EVALUATION OF MERISTEM TIP CULTURE, CHEMOTHERAPY AND THERMOTHERAPY ON THE REDUCTION OF CASSAVA BROWN STREAK VIRUS IN INFECTED CASSAVA (GUZO VARIETY) CUTTINGS

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DECLARATION

This research thesis is my original work and has not been presented for a degree award in any institution

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DEDICATION

I dedicate this thesis to the Lord for his gracious providence; my beloved parents Philip Ndemwa and Virginia Ndemwa.

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ABBREVIATIONS AND ACRONYMS

BAP	Benzylaminopurine
Bp	Base pairs
cDNA	Complementary De-oxyribo Nucleic Acid
CBSV	Cassava brown steak virus
CMD	Cassava mosaic disease
CTAB	Cetyl trimethylammonium bromide
DNA	De-oxyribo Nucleic Acid
dNTPS	Dinucleotide TrisPhosphate
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and Agriculture Organization
GA_3	Gibberellic Acid
IBA	Indole-3-butyric Acid
mRNA	Messenger ribonucleic acid
KEPHIS	Kenya Plant Health Inspectorate Services
µl	Microlitre
PCR	Polymerase Chain Reaction
PQBS	Plant quarantine and bio-security station
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-PCR
TBE	Trisbase Boric Acid EDTA
Tris HCl	Tris Hydrochloride

ABSTRACT

Cassava brown streak disease (CBSD) is an economically important disease of cassava (*Manihot esculenta crantz*) caused by *Cassava brown streak virus* (CBSV) which was the target in this study and *Ugandan cassava brown streak virus* (UCBSV). This disease is spreading fast in several countries in the East and Central Africa. The general objective of this research was *in vitro* production of CBSV-free cassava from infected cassava. Specifically this research aimed at (i) evaluating the effect of meristem tip sizes (ii) determine the effects of varying concentration levels of selected chemicals (iii) determine the efficacy of combining thermotherapy with meristem tip culture and chemotherapy on the reduction of cassava brown streak virus from infected cassava. CBSV infected cuttings of Guzo variety collected from the Coast province of Kenya established and maintained in a greenhouse at the Plant Quarantine and biosecurity Station (PQBS) in Kenya Plant Health Inspectorate Service's (KEPHIS) in Muguga were used as test plants. Leaves were sampled from eighteen cassava plants of Guzo variety and virus indexing was done using (RT-PCR) with virus specific primers and those that tested positive for CBSV were used as initiation materials. From the *in vitro* plantlets established the second subcultured materials were subjected to the virus reduction procedures. *In vitro* meristems (0.5mm, 1mm, 2mm and 10mm (control) were obtained and cultured in modified Murashige and Skoog (MS) media. For chemotherapy, nodes were cultured in MS media supplemented with antivirals (ribavirin and salicylic acid) at 10mg/l, 20mg/l, 30mg/l and control of 0mg/l. In the combination treatments, plants were subjected to thermotherapy at 38ºC then meristem tips (1.0mm) were excised and heat

treated plants at 38ºC were subjected to ribavirin treatments at (10mg/l, 20mg/l and 30mg/l). Data collected was analysed using genstat. The regeneration of plants established from 0.5mm was 63% while those from 2mm was 88% with the proportion of virus-free plants decreasing with increase in meristem tip size. Meristem tip size of 0.5mm resulted in the highest % (88.23) of virus-free obtained. In chemotherapy, survival of shoots was observed to decrease with increase in the antiviral concentrations. Ribavirin 10mg/l recorded the highest value of survivals compared to the other treatments. On the other hand salicylic acid exhibited the least survivals compared to ribavirin. The number of virus-free plants obtained 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. *In vitro* plants that had been thermo treated and then subjected to chemotherapy did not give the expected results since complete death of the plants was recorded. Based on efficiency of virus reduction in terms of CBSV-free plants obtained and the number of plants surviving after the treatment, thermotherapy at (38°C) combined with meristem tip culture can be used for production of CBSV-free cassava. Farmers are therefore 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. There is need for studies to be done that will show effects of these cleaning methods on the reduction of Ugandan cassava brown streak virus (UCBSV) from infected guzo plants and evaluate the effect of chemotherapy by

subjecting virus infected cassava plants to lower antiviral concentrations while increasing duration of treatment to evaluate the effect of CBSV reduction from infected cassava.

1.0 CHAPTER ONE

1.1 INTRODUCTION

1.1.2 Background of study

Cassava is an important crop in food security across Sub-Saharan Africa. Internationally cassava is ranked among the top ten most significant food crops produced in the developing countries as a major source of carbohydrates (FAO, 2012). The total world production in the year 2011 was 250.2 million tonnes, with 145 million tonnes being produced in Africa alone (FAO, 2012). In addition cassava is a cash crop, feed crop, and a source of raw materials for industrial uses such as starch and alcohol production. Thus, cassava plays as an important source of income in the rural marginal areas (FAO, 2011).

1.1.3 Origin and botany of cassava

Cassava is thought to have originated and domesticated in the Amazon region in South America. It could have been domesticated more than once over time and in distant sites across some Brazilian states (Allem, 2002). The Portuguese brought cassava to Africa in the 16th Century; reaching the west coast of Africa via the Gulf of Benin and the river Congo, the east coast via the islands of Madagascar and Zanzibar (Jennings, 1976; Jones, 1989).

In East Africa, Stanley first reported cassava around Lake Victoria in 1878. The crop was however established in Uganda and Western Kenya in the 19th Century (Martin, 1970).

Currently cassava is cultivated in all the tropical countries of the world, including some isolated and remote islands of the Pacific (Hillocks, 2002). Cassava is a perennial crop that thrives in the tropics. Common names given to cassava include topioca, manioc and yucca. It belongs to the species *Manihot esculenta crantz*, family Euphorbiaceae and requires at least 8 months of warm weather to produce a crop. Examples of wild species of cassava include *M. glaziovii*, *M. pseudoglaziovii*, *M. aesculifolia*, *M. pilosa*, *M. dichotoma*, *M. pohlii*, *M. neusana* and *M. anomala* (Lebot, 2009).

The cultivated species, *M. esculenta,* is placed in the *Manihot* section of the genus. This section contains low-growing shrubs adapted to savannah and grassland. *Manihot* species range in nature from herbs to shrubs, small trees and climbing vines (Lebot, 2009). All species have tuberous roots rich in starch but can produce toxic cyanogenic glucosides. Stem skin colour varies from very light grey, brown, yellowish or reddish (FAO, 1990). Cassava can be propagated either by stem cuttings or from botanical seed (true seed) although the former is the most common practice used by farmers for multiplication and planting purposes.

1.1.4 Production and utilization of cassava

Cassava is grown in 39 African countries among them Nigeria, Democratic Republic of Congo, Ghana, Tanzania, Kenya, Uganda, Rwanda, Burundi, Mozambique (FAO, 2001). Africa produces more cassava than the rest of the world combined; production exceeds 104 million tonnes annually. In Kenya, cassava is grown on over 90,000 hectares with an annual production of about 540 000 tons (Munga, 2009). Cultivation is mainly in Western Kenya. The annual production in 2012 was 893,122 tonnes (FAOSTAT, 2012).

Cassava is the dietary staple food of the world's poorest tropical and subtropical countries, and has been estimated to provide 37, 12 and 7 per cent of the energy in the diet of the tropical areas of Africa, America and Asia; respectively (Holzman and John, 1986). Cassava is second to maize as Africa's most important staple crop first in total fresh weight production as well as per capita calories consumed (Nweke *et al.,* 2002). The roots are a source of carbohydrates, vitamins, minerals and proteins (Jain, 2009). Cassava is mainly utilized as fresh tubers or leaves for consumption in Africa (Howler *et al.,* 2001).

The utilization of cassava in Kenya is at farmer level and processing level. At farmer level, whole tuber is consumed as boiled, fried, roasted or raw. About 51% of farmers use it as flour from grounded dried cassava chips. The flour can also be used in preparing 'Ugali' mostly when mixed with finger millet flour. Some farmers also utilize the young leaves as vegetables. Cassava can be processed into crisps or flour after

processing and it is also fed to livestock such as cattle, goats and chicken by about 55% of farmers growing it (Kiura *et al*., 2007). Cassava flours are also used to prepare deep-fried savoury or sweet cakes (Dixon, 1999). The crop is mainly grown for human consumption although there is great potential for use in the feed industry (Njeru and Munga, 2002).

Broad utilization of cassava products can be a channel of rural industrial development hence raising the income of producers, processors and traders in the cassava production sector. Cassava is increasingly gaining potential as a major bio energy crop because it can give a high yield of starch and total dry matter (Jain, 2009). The starch also has the potential of being used in textile industries. Cassava therefore holds great promise for feeding Africa's expanding population, increasing farm incomes to reduce poverty and generating foreign exchange, as an industrial crop (Nweke *et al.*, 2002).

1.1.5 Constraints to cassava production

The biggest constraints to cassava production are pest attack (49.6%) followed by drought (22.9%), weeds (14.7%), and shortage of clean planting materials (12.8 %) (Ntawuruhunga and Legg, 2007). Pests cause damage to cassava directly as they feed on the leaves or stems, reducing the canopy area of the plant, the leaf life and therefore its photosynthetic capacity (Lebot, 2009). The major arthropod pests of cassava in Africa are the cassava green mite, the cassava mealy bug, and the variegated grasshopper. Nematodes are often responsible for indirect damage as they open the way to various bacterial or fungal infestations. The most frequently encountered nematodes belong to

the genera *Meloidogyne, Pratylenchus, Heliocotylenchus, Rotylenchulus, Criconemoides, Scutellonema* and *Xiphinema* (McSorley *et al*., 1983; Jatala and Bridge, 1990; Lebot, 2009).

Crops that are vegetatively propagated are prone to virus infection and cassava is no exception (Gibson and Otim-Nape, 1997; Chellappan *et al*., 2004). Cassava being vegetatively propagated is prone to damage by viruses that build up in successive cycles of propagation (Calvert and Thresh, 2002). Viral diseases are the major production constraints of cassava production constituting a great economic importance in cassava production. This could be contributed by the widespread movement of germplasm throughout the world. At least 16 different viruses have been isolated from cassava Hillocks and Thresh (1999).

In Africa alone, six viruses were isolated affecting cassava (Hillocks *et al.,* 1999); Cassava mosaic disease caused by gemini viruses (Thresh *et al*., 1998) (including its variants African cassava mosaic, Eastern African cassava mosaic virus). Other viruses also include South African cassava mosaic virus, cassava ivorian bacilliform virus, Cassava kumi virus, cassava Q virus, Cassava common mosaic virus (*petoxivirus*) and Cassava brown streak virus caused by *potyviridae ipomovirus*. In Kenya, the main viral diseases constraining cassava production are the Cassava mosaic virus disease (CMD) caused by Cassava mosaic geminiviruses (CMGs) and Cassava brown streak virus disease (CBSD) caused by two strains of viruses the highland Ugandan strain UG-CBSV and the coastal lowland strain *cassava brown streak virus* (CBSV) (Monger *et al*., 2001; Munga and Njeru, 2002). *Cassava brown streak virus* (CBSV*)* belongs to the family

Potyviridae, containing the largest number of single-stranded RNA viruses infecting plants (Monger *et al.*, 2009). This virus family is divided into the genus *Bymovirus* with bipartite genomes and the genera *Ipomovirus*, *Macluravirus*, *Potyvirus*, *Rymovirus*, and *Tritimovirus*. CBSD makes the storage roots of cassava unsuitable for consumption and poses a major threat to commercialization and utilization of the crop.

