Evaluation of *in vitro* protocols for the elimination of *banana streak virus* in tissue culture produced banana plantlets

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this work to my beloved family; my husband Paul Wanjau, and daughters Esther Mukami and Josephine Wairimu. My dear late parents Josephine Njoki, Jonah Mungai and my entire family. Without your encouragement and support, this journey would have been long and tough. You put your heart in all that I did, supported, facilitated, encouraged and prayed for me.

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LIST OF ABBREVIATIONS

BanMMV	Banana mild mosaic virus
BBrMV	Banana bract mosaic virus
BBTV	Banana bunchy top virus
BDBD	Banana die back disease
BDBV	Banana die back virus
BSV	Banana streak virus
CMV	Cucumber mosaic virus
DNA	Deoxy ribonucleic acid
ELISA	Enzyme link immuno sorbent assay
IC-PCR	Immune capture - polymerase chain reaction
ISEM	Immunosorbent electron microscopy
KARI	Kenya Agricultural Research Institute
MS	Murashige and Skoog
PCR	Polymerase chain reaction
TAS-ELISA	Triple antibody sandwich–enzyme linked immunosorbent assay
TC	Tissue culture

ABSTRACT

Tissue culture (TC) propagation of banana crop has been used in many laboratories for mass production of planting materials which are free from plant pathogens. The Jomo Kenyatta University of Agriculture and Technology (JKUAT) commercial banana TC laboratory which currently supplies banana planting material all over the country, and parts of East Africa, utilizes the TC technique for the mass propagation of banana plantlets for sale to farmers. However, the TC process does not eliminate banana viruses. *Banana streak virus* (BSV) reduces banana yield from 6% to 15% and is also the most prevalent banana virus in Kenya. This poses a big danger since the plantlets in supply could be BSV infected. Therefore, this research was done to evaluate *in vitro* protocols for elimination of BSV with the aim of producing BSV-free TC banana planting materials for farmers. Asymptomatic and symptomatic leaf and shoot samples were randomly collected from Kenya Agricultural Research Institute (KARI) Kisii, KARI Thika and JKUAT banana mother orchards that mainly supply JKUAT commercial TC laboratory with initiation materials. The leaf samples were screened for presence of BSV using polymerase chain reaction (PCR) technique, while the suckers were used for initiation materials which were taken through TC process up to the second subculture. The second subcultered plantlets were used in evaluation of the *in vitro* protocols for elimination of BSV. Three viral elimination techniques namely chemotherapy (ribavirin, and salicylic acid), thermotherapy and meristem tip culture were evaluated in attempts to eliminate BSV. All the costs incurred in the production of BSV-free plantlets were determined using the year 2012 market price in Kenya

shillings to determine which of the virus elimination methods was cost effective. Of the 30 samples collected from the three mother orchards, 15 asymptomatic and 15 symptomatic, 21 were detected with BSV using PCR. In chemotherapy, 0mg/l (control), 10 - 40 mg/l were used and they gave 0 - 90% virus elimination using ribavirin and 0 - 90% virus elimination using salicylic acid respectively. In thermotherapy, 27°C (control), 32 - 38°C all for 10 days were used which resulted in between 0% and 90% virus elimination. Meristem tip culture using tips ranging between 1, and 5mm (control) resulted in 0%, and 70% virus elimination. Best BSV elimination and explant regeneration rates were observed at 20mg/l of salicylic acid and at 36°C. However, thermotherapy can be time consuming and laborious in laboratories which do not have automated systems. Production of one BSV– explant using the four virus elimination methods was Ksh 125.5, 125.6, 127.9, and 130.4, using salicylic acid, ribavirin, meristem tip culture and thermotherapy, respectively. Chemotherapy using salicylic acid at 20mg/l was considered the most effective since it was cheaper, in terms of implementation, less laborious, and it had the highest regeneration and virus elimination rates. Therefore all infected initiation materials to be used in commercial laboratories should subjected to chemotherapy using salicylic acid (20mg/l) to ensure that all banana planting materials released for sale are BSV-free.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Banana *(Musa*) is the fourth most important food crop worldwide based on gross value of production (Singh *et al.,* 2011). It is a subsistence and cash crop for many smallholder farmers, particularly in West, Central and East Africa (Singh *et al.,* 2011).

Presently, banana is grown in around 150 countries across the world on an area of 4.84 million per hectare producing 95.6 million tonnes (FAOSTAT, 2011). Asia, Africa and Latin America are the major banana producing continents. Among the major producers, India alone accounts for 27.43 % (26.2 million tonnes) followed by Philippines, producing 9.01 million tonnes and China, Brazil and Ecuador, with production ranging from 7.19 to 8.21 million tonnes (FAOSTAT, 2011; Singh *et al*., 2011).

The commercial cultivation of banana in Kenya involves a large number of small-scale growers (Wangai *et al.,* 2002). It is produced for local consumption and for export. The year-round fruit habit of the crop ensures food security at household level with a potential of sustaining food supply to urban markets especially in periods between cereal crop harvests. This potential coupled with the environmental conservation attributes of the plants makes banana an ideal crop for economic growth and sustainability of the agricultural resource base (Wangai *et al.,* 2002).

In Kenya, banana is a major fruit crop for both subsistence and commercial use. In priority setting exercise for horticultural crops research undertaken in the year 1996, banana was ranked as the most important crop among the fruit crops. It is estimated to cover 74,000 hectares, about 2% of total arable land, (Moald, 1997) ranging from 1000m to 1800 m above sea level. In terms of production, over a million tons are obtained per year. Nyanza and Western Provinces account for 64.4% of production while Central and Eastern Provinces account for 26% of production. Hence the rest of the Provinces in the country can be classified as being minor producers, with Rift Valley Province accounting for 3.9% and Coast Province accounting for 5.5%. The crop is predominantly grown by small-scale farmers who have an average holding of 0.3 hectares making up to 13% of the total farm area (Qaim, 1999). Dijsksra and Magori (1994) reported that banana accounted for over 70% of farmers' incomes in Kisii. In the recent years, banana has become an important cash crop for semi-intensive medium scale farmers who supply to the urban markets in the country. This is more where the income from traditional cash crops, especially coffee, has drastically reduced. Continuous availability of harvestable bunch from a banana stool is especially important for farmers, because it contributes to the year round security of food and income (FAO, 2007).

The commonly grown varieties are East Africa highland (cooking varieties) and apple bananas in Western and Nyanza Provinces while Cavendish and Kampala varieties are grown in Central and Eastern Provinces (Nguthi *et.al.,* 1999).

1.2 Constraints to banana production

Banana is vulnerable to bacterial, fungal and viral diseases, nematodes and banana weevils. Viral diseases are among the major banana production constraints **(**Gambley and Thomas, 2001). In East Africa, the main constraint to banana production is the lack of clean banana plantlets and therefore there is immense exchange of infected suckers (INIBAP, 2000) this is mainly contributed to lack of information by the farmers. Another major constraint facing banana production is the exceedingly high sterility polyploidy of the clones of primary interest hindering improvement programs. This is because banana does not produce mature seeds, they are sterile and seedless because they have odd polyploidy in which one set of chromosomes (A or B) has no homologous set to pair up with during synapsis of meiosis. Also the low seed set of banana hinders hybrid plant production of the most common triploid clones due to embryo mortality, dominancy and disruption of embryo (Thomas *et al.,* 2000). *Banana streak virus* (BSV) is one of the recent constraints of banana production in Kenya since it is prevalent in all banana growing zones and it infects all the banana cultivars (Karanja *et al.,* 2008).

Banana streak disease was first reported in the Cote d'Ivoire in 1966. Today, the disease is found in all countries where banana is grown (Lockhart and Jones, 2000). Banana streak virus (BSV) is a *Pararetrovirus* – that is, a virus with circular dsDNA in virions, which replicates via a longer – than – genome length ssRNA (+) intermediate, via reverse transcription (Lockhart and Olszewski, 1993). The virus causes a wide range of symptoms and its damage ranges from mild to severe. Recent

studies in Kenya have shown that BSV is prevalent in the entire banana growing regions and affects all the popular cultivars grown by farmers (Wangai *et al*., 2002).

Most BSV isolates exhibit broken or continuous chlorotic streak or fine spindle-shaped spots, which are first chlorotic, then become black streaking in older leaves. Some isolates produce severe necrosis causing heart rot of spindle leaves. Bunches may be reduced in size and symptomless infection occurs frequently. Symptoms are often confused with those of *Cucumber Mosaic Virus* (CMV) (ISAAA, 1999).

BSV is transmitted in a semi persistent manner by citrus mealy bug, *Planococcus citri*. The virus is principally transmitted via suckers and tissue-cultured plantlets derived from infected sources (Lockhart and Jones, 2000). It is possible that infected sugarcane may be a source of virus for banana infection (Tsao, 1998; Wu and Su, 1990). As vegetative propagation appears to play a major role in transmission of BSV, the most effective means to control the disease is to ensure that source plants used for propagation are virus-free (Dale, 1987).

Infection of cultivated *Musa* sp. by BSV affects plant yield and performance. It also sets a problem in transferring infected germplasm from one region to another, as well as the danger of transferring BSV infection in *Musa* breeding programs (INIBAP, 1998). Given the widespread occurrence and potential threats posed by BSV infection in cultivated *Musa* sp., it has become imperative to develop effective and reliable methods for detecting BSV in field samples, breeding lines and TC stock (ISAAA, 1999).

The principal problem in developing reliable indexing methods for BSV is the extreme serological and genomic variability among strain of the virus. As a result, no single antiserum or nucleic acid probe is capable of detecting the wide range of the virus isolates. Further compounding this situation is the possibility that BSV might possibly exist as DNA rather than virions in the apical meristems and embryonic tissues of the plants (INIBAP, 1998).

In some *Musa* cultivars, BSV can be activated by stresses associated with TC and/or crossing to give episomal infections (INIBAP, 1997). Another feature of BSV infections is that symptoms can be transient with symptom suppression at higher temperatures. This resembles the situation with another pararetrovirus, cauliflower mosaic virus, in which symptom suppression is associated with perturbation of the virus replication cycle (INIBAP, 1998).

Serological detection of BSV is complicated by the occurrence of a wide degree of serological diversity among viral strains. An antiserum raised against many strains is capable of detecting all known strains by ISEM in partially purified extract. Specific primers to the virus sequence have also been very effective in detecting the virus (Tsao, 1998; Wu and Su, 1990).

1.3 Problem statement

Despite banana being an important crop, BSV infection has remained a limiting factor to attainment of high yields. The economic yield loss impact is about 60 tons per hectare (FAOSTAT, 2006). This is therefore an already delicate food security threat in

Kenya. Although TC has been shown to eliminate most plant disease causing organisms, TC does not eliminate virus (Wangai *et al*., 2002). This has therefore left ambiguity on methods used for and protocols used in propagation of TC banana.

The introduction of TC techniques for banana propagation was perceived as having the potential to help reverse the situation since it would ensure timely availability of "clean" planting material. Using TC, the production of planting material can be achieved as per needs. Micropropagated plants exhibit uniform growth and maturity enabling one time and timely harvesting. Intercropping banana with short duration legumes adds to the farmer's income and soil fertility. Using TC, it is possible to develop planting material which is free from sucker borne diseases and pests. Production of plants in test tubes facilitates safe movement and easy handling of germplasm between laboratories within and across countries. Since the micropropagation based progeny is genotypically and phenotypically similar to the mother plant, which is often a superior selection, the yield and returns are expectedly higher (Thomas *et al.*, 2000).

