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Elimination of Cassava Brown Streak Virus from Infected Cassava

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Abstract
Cassava brown streak disease (CBSD) is an economically important disease of cassava (*Manihot esculenta crantz*) caused by *Cassava brown streak virus* (CBSV). Use of clean planting material is one of the strategies for disease management. However, obtaining clean planting material for some farmer-preferred varieties is often difficult. This research was aimed at evaluating the effect of meristem tip sizes, effects of varying concentration levels of ribavirin and salicylic acid and determining the efficacy of thermotherapy in combination with either meristem tip culture or chemotherapy in the elimination of CBSV from infected cassava. CBSV infected cuttings of Guzo variety collected from Coast province of Kenya were established and maintained in a greenhouse at the Plant Quarantine Station in Kenya Plant Health Inspectorate Service in Muguga were used as test plants. Cassava leaves were sampled from eighteen cassava plants of Guzo variety and virus indexing was done using Reverse Transcriptase-Polymerase Chain Reaction with virus specific primers and those that tested positive for CBSV were used as initiation materials. From the i*n vitro* plantlets established, the second sub-cultures were subjected to the virus elimination procedures. *In vitro* meristems (0.5mm, 1mm, 2mm and 10mm) were obtained and cultured in modified Murashige and Skoog media. For chemotherapy, nodes were cultured in MS media supplemented with antivirals at 0mg/l, 10mg/l, 20mg/l, 30mg/l. In the combination treatments single nodal plantlets were subjected to thermotherapy at 38ºC for twenty one days then excised meristem tips (1.0mm) with some plants being subjected to ribavirn treatments at (10mg/l, 20mg/l and 30mg/l). Data was analysed using Genstat 13th edition (2013). The regeneration of plants established from 0.5mm was 63% while 2mm was 88%. In chemotherapy survival of shoots was observed to decrease with increase in the antiviral concentrations. Ribavirin at 10mg/l recorded the highest rate of survival compared to the other treatments. On the other hand salicylic acid exhibited the least survival rate compared to ribavirin. The number of plants testing negative was observed to increase with increase in concentration for both chemicals. At 30 mg/l of ribavirin and salicylic; 88.8% and 100% of virus free plantlets were produced respectively. Thermotherapy (38°C) combined with meristem tips (1mm) resulted in 68% of regenerated plants with 84% being virus free. *Invitro* plants that had been thermo treated and then subjected to chemotherapy did not give the expected results since all plants died. Thermotherapy at (38°C) for a period of twenty one days combined with meristem tip culture can be used for production of virus free cassava.
Keywords: Cassava brown streak virus, thermotherapy, chemotherapy, meristem tip culture, Virus elimination.

1.0 Introduction

CBSD is spreading fast in several countries in East and Central Africa (Hillocks and Jennings, 2003). Nearly all cassava varieties that have been bred for resistance to CMD are susceptible to CBSD (Hillocks and Jennings, 2003). Coat protein (CP)-encoding sequences of coastal lowland CBSV isolates (*Monger et al.,* 2001) and complete CP sequences of highland UG isolates from East Africa are available, revealing that these isolates belong to two phylogenetically different strains. Both have (+) ss RNA genomes, which belong to the genus *Ipomovirus* in the family *Potyviridae,* and produce generally similar symptoms in infected plants. It is difficult to recognize CBSD symptoms because of their variability and poor expression on leaves (Ntawuruhunga and Legg, 2007). This makes the ability of the farmer to select planting material only from healthy mother plants, and then rogue plants that show symptoms soon after sprouting as a control measure for CBSV difficult to practice. The effect of this constraint has led to reduction of yields in Kenya to 5-10t/ha against a potential of about 32t/ha (Munga and Thresh, 2002). As a result, cassava production has declined drastically since 1995; some areas have experienced almost total crop failure, prompting farmers to abandon production, especially of highly susceptible varieties (Obiero *et al.,* 2007). The use of infected plant cuttings has been reported to be the main avenue for disease spread in the affected regions (Munga and Thresh, 2002). Unlike bacterial and fungal diseases, viral diseases have no effective chemical control on infected plants, Lebot (2009). The supply of virus-free planting materials is therefore important for sustainable crop production and is a prerequisite for the international exchange of germplasm to avoid risks of introducing diseases to uninfected areas Lebot (2009).