1.2 Statement of problem and justification of the study

CBSD is spreading fast in several countries in the East and Central Africa (Hillocks and Jennings, 2003). Nearly all cassava varieties that have been bred for resistant to CMD are susceptible to CBSD (Hillocks and Jennings, 2003). The vegetative nature of cassava propagation contributes to the spread of CBSV and can be easily spread from one field to another in most cassava growing areas if farmers continue to use infected stem cuttings as planting materials (Hillocks and Thresh, 2000). Farmers often find it 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 to rogue out any plants that show symptoms soon after sprouting as a control measure for CBSD difficult to practice. The use of infected plant cuttings has been reported to be the main avenue of 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, thus causing heavy yield losses in most vegetatively propagated plants (Lebot, 2009).

This therefore poses a huge threat in spreading of CBSV and introduction into areas free from the disease within the country and its neighboring countries. The effects of this constraints has recorded reduction of yields in Kenya from 5-10t/ha while the potential is 32t/ha (Munga, 2000). As a result, cassava production has declined drastically since 1995; some districts have experienced almost total crop failure, prompting farmers to

abandon production, especially of highly susceptible varieties (Obiero *et al*., 2007; Hillocks *et al*., 2001).

The supply of virus-free planting materials is therefore important for sustainable crop production. This situation also has clear implications on germplasm exchange within Kenya and its neighboring regions such as north-western Tanzania, Rwanda, Burundi and eastern DRC. Recognizing the importance of this situation, it was proposed that only virus-tested tissue culture materials be used for inter country cassava germplasm movement (Ntawuruhunga and Legg, 2007). Notably, introductions of germplasm to both Rwanda and Burundi, from the Kenya Plant Health Inspectorate Service (KEPHIS) Plant Quarantine Station at Muguga, Nairobi supported by EARRNET (East Africa Regional Research Network) have been in tissue culture form (Ntawuruhunga and Legg, 2007). In addition the production of virus-free plants is a prerequisite for international exchange of clonal material to avoid risks of introducing diseases to uninfected areas (Lebot, 2009). This is because the most viable methods for obtaining virus-free stocks is by viral eradication using tissue culture techniques usually aided by thermo-and/or chemotherapies (Mellor and Stace-Smith, 1970; Pannatoni *et al.,* 2013).

Production of cassava through micropropagation alone does not guarantee freedom from diseases; the combination of an efficient *in vitro* protocol for virus elimination and a highly sensitive diagnostic technique can allow for the production of virus-free plantlets (Cha-um *et al.,* 2006). The virus (CBSV) is poorly understood considering that it has received little attention in the past, which therefore implies that the techniques for CBSV

elimination are yet to be developed and optimized. Propagative clean materials produced by these methods can provide clean planting materials for farmers, recover fully infected genotypes in the field that are highly preferred by farmers and used for safe germplasm conservation and movement.

1.3 Objectives of the study

1.3.1 General objective

Production of CBSV-free *in vitro* cassava plants from infected cassava (guzo)

1.3.2 Specific objectives

1. To evaluate the effect of meristem tip sizes on the reduction of CBSV from infected cassava.

2 To determine the effects of varying concentration levels of selected chemicals on the reduction of CBSV from infected cassava.

3 To determine the efficacy of combining thermotherapy with meristem tip culture and chemotherapy on the reduction of CBSV from infected cassava.

1.4 Null hypothesis

1. The size of the meristem tip has no effect on the reduction of CBSV from infected cassava *in vitro.*

2. The concentration of selected chemicals has no effect on the reduction of CBSV from infected cassava in *vitro.*

3. Thermotherapy in combination with meristem tip culture and chemotherapy has no effect on the reduction of CBSV from infected cassava *in vitro.*

2.0 CHAPTER TWO

2.1 LITERATURE REVIEW

2.1.1 Cassava Brown Streak Disease (CBSD)

2.1.2 Symptoms of CBSD on cassava

There are a number of different symptoms of CBSD depending on variety, crop age and weather conditions (Hillocks *et al*., 1999). On the leaves the disease appears as a feathery chlorosis on either side of the smaller veins (Plate 2.1b). These foliar symptoms are less conspicuous and farmers are often unaware of the problem until the roots are harvested and the corky, yellow-brown necrotic root becomes evident.

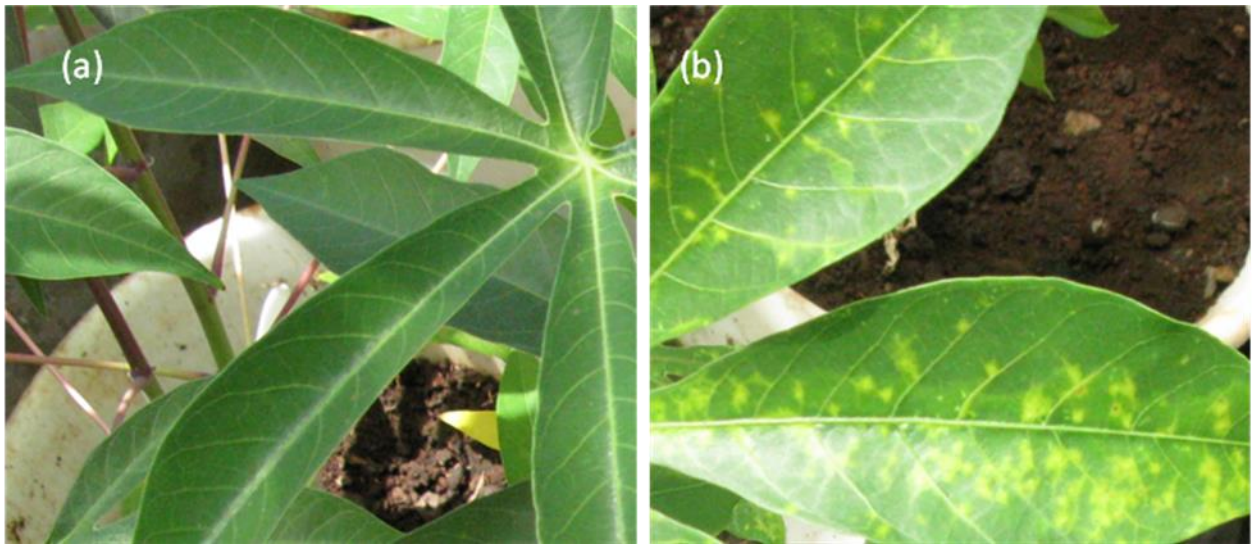

Plate 2.1: Healthy cassava plant (a) and foliar symptoms of CBSV infected cassava (b)

2.1.3 Aetiology of CBSD

CBSD was first reported and described in Tanzania in the 1930s (Storey, 1936). Brunt *et al.,* (1990) describe two brown streak viruses, a 'carlavirus' and a 'potyvirus'. They stated that the virus was transmitted by *Bemisia tabaci* although this was speculation and had not been demonstrated experimentally. The role of the two types of particle associated with CBSD symptoms had not been determined and the aetiology of the disease remained uncertain until the reports of Monger *et al*., (2001), which supported the view that CBSD is caused by a virus in the genus *Ipomovirus* in the family *Potyviridae*. This was also confirmed after successful CBSV transmission by *B. tabaci* reports by Mware *et al.*, (2009) but this does not preclude the possibility that under suitable conditions, *Aleurodicus dispersus* whose population has been directly correlated with CBSD incidence may also transmit the virus (Mware *et al.,* 2009). Cassava brown streak virus disease (CBSD) is caused by two strains of viruses the highland Ugandan strain UG-CBSV and the coastal lowland strain CBSV (Monger *et al*., 2001; Munga and Njeru, 2002). 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. They both have (+) ss RNA genomes, belonging to the genus *Ipomovirus* in the family *Potyviridae*, and produce generally similar symptoms in infected plants (Monger *et al.,* 2001).

2.1.4 Epidemiology and spread

CBSD is spread both through by a whitefly vector *Bemisia tabaci* (Storey, 1993; Maruthi *et al.,* 2004) and propagation of infected cuttings (Munga and Thresh, 2002). Successful CBSV transmission by *B. tabaci* was reported by Maruthi *et al.,* (2004) and Mware *et al.,* (2009) but this does not preclude the possibility that under suitable conditions, *Aleurodicus dispersus* whose population has been directly correlated with CBSD incidence may also transmit the virus (Mware *et al*., 2009). Ability of whitefly to transmit CBSV may explain its contribution in the spread of CBSD in cassava growing areas in Coastal Kenya (Mware *et al.,* 2009). This suggests that all parts of the region already affected by the CMD pandemic with concomitant super-abundant whitefly populations may be particularly vulnerable to CBSD spread (Mware *et al.,* 2010).

CBSD was reported present in all coastal districts namely in Kenya, Kwale, Mombasa, Malindi, Kilifi although the incidence was low (Storey, 1939; Bock.1994; Maruthi *et al,* 2004). Results of low rates of natural spread were later confirmed to be inconsistent with incidences observed up to 64% (Alicia *et al* 2007) due to the wide spread of the vector. These reports were confirmed by Mware *et al*., (2009). This could be possibly due to the abundance of the whitefly vector *Bemisia tabaci* (Mware *et al*., 2009). Currently the disease has also been reported in parts of, Western and Nyanza provinces in the country.

2.2 Detection of CBSV

Although accurate description of symptoms is necessary to describe the disease, virus diagnosis should not be based on symptoms alone because several unrelated viruses

cause similar symptoms and some viruses or same virus or its strain can result in different symptoms on the same host or different host species (CIP, 2007). Procedures to detect the presence of viruses include;

Use of indicator plants; Prior to the advent of antigen-antibody reaction detection systems, earlier methods of detecting viruses relied on the use indicator plants. The development of necrotic spots on the leaves of the indicator plants is a typical symptom indicating the presence of viruses. For example in sweet potato, its wild relative, Brazilian morning glory (*Ipomoea setosa*) is still used as the indicator host (CIP, 2007). Similarly, in many fruit trees, the appropriate rootstocks are used for indexing of various virus strains (Arthofer, 2001). Mechanical inoculation with sap from older, lower leaves of CBSV infected cassava will produce local lesions in *Chenopodium quinoa* and vein yellowing, mottling and distortion in *Nicotiana benthamiana* (FAO, 1990). The detection of plant diseases has changed radically in the last 20 years. A number of indicator plant based tests have been replaced with nucleic acid based techniques.

ELISA; The basic principle of ELISA technique involves immobilizing the antigen onto a solid surface or captured by specific antibodies bound to the surface, and probing with specific immunoglobulin's carrying an enzyme label (IITA, 2009). ELISA detects pathogens from their protein material. The enzyme retrained in the case of positive reaction is detected by adding a suitable substrate. The enzyme converts to a product which can then be recognized by color (IITA, 2009).

ELISA has been the most widely method used for virus and pathogen detection in plants. Various alternative formats, substrates, and antibody binding modifications have been developed over the years to increase specific sensitivity (Agdia, 2002; Bioreba, 2002). ELISA is commonly used in detection of sweet potato viruses i.e. SPMMV, SPFMV, SPLV, SPCSV, SPVG among others through DAS ELISA (direct double sandwich which is one of the most sensitive serological techniques and NCM ELISA (nitrocellulose membrane) (Clark and Adams, 1977; CIP, 2007).