Tissue culture remains a useful technique for the production of clean planting material free of nematode, weevil and fungal diseases in many laboratories across the world. Despite its apparent potential to eliminate these shortcomings, TC technique is not able to eliminate BSV and is even believed to activate BSV infection in some banana varieties (Harper *et al.*, 2005).

Recent studies in Kenya have shown that BSV is found in all the banana growing regions and all the popular cultivars grown by farmers are susceptible, it is also considered the most prevalent banana viral disease (Karanja *et al.*, 2008). Diagnostic methods for BSV are inadequate because of the considerable genetic and serological diversity amongst BSV isolates and the presence of integrated BSV sequences in some banana cultivars which leads to false positives (Harper *et al.*, 2005). Therefore, for one to do proper diagnostics, good procedure has to be used to eliminate these false positives for instance the use of a templiphi kit or use of PCR using the specific primers for different strains.

JKUAT TC laboratory is one of the biggest TC laboratories in Kenya with approximately two million plantlets produced per year. The propagation method in use at the JKUAT commercial laboratory is micropropagation via TC method of propagation for mass propagation of plantlets; these plantlets are free from fungal and bacterial diseases but are not free from banana viruses.

1.4 Justification

The year-round fruiting habit of the banana crop ensures food security at household level with a potential of sustaining food supply to urban markets especially in periods between cereal crop harvests. Since banana has many environmental conservation attributes it makes an ideal crop for economic growth and sustainability of the agricultural resource base. Tissue culture facilitates production of large number of plantlets/unit time, thus helping in rapid introduction and dissemination of new

varieties. It also ensures that limited numbers of mother plants are required for raising a large number of progeny plants which can be maintained with required care at a limited cost. TC results in a high degree of genotypic and phenotypic uniformity of the progeny plants.

Whilst TC banana plantlets are produced in a sterile environment, and are free from fungal and bacterial diseases, BSV can still be transmitted through the *in vitro* plantlets (ISAAA, 1996). The stage and condition of suckers prior to initiation plays a major role in further multiplication potential and in production of disease free plantlets. Therefore, the source plants for TC must completely be free from viruses. Virus indexing technology and subsequent elimination of the virus using an appropriate technique is an essential component of virus –free seedling production (FAOSTAT, 2006). To produce healthy planting material, national TC laboratories are encouraged to micropropagate virus-tested germplasm for distribution.

1.5 Research Objectives

1.5.1 General objective

To evaluate virus elimination *in vitro* protocols, for production of *Banana Streak Virus*-free TC banana planting materials for farmers

1.5.2 Specific objectives

i) To determine incidence of *Banana streak virus* in mother orchards supplying the JKUAT commercial tissue culture laboratory with initiation materials

ii) To evaluate *in vitro* techniques for the elimination of *Banana streak virus* from infected banana

ii) To conduct a cost – margin analysis for cleaning and the production of BSV - free *in vitro* plantlets

1.6 Hypotheses

i. Banana plantlets used at JKUAT TC laboratory are not infected with *Banana streak virus*

ii. Elimination of *Banana streak virus* through a well developed *in vitro* technique is not possible

iii. BSV–free plantlets cost the same as TC plantlets

1.7 Expected outputs

i) The incidence of *Banana streak virus* in orchards supplying JKUAT commercial TC laboratory with initiation materials will be known

ii) A protocol for cleaning banana plantlets from *BSV* will be developed

iii) Cost- margin analysis will be performed

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and botany of banana

Banana belongs to *Plantae* kingdom, *Musaceae* family and *Musa* genus. *Musa* has two major species namely *Musa acuminata* and *Musa balbisiana* (IITA, 1998). Edible *Musa spp.* originated in South - Eastern Asia, from Eastern India and South to Northern Australia. Banana is likely to have been first domesticated in Papua New Guinea. Early Filipinos probably spread the banana eastward to the Pacific Islands and Hawaii. Banana is known to have arrived in East Africa around 500 A.D. Today, banana is the largest cultivated fruit throughout the world (FAOSTAT, 2010; FAO, 2009).

Banana and plantains are mostly sterile triploid hybrids between the species *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). All cultivated commercial bananas are triploid, 2n=3x=33 with genome constitution of AAA (mainly the sweet dessert banana), AAB, ABB (mainly but not exclusively starchy plantains eaten after cooking). There are also seedless cultivated AA and AB diploids, Tetraploid (2n=4x=44) with genome constitution of AAAA, AAAB, AABB, ABBB and sterile, except a few parthenocarpic AA and AB diploids (ISAAA, 1999) and most banana cultivars are hybrids of these species. Banana cultivars vary greatly in plant and fruit size, plant morphology, fruit quality, and disease and insect resistance. Most bananas have a sweet flavor when ripe; exceptions to this are cooking bananas and plantains. Plantains are hybrid bananas in which the male flowering axis is either degenerated,

lacking, or possess only relicts of male flowers. Plantains are always cooked before consumption and are higher in starch than bananas. The two groups of plantains, French and Horn, produce fewer fruit per plant than sweet bananas. The groups differ in whether the male parts of the inflorescence are persistent or absent.

Banana and plantain are large, herbaceous monocots, reaching 25 ft in some cultivars, but generally 6-15 ft tall in height. It has a pseudostem, with a cylindrical aggregation of leaf stalk bases with leaves of up to 9 ft long and 2 ft wide, which tear along the veins in windy conditions, giving a feathered or tattered look. The perennial portion of the plant the rhizome (corm), produces suckers, which are thinned to three or four per plant - one "parent" sucker for fruiting and one "follower" to take the place of the parent after it fruits (FAOSTAT, 2005). It has roots, which serves as a storage organ for the plant with maturity time ranging from 9 to 12 months (IITA, 1998).

2.2 Banana production

Banana is the second largest food-fruit crop of the world produced in the tropical and subtropical regions of mostly the developing countries (Singh *et al*., 2011). The crop is a staple food for 400 million people in the tropics, a major staple food and a source of income for over 20 million people in Eastern Africa. Total world production is estimated at around 97 million tonnes, of which approximately one third is produced in subsistence farms in Africa (FAOSTAT, 2011). Banana is grown in 150 countries worldwide (Table 1) on 11 million acres. Worldwide, the average yield is about 14,000 kg acres- (FAOSTAT, 2011; Singh *et al*., 2011).

Table 1: Top banana producing nations (million metric tons) in 2009

Country	Production in million metric tons	Country	Production in million metric tons
India	21.77	Guatemala	1.57
China	8.04	Vietnam	1.36
Philippines	7.48	Kenya	1.19
Brazil	7.10	Bangladesh	1.00
Ecuador	6.00	Honduras	0.91
Indonesia	5.46	Egypt	0.88
Tanzania	3.50	Papua new guinea	0.87
Costa Rica	2.08	Cameroon	0.86
Thailand	2.00	Uganda	0.62
Mexico	1.96	Burundi	0.60

***Source**: Food and Agriculture Organization of the United Nations, (2009).*

In Kenya, banana crop is predominantly grown by peasant farmers for home consumption and for the national market. Apart from being the most popular fruit in the country, the cooking banana varieties also serve as an important staple food. The crop covers 1.7% of Kenya's total arable land (FAO, 2009).

The average banana yield in Kenya is 5.7 tons per acre (14 tons per hectare). Banana achieves about one third of the crops potential under favourable conditions of the humid tropics. Apart from the low input levels; the oppressive infestation of banana with various pests and diseases is the main determinant of this yield gap (INIBAP, 2000). Most pathogens are spread through infected banana suckers being used by farmers for plant propagation, due to lack of clean planting material, and also because of the farmers limited knowledge.

The commercial cultivation of banana in Kenya involves a large number of small-scale growers (IITA, 1998). Banana is grown in various agro ecological zones of the country, from the coast up to an altitude of about 1500 meters above sea level in the western highlands. Cultivation takes place predominantly under rain fed conditions in areas that receives an annual precipitation of 1000 millimeters (FAO, 2009). In Kenya, banana is grown in fourteen counties namely Mt Elgon, Bungoma Kakamega, Kisii, Nakuru, Maragua, Nyeri, Kiambu and Kirinyaga, Embu and Meru, Taita Taveta, Kilifi and Kwale (Wangai *et al*., 2002;).

2.3 Banana varieties

In Kenya, a wide range of banana varieties is grown. The first obvious separation between varieties can be made between the ripening (dessert) banana, on one hand, and the cooking types on the other. Some of the varieties such as FHIA are also used for both purposes (FAO, 2009).

According to FAO (2009), about half of the Kenyan banana production is of the ripening type, whereas the other half is made up of cultivars mostly used for cooking. Among the ripening varieties, there are some which have been used for a long time in Kenya, the most prominent of which are gros Michel (AAA), locally known as Kampala, apple banana (AB) and many others. Other ripening types have been introduced more recently, especially those of the Cavendish subgroup (AAA), such as Dwarf Cavendish, Lacatan, Valery, Grand Nain, Williams hybrid, Paz, and others, generally with a fairly good acceptance among banana producers and consumers (ISAAA, 1999).

Many of the cooking varieties used in Kenya belong to the subgroup of East African highlands banana (EAH-AAA). Though not all of them have been genetically identified, although in cytological analysis, they turn out to be very similar. Local names of popular cooking varieties include matoke, kiganda, mutahato, bokoboko among others. A commonly used local double purpose cultivar is muraru. KARI (Kenya Agricultural Research Institute) estimates that around thirty five different banana varieties are commonly used in Kenya (ISAAA, 1999).

2.4 Utilization of banana

Banana is produced for different purposes in Kenya. Apart from banana being utilized as a boiled fruit or as dessert, there are various methods of processing such as banana chips, banana puree (utilized as baby food), banana flour and powder, banana juice or even banana alcohol. Banana products and by – products have other uses and applications such as in making baskets, carpets, banana paper, animal feed, wrapping food, in intercropping, environment protection against soil erosion, for tourist attraction, in textile industry and as medicine in treating gastric ulcers, diarrhea, aid to digestion, stress, anxiety, prevention and cancer and heart disease. It is a good source of energy and micronutrients, prevents bone breakdown (Table 2), diabetes, and decreases the risk of age-related muscular degeneration (FAOSTAT, 2011). The dessert- type varieties constitute the most popular fruit among urban and rural consumers, and the cooking type varieties are an important staple food, particularly for

rural households. The processing of banana for banana beer, which is quite common in other countries of Eastern Africa, is rare in Kenya.

The crop is mostly cultivated on comparatively small farms with an average banana holding of 0.3 hectares. Although it is still a semi-subsistence crop, commercialization has expanded in recent years. Reasons for this are the higher market demand due to the rising degree of urbanization as well as diminishing farm incomes from the more traditional cash crops and export crops notably coffee.

Table 2: Nutritional value per 100g energy in one banana

Element	Amount (mg)	Element	Amount (mg)
Carbohydrate	22.84	Vitamin B 6	0.367
Sugars	12.23	Folate (Vit B 9)	2.0
Dietary fibre	2.6	Vitamin C	8.7
Fat	0.33	Calcium	5.0
Protein	1.09	Iron	0.26
Vitamin A	3.0	Magnesium	27.0
Thiamine (vitB1)	0.031	Phosphorous	22.0
Niacin (vitB3)	0.665	Potassium	358.0
Pantothenic acid	0.334	Zinc	0.15

Source: (FAOSTAT, 2011).

