P<0.001) (Table 9.0)

Table 9. Effect of meristem tip size on BSV elimination and explants survival

Size (mm)	BSV elimination	Explants survival
1	89.00±1.202[a]	32.44±0.377[b]
2	62.56±0.766[a]	100.00±0.00[a]
3	43.00±3.00[b]	100.00±0.00[a]
4	0.56±0.242[c]	100.00±0.00[a]
5	0.11±0.11[c]	100.00±0.00[a]

The size of the explant used had a significant effect on both the BSV elimination and survival of the explants in all the varieties (Df=4; P<0.001).

4. DISCUSSION

All the three methods evaluated for elimination of Banana streak virus were effective though at different specifications. Heat therapy gave the best results at 36°C, while chemotherapy gave the best results at 20mg/l for both chemicals used separately and meristem tip culture at 1 mm .

The different specific primers pairs (Table 1.0) used to detect different strains of BSV amplified all the BSV strains present except for BSV Cavendish. Results of this work showed that 50% of the samples collected were BSV infected (Table 2.0). These results are in agreement with previous studies carried out in Kenya which showed that BSV is prevalent in all banana growing regions and that all popular cultivars grown by farmers are susceptible [15], [16], [3].

In meristem tip culture, the size of the explant used had a significant effect on both explant survival and BSV elimination. The smaller the size of the meristem used the higher the BSV elimination (Table 8.0 and 9.0).

The success of heat therapy depends on selecting the temperature and the duration of the treatment for the elimination of the virus and also with the survival of the plant. In this study, all explants exposed to temperatures of 27°C, 32°C, 34°C and 36°C survived after being put in an incubator for 10 days, but at 38°C all the explants were scorched by the heat and therefore no plantlet could be regenerated from this treatment. The highest virus elimination percent was at 36°C (Table 6.0 and Table 7.0).

The results demonstrate that ribavirin and salicylic acid at concentration of 10mg/l enhanced growth differentiation of propagated meristems. It can be concluded that the use of ribavirin and salicylic acid separately at 20 mg/l combined with TC process can increase the percentage of virus-free plants. It was noted that the higher the chemical concentration , the higher the virus elimination percentage (Table 5.0). This results are in agreement with those obtained by Qiaochunand, et al. [10].

Although, thermal treatment at 36°C resulted in high viral elimination and survival rate of explants treated, the technique can be cumbersome for commercial laboratories leaving 20mg/l of salicylic acid as the best option. This is because it is easy to incorporate the chemical into the medium used in TC and it is far much cheaper than thermotherapy which requires an incubator and frequent monitoring.

When TC procedures without any of the three virus elimination techniques was used as control, there were no viral elimination which is in agreement [15], [3] that TC technique does not eliminate *Banana streak virus*. Therefore, though TC has very many advantages over the conventional methods of mass propagation, it does not eliminate BSV. Therefore, a combination of TC with a reliable virus elimination method is required to be put in place to be able to eliminate BSV.

5. CONCLUSIONS AND RECOMMENDATIONS

This study demonstrated that *Banana streak virus* can be eliminated using chemotherapy, thermotherapy and meristem tip culture. Chemotherapy using salicylic acid at 20mg/l was selected as the best method with up to 90% BSV elimination. The method is also easy to implement since it is incorporated into the medium. It is recommended that *banana streak virus* free orchards be established and continuous checks be done to ensure only BSV plantlets are in circulation.

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