PCR; The Polymerase Chain Reaction (PCR) is highly sensitive and specific it can detect pathogens in extremely low amounts (Henson and French 1993; Hadidi *et al.* 1995; Candresse *et al*. 1998). PCR detects pathogens from their genetic material. It utilizes short DNA sequences (primers) to amplify a specific target DNA sequence. Each primer hybridizes to opposite strands of the target sequence and oriented so that DNA synthesis by Taq polymerase proceeds across the target sequence effectively doubling the amount of the target sequence. Since the extension products are also complementary to and capable of binding primers the cycle can be repeated after a denaturation step. Repeated cycles result in an exponential increase in target DNA to a point where there are enough copies of the sequence for it to be visualized. (Lunello *et al,* 2004; Rebecca and Lynn, 2010)

Coat protein (CP)-encoding sequences of coastal lowland isolates (Monger *et al.,* 2001) known as CBSV isolates and complete CP sequences of highland known as UG isolates from East Africa are available, revealing that these isolates belong to two

phylogenetically different strains (Mbazibwa *et al.*, 2009). Therefore current research has made it possible to detect the two strains of viruses whereby primers are available that are specifically designed to detect the lowland and highland strains. This is achieved through reverse transcriptase-polymerase chain reaction (RT-PCR) which can detect minute amounts of target RNA and is suitable for detecting viruses in very small tissue samples with extremely low virus titer, as found in meristem-tip culture (Dovas *et al*. 2001). Recently, a novel real-time quantitative PCR assay (TaqMan technology) was developed for the detection and quantification of plant viruses (Dietzgen *et al.*, 1990; Roberts *et al*. 2000). Real time PCR technology is based on the theory that there is quantitative relationship between amount of starting target sequence and amount of product at any given cycle.

In real time PCR assay an additional probe is used. The probe is labeled with a reporter fluorescent dye and quencher dye. The system exploits the process known as fluorescent resonance energy transfer during which the quencher absorbs florescence from the reporter. While the probe is intact the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye. The reporter dye molecules are cleaved from their respective probes with each cycle effecting an increase in florescence intensity propositional to the amount of amplicon produced (Rebecca and Lynn, 2010). In addition to sensitivity and specificity, this technique has certain advantages; it reduces the risk of cross-contamination, provides higher throughput, and enables quantification of virus load in a given sample.

In conventional PCR a set of forward and reverse primers are used to produce an amplicon, for instance in this study sets of primers used were CBSV10 (5"ATCAGAA TAGTGTGACTGCTGG-3') and CBSV 11 (5"CCACATTATTATCGTCACCAGG-3') (Monger *et al.,* 2001), which amplify ~230 bp length nucleotides. Unlike real-time PCR where results are real-time and visualized on a computer conventional PCR products are visualized in agarose gel electrophoresis.

2.3 Control of CBSD

Management options for CBSD include; phytosanitation, the use of less-susceptible cultivars, use of clean planting materials and vector control (Lozano *et al.,* 1981; Ntawuruhunga and Legg, 2007).

Phytosanitation relies on the ability of the farmer to select planting material only from clean mother plants, and then to rogue out any plants that show symptoms soon after sprouting (Lebot, 2009). The drawbacks to this approach are: farmers often find it difficult to recognize CBSD symptoms because of their variability and poor expression on the leaves (Ntawuruhunga and Legg, 2007).

The use of resistant or tolerant cultivars which is a long term management option although this is challenged by the fact that lengthy time is required for breeding. Application of selective insecticides against CBSV transmitting vectors (*Bemisia tabaci*) only when damage is severe and the plant cannot recover without the aid of the application (Lozano *et al.,* 1981).

Observation of quarantine measures to avoid the dissemination of pests and viruses in areas where they do not yet occur (Lozano *et al.,* 1981; Lebot, 2009). This is best achieved through the use of clean planting materials that are disease-free (Ntawuruhunga and Legg, 2007).

2.3.1 Use of clean planting material as CBSD management strategy

Viruses vary in the ease with which they can be eradicated. One of the most viable methods for obtaining virus-free plant materials is through tissue culture techniques, aided by thermo or chemotherapies (Mellor and Stace-Smith, 1970, Pannatoni *et al.,* 2013). Meristem-tip culture combined with thermotherapy can eliminate viruses with varying degrees of success (Manganaris *et al*., 2003). Improved virus elimination can occur also if chemotherapy is combined with thermotherapy with subsequent meristem tip culture (Kartha and Garmborg, 1986; Luciana *et al.*, 2007).

Meristem tip culture; The plant meristem is a zone of cells with intense divisions, situated in the growing tip of stems and roots. The virus travels through the plant vascular system, which is absent in the meristem. Moreover, the cell-to-cell movement of the viruses through plasmodesmata cannot keep up with the growth and elongation of the apical-tip (Morel and Martin, 1952). The high metabolic activity of meristematic cells, usually accompanied by elevated endogenous auxin content in shoot apices may also inhibit virus replication (Belkengren and Miller, 1962; Boxus, 1976). Meristem-tip is a meristematic dome with the first pair of sub adjacent leaf primordia, ranging in length from about 0.1 to 0.5 mm. The size of the meristem, however, varies from species to species. If explants are too big, they are likely to contain virus particles in the

associated vascular tissue. To obtain a high yield of virus-free plantlets the meristem size has to be taken into account; the smallest explants are those that typically will be the least successful during *in vitro* culture, but will produce the highest proportion of virus-free material when entire plants are reared in the glasshouse or field (Manganaris *et al.*, 2003). Virus-free plants have been obtained by thermo-therapy and meristem culture in several species such as bananas, potatoes, nectarine, and garlic (Boxus, 1976; Robert *et al*., 1997; Manganaris *et al.*, 2003).

Thermotherapy; The most commonly employed method of virus elimination from infected plants is the combination of thermotherapy and meristem tip culture. Conventionally, viruses are eliminated by thermotherapy of whole plants in which plants are exposed to temperatures between 35-40°C for a few minutes to several weeks depending on the 'host-virus combination (Kartha and Garmborg, 1986). Successful treatment times may vary from weeks to months depending on the plant cultivars and specific virus (Qiaochunb and Jari, 2009). Plants can then be maintained under these conditions after which time meristem tips are excised for tissue culture (Qiaochun and Jari 2009). Currently there are more sophisticated thermo chambers that are being used for *in vitro* thermotherapy.

In cassava production, positive yield responses after thermotherapy to obtain clean planting materials have been reported in Cuba (Garcia *et al*., 1993), Cameroon (Zok *et al.*, 1992), and Peru (Delgado and Rojas, 1992). Thermotherapy and meristem tip culture, although, are reported to eliminate plant viruses when each is applied alone,

there is evidence that they fail to completely free some species of plants of some viruses (Dodds *et al.,* 1989; Griffiths *et al.,* 1990). According to these authors, potato virus s (PVS) and potato virus X (PVX) are difficult to eliminate by thermotherapy or meristem-tip culture alone. In such cases, thermotherapy can complement meristem-tip culture to produce virus-free plants. Leonhardt *et al.,* (1998) also reported similar effect on potato, suggesting a temperature of 37°C for a minimum of 30 days as adequate for virus elimination in potato plants cultivated *in vitro* from single node cuttings. Some examples of plants virus cleaned using thermotherapy include; nectarine at temperatures of 35°C by Manganaris *et al.,* (2003), garlic at temperatures of 30-36°C for cycles of 7 days in coupled with meristem culture (Robert *et al*., 1997). Other crops also include bananas, sweetpotatoes, and carnation.

Chemotherapy; Chemotherapy involves the use of chemicals such as antibiotics, plant growth regulators, amino acids, purine and pyrimidine analogues to inactivate viruses or inhibit replication or movement of viruses in tissues (Seal and Coater, 1998). These chemicals can either be sprayed on growing plants prior to excision of meristems or incorporated into tissue culture media.

Chemotherapy is considered an alternative method for regenerating virus-free plants to classical thermotherapy which is time consuming and requires intensive equipments. Viral chemotherapy is centered on purine and pyrimidine analogs, with the presumption that the synthesis of the nucleic acid of the virus could be inhibited by such. These chemicals are filter sterilized and supplemented into meristem culture media or

autoclaved with the medium depending on the nature of the chemical (Nascimiento *et al.,* 2003). The chemicals act as competitive inhibitors, and could interfere either with the incorporation of nucleotides into nucleic acids or with the formation of the bases from their precursors molecules (Pannatoni *et al.,* 2013).

Chemotherapy directed to plant viral diseases has evolved significantly. Not only substances that inhibit viral replication, but also those that induce resistance, have been discovered. These compounds express an antiviral effect, but none of them present a satisfactory selective action that would enable them to be used in large-scale therapy of plant viral diseases (Hansen and Lane, 1985). The most widely used is ribavirin also known as Virazole. Ribavirin has been reported to eliminate major viral pathogens from potato and other crops (Qiaochun and Jari, 2009). Ribavirin showed eradication of potato viruses with greatest proportion of healthy plants obtained with filtered and autoclaved ribavirin, resulting in PVY eradication in the order of 55.5% and 41.7% at 20mg/l, respectively. A high percentage of elimination was also verified for the filtered and autoclaved azacytidine antiviral, at 37.5% and 33.3% respectively (Nascimento *et al.,* 2003).

Cryotherapy; With some plants, thermotherapy followed by meristem tip culture does not result in any virus-free plantlet. An approach based on cryotherapy was found to solve this problem, (Wang and Valkonen, 2009). Cryotherapy is a novel application of plant cryopreservation techniques that allows pathogen eradication at a high frequency. It eliminates plant pathogens such as viruses; phytoplasmas and bacteria by briefly

treating shoot tips in liquid nitrogen. In cryotherapy infected cells are eliminated by the lethal effects of the ultra low temperature. The number of regenerated shoot tips might be lower after cryotherapy than shoot tip culture but the difference is compensated for by the easier excision of larger shoot tips (Wang *et al.,* 2006).

Cryotherapy has the potential to replace more traditional methods like meristem culture (Qiaochun and Jari, 2009). Besides its traditional use for the conservation of genetic resources, it has been proven that cryotherapy can be successfully applied to eradicate virus from plum, banana and grape (Brison *et al*., 1997; Wang *et al*., 2003).

3.0 CHAPTER THREE

3.1 GENERAL METHODOLOGY

3.1.1 Source of cassava material

Stems of popularly grown cassava (Guzo variety) showing CBSD symptoms were collected from the coast province. The stems were established and maintained in a greenhouse in the Kenya Plant Health Inspectorate Service's (KEPHIS), Plant Quarantine Station (PQBS) at Muguga and used as test plants for the study.

3.1.2 Selection of infected cassava plants

Selective sampling was done on the established eighteen plants exhibiting CBSD symptoms which are characterized by chlorosis on the leaves of smaller veins of Guzo variety. A positive control (tested infected variety of Kibandameno) and negative control (tested virus-free variety of TME-14) was included for the virus test. Three young leaves from each cassava plant were sampled and stored in self-sealable plastic bags for preserving moisture for virus indexing using reverse transcriptase-polymerase chain reaction (RT-PCR).