2.5 Banana propagation

Banana is a crop with dual propagation abilities, sexual through seeds and asexual through suckers. Seed propagation is common in wild species which are diploid and undergo normal meiosis, fertilization and seed set.

The extent of seed set, germ inability and dormancy depends on the species. In *Ensete*, the only other genus of *Musaceae*, seed propagation is the only means of perpetuation since sucker production is absent. All cultivated commercial bananas are triploid and sterile, except a few parthenocarpic AA and AB diploids. Sucker propagation is the only natural means of their perpetuation; artificial methods of propagation include macropropagation and micropropagation (INIBAP, 2000).

2.5.1 Macropropagation

Macropropagation is an excellent option for propagation of banana explants. This is a simple method because of the ease of multiplication, saves cost of producing planting material and has the potential of producing 50-60 shoots per sucker in 4-5 months. Macropropagation is achieved by two methods and could be adopted either in the field conditions (*in situ*) or in the nursery (*ex situ*). It involves decapitation, decortication and hardening (INIBAP, 2000).

The production of suckers using conventional means is highly season dependent and, hence, availability of planting material in a given season is often a limiting factor. The planting season in most of the banana producing areas starts with the onset of rains which creates a heavy demand for the planting material often leading to supply of substandard material (Thomas *et al.*, 2000).

2.5.2 Micropropagation

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants under aseptic conditions using modern plant tissue

culture methods (INIBAP, 2000). The rate of multiplication in banana is restricted to 5-20 suckers per plant during its growth period, which makes it difficult to obtain sufficient amount of planting material of a clone of choice. Micropropagation facilitates production of large number of plantlets/unit time, thus helping in rapid introduction and dissemination of new varieties. The rapid multiplication technology ensures that limited numbers of mother plants are required for raising large number of progeny plants. These few mother plants can be maintained with required care at a limited cost. Being a vegetative reproduction method, micropropagation results in a high degree of genotypic and phenotypic uniformity of the progeny explants (Thomas *et al.,* 2000). The limited variation seen sometimes can be overcome by following appropriate micropropagation, genetic fidelity testing and introduction of protocols. Using micropropagation, the production of planting material can be achieved as per needs. Micropropagated plants exhibit uniform growth and maturity enabling one time harvesting. Using TC, it is possible to develop planting material which is free from sucker borne diseases and pests. Use of healthy planting material complemented with integrated pest management program is the key to a good crop stand in field (INIBAP, 2000).

Production of plants in test tubes facilitates safe movement and easy handling of germplasm between laboratories within and across countries. Since the micropropagation based progeny is genotypically and phenotypically similar to the mother plant, which is often a superior selection, the yield and returns are expectedly higher (Thomas *et al.,* 2000).

2.6 Viral Diseases of Banana

Banana is affected by six known, relatively well-characterized viruses (Diekmann and Putter, 1996). These are *Banana bunchy top virus (*BBTV) genus *Nanavirus*, *Banana streak virus* (BSV) genus *Badnaviruses*; *Cucumber mosaic virus* (CMV) genus *Cucumovirus*; *Banana bract mosaic virus* (BBrMV) genus *Potyvirus*, *Banana mild mosaic virus* (BanMMV) and *Banana dieback virus (*BDV) (Lockhart, 1995; Thomas *et al*., 2000; Gambley and Thomas, 2001).

2.6.1 Banana Streak Disease (BSD)

Viral leaf streak of banana, caused by *Banana streak virus* (BSV), is a recently-described disease which occurs in most banana-producing countries. The disease, which is known to affect only Musa and Ensete, is spread by vegetative propagation, mealy bugs and through seed. Viral leaf streak of banana was first recognized as a distinct syndrome in Cote d Ivore in 1978, but was initially considered to be caused by a strain of CMV. The true nature of the causal agent was first established in 1986. Since then, *Banana streak virus* has been shown to occur worldwide, infecting a wide range of *Musa* genotypes, including AA, AAA, AB, AAB, ABB and BB but the genotypes with AB combinations are more prone to BSV than the AA genotypes since the endogenous activatable BSVs have only been detected in the B-genome of various banana accessions (Singh *et al*., 2011).

The disease symptoms varies and are thought to depend on a variety of factors such as virus isolate, host genotype, level of management and environmental conditions (Lockhart, 1986; Lockhart and Jones, 2000; Dahal *et al*., 1998*a*).

Banana streak virus is a member of the family *Caulimoviridae,* genus badnavirus 7.5 kbp encapsidated in bacilliform particles approximately 120 X 30 nm (Van Regenmortel *et al*., 2000). BSV is the most widely spread virus of *Musa*. This virus is a major constraint, not only to *Musa* production worldwide, but also to the international movement of germplasm. Recent molecular studies on BSV have indicated that this virus has several unique features not found in most other plant viruses.

Strains of the virus differ in symptoms produced, and are serologically and genomically heterogeneous. This has led to problems in developing reliable indexing methods for virus identification and disease control. Integration of segments of viral genome into *Musa* nuclear DNA may limit the usefulness of PCR amplification for BSV detection in *Musa* (Daniells *et al*., 2001). The current thinking is that there are three forms of BSV (an encapsidated episomal form, an unencapsidated episomal form and an integrated form).

Encapsidated episomal form is the conventional form of the virus with the DNA viral genome encapsidated in the viral coat protein. This form is found in all cases of BSV symptom expression and sometimes in asymptomatic plants. The genome of an strains of BSV has recently been sequenced confirming that it is a badnavirus, with the

genome organization characteristic for the mealy bug-transmitted genus of this subfamily (Anthony *et al*., 2010).

Unencapsidated episomal form is one of the features of BSV infections in the periodic appearance and disappearance of symptoms. In this symptom suppression phenomenon, BSV resembles the behaviour of another pararetrovirus, cauliflower mosaic virus (CaMV), in certain hosts (Anthony *et al*., 2010).

Recent developments of the BSV problem led to the conclusion that there are integrated forms of BSV which, in some cultivars, could be activated by certain stresses to give episomal infections. There are several lines of evidence confirming that there are in fact BSV-like sequences integrated into the *Musa* genome (Anthony *et al*., 2010). Southern blots of *Musa* chromosomal DNA probed with BSV sequences show hybridization with molecules of much higher molecular weight than that of the BSV genome (Anthony *et al*., 2010).

2.6.2 Economic importance of banana viruses

In Uganda, a serious outbreak of the *Banana streak virus* was reported in 1996, with some plantations containing 100% infected plants (Tushmereirwe *et al*., 1996). The CMV is currently considered an emerging threat, especially where cucurbitaceous vegetables are cultivated as intercrops in banana plantations causing 45-65% losses (Estelitta *et al.,* 1996). Yield losses of up to 40% have been recorded in Philippines on cultivar Cardaba and cultivar Lacatan (Magnaye, 1994) in plants infected by BBrMV. BBTV caused devastating epidemics, i.e. in 1992, the disease was widespread in

Pakistan with disease incidences up to 100%, and that about half of the plantations had been destroyed (Soomro *et al*., 1992; Khalid *et al*., 1993). BSV and CMV are important limiting factors to banana production in Kenya (Wangai *et al*., 2002). East Africa is currently free of several key viral diseases such as BBrMV but BBTV has recently been identified in Rwanda posing a significant threat to neighbouring countries. Of particular concern is the spread of infected planting material between farmers, who have a tradition of exchanging banana suckers for propagation. But, with the rise in multiplication of TC bananas, this too has the potential to increase the distribution of potentially virus-infected plantlets (Wangai *et al*., 2002).

2.6.3 Detection and diagnosis of BSV

Detection of BSV is problematic due to the serological and genomic heterogeneity of virus strains (Lockhart and Olszewski, 1993), the erratic appearance of symptoms (Dahal *et al.,* 1998) and the uneven distribution of the virus in plants.

Several detection methods been used to confirm the presence of BSV in banana germplasm. Virus detection methods include: - observation of symptoms, examination of tissues by electron microscope (TAS- ELISA, Electron microscopy, and ISEM), the use of indicator plants, serology and nucleic acid hybridization (IC-PCR, and PCR) and recently the use of rolling circle technique like templiphi (Anthony *et al*., 2010). Each method has certain advantages and disadvantages. To establish a program for producing virus–free banana plantlets, the viruses presenting a country

have to be known so that a suitable method of virus detection can be selected (Diekmann and Putter, 1995).

2.6.4 Virus elimination from infected plants

There are various virus elimination methods in use today for eliminating virus from plant samples namely chemotherapy, thermotherapy cryotherapy, meristematic tip culture and electrotherapy.

Chemotherapy is the use chemicals for virus eradication (Kartha, 1986). Success has been reported utilizing chemicals to cure plants of pathogenic viruses. Ribavirin and salicylic acid are potent viricides that have been reported to eliminate major viral pathogens from potato and other crops (Qiaochunand *et al*., 2009). In most instances, chemotherapy is more effective if incorporated into TC medium that allows for the plant to uptake the chemical uniformly through the root system (Anthony *et al*., 2010).

Thermotherapy is the use of high temperatures to eliminate viruses from plants. It is the most commonly employed method of elimination of viruses from infected plants in combination with meristematic tip culture (Anthony *et al*., 2010; Qiaochunand *et al.*, 2009). The success of thermotherapy depends on the target virus's inability to multiply and move readily within plants being exposed to air temperatures of 35ºC to 40ºC. Successful treatment times may vary from weeks to months depending on the plant cultivars and specific virus (Qiaochunand *et al*., 2009). Thermotherapy was very effective in reducing virus titer in geraniums, though they are not heat tolerant, and will die after 5-6 weeks at 38ºC (Qiaochunand *et al.,* 2009). Gradual acclimatization

period allows the plant to adjust to the higher temperatures. Plants can then be maintained under these conditions for as long as possible (3-4 weeks), after which time meristematic tips are removed for tissue culture (Qiaochunand *et al*., 2009).

Meristem tip culture technique involves the use of apical dome or shoot tip with a few leaf primordia of the size less than 1 mm in length as the explant. The application of meristematic-tip culture to eradicate viruses was initially based on the concept of meristems "immunity" towards viruses (Morel, 1948). Different researchers have shown that the probability of obtaining virus-free plants is inversely related to the size of the meristematic tissue (Faccioli and Marani, 1998).

Cryotherapy of shoot tips based on cryopreservation techniques is a new method for pathogen eradication. Cryopreservation refers to the storage of biological samples at ultra-low temperature in liquid nitrogen (−196°C), and is considered an ideal means for long-term storage of plant germplasm. It eliminates plant pathogens such as viruses, phytoplasmas and bacteria by briefly treating shoot tips in liquid nitrogen using cryopreservation protocols. Healthy plants are regenerated from the surviving pathogen-free meristematic tissue. The method facilitates treatment of large numbers of samples and is independent on shoot tip size (Qiaochunand *et al.*, 2009; Brisn *et al*., 1997; Helliot *et al*., 2002, Wang *et al*., 2003).