Recognizing the importance of this situation, it was proposed that only virus-tested tissue culture materials be used for inter country germplasm movement (Ntawuruhunga and Legg, 2007). Notably, recent introductions of germplasm to both Rwanda and Burundi, from the Kenya Plant Health Inspectorate Service Plant Quarantine Station at Muguga, Nairobi supported by East Africa Regional Research Network have been in tissue culture form (Ntawuruhunga and Legg, 2007).This is because tissue culture techniques offer the most viable methods for obtaining virus-free stocks by viral eradication usually aided by meristem tip culture, thermo- and/or chemotherapies (Mellor and Stace Smith, 1970).

The plant meristem is a zone of cells with intense divisions, situated in the growing tip of stems and roots. In plants, viruses are rapidly disseminated through the vascular system which is absent in the meristematic tissues, those located in the phloem cannot invade the meristematic tissues because there is no cell differentiation in this zone (Alam *et al.,* 2010). Meristem culture *in vitro* has been used for many decades to eliminate plant viruses (Faccioli and Marani, 1998) in several species (Mervat and Ashoud, 2009; Mohammad *et al.,* 2009; Manganaris *et al.,* 2003).

Chemotherapy is centered on base analogs, with the presumption that the synthesis of the nucleic acid of the virus could be inhibited by such molecules (Panattoni *et al.,* 2013). Valuable contributions have been provided by investigations of antiviral chemotherapy performed in clinical medicine (Panattoni *et al.,* 2013). The potential similarities between animal and plant hosts' metabolic pathways present in both, has been the starting point for experimentations on phytoviruses (Panattoni *et al.,* 2013). In this regard the discovery of ribavirin presented a defining moment in research (Sidwel *et al.,* 1972). Ribavirin compound is a guanosine analog with broad-spectrum activity against animal viruses and appears also to be active against plant virus replication in whole plants (Sidwel *et al.,* 1972). The efficiency of ribavirin in the elimination of plant viruses is documented in some crops (Fletcher *et al.,* 1998; Panattoni *et al.,* 2013; Nascimiento *et al.,* 2003) and depends on the utilized concentration, host plant and type of infected tissue (Paunovic *et al.,* 2007). Salicylic acid on the other hand functions by inhibiting catalase and ascorbate peroxidase enzymes which results in elevated levels of hydrogen peroxide and other reactive oxygen species (ROS) derived from hydrogen peroxide which then activates the plant defence-related genes such as pathogen related (PR)-1 gene against pathogens and diseases (Gafney *et al.,* 1993). Salicylic acid is a potential antiviral that can be used to eliminate viral pathogens (Gafney *et al.,* 1993).

Plant thermotherapy is described as achieving a cellular environment which is progressively less adequate for virus vitality (Mink *et al.,* 1998). The absence of mosaic symptoms on the leaves of rooted explants and effects after subjecting diseased donor cassava explants to heat treatment for atleast 30 days at 35°–38°C has been reported by (Adejare and Couts, 1981; Chellapan *et al.,* 2005). Improved virus elimination can occur also if chemotherapy is combined with thermotherapy (Luciana *et al.,* 2007). Joint effects of thermo at 37°C and ribavirin applied to *in vitro* plants was highly efficient in eliminating potato virus Y resulting in 83.3% of virus free potato plants (Nascimiento *et al.,* 2003). Further reports support the use of thermotherapy together with the addition of antiviral agents into the growth medium as the best treatments for virus elimination in potato (Fletcher *et al.,* 1998; Griffiths *et al.,* 1990). Thus, the aim of the study was to optimise *in vitro* techniques for CBSV elimination from infected cassava.

2.0 Materials and Methods

2.1 Test cassava plant materials

Thirty stems of popularly grown Kenyan cassava of Guzo variety exhibiting CBSV symptoms were collected from the Coast province. The plants were confirmed to be infected through reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method modified from (Lodhi *et al.,* 1994).