3.1.3 RNA extraction

From the leaves sampled, RNA was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method modified from Lodhi *et al.,* 1994. Leaves of approximately 100 mg were measured and put into a mortar then 1ml of CTAB extraction buffer containing (0.2% (V/V) 2-mercaptoethanol was added prior to extraction. The samples were then ground into a fine paste. From the extract produced 750µl was then transferred into a 1.5 ml micro-centrifuge tube and incubated at 60°C for 30min. The

extract was then mixed with an equal volume (750µl) of chloroform: isoamyl alcohol (24:1), vortexed briefly and centrifuged at 14,000 revolutions per minute for 10 min. The top aqueous solution (500µl) was then transferred into a new 1.5ml tube. Nucleic acids were precipitated by adding 0.6 volumes (300µl) of ice cold isopropanol and the tubes incubated at -20°C for 30 min. After incubation, the samples were centrifuged at 14,000 revolutions per minute for 10 min and the supernatant discarded. The pellets were then resuspended in 500µl of 70% ethanol by vortexing and incubation at -20°C for 10 min. This was centrifuged for 5 min at 14,000 revolutions per minute. Ethanol was poured off and pellets dried. The pellets were then resuspended in 100µl of 1x TE buffer on ice for about 30 min and stored at -20°C.

3.1.4 Reverse transcriptase polymerase chain reaction using CBSV 10/11

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') (Monger *et al.,* 2001) which amplify ~230 bp length nucleotides. The 10 µl PCR reaction mix contained 6.85µl of sterile nuclease-free 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 cycle 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 the products visualized on a UV transimilluminator.

3.1.5 Visualization of the PCR products

A 1% agarose gel was prepared by mixing 1.0 g of agarose with 100ml 1x TBE (Trisbase Boric Acid EDTA) buffer. The solution was then heated in a microwave for proper mixing. The gel was left to cool to approximately 60°C then 3 µl of nucleic acid gel stain 10,00x Gelred [Biotium) was added. The gel was then cast in trays and combs carefully placed and solidification allowed to take place. The PCR products obtained were then mixed with 1 µl of the loading dye (orange G) and 5µl of the sample loaded in each well.

3.2 Multiplication of CBSV positive cassava plants

Media preparation; The culture medium used for initiation of CBSV infected cassava was prepared with 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).

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% hypochlorite solution for 20 minutes and 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.

3.3 Results

The PCR products were visualized as illustrated in section (3.1.5**).** From the 18 plants tested, six were confirmed positive for CBSV (Plate 3.1).

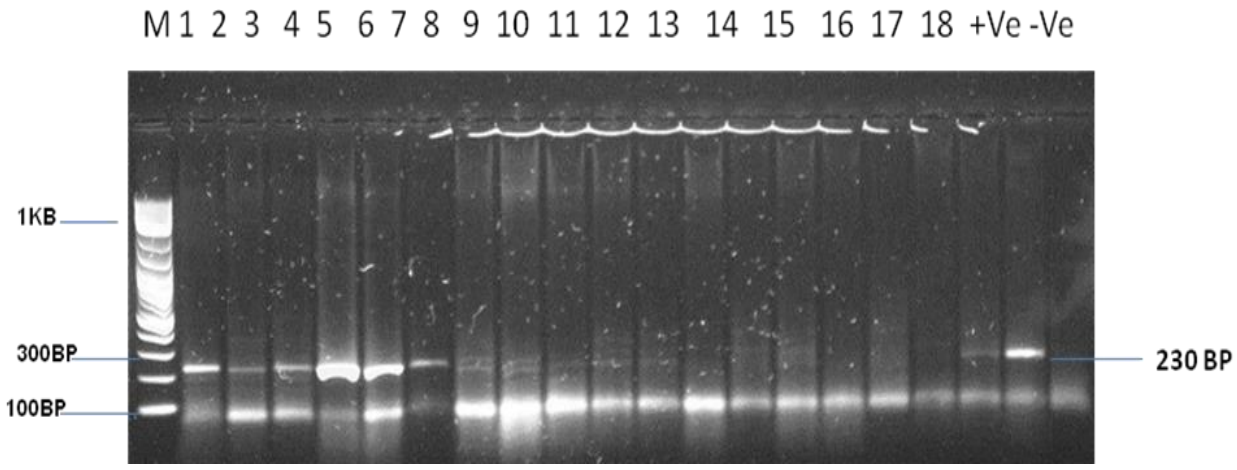

Plate 3.1: Agarose gel-electrophoresis of CBSV detection on infected cassava in a 1% (w/v) agarose gel; lane 1; 1kb ladder; lane 1-18 infected cassava; lane19 (positive control) lane 20 (negative control)

4.0 CHAPTER FOUR

4.1 EVALUATION OF MERISTEM TIP CULTURE FOR THE REDUCTION OF CASSAVA BROWN STREAK VIRUS FROM INFECTED CASSAVA.

4.1.2 Abstract

Meristem tip culture has been successfully employed in eliminating viral pathogens from a wide range of vegetatively propagated plants. In this study, meristem tip culture was evaluated for the production of CBSV-free cassava plants. CBSV infected cuttings of Guzo variety collected from Coast province of Kenya established and maintained in a greenhouse at the Plant Quarantine Station in Kenya Plant Health Inspectorate Service's (KEPHIS) in Muguga were used as test plants. Cassava leaves were sampled from eighteen cassava plants of Guzo variety and virus indexing was done using (RT-PCR) with virus specific primers and those that were confirmed positive for CBSV were used as initiation materials for the prospective test plants. From the initiated tissue culture materials, the 2nd subcultured plants were subjected to the cleaning methods. A completely randomized design was used to subject the tissue cultured material to the different treatments (where by each treatment was replicated 3 times). *In vitro* meristem tips varying in sizes (0.5mm, 1mm and 2mm long) were excised from *in vitro* plantlets and cultured on modified MS media. Nodal plantlets measuring 10mm were used as controls. The regeneration of plants from 0.5mm, 1mm and 2mm was 63%, 72%, and 88%, respectively. There was a significant difference ($P<0.01$) in the number of plants that survived among the treatments. Virus-free plantlets (15, 18, 24) obtained were observed to decrease with increase in meristem tip size (0.5mm, 1mm, 2mm) while that

of positive plants increased with increase in meristem tip size (2, 7, 12) respectively. Meristem tip size of 0.5mm resulted in the highest percentage (88.23) of virus-free plants obtained and is recommended for virus reduction of CBSV from infected cassava.

4.1.3 Introduction

The plant meristem is a zone of cells with intense divisions, situated in the growing tip of stems and roots. Shoot tips used for micro propagation are anatomically defined as structures that consist of the apical or lateral shoot meristem with three to four leaf primordia (Takada and Tasaka, 2002). 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. Viruses that infect non-vascular tissues are disseminated from cell to cell through the plasmodesmata. This is a slow process which makes it relatively difficult for viruses to infect the rapidly dividing cells. Several other reasons such as high metabolic activity in meristematic cells makes viruses unable to take over control of the host biosynthetic machinery hence viral replication is hindered, competition between synthesis of nucleo proteins for cellular division and viral replication with the presence of inhibitor substances make meristem tip useful to obtain virus-free plantlets (Alam *et al.,* 2010). Another advantage is the genetic stability inherent in the technique thus somaclonal variations are minimized (Alam *et al.,* 2010). Morel and Martin (1952) first demonstrated the elimination of viruses from dahlia using meristem culture. Since then the use of meristem culture *in vitro* has been used for many decades to eliminate viruses from vegetatively propagated plants (Faccioli and Marani, 1998). Virus-free plants have

been obtained by meristem culture in several species (Boxus 1976; Robert *et al*., 1997; Manganaris *et al.,* 2003).

The regeneration ability of a meristematic tissue is positively proportional to the size of shoot tip but pathogen eradication is more efficient using small shoot tips. Hence pathogen eradication using meristem culture is challenged by the difficulty of excising very small meristems and ensuring the survival of tiny meristems (Faccioli and Marani, 1998; Wang and Jari, 2009). The smallest explants are those that typically, will be the least successful during *in vitro* culture, but will produce the highest proportion of virus-free material when entire plants are reared in the glasshouse or field (Manganaris *et al.*, 2003). Apart from the indicated factors, elimination of viruses also depends on their concentration in plant tissue and the physiological condition of the mother plant from which the meristem has been isolated (Verma *et al.,* 2004).

Other obstacles to production of virus-free plants using meristem culture include; meristem position on a plant, protocol applied during detection (different sensitiveness of the applied methods) period of recovery and growth of produced plants (Cha-um *et al.,* 2006). Furthermore the distribution of viruses in shoot tips may vary depending on a virus host combination. Therefore various sizes of shoot tips have to be excised and tested to determine the optimal size range supporting efficient virus elimination and also the rate of plant regeneration (Milosevic *et al.,* 2012). Reports on meristem tip culture to eliminate CBSV in cassava are still limited. Therefore this study was carried out to

determine the effect of meristem tip sizes for the reduction of CBSV from infected cassava.

4.2 Methodology

4.2.1 Selection and multiplication of CBSV infected plants

CBSV positive plants were selected, tested, initiated *in vitro* and used as prospective test plants for meristem tip culture. This is captured in chapter 3 on general methodology.

4.2.2 Meristem tip culture

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 6-Benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA) hormones.

4.2.3 Experimental design and data collection

A completely randomized design was used to subject the tissue cultured material in the different treatments. Each treatment had a total number of 15 plants replicated three times to create a total sample size of 45 plants subjected to (0.5 mm, 1 mm, 2 mm, and

10 mm) sizes. The number of surviving plantlets, CBSV-positive and CBSV-negative plants after virus testing was recorded.

4.2.4 Data analysis

ANOVA using genstat software was used to calculate the significance at ($P \leq 0.01$) for the plants that survived subjected to meristem tip culture.

Virus reduction was determined as the proportion of CBSV-negative plants obtained expressed as a %.

4.3 Results

4.3.1 Effect of meristem tip culture for the reduction of CBSV from infected cassava

After a period of 7-10 days, shooting was observed in most of the meristems while some turned brown and failed to grow consequently leading to death. The death of meristems was more evident from meristems established at 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.

4.3.2 Survival and CBSV reduction from regenerated meristems

The size of the explants influenced the number of plants regenerated, virus-free and positive plants established (Table 4.1). The number of plants regenerated from 0.5mm,

1mm and 2mm was 17, 25 and 36 respectively (Table 4.1). There was a significant difference ($P<0.01$) in the number of plants that survived among the treatments. All the control plants were positive for CBSV. Meristem tip size of 0.5mm had the highest percentage (88.2%) of virus-free plants (Table 4.1).

Table 4.1: Survival and virus reduction (%) of plants subjected to 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

The least significant difference between any of the means of at $P\leq0.05$ indicates that each meristem tip size produced a significantly different survival from each other (Table 4.2).

Table 4.2: Means of survivals of plants subjected to meristem tip culture and the least significant difference.

Meristem size (mm)	**Mean survival (%)**
10	100.0
0.5	37.7
1	55.7
2	80.1
$LSD_{0.05}$	19.3

4.3.3 PCR detection for CBSV *in vitro* meristems

The PCR products of the survived putative virus-free material were visualized in an agarose gel (Plate 4.1). At 0.5mm, 1mm, 2mm sizes of meristems, 2, 7, and 12 respectively number of plants tested negative for CBSV. A 1kb molecular marker was used thus perfectly giving the expected PCR product in the positive control (Plate 4.1).