Electrotherapy is the use of electrical pulses to eliminate viruses from plant tissue and has recently received much attention. Lozoya-Saldana *et al.* (1996) reported on the elimination of PVX from different clones of *Potato virus X* from different clones of

potato (Faccioli *et al*., 1996). For banana, (Cv. W. Bungulan (AAA), Hernandez *et al.* **(**1997) and Qiaochunand *et al.* (2009) reported BSV elimination in approximately 40-80% of regenerated plants.

2.7 Diversity of *Banana streak virus* strains

Five BSV isolates have been cloned and sequenced part of the genomes. They have been designated as BSV-RD, BSV-Cav, BSV-Mys, and BSV-GF, BSV-Onne. These isolates originated from banana cultivars Red Dacca, Williams, Mysore, and Gold finger and from Nigeria respectively. All clones contained a sequence covering part of open reading frame III and the intergenic region of the badnavirus genome. The BSV-RD sequence was virtually identical to that of BSV-Onne, differing by only two nucleotides over 1,292 bp. However, BSV-Cav, -Mys, and -GF were divergent in nucleotide sequence. Phylogenetic analyses using conserved sequences in the ribonuclease H domain revealed that all BSV isolates were more closely related to each other than to any other badnavirus. BSV-Cav was most closely related to BSV-Onne, and there was 95.1% identity between the two amino acid sequences. Other relationships between the BSV isolates were less similar, with sequence identities ranging from 66.4 to 78.2%, which is a magnitude comparable to the distance between some of the recognized badnavirus species. Immunocapture-polymerase chain reaction assays have been developed, allowing specific detection and differentiation of the four isolates of BSV (Karanja *et al*., 2008; Harper, 1999).

2.8 Cost margin analysis of cleaning and the production of virus-free *in vitro* plantlets

Banana is globally ranked fourth, next to rice, wheat and maize in terms of gross value of production. It is a major staple food crop for millions of people as well as provides income through local and international trade (Singh *et al.,* 2011).

Several studies have been carried out to estimate the costs and returns to identify the factors influencing the cost of production, and to assess the resource use efficiency of TC banana so as to identify the factors determining the adoption of TC banana and the risks involved (Anthony *et al*., 2010)

Results of economic analysis in India indicated that the cost of production per bunch was higher in TC bananas (0.84 US Dollar) than in sucker raised plants (0.65 US Dollar) (Table 3). This is mainly due to the high cost of TC bananas, plant protection chemicals and labour. However, the gross and net income from TC bananas is higher than those from conventional planting material which indicate the economic advantage of TC bananas. This has been attributed to the efficient utilization of resources by TC bananas (Sighn *et al*., 2011). Profit analysis revealed that gross income and bunch weight have a positive and significant influence on the adoption of TC bananas.

Table 3: Comparative income from TC and sucker –propagated banana

	TC produced plantlet (US Dollar)	Sucker produced plantlet (US Dollar)
	2663	2416
Mean price received (bunch)	1.90	1.52
Gross income / ha	5,066.00	3,743.00
Total expenses	2,820.80	2,165.90
Net income /ha	2,245.20	1,577.10
Cost of production/ bunch	1.05	0.87
Net income / bunch	0.84	0.65

Source: Singh *et al*. (2011).

However, TC procedures do not eliminate *Banana streak virus* posing a big danger because most farmers are relying on TC bananas which for a long time are believed to be disease free. Therefore, three different methods of BSV elimination were used to eliminate the virus and later the cost margins analysis was done for production of one virus-free plantlet. After production of BSV-free plantlet, the cost of production of one BSV plantlet was compared with the cost of production of tissue-cultured explant (Singh *et al*., 2011).

CHAPTER THREE

3.0 INCIDENCE OF BANANA STREAK VIRUS IN MOTHER ORCHARDS SUPPLYING EXPLANTS TO THE JKUAT TISSUE CULTURE LABORATORY

Abstract

The banana industry in Kenya is threatened by the presence of *Banana streak virus* (BSV)**.** The JKUAT commercial banana laboratory uses tissue culture (TC) technique for mass propagation of plantlets which are free from most disease causing organisms for commercial purposes. However, the TC technique does not eliminate viruses. It is therefore key to index all initiation materials used for mass propagation. Tissue culture initiation materials from two KARI and JKUAT orchards and JKUAT greenhouse were sampled using sterile surgical blades, packed in well labeled packing bags and put in a cool box for transportation to JKUAT molecular biology laboratory for indexing using the polymerase chain reaction (PCR) technique. Out of the samples collected, 50% from each orchard showed BSV symptoms. However, when PCR was performed, BSV was detected in all the orchards studied at 40%, 80%, 90%, and infection levels from KARI Thika, JKUAT and KARI Kisii orchards respectively. These results indicate infection of TC initiation material with BSV. Hence this calls the development for BSV elimination procedures from banana plants. An *in vitro* method for eliminating this virus is recommended and also BSV-free mother orchards should be established to increase banana yields in Kenya.

3.1 Introduction

In Kenya, the banana crop is grown for subsistence consumption and for the national market (FAO, 2007). It is also perceived as a major avenue to alleviate food insecurity in the region (ISAAA, 1999). Despite this, optimum banana yields are lowered by *banana streak disease* (BSD) caused by *Banana streak virus* (BSV). Recent studies in Kenya have shown that BSV is prevalent in all the banana growing regions and that all popular cultivars grown by farmers are susceptible (Karanja *et al*., 2008).

Banana streak virus-infected *Musa* plants frequently express broken or continuous chlorotic or necrotic streaks on the leaves, stunting of diseased plants and occasionally heart-rot of the pseudostem and plant death. However, the disease symptoms vary and are thought to depend upon a variety of factors such as virus strain, host genotype, level of management and environmental conditions (Lockhart, 1986; Dahal *et al*., 1998*a*; Lockhart and Jones, 2000;). *Banana streak virus* causes yield loss between 6 to 15% (Dahal *et al*., 2000; Daniells *et al*., 2001).

Diagnostic methods for BSV are inadequate because of the considerable genetic and serological diversity amongst BSV strains. Furthermore the presence of integrated BSV sequences in some banana cultivars leads to false positives (Wangai *et al*., 2002)**.** Therefore, a good procedure has to be used to eliminate these false positives for

instance the use of a templiphi kit or use of PCR using specific primers for different virus strains (Wangai *et al.*, 2002)**.**

The JKUAT banana TC laboratory is one of the biggest TC laboratories in Kenya, producing over two million plantlets per year (Kahangi *et al.,* 2006). The propagation method in use at the JKUAT commercial laboratory is micropropagation through TC for the mass propagation of suckers. This method ensures production of sufficient planting materials which are free from most disease causing organisms but are not free from banana viruses. The JKUAT TC laboratory supplies planting materials all over the country and the East African region including Ethiopia, Southern Sudan and Somalia (Kahangi *et al.,* 2006).

Approximately up to 100, 100, and 400 pieces of initiation materials from the KARI Thika, KARI Kisii and JKUAT orchards respectively are supplied to the TC laboratory every month. It is therefore paramount to index for the presence of this injurious virus in initiation materials before using them for massive TC plantlets production. This work was done to determine the incidence of BSV in mother orchards supplying initiation materials for TC work.

3.2 Materials and Methods

3.2.1 Study design and sampling

A survey on three main orchards supplying suckers for initiation to the JKUAT commercial banana TC laboratory (KARI Thika, KARI Kisii and JKUAT orchards), and the JKUAT greenhouse was done to determine the BSV incidence (Table 4).

Table 4: Sampling sites and their altitude in meters, rainfall (mm), number of varieties and number of plants in the specific orchards

Orchard	Altitude (M)	Rainfall (mm) per annum	No. of varieties	No of plants *
JKUAT	1,525	200-2,000	10	1,100
KARI KISII	1,500	200-4,000	35	2,100
KARI THIKA	1,500	200-2,000	20	1,800
JKUAT green-house	1,525		10	20,000

Source: (FAO, 2009) * approximate

3.2.2 Sampling

3.2.2.1 Leaf Sampling in the orchards

The three varieties sampled were Nusu Ng'ombe, Fhia18 and Kampala from KARI Kisii, KARI Thika and JKUAT orchard respectively. Ten samples (five symptomatic and asymptomatic) were randomly selected with three replicates per sample were collected. From each plant, sterile surgical blades were used to cut three leaf samples, which were packed in well labeled packing bags and put in a cool box for transportation to JKUAT molecular biology laboratory where they were used for detection of BSV by PCR. These three varieties were also sampled from the JKUAT greenhouse in order to assess the BSV infection levels in supply.

3.2.2.2 BSV symptom description

Mild symptoms were characterized by chlorotic and necrotic streaks on the lamina tissue in some leaves and severe symptoms were chlorotic, necrotic streaks on the tissue, distorted bunch, splitting of the pseudostem, heart rot and plant death (Singh *et al*., 2011).

3.2.2.3 Determination of BSV incidence

The incidence of BSV in collected samples (both visual and molecular methods of viral detection) from the three orchards was calculated as follows in every orchard.

$$\text{Incidence of BSV} = \frac{\text{Number of BSV- Infected samples}}{\text{Number of samples collected}} \times 100$$

3.2.3 Nucleic acid extraction from banana leaf samples

Total nucleic acids extraction from the leaf samples was done using the CTAB method as described by Doyle and Doyle (1990). Leaf samples weighing 0.4 g were ground in 3 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 1.4 M NaCl, 80 mM Na_2SO_3, 2% PVP 10 and 2% CTAB) using a mortar and pestle. Samples were incubated at 65°C for 15 min in a water bath, and then centrifuged for 5 min at 13,000 rpm. The supernatant was subsequently mixed with an equal volume of chloroform: isoamyl alcohol (24:1) and the mixture centrifuged at 13,000 rpm for 5 minutes. The chloroform: isoamyl alcohol step was repeated and the supernatant was mixed with an equal volume of cold isopropanol and incubated at room temperature for 5 minutes. Nucleic acids were pelleted by centrifugation for 5min at 13000 rpm as described

above and the pellets washed with 70% ethanol, air-dried and resuspended in 50 µL of sterile distilled water. (Appendix 1)

3.2.4 Quantification of nucleic acid and gel electrophoresis

Nucleic acid quantification was done by observing the bands on 0.8% agarose gel (0.8 g agarose gel dissolved in 100ml of Tris boric EDTA (1 X TBE)) to confirm presence and quality of nucleic acid. The purity and nucleic acid concentration was determined by measurement of the absorbance at 260 and 280 nm in a spectrophotometer.

3.2.5 Polymerase chain reaction

Five specific primer pairs (Table 5) for detecting each BSV strain were used for detecting the presence of the various BSV strain in the banana leaves. PCR mixture (20 µL) containing 10 µl 2x GoTaq Green Master Mix (Promega Corp, Madison, WI), 5 ρmol of each primer (Table 4), 1 µl of nucleic acid extract and water to final volume. PCR cycling conditions included an initial denaturation of 94°C for 2 min followed by 35 cycles at 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 2 min. Reaction products were analyzed by agarose gel electrophoresis and amplicons visualized in 1.5% agarose gel (Appendix 2)

Table 5: Primer pairs used in the amplification of various BSV strains

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

Adopted from Geering *et al. (*2000) and Harper *et al.* (1999a, 2002)
BSV-RD - BSV Red Dacca, BSV- Cav- BSV Cavendish, BSV- GfV- BSV Gold Finger, BSV- BSOEV- BSV – Mysore, Badna - degenerate primer
[1]Represents the name of the virus strain from which primers was designed
[2]Represents the gene bank number of the sequence used for primer design

3.3 Results

3.3.1 Incidence of BSV by visual observation and in PCR

During sampling, symptoms of broken and continuous chlorotic, necrotic streaks on the leaves and stunting of diseased plants were observed in all the orchards, with an incidence of 50%. Of all the study sites, samples from KARI Kisii and JKUAT orchards had the most severe symptoms; samples from KARI Thika orchard did not show distinct symptoms, while those from JKUAT greenhouse had no observable symptoms (Plate 1; Table 6).