The extracted RNA was then subjected to a one step RT-PCR for virus detection using primer set CBSV 10 (5'ATCAGAA TAGTGTGACTGCTGG-3') and CBSV 11 (5'CCACATTATTATCGTCACCAGG-3') (19) which amplify ~230 bp length nucleotides. The 10 µl PCR reaction mix contained 6.39µl of sterile distilled water, 1 µl of 10x MMLV buffer, 0.3 µl dNTPs (2mM), 0.08 µl of Taq polymerase (5U/µl), 0.15µl of the primer mix, and 2 µl of RNA template. Thermal cycling conditions comprised of Pre-PCR program for generating the cDNA in 1 cycle at 42°C for 30 min 94°C for 2 min, 52°C for 2 min and 72°C for 3min. The PCR regime for cDNA multiplication; included 30 cycles of 94°C for 30 min, 52°C for 30 sec 72°C for 1 min and stored at 4°C. Gel electrophoresis was done in 1x TBE at 100V for 1hr and visualized the products on a UV transimilluminator.

2.2 Multiplication of CBSV positive cassava plants

2.2.3 Media preparation; The culture medium used for initiation of CBSV infected cassava plants was prepared using MS medium (Murashige and Skoog, 1962) supplemented with 30 g/litre of sucrose; 7.0 g/litre of agar and 0.1 mg/litre of (gibberellic acid - GA3).The meristem media was supplemented with 30 g/litre of sucrose; 7.0 g/litre of agar, BAP 0.1mg/l, NAA 0.15mg/l and 0.03 mg/litre of GA3.

2.2.4 Sterilization and initiation of explants; About 2-3 nodes of the apical buds were cut from the CBSV positive cassava plants using clean sterile blades. The node cuttings were washed three times using tap water

containing 2 drops of tween 20 to remove excess debris and sequentially rinsed with distilled water. The explants were then soaked in 20% sodium hypochlorite solution for 20 minutes. After 20 minutes the explants were again rinsed 3 times with sterile distilled water. The edges of the scorched ends of the nodes were carefully cut under sterile conditions and each node was individually cultivated in the modified MS media and incubated in a growth room under a temperature regime of 24 ± 1^{o}C under 16-hour photoperiod provided by fluorescent bulbs with light intensity of 1500 lux. Transfer to fresh medium was done after 6 weeks.

2.3 Meristem tip excision

Under a binocular dissecting microscope, leaflets from the *in vitro* second subcultures surrounding the apical tip were removed until only the apical dome and a few primodium leaves remained. Using sterile needles meristem tips were cut and size determined using the microscope lens ruler. Different sizes of the meristems 0.5 mm, 1 mm, 2 mm and a node of 10 mm which was used as a control were individually cultured in testubes containing the modified MS media and were incubated in the growth room. The meristems were left to establish for a period of 4 weeks before transferring onto MS media without BAP and NAA hormones.

2.4 Chemotherapy

Single nodal cuttings from the second subcultures were transferred to media supplemented with ribavirin and salicylic acid at concentrations of 0, 10, 20 and 30 mg/l for two weeks before transferring onto media without antiviral compounds.

2.5 Thermotherapy at 38 °C combined with meristem tip culture

Single nodal cuttings from the second subcultures were cultured in modified MS and incubated for 14 days at 24±1°c for establishment and later transferred to the thermotherapy chamber at temperatures of 38^{o}C at humidity of 80% for a period of 21 days. These temperatures were maintained under photoperiod cycle of 16/8 hr light /dark. *In vitro* meristems (1.0mm) were then excised and cultured in modified MS media supplemented with 30 g/litre of sucrose; 7.0 g/litre of agar and 0.002 mg/litre of GA3 0.1mg/l BAP and 0.15mg/l NAA). After a period of three weeks the meristems were subcultured onto MS without BAP and NAA hormones. Nodal plantlets of 10mm size were used as controls incubated at 24±1°c under photoperiod cycle of 16/8 hr light /dark.

2.6 Thermotherapy at 38 °C combined with chemotherapy

Single nodal cuttings from the second subcultures were cultured in modified MS and incubated for 14 days at 24±1°c for establishment and later taken to the thermotherapy chamber at temperatures of 38^{o}C and 80% humidity for a period of 21 days. From the heat treated plants, nodal cuttings were cultured in modified MS supplemented with 10, 20, and 30mg /l. Incubation was then done for a period of 2 weeks. Nodal plantlets of 10mm size were used as controls incubated at 24±1°c under photoperiod cycle of 16/8 hr as light /dark.

3.0 Experimental design and data collection and analysis

A completely randomized design was used to subject the tissue cultured material in the different treatments where by each sample was replicated three times. Each treatment had a total number of 15 plants replicated three times totaling 45 plants subjected in (meristem tip culture, chemotherapy and thermotherapy combined with meristem tip culture and chemotherapy). The number of survival, positive and clean plants was recorded. Virus elimination (%) rates were calculated through the division of negative plants obtained by number of survived plants subjected to each treatment multiplied by a hundred. ANOVA analysis using genstat software was used to calculate the least significant differences and standard errors in the survivals.