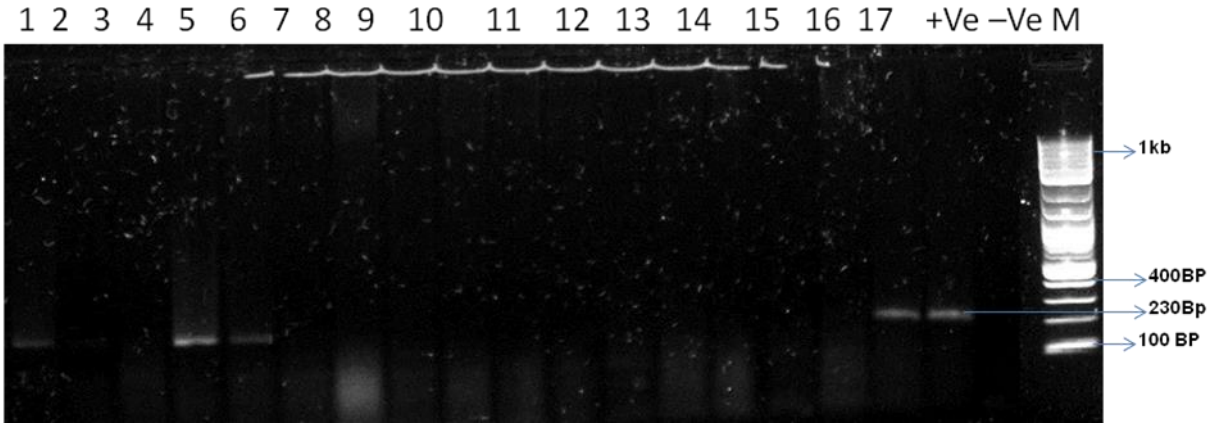

Plate 4.1: Agarose gel-electrophoresis of CBSV detection of *in vitro* plantlets established from meristems at 1% (w/v) agarose gel; lane 1-17 *in vitro* meristems at the

different treatment sizes lane18 (positive control). Lane 19 (negative control) lane 20 1kb ladder

4.4 DISCUSSION

The effect of different sizes of meristem tips on the reduction of CBSV from infected cassava was evaluated. Meristem tip culture on CBSV reduction was influenced by the size of explant. The larger the size of meristem cultured, the greater was the number of regenerated plants, but the number of virus-free plantlets obtained was inversely proportional to the size of cultured tip and this is in agreement with Faccioli and Marani, 1998. More number of plants were established from larger meristems of 1mm and 2 mm and this agrees with earlier observations by Manganaris *et al.,* (2003) and Cha-um, (2006) who showed that meristems of 1.3-2.0mm favored shoot survival growth.

Meristem tips of 0.5mm had the highest percentage (88.23) of virus-free plants obtained which is in agreement with the findings of Fayek *et al .,* (2009) who reported that the smallest explants are those that typically, will be the least successful during *in vitro* culture, but will produce the highest proportion of virus-free material. Controls obtained from nodal plantlets (10mm) resulted in all positive plants this was expected since they contain the plant vascular system which is absent in the meristem (Morel and Martin, 1952) thus meristems are highly protected from infection. This was followed by meristem tip sizes 2mm and 1mm having higher numbers of positive plants of 12 and 7 respectively in agreement with Manganaris *et al.,* (2003) who reported that if explants are too big they are likely to contain virus particles in the associated vascular tissue. Therefore larger meristems were effective in terms of shoot survival but allowed some

percentage of the virus to escape producing virus positive plants. Although excision and regenerating small *in vitro* meristems is low, larger meristems can be excised and enhanced through thermotherapy to improve plant regeneration from explants (Appiano and Pennazio 1972).

Based on these findings smaller meristems of 0.5 mm were found to be optimal for the reduction of CBSV from infected cassava in terms of virus-free plants produced. This is agreement with (Kartha and Garmborg, 1986; Cha-um *et al.,* 2006; Fayek *et al.,* 2009) whereby meristems sizes of 0.3-0.5mm were found optimum in production of CMD free cassava in the Nigerian cultivars, the production of disease-free sugarcane varieties using meristem culture, elimination of grapevine fan leaf virus from infected grapevine plantlets using meristems of 0.5mm. There are many publications that also recommend on 0.1-0.5mm in length of meristems sizes for elimination of pathogenic virus diseases (Kartha and Garmborg 1986; Alam *et al.,* 2004; Cha-um *et al.,* 2006).

5.0 CHAPTER FIVE

5.1 EFFECTS OF CHEMOTHERAPY ON THE REDUCTION OF CASSAVA BROWN STREAK VIRUS FROM INFECTED CASSAVA

5.1.1 Abstract

Chemotherapy is the application of different antiviral analogs in tissue culture which work by hindering viral replication in explants. Chemotherapy was evaluated for the production of CBSV-free cassava plants from CBSV infected cassava. CBSV infected cuttings of Guzo variety collected from Coast province of Kenya established and maintained in a greenhouse at the Plant Quarantine Station in Kenya Plant Health Inspectorate Services (KEPHIS) in Muguga were used as test plants. Cassava leaves were sampled from eighteen cassava plants of Guzo variety and virus indexing was done using (RT-PCR) with virus specific primers and those that were confirmed positive for CBSV were used as initiation materials for the prospective test plants. From the initiated tissue culture materials the 2^{nd} subcultured plants were then subjected to MS (Murashige and Skoog) media supplemented with 0, 10, 20 and 30mg/l of ribavirin and salicylic acid. A completely randomized design was used to subject the tissue cultured material to the different treatments (where by each treatment was replicated 3 times). The plants were subjected to ribavirin and salicylic acid treatments for a period of two weeks then transferred onto media without antiviral compounds. Survival of shoots decreased with increase in the antiviral concentrations. There was a significant difference ($P<0.01$) in the number of plants that survived among the treatments. Plants subjected to 10mg/l ribavirin resulted in the highest survival rates while salicylic acid treatment exhibited

least growth due to phytotoxic effects in general. The number of virus-free plants obtained was observed to increase with increase in concentration. Treatments of 30 mg/l of ribavirin and salicylic acid resulted in 88.8% and 100% respectively of virus-free plants produced. Based on survival and virus elimination, ribavirin treatment at 10 mg/l is recommended for CBSV reduction in cassava.

5.1.2 Introduction

Chemotherapy employs the application of different antiviral analogs such as antibiotics, plant growth regulators, amino acids, purine and pyrimidine to retard or hinder viral replication in explants (Seal and Coater, 1998). Antiviral chemicals can be used as additives in the culture medium (Kartha and Garmborg, 1986) or the chemicals may be sprayed on growing plants.

Chemotherapy is considered an alternative method for regenerating virus-free plants to classical thermotherapy which is time consuming and requires intensive equipments. Viral chemotherapy is centered on purine and pyrimidine analogs, with the presumption that the synthesis of the nucleic acid of the virus could be inhibited by such molecules. The chemicals act as competitive inhibitors, and could interfere either with the incorporation of nucleotides into nucleic acids or with the formation of the bases from their precursor molecules (Panattoni *et al.*, 2013).

The development of research in the field of chemotherapy has not been active as the work conducted on thermotherapy (Panattoni *et al.*, 2013), although valuable contributions have been provided by extensive investigations of antiviral chemotherapy performed in clinical medicine. In this regard the discovery of ribavirin (Sidwell *et al.*,

1972; Huffman *et al.,* 1973) represented a defining moment in research marking a different route of investigation in the study of new chemical synthesis analogues of nucleoside or precursors of RNA bases. To date, more than 40 antiviral molecules synthesized in this way are available on the market for clinical application (Panattoni *et al.,* 2013).

Keeping in mind the relevant differences between animal and plant hosts, the potential similarities between metabolic pathways present in both have been the starting point for experimentation on phytoviruses (Panattoni *et al.,* 2013). 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 (Sidwell *et al.,* 1972; Jason and Craig, 2005). The efficiency of ribavirin in the elimination of plant viruses is documented in some crops (Chen and Sherwood, 1991; Fletcher *et al*., 1998; Panattoni *et al.,* 2013) and depends on the utilized concentration, host plant and type of infected tissue (Paunovic *et al*., 2007). Ribavirin showed eradication of potato viruses with greatest proportion of healthy plants obtained with filtered and autoclaved ribavirin, resulting in PVY eradication in the order of 55.5% and 41.7% at 30 mg/l and 20mg/l, respectively (Nascimento *et al.,* 2003). Ribavirin has also been successfully used in eliminating Indian citrus ringspot virus (Sharma *et al.*, 2007).

Salicylic acid on the other hand, functions by inhibiting catalase and ascorbate peroxidase enzymes. The inhibition of catalase enzyme results in elevated levels of hydrogen peroxide or 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.

Salicylic acid is a potential antiviral that has been reported to eliminate major viral pathogens in potatoes, bananas and other crops (Qiaochun and Jari, 2009). Salicylic acid applied exogenously to plant induces resistance as well as plant proteins which typically accompany systemic acquired resistance (Ward *et al.,* 1991; Gafney, 1993). The systemic acquired resistance (SAR) involves a signal that spreads systematically from the site of the hypersensitive response to the rest of the plant. Infection by a pathogen causes host cell to trigger a signal to be emitted throughout the plant, which then triggers the activation of defense genes in uninfected tissues and results in the plant showing enhanced resistance to subsequent infection by an array of pathogens (Glazebrook *et al.,* 1997).

The fact that in plant virology less resources are available in chemotherapy and molecular characterization of many phytoviruses means that fewer results than in medicine are available (Panattoni *et al.,* 2013) hence need for the present work which was developed to evaluate the efficacy of ribavirin and salicylic acid at varying concentrations for the reduction of CBSV from infected cassava.

5.2 Methodology

5.2.1 Selection of infected cassava plants

CBSV positive plants were selected, tested, initiated *in vitro* and used as prospective test plants for meristem tip culture. This is captured in chapter three on general methodology.

5.2.2 Chemotherapy

From the initiated *in vitro* cassava plantlets; using single nodal cuttings the 2nd subcultures were then transferred to media supplemented with ribavirin and salicylic acid at concentrations of 0, 10, 20 and 30 for two weeks before transferring to media without antiviral compounds. The plants were then maintained in a growth room, under a temperature regime of 24 ± 1^{o}C and a 16-hour photoperiod provided by fluorescent bulbs with light intensity of 1500 lux.

5.2.3 Experimental design and data collection

A completely randomized design was used to subject the tissue cultured material in the different treatments. Each treatment had a total number of 15 plants replicated three times to create a total sample size of 45 plants subjected to (0 mg/l, 10mg/l, 20 mg/l and, and 30 mg/l). The number of surviving plantlets, CBSV-positive and CBSV-negative plants after virus testing was recorded.

5.2.4 Data analysis

ANOVA using genstat software was used to calculate the significance at ($P \leq 0.01$) for plants that survived after subjecting to chemotherapy.

Virus reduction was determined as the proportion of CBSV-negative plants obtained expressed as a %.

5.3 Results

5.3.1 Effects of chemotherapy for the reduction of CBSV from infected cassava

Comparison of shoot growth chemo treated plants and the non-treated controls indicated delay in plant development by approximately one month. The phytotoxic effect odue to the antiviral compounds was evident through defoliation of the plant leaves in a week's time with plants subjected to ribavirin and salicylic acid at 30mg/l being more affected. Control plants grew better and differed from all other treatments (Plate 5.1).