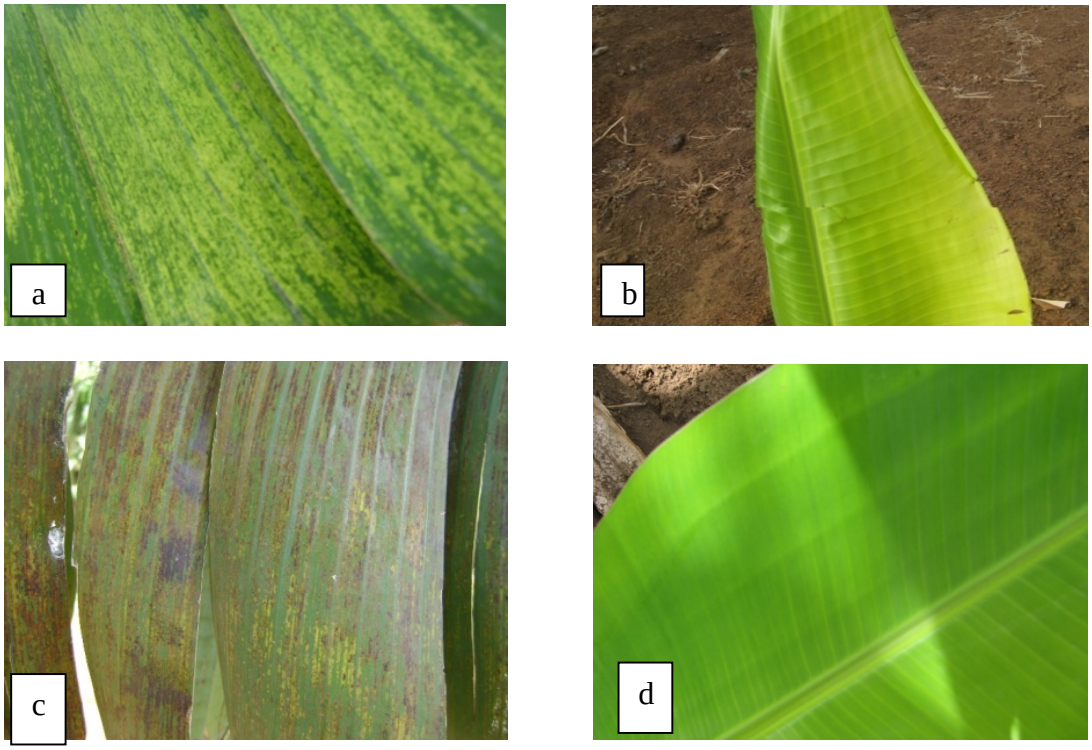

Plate 1: Severe streaks due to BSV infection in Kampala variety (a); yellowing leaves and folding of leaf edges due to mild BSV infection in Fhia 18 variety, (b); severe yellow and black streaks due to BSV infection in Nusu Ng'ombe variety (c); and a healthy banana leaf (d).

Table 6: Incidence (%) of BSV in samples collected by visual observation and PCR detection methods

Orchard	Variety	Visual BSV incidence (%)	BSV incidence by PCR (%)
JKUAT	Kampala	50	80
KARI THIKA	Fhia 18	50	50
KARI KISII	Nusu ngombe	50	90
JKUAT greenhouse	Nusu Ng'ombe	0	70
	Kampala	0	40
	Fhia 18	0	30

From each orchard the variety normally collected for initiation was sampled

In all the three varieties there was 50% BSV incidence through visual detection while 50, 80 and 90% BSV incidence was detected in Fhia 18, Kampala and Nusu ngombe varieties respectively using PCR (Appendix 3). Discontinuous yellow streaks and yellow dots with green and yellow patches were observed on upper and lower sides of the leaf samples in five samples (Plate 1). Five Samples did not show any symptoms. Severe BSV symptoms were observed in three samples. However, when PCR was performed, BSV strains could not be detected in two, five and one out of ten samples from Kampala, Fhia 18 and Nusu Ng'ombe varieties, respectively (Table 7). Dual BSV strain infection was detected in three samples (Table 7).

At the JKUAT greenhouse**,** three varieties, Kampala, Nusu Ng'ombe and Fhia 18 (Table 7) which had their origin from the three mother orchards, were sampled. Unlike in the mother orchards, there were no symptoms observed in green house samples

(Plate 1d). When the samples were tested via PCR, there were 40, 70 and 30% BSV infections recorded from Kampala, Nusu Ngombe and Fhia 18 varieties respectively (Table 7).

Table 7: Number of *Banana streak virus* strains detected in samples from three orchards and JKUAT greenhouse using specific primers for *BSV* strains

	No. of BSV infections per variety							
Variety	Mys	Badna	GF	Cav	RD	Dual infection		Infection (%)
Kamp	4	8	1	-	4	1		80%
N/NG	1	9	3	-	8	-		90%
Fhia 18	3	5	-	1	3	3		50%
Greenhouse Kamp	-	4	-	-	4	-		40%
N/NG	-	7	-	-	7	-		70%
Fhia 18	-	3	1	-	2	2		30%

Key: In Kampala variety, there were 4, 8, 1 and four amplicons from Mysore, Badna, Gold Finger and Red Dacca respectively. In Nusu Ng'ombe, there were 1, 9, 3 and 8 for Mysore, Badna, Gold Finger and Red Dacca respectively. While in Fhia 18, there were 3, 5, 1 and 3 amplicons for Mysore, Badna Cavendish and Red Dacca respectively.

Five specific primers (Table 5) were used in detecting the presence of different BSV strains in variety Kampala. Ten samples of Kampala variety were tested in a PCR and the PCR products visualized in a 1.5% Agarose gel (Plate 2). BSV-Mysore and Red Dacca specific primer detected the virus in four samples each while BSV- Gold Finger strain was detected only one sample. When the degenerate primer Badna was used, the

virus strain was detected in eight samples. Double infections of BSV strains were in three samples. Only one Sample had three BSV strains the same sample had severe BSV symptoms as shown in plate 1a. All the samples which showed symptoms in the field were confirmed to have the virus using PCR.

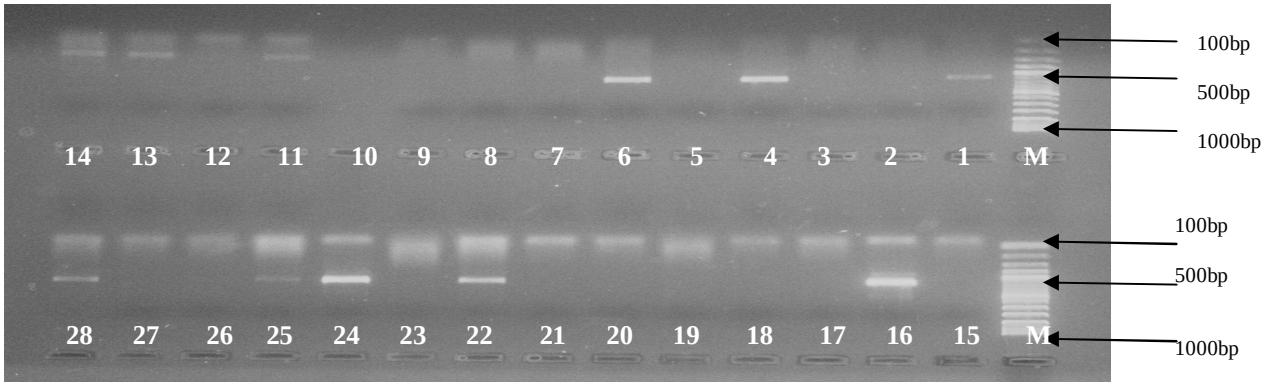

Plate 2: Detection of *Banana streak virus* (BSV) in banana variety Kampala samples. Four specific primers detected the virus in eight out of the ten samples tested, BSV Mysore (589bp), BSV –RD (522bp), BSV Gold Finger (476bp).
Key**:** 1-10 (BSV Mysore), 11-20 (BSV Red Dacca), 21-28 (BSV Gold Finger)

When PCR was performed on Fhia 18 samples, only Red Dacca and Gold Finger strains were detected. Out of the ten samples amplified, five had at least one BSV strain infection (Table 7). BSV-Red Dacca had a 522bp fragment amplified in four samples; BSV Gold Finger amplified a 476bp fragment in three samples. There were two samples which had shown symptoms in the field but when tested via PCR, they were not infected, while two other samples which had not shown any observable symptoms in the field were confirmed to have BSV strains through the PCR.

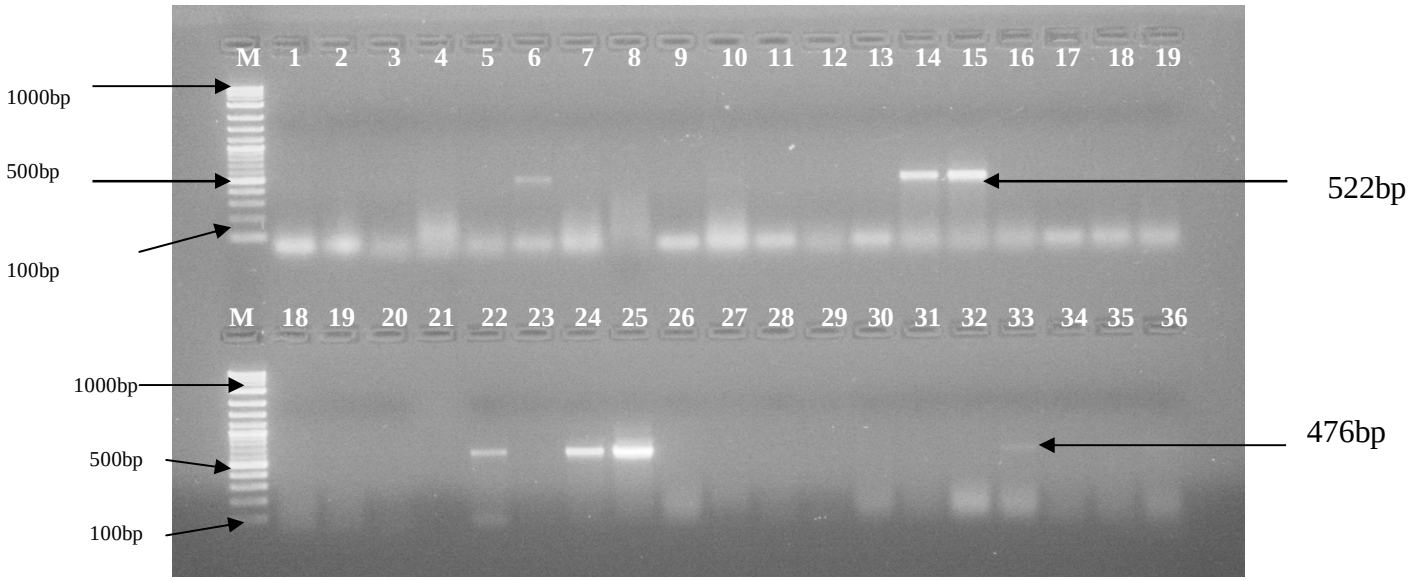

Plate 3. Detection of *Banana streak virus* (BSV) in banana variety Fhia 18**.** Four specific primers detected the virus in eight out of the ten samples tested, BSV Mysore (589bp), BSV –RD (522bp), BSV Gold Finger (476bp).