4.0 Results

4.1 PCR amplification of extracted RNA from the selected Guzo test plants

Out of eighteen plants tested, six were confirmed positive for CBSV (sample number 1-6) (Plate 1). These plants were subsequently used for the different treatments.

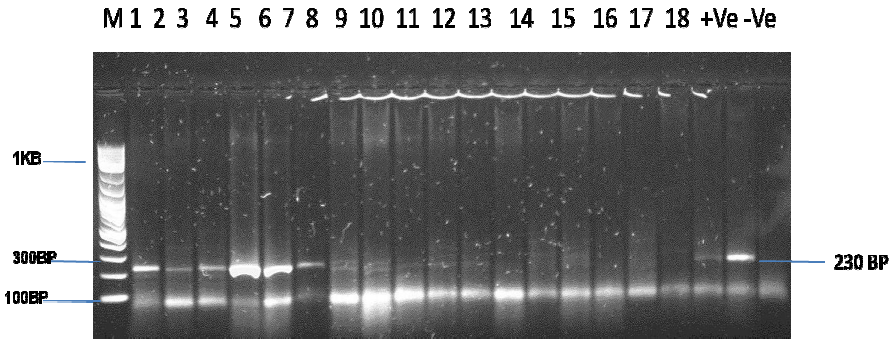

Plate1. Agarose gel-electrophoresis of CBSV detection in infected cassava in a 1% (w/v) agarose gel; lane 1; 1kb ladder; lane 1-18 tested infected cassava; lane19 (positive control) lane 20 (negative control)

4.2 Effects of meristem tip culture on CBSV elimination from infected plants

After a period of 7-10 days, shooting was observed in most of the meristems while some turned brown and failed to grow. The death of meristems was more evident for meristems that were 0.5mm with most of the explants producing callus. Single cultures of meristem derived plantlets exhibited slow growth compared to controls established from nodal cuttings. Cassava plantlets derived from meristems took a period of 10 weeks to establish into complete plants attaining a size of 4-5cm while the controls took a period of 6 weeks to establish into complete plants. The number of plants regenerated from 0.5mm, 1mm and 2mm was 17, 25 and 36 respectively (Table 1). There was a significant ($P<0.01$) difference in the number of plants that survived among the treatments. All the controls plants were positive for CBSV. Meristem tip size of 0.5mm had the highest percentage (88.2%) of virus free plants (Table

Table 1: Survival and virus elimination (%) of regenerated plants from meristem tip culture

Meristem tip size (mm)	**Initiated explants (No)**	**Regenerated plants (No)**	**Positive plants (%)**	**Negative plants (%)**
0.5	45	17	11.7 (2)	88.2 (15)
1	45	25	28.0 (7)	72.0 (18)
2	45	36	33.1 (12)	66.7 (24)
10(Control)	45	45	100.0 (45)	0.0 (0)

Analysis based on survival ($P\leq0.01$) (S.E-standard error) 0.122
Data in parenthesis are actual number of plants

4.3 Effects of chemotherapy on elimination of CBSV from infected cassava.

The concentration of each antiviral compound influenced the number of plants regenerated. There was a significant ($P<0.01$) difference in the number of plants that survived among the treatments. Plants regenerated from ribavirin at 0mg/l, 30mg/l was 45 and 9 respectively while that of salicylic 0mg/l, 30mg/l was 14, 1 (Table 2 and Table 3). The proportion of virus free plants obtained was observed to increase with increase in concentration for both antivirals. At 30 mg/l of ribavirin treatment, 88.9% virus free plants was recorded while at 10mg/l, 68.8% virus free plants was recorded (Table 2). Controls obtained from (0mg/l) resulted in all positive plants obtained.