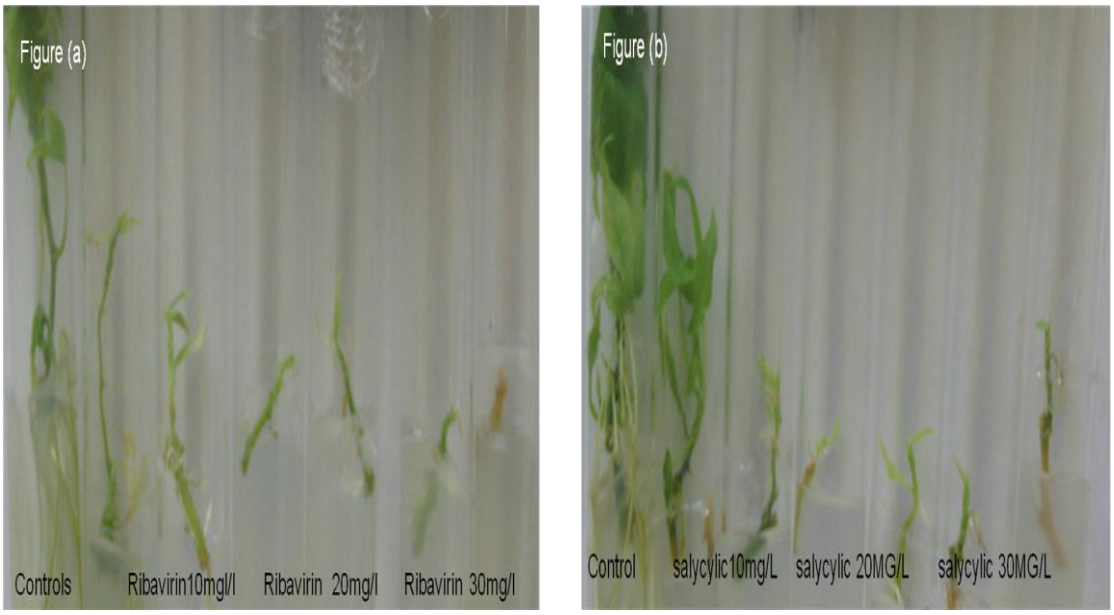

Plate 5.1: Plants subjected to different concentrations of antivirals figure (a) Ribavirin 0mg/l,10mg/l,20mg/l,30mg/l) and figure (b) Salicylic acid 0mg/l,10mg/l,20mg/l,30mg/)

5.3.2 PCR detection for CBSV in plants subjected to ribavirin and salicylic acid treatments

The PCR products of the survived putative virus-free material amplification was visualized in an agarose gel. From the number of plantlets tested at 10mg/l, 20mg/l and 30mg/l of ribavirin, 21, 22 and 8 number of plants respectively tested negative for CBSV (Plate 5.1). From the plantlets tested at 10mg/, 20mg/l and 30mg/l of salicylic, 11, 10 and 1 number of plants respectively tested negative for CBSV (Plate 5.3). A 1kb molecular marker was used thus perfectly giving the expected PCR product in the positive control (Plate 5.2 and Plate 5.3)

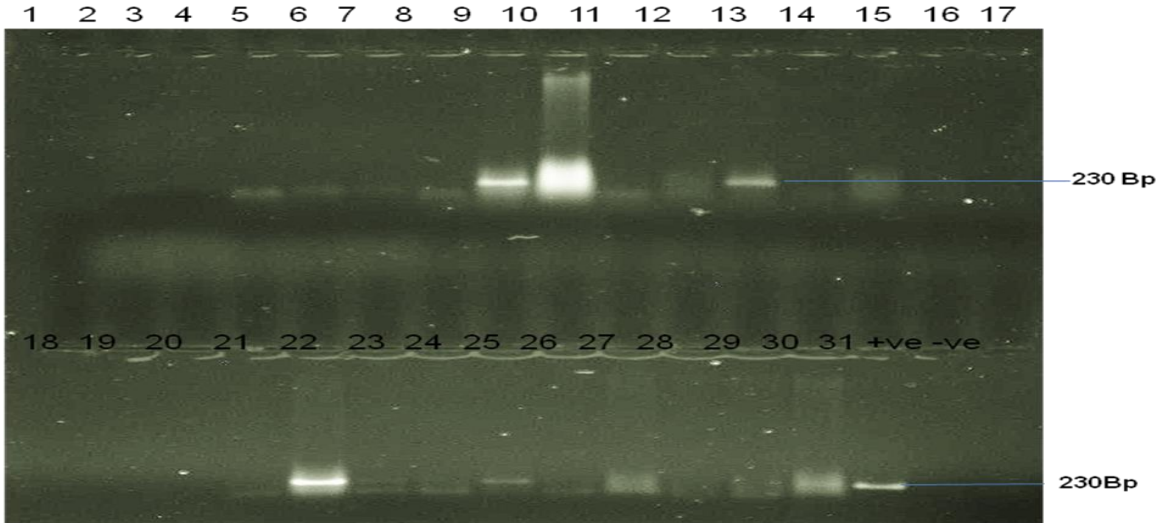

Plate 2.2: Agarose gel electrophoresis of CBSV detection of *in vitro* cassava treated with ribavirin at a 1% (w/v) agarose gel; lane 1- 17 10mg/l; lane 18- 24mg/l lane 25-31 30mg/l lane 32 +ve control; lane 33-ve control

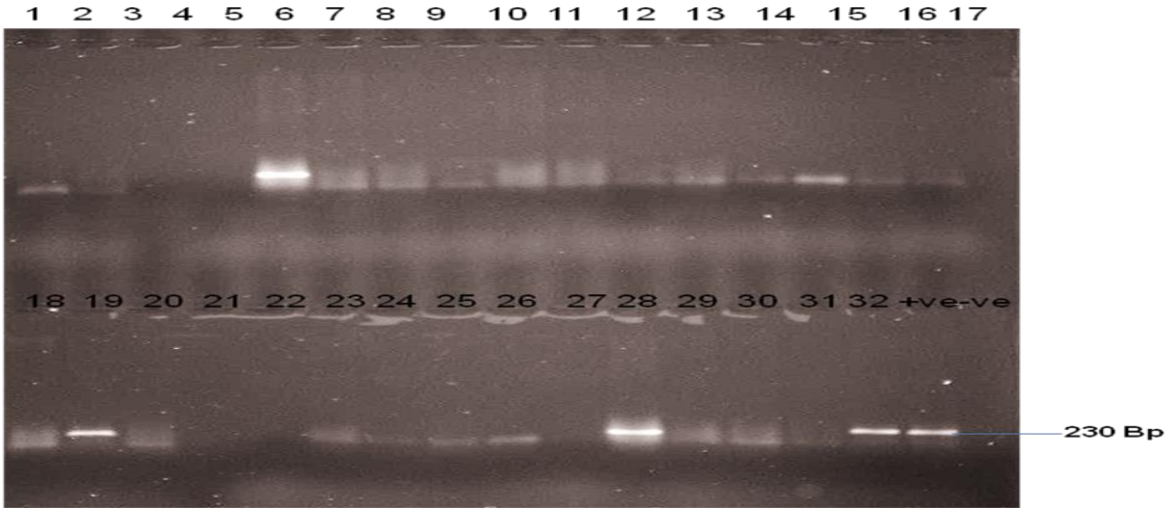

Plate 5.3: Agarose gel electrophoresis of CBSV detection of *in vitro* cassava treated with salicylic at a 1% (w/v) agarose gel; lane 130mg/l; lane 2-19 20mg/l; lane19-32 10mg/l ;lane 33 +ve control; lane 34 –ve control

5.3.3 Survival and CBSV virus reduction of regenerated plants

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 45 and 1 respectively (Table 5.1 and Table 5.2).

Table 5.1: Survival and virus reduction (%) of plants subjected to ribavirin treatment

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

Analysis based on survival ($P \leq 0.01$) (S.E-standard error) 0.1386

Data in parenthesis are actual numbers of plants

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 were produced while at 10mg/l, 68.8% virus-free plants were produced (Table 5.2). Controls obtained from (0mg/l) resulted in all positive plants obtained.

Table 5.2: Survival and virus reduction (%) of plants subjected to salicylic acid treatment

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

Analysis based on survival ($P \leq 0.01$) (S.E-standard error) 0.0467

Data in parenthesis are actual numbers of plants

The least significant difference between any of the means at $P \leq 0.05$ indicates that at each concentration of ribavirin and salicylic acid, the plants survivals are significantly different from each other (Table 5.3 and Table 5.4).

Table 5.3: Means of survivals from plants subjected to ribavirin treatment and the least significant difference.

Ribavirin concentration (Mg/l)	**Mean survival (%)**
0	100.0
10	69.0
20	55.5
30	31.1
$LSD_{0.05}$	14.8

Table 5.4: Means of survivals from plants subjected to salicylic acid treatment and the least significant difference.

Salicylic acid concentration (Mg/l)	**Mean survival (%)**
0	100.0
10	31.1
20	28.8
30	0.0
$LSD_{0.05}$	15.1

5.4 Discussion

The effects of ribavirin and salicylic acid at varying concentrations were evaluated for the reduction of CBSV from infected cassava. CBSV reduction from infected cassava was influenced by the antiviral compound and level of concentration. Survival of shoots was observed to decrease with increase in the antiviral concentration. Toxicity to plants and antiviral activity was more pronounced in higher concentration of 30mg/l in both salicylic acid and ribavirin as evidenced by defoliation of leaves. This was in agreement with Klein and Livingston (1982) that at higher concentrations of ribavirin, higher mortality and phytotoxicity is detectable. This was also verified by Griffith *et al.,* 1991 and Nascimiento *et al.,* (2003).

Increasing concentrations of ribavirin typically increases the effectiveness of virus elimination (Mellor and Smith, 1977), but slowed growth and phytotoxicity may be evident at high concentrations similar to the present study in ribavirin and salicylic acid treatments at 30mg/l. This concentration however needs to be below the concentration at which ribavirin is not highly phytotoxic to the plant which was in the case of ribavirin at 10mg/l having a higher survival and effective in CBSV reduction.

The concentrations of many antiviral chemicals required during chemotherapy to inhibit virus multiplication are very close to the toxic concentration for the host plant (Panattoni *et al* .,2013) thus at higher concentrations of 20mg/l and 30mg/l, the proportion of clean plants produced relative to those that survived was higher. This can be explained by the fact that when mutation rates of viral RNA exceeds critical threshold a virus may

experience decreased infectivity and extinction of the virus population (Mellor and Smith, 1977; Panattoni *et al.*, 2013).

The role of salicylic acid in the compatible host –virus interactions was investigated previously in the elimination of tobacco mosaic virus which resulted in reduced viral accumulation and delayed appearance of the disease symptoms (Gaffney *et al.*, 1993). This was also observed in the ability of salicylic acid to effectively eliminate CBSV in the cassava at concentrations of 10mg/l and 20mg/l. The mechanisms of salicylic acid of causing oxidative stress resulting into RNA mutation rates hence causing early senescence, was similarly evident in older leaves of CBSV infected cassava plants subjected to the salicylic treatments exhibiting high defoliation of leaves. Salicylic acid treatment was observed to offer high effectiveness in terms of CBSV virus reduction but poor in cassava shoot survival.