Only BSV-Red Dacca (522bp) and Gold finger (476bp) BSV specific primers detected the virus in Fhia 18 variety (AAAB). In Nusu Ng'ombe variety, out of the samples collected, 50% were showing BSV symptoms. The variety had the severest BSV symptoms observed, characterized by broken and continuous chlorotic or necrotic streaks on the upper and lower side of the leaves (Plate 1c).

In Nusu Ng'ombe variety, PCR analysis using the five BSV specific primers, nine out of the ten samples tested, had at least one or more BSV strains detected (Plate 4). Out of the five samples showing BSV symptoms, only one 56k (in well 6, 16 and 26) tested negative (Table 7), whereas all the symptomless samples were confirmed to have BSV. No BSV– Cavendish strain was detected in this variety (Table 7),whereas the most prevalent BSV strain from Nusu Ng'ombe variety was Red Dacca, which

was tested in seven out of ten samples tested. BSV- Gold Finger amplified in three samples (Table 7). Dual infections were detected in three samples whereas the other six had only one infection.

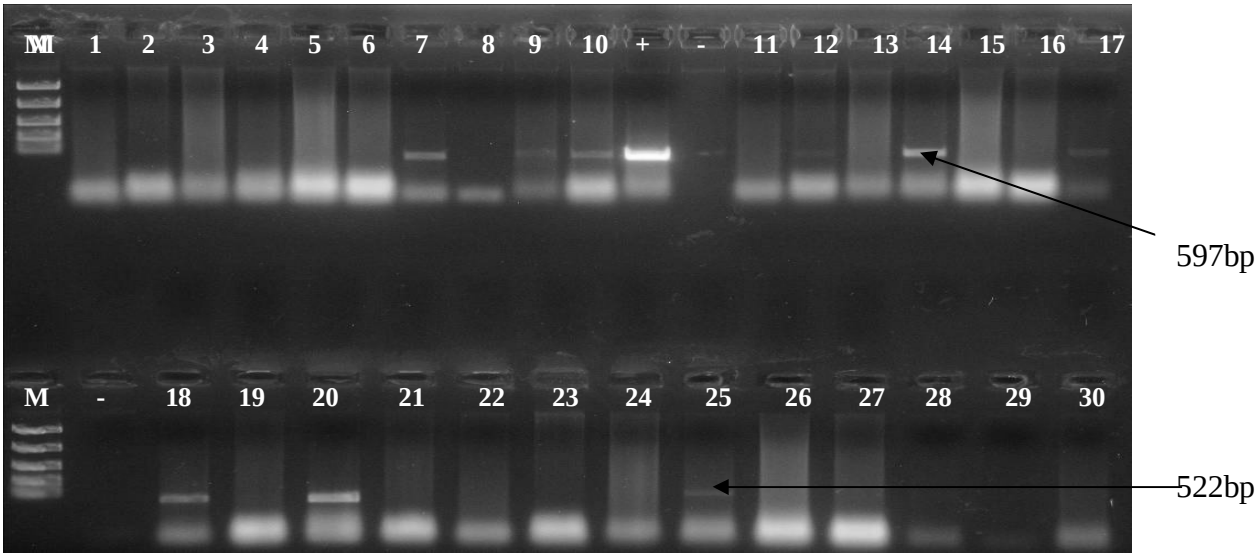

Plate 4. Detection of BSV in Nusu Ng'ombe variety using different primers. Samples 1-10 were amplified using specific primer BSV- GF (476bp), samples 11-20 were amplified using BSV-RD specific primer (522bp) and samples 21-30 were amplified using BSV- MYS (597bp)

BSV incidences in the greenhouse were lower than in the mother orchard with seven out of ten in Nusu Ng'ombe variety tested BSV-positive compared to the nine out of ten sampled from KARI Kisii. Kampala from the greenhouse had four out of ten infected compared to eight from JKUAT mother orchard and Fhia 18 had three out ten BSV infections compared to the four from KARI Thika mother orchards (Appendix 4).

To determine BSV levels in samples collected from the JKUAT greenhouse, the three varieties which had their origin from KARI Kisii, KARI Thika and JKUAT orchard respectively were sampled for indexing. These samples showed no symptoms (Table 6) at the time of sampling. However, PCR results showed that some of the samples were BSV infected (Plate 5). Out of the thirty samples tested, fourteen were BSV positive using PCR. The most prevalent strain in the greenhouse was Red Dacca. Nusu Ng'ombe variety had the highest BSV infection with all the samples except three testing positive for Red Dacca BSV strain in PCR. In Kampala, only four out ten samples tested were confirmed to have the BSV strain and like in Nusu Ng'ombe the most prevalent viral strain was Red Dacca (Table 6). In Fhia 18, only three out of ten tested positive, out of this one had the gold finger strain, while two tested positive to Red Dacca (Plate 5). Results from the greenhouse samples, showed no double infection unlike in the samples from the orchards.

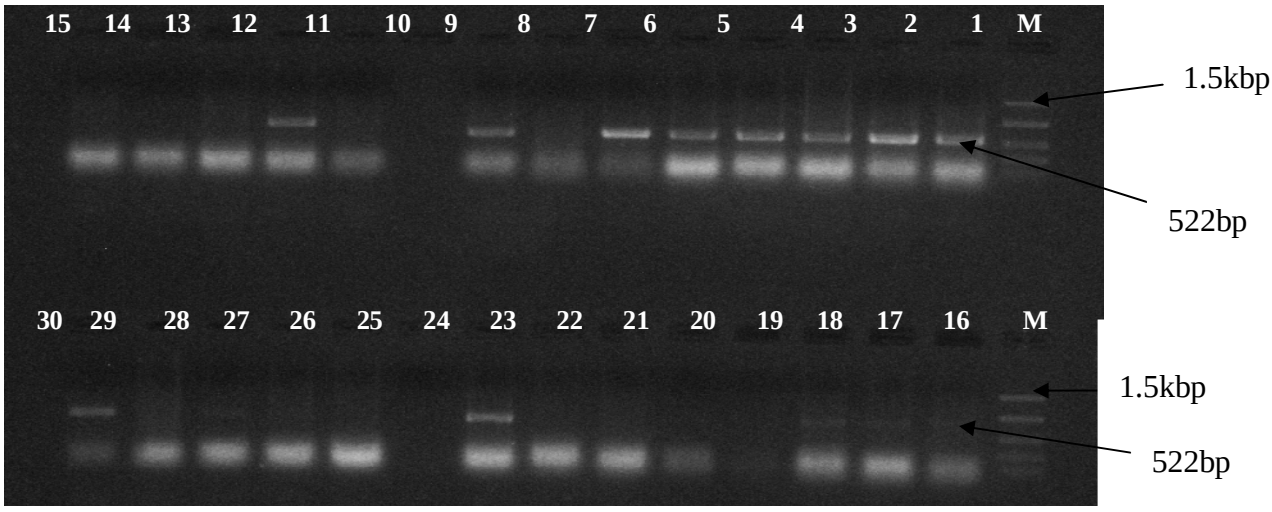

Plate 5**.** Lanes 1-10 shows samples from Nusu Ng'ombe variety from KARI Kisii orchard, 11-20 Kampala variety from JKUAT orchard, 21-30 Fhia 18 from KARI Thika orchard.

3.4 Discussion

The objective of this study was to determine incidence of BSV in initiation material in use at the JKUAT banana commercial laboratory. To achieve this, samples from three banana varieties from three main orchards supplying JKUAT with initiation material and from JKUAT greenhouse were indexed.

The different specific primers used to detect different strains of BSV amplified all the strains present except for BSV Cavendish. Results of this work showed that 50% of the samples collected were BSV infected, in the three mother orchards supplying the JKUAT commercial banana laboratory with initiation material. These results are in similar with previous studies carried out in Kenya which showed that BSV is prevalent in all banana growing regions and that all popular cultivars grown by farmers are susceptible (Wangai *et al*., 2002; Harper, 2008; Karanja, 2008). Detection of various BSV strains in this study agree with results of previous work done in Kenya and other regions, indicating that BSV has many strains (Geering *et al.*, 2000; Harper *et al.*, 2005; Karanja *et al*., 2008).

Results in this study indicate that fifteen samples and all greenhouse samples had one BSV strain while seven had two BSV strains which agree with earlier work by Karanja *et al*. (2008) that BSV has many strains and a sample can be infected by more than one strain. It was also noted that symptom expression was more severe in those cultivars with more than one BSV strains in a plant. These results confirmed some

work by Dahal *et al.* (2000) who noted that the more the number of BSV strain infecting a sample, the severe are the symptoms.

Though samples from JKUAT greenhouse did not show any observable symptoms, the virus was detected in all varieties used. BSV infection in Kenya was also reported in earlier studies done in Kenya (Lockhart and Olszewski, 1993; Harper *et al*., 2005) and that BSV can hide in the banana genome especially in integrated genome.

The contradictory results in the presence of symptoms and lack of PCR amplification in some samples using the specific primers could be attributed to other factors like presence of a different virus strain or other viruses that show similar symptoms as BSV in banana such as *Cucumber mosaic virus*. These results concur with observations by Harper *et al*. (2002). Visual inspection alone may not be sufficient method of BSV detection because there can be presence of BSV strains in asymptomatic plants (latent infection) as well as long BSV incubation periods before symptom expression (Lockhart and Jonnes, 2000).

Samples from the JKUAT greenhouse did not show any observable BSV symptoms, unlike in the mother orchards where the symptoms could be clearly traced. This is because the leaves were very young concluding that for BSV detections older leaves should be used in order to capture the virus faster. These results are in agreement with results of Harper *et al*. (2002). The fact that PCR could detect the virus in the some samples from the greenhouse indicates the need for more effective detection methods for indexing TC materials for distribution to the farmers.

Results in this study clearly show that the mother orchards supplying the JKUAT commercial banana TC laboratory are BSV infected and therefore the material in circulation from the JKUAT laboratory have incidences of BSV. *Banana streak virus* free orchards should be established in order to ensure that all the materials used for initiation work are BSV-free. Continuous checks, in these BSV-free stocks should be done to ensure that there are no re-infections of BSV. Good orchard managements such as weeding, removing of old leaves watering, thinning of the banana plants to destroy the hiding places of mealy bugs should be done to avoid BSV reinfections.