Table 2: Survival and virus elimination (%) of regenerated plants from ribavirin treatment

Antiviral concentrations	**Initiated explants (No)**	**Regenerated plants (No)**	**Positive plants (%)**	**Negative plants (%)**
Ribavirin				
10mg/l	45	21	31.3 (10)	68.8 (21)
20mg/l	45	15	12 (3)	88 (22)
30mg/l	45	6	11.12 (1)	88.9 (8)
Control	45	45	100 (45)	0.00 (0)

Analysis based on survival ($P\leq0.01$) (S.E-standard error) 0.138
Data in parenthesis are actual number of plants

Table 3: Survival and virus elimination (%) of regenerated plants from salicylic acid treatment

Antiviral concentrations	**Initiated explants (No)**	**Regenerated plants (No)**	**Positive plants (%)**	**Negative plants (%)**
Salycylic				
10mg/l	45	14	(3) 21.42	(11) 78.6
20mg/l	45	11	(1) 9.1	(10) 90.9
30mg/l	45	1	(0) 0	(1) 100
Control	45	45	(45)100	(0) 0.00

Analysis based on survival ($P\leq0.01$) (S.E-standard error) 0.138
Data in parenthesis are actual number of plants

4.4 Effects of thermotherapy combined with either meristem tip culture or chemotherapy in elimination of CBSV from infected cassava

Thermotherapy combined with meristem tip culture resulted in 68% of regenerated plants with 84% being virus free with thermotherapy combined with chemotherapy having no survivals. Meristem tip culture combined with thermotherapy had the highest percentage (87.0) of virus free plants obtained (Table 4). Controls obtained from nodal plantlets resulted in all positive plants obtained.

Table 4: Survival and virus elimination (%) of regenerated plants from thermotherapy combined with both meristem tip culture and chemotherapy

Treatment	Initiated explants (No)	Regenerated plants (No)	Positive plants (%)	Negative plants (%)
Meristem Tip Cultute+ Thermotherapy	45	31	(5) 15.6	(27) 84.4
Chemotherapy+ Thermotherapy Ribavirin 10mg/L	45	0	(0) 0	(0) 0
Chemotherapy+ Thermotherapy Ribavirin 20mg/L	45	0	(0) 0	(0) 0
Chemotherapy+ Thermotherapy Ribavirin 30mg/L	45	0	(0) 0	(0) 0
Control	45	45	(45) 100	(0) 0

Analysis based on survival (P≤0.05) (S.E-standard error) 0.048
Data in parenthesis are actual number of plants

5.0 DISCUSSION

A number of techniques were evaluated for the ability to eliminate CBSV from infected cassava plants. These include meristem tip culture, chemotherapy, thermotherapy and a combination of each of these with thermotherapy. The effectiveness of meristem tip culture on the elimination of CBSV was influenced by the size of meristem tip that was cultured. The larger the size of meristem cultured, the greater was the number of regenerated plants. However the number of virus-free plantlets obtained was inversely proportional to the size of cultured tip in agreement with (Faccioli and Marani, 1998; Milosevi *et al.,* 2012). More plants were established from larger meristems of 1mm and 2mm which is in agreement with earlier observations (Manganaris *et al .,* 2003; Cha-um *et al .,* 2006) that larger meristems of 1.3-2.0mm favored shoot survival and growth. Meristem tips of 0.5mm had the highest (88.2%) proportion of virus-free plants obtained. This is in agreement with earlier work in (Manganaris *et al.,* 2003; Mohammad *et al.,* 2009) which reported that meristems sizes of 0.3-0.5mm were found to be optimum in production of CMD free cassava in the Nigerian cultivars. The production of disease-free sugarcane varieties using meristem culture and elimination of grapevine leaf roll-associated virus-1 and grapevine fan leaf virus from infected grapevine plantlets was achieved using meristems tip sizes of 0.3-0.5mm. As expected control controls plants obtained from nodal plantlets (10mm) resulted in plants that tested positive for CBSV.