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; Griffith *et al.*, 1990; Nascimiento *et al.*, 2003). Levels of phytotoxicity have to be tolerated to achieve the eradication of viruses (Robert and Clark, 1982; Griffith *et al.*, 1990). With this in consideration, 10mg/l of ribavirin was found to be optimum for the CBSV reduction from infected cassava *in vitro.* This is in agreement with Robert and Clark (1982) whereby potato virus X virus in potato could not be detected in over 80% of plantlets developed from cultures treated with 10mg/l of ribavirin.

6.0 CHAPTER SIX

6.1 CASSAVA BROWN STREAK VIRUS REDUCTION FROM INFECTED CASSAVA THROUGH THE COMBINATION OF THERMOTHERAPY WITH CHEMOTHERAPY AND MERISTEM TIP CUTURE

6.1.2 Abstract

Thermotherapy, chemotherapy and meristem tip culture have been used either alone or in combination to eliminate viruses from plants**.** CBSV infected cuttings of Guzo variety collected from Coast province of Kenya established and maintained in a greenhouse at the Plant Quarantine Station in Kenya Plant Health Inspectorate Service (KEPHIS) in Muguga were used as test plants. Cassava leaves were sampled from eighteen cassava plants of Guzo variety and virus indexing was done using (RT-PCR) with virus specific primers and those confirmed to be positive for CBSV were used as initiation materials for the prospective test plants. From the initiated tissue culture materials the 2nd subcultures were subjected to heat treatment at 38°C for 21 days and later subjected to ribavirin treatment at varying concentrations of (10mg/l, 20mg/l, 30mgl) then left to establish for 14 days. *In vitro* meristems of 1mm were excised from heat treated plantlets at 38°C for 21 days and cultured in modifies MS media. A completely randomized design was used to subject the tissue cultured material to the different treatments (where by each treatment was replicated 3 times). Nodal plantlets of 10mm size not subjected to any treatment were used as controls. There was a significant difference ($P<0.01$) in the number of plants that survived among the treatments. Thermotherapy combined with chemotherapy resulted in complete mortality of plants due to the high stress levels from the high temperatures combined with the phytotoxic effect of the ribavirin.

Thermotherapy followed by excision of meristem tips (1mm) resulted in 68.8% of shoot survival with 84% being virus-free. Thermotherapy combined with meristem tip culture was successfully employed to produce CBSV-free cassava plants.

6.1 Introduction

The most viable methods for obtaining virus-free stocks is by viral eradication using tissue culture techniques, aided by thermo or chemotherapies (Mellor and Stace-Smith, 1970 ; Pannatoni *et al.,* 2013). Plant thermotherapy is described as achieving a cellular environment which is progressively less adequate for virus vitality (Pennazio, 1995). Similar interactions are also reported by (Mink *et al.,* 1998) who discussed the effects of heat treatment on the functionality of viral movement. In fact the different ability for the movement of viral particles in plant tissues influenced the choice of elimination (Pannatoni *et al.,* 2013) with thermotherapy as the most effective against viruses characterized by parechymatic localization (Panattoni *et al.,* 2013) compared to meristem culture which is more suitable for phloemetic viruses that are limited to vascular tissues (Grout,1990).

Developments over the last 20 years in research have suggested that heat treatment effects triggered the natural antiviral response produced by infected plant, particular reference being to virus induced gene silencing (VIGS) induced by the presence of viral RNA in infected plants (Ruitz *et al.,* 1998). Chellapan *et al.,* (2005) did some research to better define the mechanisms that determine the influence of temperature on the antiviral silencing for *Geminiviruses* (ssDNA) by applying heat treatment (25◦C to 30◦C) to

cassava (*Manihot esculenta)* and tobacco (*Nicotiana tabacum*) plants infected by cassava mosaic disease and they achieved similar results confirming the close relationship between temperature and the VIGS.

Heat treatment of stock plants prior to meristem tip culture is often used to enhance virus elimination (Mink *et al.,* 1998). Temperatures ranging from 34°C to 40°C for periods ranging from days to weeks are efficient for viral eradication (Betti, 1991). Meristem tip culture has been used effectively in combination with thermotherapy to obtain clean nectarine at 35°C with meristems ranging from 1.8mm-2mm (Manganaris *et al*., 2003). Application of meristem culture combined with thermotherapy is reported to increase the survival rate of *in vitro* explants (Manganaris *et al*., 2003); since larger tips can be obtained from heat-treated plants while ensuring virus-free plant production. Adejare and Coutts (1981) also reported the absence of mosaic symptoms on the leaves of rooted explants when they subjected diseased donor cassava explants to heat treatment for at least 30 days at 35°–38°C, and cultured meristems on modified MS (Murashige and Skoog, 1962) medium. Successful elimination of sweet potato featherly mottle virus SPFMV from Egyptian Abees sweet potato cultivar by heat therapy and meristem tip culture has also been reported (Mervet *et al.,* 2009).

Improved virus elimination can also occur if chemotherapy is combined with thermotherapy with subsequent meristem tip culture (Kartha and Garmborg, 1986; Spiegel *et al.,* 1993; Luciana *et al.,* 2007). Joint effects of thermotherapy at 37°C and ribavirin applied to *in vitro* plants were 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 to the growth medium as the best treatments for virus elimination in potato (Fletcher *et al.*, 1998, Griffith *et al.*, 1990).

6.2 Methodology

6.2.1 Selection of infected cassava plants

CBSV positive plants were selected, tested, initiated *in vitro* and used as prospective test plants for meristem tip culture. This is captured in chapter three on general methodology.

6.2.2 Thermotherapy at 38 °C combined with meristem tip culture for the reduction of CBSV from infected cassava

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 38ºC 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.

6.2.3 Thermotherapy at 38 °C combined with chemotherapy for reduction of CBSV from infected cassava

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 38ºC 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.

6.2.4 Experimental design and data collection

A completely randomized design was used to subject the tissue cultured material in the different treatments. Each treatment had a total number of 15 plants replicated three times to create a total sample size of 45 plants. The number of surviving plantlets, CBSV-positive and CBSV-negative plants after virus testing was recorded.

6.2.5 Data analysis

ANOVA using genstat software was used to calculate the significance at ($P \leq 0.01$) for the plants that survived subjected to thermotherapy combined with meristem tip culture and chemotherapy.

Virus reduction was determined as the proportion of CBSV-negative plants obtained expressed as a %.

6.3 Results

6.3.1 Effects of thermotherapy combined with meristem tip culture and chemotherapy for the reduction of CBSV from infected cassava

Plants that were subjected to meristem tip culture and thermotherapy at 38^{o}C exhibited slight chlorosis due to the high temperatures compared to the controls left at room temperatures of 24^{o}C (Plate 6.1a). Plants that were subjected to heat treatment in combination with chemotherapy dried up resulting in mortality of all plants subjected to this treatment (Plate 6.1b).

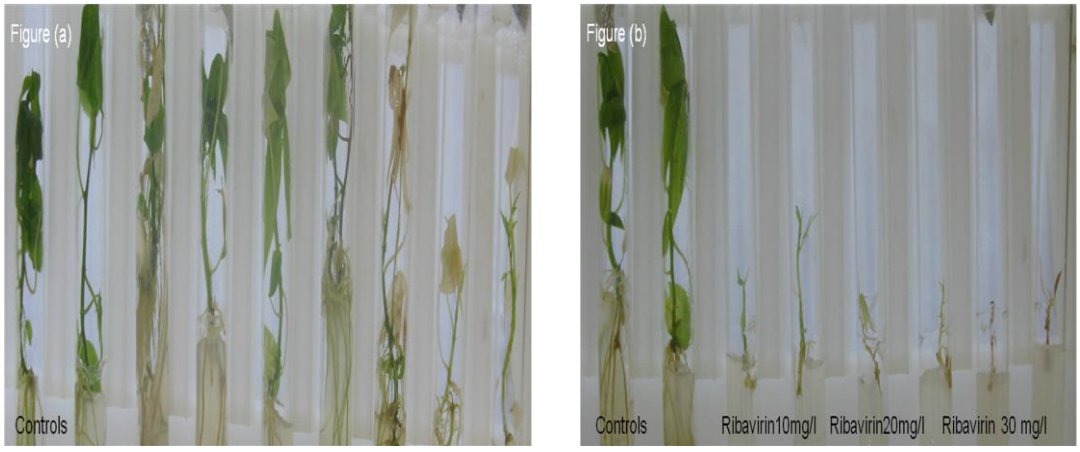

Plate 6.1: (a) Heat treated plants at 38^{o}C (b) Plants subjected to thermotherapy in combination with chemotherapy

6.3.2 PCR detection for CBSV in plants established from meristems excised from heat treated plants

The PCR products of the survived putative virus-free material amplifications were visualized in an agarose gel as illustrated in Plate 6.2. Out of 31 the plants tested in 27 were CBSV-free (Plate 6.2).

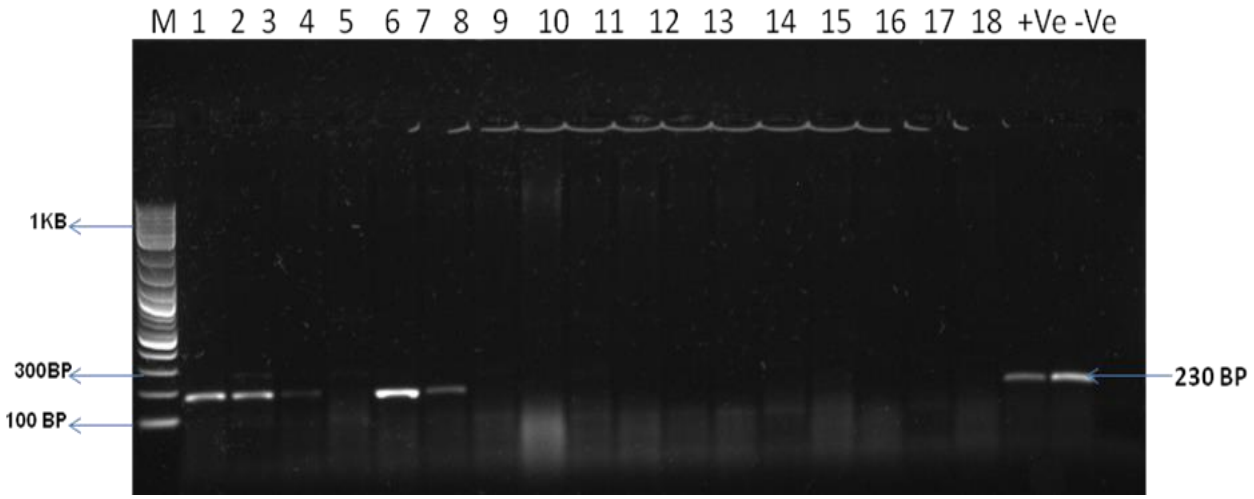

Plate 6.2: Agarose gel electrophoresis of CBSV detection of in vitro meristem excised from heat treated plants at 38 ^{o}C at 1% (w/v) agarose gel; lane 1 1kb ladder lane; 2-18 in vitro meristems; lane19 (positive control). Lane 20 (negative control)

6.3.3 Survival and virus reduction of regenerated plants

Thermotherapy combined with meristem tip culture resulted in 68% of regenerated plants with 84% being virus-free (Table 6.1). Thermotherapy combined with chemotherapy resulted to no survivals. Controls obtained from nodal plantlets resulted in all positive plants obtained.