Since BSV has many strains which are always having mutations, the use of the various specific primers was necessary in this work in order to capture all the BSV strains through PCR. An urgent need to index all the initiation materials used at the JKUAT laboratory and also in other laboratories supplying farmers with planting materials. TC Banana plantlet propagators should follow-up with their farmers to see the extent of damage through BSV and see how they can compensate their losses

CHAPTER FOUR

4.0 EVALUATION OF DIFFERENT METHODS FOR EFFECTIVENESS IN ELIMINATION OF *BANANA STREAK VIRUS* FROM BANANA

Abstract

Three virus elimination techniques were evaluated with the aim of determining the effective method in eliminating *Banana streak virus* from infected plants at the JKUAT commercial banana tissue culture (TC) laboratory. These were chemotherapy, meristem tip culture and thermotherapy. Sucker samples were collected from the three mother orchards supplying the JKUAT commercial laboratory with initiation materials. From each orchard, one banana variety was chosen and from each, ten samples with replicates of three were sampled, well labeled and transported to JKUAT in gunny bags. The outer coverings were excised using a sharp clean knife leaving a $5cm^3$ corm. The corms were taken through the TC procedure up to the second subculture stage after which they were subjected to the virus elimination techniques. In chemotherapy, 0mg/l (control), 10, to 40 mg/l of salicylic acid and ribavirin were separately used. In thermotherapy, 27°C (control), 32°C, to 38°C for 10 days were used. Meristem tip culture at 1 to 5mm (control) was used. All these three virus elimination methods, gave 0 to 90% virus eliminations. Concentrations of ribavirin and salicylic acid at 10mg/l and 20mg/l encouraged regeneration of shoots. While at 40mg/l there were very high mortality rates using both chemicals. Of all the virus elimination methods 20mg/l of salicylic acid and thermotherapy at 36°C had the highest virus elimination percent. However, thermotherapy is laborious especially in

laboratories which are not connected to an automatic generator. Therefore 20 mg/l of salicylic is recommended because it requires low input, easy to implement and is not harmful at low concentrations. It is the most recommended to be used in the JKUAT and other banana commercial laboratories.

4.1 Introduction

Tissue culture remains a useful technique for the production of "clean" planting material in many laboratories across the world. Despite the apparent potential for TC to eliminate most banana pathogens, it is not able to exclude viruses including BSV and is even believed to activate BSV infection in some banana varieties (Harper *et al.*, 2005). Viral diseases are the major banana production constraints due to the widespread exchange of *Musa* germplasm in form of *in vitro* plantlets throughout the world **(**Gambley and Thomas, 2001).

The JKUAT TC laboratory is one of the largest TC laboratories in Kenya with approximately two million plantlets being produced per year. Tissue culture technique is used at JKUAT for mass production of banana planting materials. These planting materials are free from fungal and bacterial diseases but may not be free from viruses. This poses a great danger since the planting materials in circulation might already be BSV-infected. Therefore, a technique for eliminating *Banana streak virus* is urgently needed to ensure that all the planting materials are BSV- free.

There are many BSV elimination methods in use today which include chemotherapy, thermotherapy, cryotherapy, electrotherapy and meristematic tip culture (Qiaochunand *et al.*, 2009). Chemotherapy is the use of chemicals to eliminate pathogens. It is an *in vitro* technique traditionally used for virus eradication (Kartha, 1986). Ribavirin and salicylic acid are potent antiviral that have been reported to eliminate major viral pathogens from potato and other crops (Qiaochunand *et al*., 2009). In most instances,

chemotherapy is more effective if incorporated into TC medium that allows the plant to take the chemical uniformly through the root system. Improved virus elimination can occur if chemotherapy is also combined with thermotherapy with subsequent meristem tip culture (Kartha, 1986).

Thermotherapy is the use high temperatures to eliminate viruses. The success of thermotherapy depends on the target virus's inability to multiply and move readily within plants being exposed to air temperatures of 30 to 40 ºC. Successful treatment time may vary from weeks to months depending on the plant cultivars and specific virus (Qiaochunand *et al.,* 2009).

Meristem tip culture involves the use of the apical dome or shoot tip with a few leaf primordia of the size less than 2mm in length as the explant. In banana meristem tip culture is considered to be the reference tool for virus eradication. The application of meristem-tip culture to eradicate viruses was initially based on the concept of meristem's "immunity" towards viruses (Morel, 1948). Different researchers have shown that the probability of obtaining virus-free plants is inversely related to the size of the meristem (Faccioli and Marani, 1998). The size of the meristem tip is critical for virus elimination. The smaller the tip, the better the chance for virus exclusion (Qiaochunand *et al.,* 2009).

In this study, three virus elimination methods namely chemotherapy, thermotherapy and meristem tip culture were evaluated to determine which one is eliminates the virus from infected banana plants and has the best regeneration

4.2 Materials and Methods

4.2.1 Multiplication of BSV infected plantlets

While the leaf samples in chapter three (3.2.7) were tested for BSV infection at molecular level, the sucker samples were taken through the normal TC processes to enhance multiplication of the explants. The infected banana plants cultivar Nusu Ng'ombe, Fhia 18 and Kampala confirmed to be BSV infected by PCR were used as sources of explants for TC. The culturing medium used in this investigation was Murashige and Skoog medium (1962).

Suckers were uprooted from the three mother orchards supplying JKUAT banana commercial laboratory using a mattock, they were packed in well labeled gunny bags per variety for transportation to JKUAT.

At JKUAT, the suckers were prepared using a sterile matchete to remove the outer covering of the sucker. Using sharp sterile knives, a corm of 5 cm^3 was prepared, put in well labeled containers with distilled water to prevent dehydration, and taken to the TC laboratory.

The explants were first washed in clean running tap water for 30 minutes and then transferred to the clean bench for sterilization. Surface sterilization was done using 70% ethanol for about 8-10 seconds after which they were sterilized for 20 minutes in 70% sodium hypochlorite where three drops of tween 20 (wetting agent) had been added. Then the explants were then rinsed twice using double distilled water**.**

4.2.2 Medium preparation

Sucrose 30g/l was weighed and added to MS medium at the time of media preparation. Hormones required depended on the stage of culture. Macronutrients, micronutrient, vitamins and hormones were dissolved in a litre of distilled water and the pH adjusted to 5.7-5.8 with NaOH and HCL, and the 2.5g/l of gelrite was added and the medium was dispensed into jars 200ml^3 (Initiation, multiplication and rooting) then autoclaved at 121°C under pressure of 1.2 ib/inch/cm^2 for 20min ***(***Appendix 5 and 6)***.*** The autoclaved medium was kept for three days at room temperature before culturing. Cultures were incubated at 26 ± 1°C under photoperiod cycles of 16 hour under light and 8 hours dark. Light intensity of 25000 lux, from white fluorescent tubes was used.

4.2.3 Initiation of explants

Using sterile surgical blades, the outer coverings which were scorched by the sodium hypochlorite were removed leaving a cube of about 2.5 cm^3. Using sterile forceps, the explants were placed in 200ml$^{\mathbf{3}}$ jam jars containing semisolid MS medium. The explants were kept in growth chambers at 26 ± 1°C and 16 hours light and 8 hours dark. The explants were sub cultured after every 4 weeks to fresh multiplication medium until they reached the 2nd subculture while those explants that were BSV infected were subjected to virus elimination methods namely chemotherapy, thermotherap**y**, and meristematic tip culture.

4.2.4. Evaluation of the effectiveness of BSV elimination methods from banana explants

The plantlets were transferred to fresh MS medium after every 4 weeks to obtain enough TC material for the purposes of eliminating the virus. After the second subculture, the plantlets were subjected to the three virus eliminating techniques namely chemotherapy, using two chemicals (ribavirin and salicylic acid) at 10, 20, 30 and 40 mg/l concentrations, thermotherapy at 32°C, 34°C, 36°C and 38°C and 27°C as control and meristematic tip culture using 1, 2, 3, 4, and 5mm size explants. Number of regenerated, dead and survived plantlets was taken and recorded in Ms excel spread sheets after four weeks.

4.2.4.1. Chemotherapy

In chemotherapy, *In vitro* meristems tips (5mm long) at the 2^{nd} subculture were excised in a laminar flow hood and cultured in MS medium supplemented with 0 (control), 10, 20, 30 and 40 mg /l of ribavirin. Another set of cultures were supplemented with 0 (control), 10, 20, 30 and 40 mg/l of salicylic acid. Then the cultures were incubated at 26 ±1°C under photoperiod cycle of 16 hours light and 8 hour dark. The light intensity used was 25000 lux; from white fluorescent tubes. After 4 weeks of incubation in the laboratory**,** the leaves were removed using sterile surgical blades and forceps in a laminar flow hood and put in well labeled packing bags then stored in a freezer for DNA extraction. DNA was extracted using Centyltrimethyl-ammonium bromide (CTAB) method according to Doyle and Doyle (1990) and DNA

visualized in 0.8% agarose gel. Once the quantity of DNA was confirmed, PCR was done as described in 3.2.7 section, to determine whether the virus had been eliminated.

4.2.4.2. Thermotherapy

In thermotherapy**,** explants at 2nd stage sub-culture with well formed shoots were incubated at varying temperatures 27°C (control), 32°C, 34°C, 36°C and 38°C for ten days. The leaves were then removed using sterile surgical blades in a laminar flow hood and placed in well labeled packing bags and then stored in a freezer for PCR detection of BSV. DNA was extracted, visualized in agarose gel before being used for PCR analysis as described above.

4.2.2.3. Meristem tip culture

In meristem tip culture**,** at the 2nd sub-culture, explants of 1, 2, 3, 4 and 5mm (control) in size were excised using clean surgical blades in the clean bench, before being inoculated onto petri dishes containing MS medium. They were left for 2 to 3 days after which they were transferred to jars containing 500mls hormone free MS medium to enhance shoot elongation, for 4 weeks. The explants were then transferred to jars containing fresh MS medium supplemented with 10mg/l NAA to enhance root formation and the leaves were put in well labeled packing bags and stored at –20° C. DNA was extracted from the leaves using the CTAB extraction method by Doyle and Doyle (1990) as described earlier.

4.2.1.6. Statistical data analysis

The number and survival rate of explants was recorded after 4 weeks. The number of surviving explants was subjected to ANOVA analysis using the statistical package for social sciences (SPSS). Mean differences were separated using Duncan's multiple range test**.**

Virus elimination techniques were evaluated to determine which among them was best in elimination of *Banana streak virus*. The following formulae was used in calculating the virus elimination rates

$$\% \text{ Virus Elimination} = \frac{\text{No of plantlets without the virus}}{\text{No of plantlets treated}} \times 100$$

4.3 Results

4.3.1 Chemotherapy to eliminate BSV

When the ribavirin and salicylic acid chemicals were used separately, 10mg/l had the highest regeneration followed by 20mg/l, 30mg/l, with 40 mg/l showing the lowest regeneration (Plate 6). However, the higher the concentration of both chemicals, the higher the viral elimination percentage in all the three varieties used (Table 8). Ten mg/l had the highest survival followed by 20 mg/l then 30 mg/l while 40mg/l had the lowest survival of 10 % in both chemicals. Concentrations of ribavirin at 30 and 40 mg/l had the highest virus elimination while 20 and 30 mg/l of salicylic acid had the highest survival. These results were observed in all the three varieties studied. Fhia 18 had the lowest regeneration compared to Kampala and Nusu Ng'ombe.