The effect of chemotherapy on CBSV elimination from infected cassava was influenced by the antiviral compound used and the level of concentration. Toxicity to plants and antiviral activity was more pronounced in higher concentrations of 30mg/l of both salicylic acid and ribavirin as evidenced by the defoliation of leaves. This high toxicity resulted into high mortality of plants hence low shoot survivals of plants at 30mg/l of ribavirin and salicylic acid. Increasing the concentrations of ribavirin typically increases the effectiveness of virus elimination (Mellor and Stace Smith, 1970), but slowed growth and phytotoxicity may be evident at high concentrations similar to the present study in ribavirin treatments at 30mg/l. The concentrations of many antiviral chemicals required during chemotherapy to inhibit virus multiplication are very close to the toxic concentration for the host plant (Nascimiento *et al.,* 2003) thus at higher concentrations of 20mg/l and 30mg/l survival rates were low but the number of clean plants produced was higher for both ribavirin and salicylic acid. This can be explained by the fact that when mutation rates of viral RNA exceeds a critical threshold; a virus may experience decreased infectivity and/or extinction of the virus population (Mellor and Stace Smith, 1970; Panattoni *et al.,* 2013). At effective low concentrations, replication of the virus is hindered. This concentration however needs to be below which ribavirin is not highly phytotoxic to the plant (Mellor and Stace Smith, 1970) which was in the case of ribavirin at 10mg/l having a higher survival and effective in CBSV elimination. Although salicylic acid-treatments had the lowest survival rates at 30mg/l, it resulted greater proportion of clean plants obtained. This is different from results of (Nascimiento *et al.,* 2003; Sharma *et al.,* 2007) having ribavirin treatments resulting in greater proportions of virus free potato compared to other antiviral compounds.

It is possible that treatment with ribavirin at concentrations that are known to be slightly phytotoxic might be regarded as desirable. In several instances, the virus titre reduction was only by treatment with ribavirin concentrations that also resulted in some tissue damage (Robert and Clark, 1982; Griffiths *et al.,* 1990; Nascimiento *et al.,* 2003). Levels of phytotoxicity have to be tolerated to achieve the eradication of viruses

(Robert and Clark, 1982; Griffiths *et al.,* 1990). When survival and virus elimination were considered 10mg/l of ribavirin was found to be optimum in CBSV elimination from cassava *in vitro.* This is in agreement with (Robert and Clark, 1982) working on potato observed that virus X in potato could not be detected in over 80% of plantlets developed from cultures treated with 10mg/l of ribavirin.

Joint effects of thermo and chemotherapies did not give the expected results. These plants completely dried up turning brown with total mortality being recorded. These results are contrasting with (Nascimiento *et al.,* 2003; Fletcher *et al.,* 1998) who found the combination of thermo and ribavirin applied to *in vitro* potato being highly efficient in elimination of potato virus Y. This can be explained by the fact that concentrations of many antiviral chemicals required during chemotherapy to inhibit virus multiplication are very close to the toxic concentration for the host plant (Paunnovic *et al.,* 2007) which can be lethal to the plants under virus elimination. It is also noteworthy that the complex interaction between the host and biological characteristics of a virus strongly interfere with the outcome and effects of virus elimination (Paunnovic *et al.,* 2007).

Meristems excised from plants subjected to thermotherapy enhanced CBSV eradication compared to the control resulting in 68.8% plant survival with 84% of the plants surviving being virus-free. These findings are not surprising since thermotherapy of *in vitro* plants prior to meristem excision has been found to give fewer virus infected meristem cultured plants in various vegetatively propagated species Acedo (2006). The combination of meristem tip culture and thermotherapy to efficiently eliminate sweet potato featherly mottle in sweet potato has been reported (Mervat *et al.,* 2009). To improve survival, application of meristem culture of 1.8mm-2mm combined with thermotherapy at 35°C is reported to increase the survival rate of *in vitro* explants (Manganaris *et al .,* 2003; Mervat *et al.,* 2009).This is because larger tips can be obtained from heat-treated plants while ensuring virus-free plant production. The use of meristem tips measuring 1mm and subjecting them to thermotherapy at 38°C *in vitro* was efficiently used in the elimination of CBSV from cassava *in vitro.*

CONCLUSIONS

Meristem tip culture combined with thermotherapy was found optimum for elimination of CBSV from infected cassava *invitro* to produce virus free cassava plants.

Farmers should therefore be strongly encouraged to use *in vitro* raised materials that have been adequately diagnosed free from CBSV. This will ultimately reduce the risk of spreading CBSV to uninfected cassava fields.

RECOMMENDATIONS

Various factors have been found to influence elimination of viruses in plants such as meristem tip sizes, effect of thermotherapy and chemotherapy, and the genotype of the plant, biological nature of the virus. Therefore there is need for studies to be done that will show effects of these cleaning methods on different cassava varieties.

There is need for studies to be done that will show effects of these cleaning methods on eliminating the Ugandan cassava brown streak virus (UCBSV) strain in infected cassava.

In addition, there is always a possibility of mutations when the plants are exposed to antiviral chemical hence this should also be investigated in subsequent virus cleaned plants.

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