Table 6.1: Survival and virus reduction (%) of plants subjected to thermotherapy combined with meristem tip culture and chemotherapy

Treatment	**Initiated explants (No)**	**Regenerated plants (No)**	**Positive plants (%)**	**Negative plants (%)**
Meristem Tip Culture+ 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≤0.01) (S.E-standard error) 0.0368

Data in parenthesis are actual numbers of plants

The least significant difference between any of the means at $P \leq 0.05$ indicates that each treatment produced a significantly different survival from each other (Table 6.2).

Table 6.2: Means of survivals from plants subjected to thermotherapy combined with chemotherapy and meristem tip culture and the least significant difference

Treatment	**Mean survival (%)**
Control	100.0
Thermo+ meristem (1 mm)	68.9
Thermo+ ribavirin 10 mg/l	0.0
Thermo+ ribavirin 20 mg/l	0.0
Thermo+ ribavirin 30 mg/l	0.0
$LSD_{0.05}$	8.6

6.4 Discussion

The effect of combining thermotherapy with meristem tip culture and chemotherapy for the reduction of CBSV from infected cassava was evaluated. Joint effect of thermo and chemotherapies did not give the expected results. These plants completely dried up turning brown and total mortality was recorded. These results differ with Nascimiento *et al.,* (2003) and 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. The combination of chemotherapy with thermotherapy caused high stress levels to the plants subjected to the treatments. 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 (Panattoni *et al.,* 2013) 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 (Panattoni *et al.,* 2013). Meristem excised from plants subjected to thermotherapy treatment enhanced CBSV eradication compared to the control resulting in 68.8% of plant survival with 84% being virus-free. These results are not surprising since thermotherapy given to *in vitro* plants prior to meristem excision has been found to give fewer virus infected 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 by Mervet *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). 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 reduction of CBSV from infected cassava *in vitro.*

7.0 CHAPTER SEVEN

7.1 GENERAL DISCUSSION, CONCLUSION AND RECOMENDATION

7.1.1 GENERAL DISCUSSION

This study aimed at *in vitro* production of virus-free cassava planting materials. Specifically by evaluating the effect of meristem tip sizes, analysing the effects of varying concentrations levels of salicylic acid and ribavirin and investigating the effect of thermotherapy in combination with r meristem tip culture and chemotherapy on the reduction of cassava brown streak virus from infected cassava.

From the results obtained the size of the explants influenced the number of plants regenerated and the reduction of CBSV from infected cassava. More plants were derived from meristems of 1mm and 2mm with the control of 10mm having the highest number of plants established. Meristem tip size of 0.5mm had the highest percentage (88.23%) of virus-free plants obtained this is in agreement with the findings of Manganaris *et al.*, (2003) and Mervet *et al.*, (2009) that the smallest explants are those that typically, will be the least successful during *in vitro* culture, but will produce the highest proportion of virus-free material.

Survival of the shoots was observed to decrease with increase in the antiviral concentrations. Ribavirin 10mg/l resulted in highest survivals compared to the other treatments. In addition salicylic acid showed least growth and survival compared to ribavirin at all concentrations. The number of virus-free plants obtained was observed to increase with increase in concentration for both chemicals. Although at 30mg/l salicylic acid resulted in 100% of negative plants only one plant survived from the experiment.

Increasing concentrations of ribavirin and increasing length of culture incubation in the presence of the compound typically increase the effectiveness of virus elimination (Mellor and Smith, 1977), but slowed growth and phytotoxicity may be evident at high concentrations (Lin *et al.,* 1990) which in this case was observed in ribavirin 30mg/l having low survivals of 20%. Although at effective low concentrations, replication of the virus is hindered. This concentration however needs to be below the concentration at which ribavirin is not phytotoxic to the plant which was in the case of ribavirin at 10mg/l having higher survivals.

Plants that were subjected to thermotherapy combined with chemotherapy (ribavirin) dried up due to high stress levels resulting in total mortality. Meristem tips (1mm) combined with thermotherapy resulted in 68.8% of plant survival with 84% being virus-free plants. These results are not surprising since thermotherapy given to *in vitro* plants prior to meristem excision has been found to give fewer virus infected meristems cultured plants in various vegetative propagated species (Acedo, 2006).

7.1.2 CONCLUSION

Meristem tip size of 0.5mm was found effective for reduction of CBSV from infected cassava, having the highest percentage (88.23%) of virus-free plants produced.

Generally salicylic acid treatment was observed to offer high effectiveness in terms of CBSV virus reduction but poor in survival of plants which rendered it not effective as a virus reduction strategy. 10mg/l ribavirin was found effective in terms of survival and CBSV reduction.

Thermotherapy combined with meristem tip culture (1mm) resulted in 68.8% of regenerated plants with 84% being virus-free. Based on efficiency of virus reduction and survival, meristem tip culture combined with thermotherapy was found optimum for reduction of CBSV from infected cassava *in vitro.*

7.1.3 RECOMMENDATION

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

There is need for studies to be done that will show effects of these cleaning methods on reduction of Ugandan cassava brown streak virus (UCBSV) from infected cassava plants (guzo variety)

Studies should also be done to evaluate the effect of chemotherapy on CBSV reduction by subjecting CBSV infected plants to lower antiviral concentrations and increasing duration of the treatment to evaluate the effect of CBSV reduction in guzo variety.

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9.0 APPENDICE

Appendix 1

CTAB extraction buffer

	In 500ml
2% CTAB	10g
100mm TRIS-HCL ph 8.0	6.05g (Tris base adjusted with HCL)
20mM EDTA	2.92g
1.4 M NACL	40.6g
Add the following to fresh aliquot of grinding buffer once per week	
	In 100ml
1% Na sulphite	1g
2% PVP-40	2g

Appendix 2

PCR master mix and thermo cycling conditions

PCR components	**Volume for 1 reaction**	**Final concentration**
PCR grade water	6.85µl	
10x MMLV buffer	1 µl	1x
2 Mm dNTPS	0.3 µl	60µm
10UM CBSV primer 10/11	0.15µl	200µm
5u/ul taq DNA polymerase	0.08 µl	0.04U/µl
200u/ul MMLV-RT	0.03 µl	0.6U/µl
Template RNA	2 µl	
Thermal cycling conditions Pre-PCR program for generating the cDNA 1 cycle of an initial denaturation at 42°C for 30 min 94°C for 2 min, 52°C for 2 min and 72°C for 3min the PCR cycle included 30 cycles of 94°C for 30 min, 52°C for 30 sec 72°C for 1 min and stored at 4°C.		

Appendix 3

Murashige & Skoog media stock solution's composition

Stock Solution		Salts/Vitamins	Gms/Lt (in stock solution)	Amount of stock solution per Litre of MS Media	Storage Conditions
1	1	NH_4NO3	33.0 gm	50ml	Refrigerate at 4°C to 10°C
	2	KNO_3	38.0 gm		
2	3	$MgSO_4.7H_2O$	37.0 gm	10 ml	Refrigerate at 4°C to 10°C
	4	$MnSO_4.4H_2O$	2.23 gm		
	5	$ZnSO_4.7H_2O$	0.86 gm		
	6	$CuSO_4.5H_2O$	0.0025 gm		
3	7	$CaCL_2.2H_2O$	44.0 gm	10 ml	Refrigerate at 4°C to 10°C
	8	KI	0.083 gm		
	9	$CoCL_2.6H_2O$	0.0025 gm		
4	10	KH_2PO_4	17.0 gm	10 ml	Refrigerate at 4°C to 10°C
	11	H_3BO_3	0.62 gm		
	12	$NaMoO_4.2H_2O$	0.025 gm		
5	13	$FeSO_4.7H_2O$	2.785 gm	10 ml	Keep in amber bottles and Refrigerate at 4°C to 10°C
	14	Na_2-EDTA	3.725 gm		
6	15	Inositol	10.0 gm	10 ml	Refrigerate at 4°C to 10°C
	16	Glycine	0.2 gm		
	17	Nicotinic Acid	0.05 gm		
	18	Pyridoxine-HCL	0.05 gm		
	19	Thiamine-HCL	0.01 gm		
7	20	Gibberellic acid	100 gm	1 ml (0.1mg/ml)	Refrigerate.

Appendix 4

Analysis of variance, table of means, stratum standard errors and coefficients of variation based on survival

OBJECTIVE 1

Analysis of variance

Variate: SURVIVAL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	4	0.72121	0.18030	2.61	
REP.*Units* stratum					
TRT	3	3.35587	1.11862	16.19	<.001
Residual	52	3.59251	0.06909		
Total	59	7.66959			

Tables of means

Variate: SURVIVAL

Grand mean 0.684

TRT	0.5mm	10mm	1mm	2mm
	0.377	1.000	0.557	0.801

Least significant differences of means (5% level)

Table	TRT
rep.	15
d.f.	52
l.s.d.	0.1926

Stratum standard errors and coefficients of variation

Variate: SURVIVAL

Stratum	d.f.	s.e.	cv%
REP	4	0.1226	17.9
REP.*Units*	52	0.2628	38.4

OBJECTIVE 2

I) RIBAVIRIN

Analysis of variance

Variate: SURVIVAL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	4	0.92269	0.23067	5.64	
REP.*Units* stratum					
TRT	3	3.71587	1.23862	30.28	<.001
Residual	52	2.12698	0.04090		
Total	59	6.76554			

Tables of means

Variate: SURVIVAL

Grand mean 0.639

TRT	ribavirin 0mg/l	ribavirin 10mg/l	ribavirin 20mg/l	ribavirin 30mg/l
	1.000	0.690	0.555	0.311

Least significant differences of means (5% level)

Table	TRT
rep.	15
d.f.	52
l.s.d.	0.1482

Stratum standard errors and coefficients of variation

Variate: SURVIVAL

Stratum	d.f.	s.e.	cv%
REP	4	0.1386	21.7
REP.*Units*	52	0.2022	31.7

II) SALYCYLIC ACID

Analysis of variance

Variate: SURVIVAL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	4	0.10453	0.02613	0.62	
REP.*Units* stratum					
TRT	3	8.10786	2.70262	63.77	<.001
Residual	52	2.20381	0.04238		
Total	59	10.41619			

Tables of means

Variate: SURVIVAL

Grand mean 0.400

TRT salycylic acid	0mg/l	10mg/l	20mg/l	30mg/l
	1.000	0.311	0.288	0.000

Least significant differences of means (5% level)

Table	TRT
rep.	15
d.f.	52
l.s.d.	0.1508

Stratum standard errors and coefficients of variation

Variate: SURVIVAL

Stratum	d.f.	s.e.	cv%
REP	4	0.0467	11.7
REP.*Units*	52	0.2059	5

OBJECTIVE 3

Analysis of variance

Variate: SURVIVAL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	4	0.08143	0.02036	1.46	
REP.*Units* stratum					

TRT	4	13.56617	3.39154	243.34	<.001
Residual	66	0.91986	0.01394		
Total	74	14.56746			

Tables of means

Variate: SURVIVAL

Grand mean 0.338

Thermo+ chermo+ribavirin 10mg/l	0.000
Thermo+ chermo+ribavirin 20mg/l	0.000
Thermo+ chermo+ribavirin 30mg/l	0.000
Thermo +meristem tip culture	0.689
Control	1.000

Least significant differences of means (5% level)

Table	TRT
rep.	15
d.f.	66
l.s.d.	0.0861

Stratum standard errors and coefficients of variation

Variate: SURVIVAL

Stratum	d.f.	s.e.	cv%
REP	4	0.0368	10.9
REP.*Units*	66	0.1181	34.9