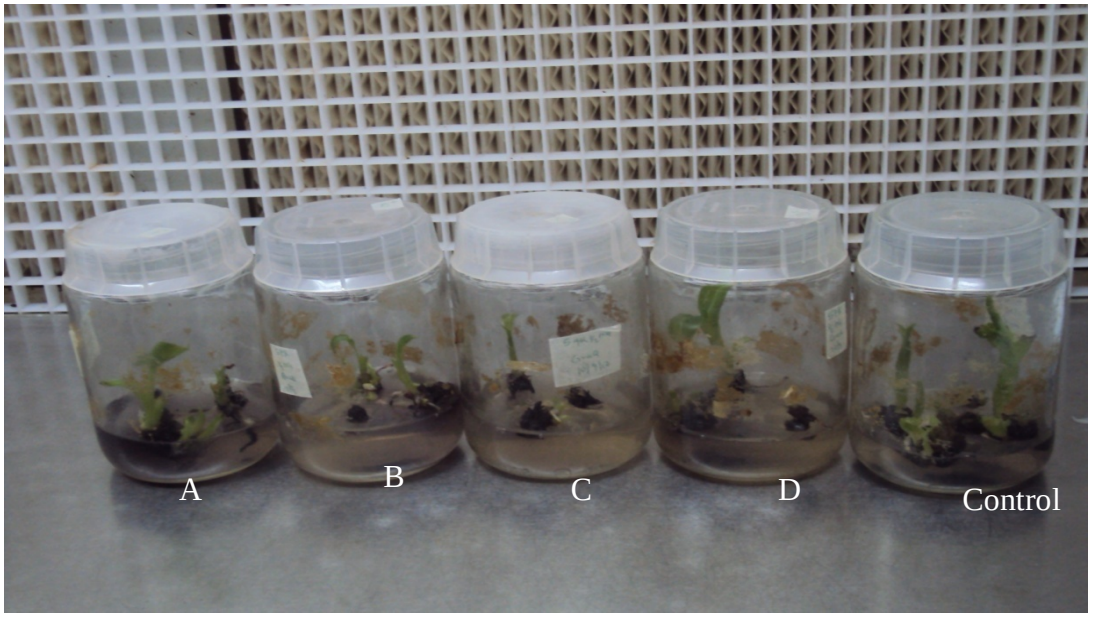

Plate 6. Regenerated plantlets after treatment with salicylic acid. A: 10mg/l; B: 20mg/l; C: 30mg/l; D: 40mg/l/ and control: 0mg/l

When the virus elimination percentages and survivals were compared, 20 mg/l of salicylic acid was the best among the treatments. There was no significant difference in 10 mg/l, 20 mg/l and 30 mg/l for both chemicals and in all the varieties used, but a higher significant difference observed at 40 mg/l since most explants died (Table 8).

Table 8: Virus elimination (%) and means of survived explants after using different concentrations of ribavirin and salicylic acid

Variety	Concentration (mg/l)	Chemical			
		ribavirin Mean	Virus elimination (%)	Salicylic acid mean	Virus elimination (%)
N/Ng	0	9.20a	0	8.00a	0
	10	8.81a	70	7.60a	70
	20	8.62a	80	7.00a	90
	30	8.60a	90	6.60a	90
	40	2.20b	90	1.00b	95
Kampala	0	9.33a	0	8.88a	0
	10	8.84a	70	7.13a	70
	20	7.00a	80	4.75b	90
	30	6.44b	90	3.25b	90
	40	2.22c	90	1.00c	90
Fhia 18	0	9.33a	0	8.88a	0
	10	8.84a	70	7.13a	70
	20	7.00a	80	4.75b	90
	30	6.44b	90	3.25b	90
	40	2.22c	90	1.00c	90

Means within a column followed by the same letter are not significally different by Duncan's multiple range test ($p>0.05$)

4.5.2 BSV elimination using thermotherapy

When the explants were subjected to 27°C, 32°C, 34°C, 36°C, and 38°C temperatures, all explants survived except at 38°C where the explants were killed by the high temperatures (Plate 7). There was no significant difference between 27°C, 32°C, 34°C and 36°C but at 38°C there was a significant drop in the survival. This was because all the samples from all varieties put at 38°C were scorched by heat and therefore no material was available to be used for PCR. It was also noted that the higher the temperature, the higher the virus elimination percentage (Table 9). The highest virus elimination was at 36°C, nine out of ten plantlets treated, tested negative in Kampala and Nusu Ng'ombe variety while eight out of ten tested negative in Fhia 18 variety (Table 9).

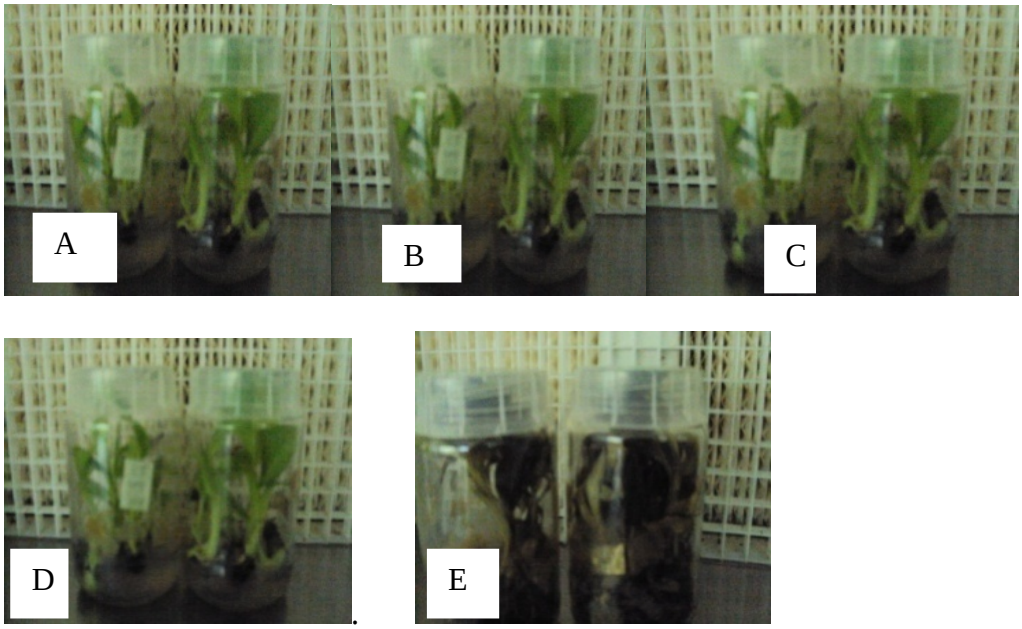

Plate 7; Plantlets in A,B,C and D survived after being subjected to 27, 32, 34, and 36°C, respectively whereas in E the plantlets died after being subjected to 38°C.

Table 9: Virus elimination (%) and means of survived explants after using different temperatures for all varieties

Variety	Temperature (°C)	Virus elimination (%)	% survival	Mean of survived plantlets
N/NG	27	0	100	10
	32	40	100	10
	34	80	100	10
	36	90	100	10
	38	0	0	0
KAMPALA	27	0	100	10
	32	40	100	10
	34	80	100	10
	36	90	100	10
	38	0	0	0
FHIA 18	27	0	100	10
	32	40	100	10
	34	80	100	10
	36	90	100	10
	38	0	0	0

The viral elimination (%) was calculated by taking the number of the BSV-free plantlets over the plantlets tested via PCR and the total was multiplied by a hundred

4.5.3 BSV elimination by meristem tip culture

Plantlets regenerated via meristem tip culture had long shoots developing from meristem tips. The highest virus elimination was achieved at 1mm followed by 2mm, 3mm and 4mm, respectively. There were no virus eliminations when 5mm and 4mm size explants were used (Table 10).

Table 10: Virus elimination (%) and means of survived plantlets at different meristem sizes for all varieties

Variety	Size (mm)	Viral elimination (%)	Mean (no)	% Survival
N/NG	1	90	10	33
	2	60	30	100
	3	50	30	100
	4	0	30	100
	5	0	30	100
	5	0	30	100
KAMPALA	1	90	10	33
	2	70	30	100
	3	50	30	100
	4	0	30	100
	5	0	30	100
FHIA 18	1	90	10	30
	2	70	30	100
	3	50	30	100
	4	0	30	100
	5	0	30	100

The viral elimination percentage was calculated by taking the percentage of the BSV-free plantlets over the plantlets tested via PCR and the total was multiplied by a hundred

When all the three techniques were compared based on virus elimination percentages, 30 and 40 mg/ l of ribavirin, 20 and 30mg/l of salicylic acid and thermotherapy treatment at 36°C emerged the highest (Table 11). But the highest among these five was at 20mg/l of salicylic acid because it is cheaper to implement and had the highest survival and virus elimination percentages.

Table 11: Evaluation of BSV elimination methods across all varieties (Kampala, Nusu Ng'ombe and Fhia 18)

Treatment	Initial No. of Plantlets	Dead plantlets (no)	Survived plantlets (no)	%Virus Elimination
Meristem tip culture				
1mm	30	20	10	90
2mm	30	0	30	60
3mm	30	0	30	50
4mm	30	0	30	0
5mm	30	0	30	0
chemotherapy				
ribavirin				
10mg/l	30	0	30	70
20mg/l	30	2	28	80
30mg/l	30	9	21	90
40mg/l	30	27	3	90
0mg/l	30	0	30	0
Salicylic acid				
10mg/l	30	0	30	80
20mg/l	30	0	30	90
30mg/l	30	9	21	90
40mg/l	30	27	3	95
Thermotherapy				
27°C	30	0	30	0
32°C	30	0	30	40
34°C	30	0	30	80
36°C	30	0	30	90
38°C	30	30	0	0

Highest percentage of virus elimination was at 30, 40 mg/l of Ribavirin, 20, and 30 mg/l of salicylic acid, and at 36°C. However, 36° C and 20mg/l of salicylic acid had the highest survival rate.

4.5 Discussion

Three different *Banana streak virus* elimination methods were evaluated in this study. They included chemotherapy, thermotherapy and meristem tip culture.

Production of BSV-free plants using meristem-tip culture depended on a number factors, the most important being meristem size. The smaller the size of the meristem tip, the higher the percentage of virus-free plantlets obtained. These results agreed with those obtained by Qiaochunand *et al.* (2009), that the smaller the size of the explant the higher the virus elimination and the lower the regeneration rates of the plantlets .

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

The results demonstrate that ribavirin and salicylic acid at the concentration of 10mg/l enhanced growth differentiation of propagated meristems. It can be concluded that using ribavirin and salicylic acid at 20 mg/l combined with TC, the percentage of virus-free plants can be increased. It was noted that the higher the chemical concentration, the higher the virus elimination percentage. This results are in agreement with those obtained Hazaa *et al.* (2006) that higher concentrations of antiviral drugs eliminates the virus but inhibits plant regeneration.

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

When TC procedures without any of the three virus elimination techniques was used as control, there were no viral elimination which is in agreement, with Wangai *et al*. (2002) and Karanja *et al*. (2008), that TC technique does not eliminate *Banana streak virus*. Therefore, though TC has very many advantages over the conventional methods of mass propagation, it does not eliminate BSV, therefore, a combination of TC with a reliable virus elimination method is required to be put in place to be able to eliminate BSV.

It was observed that the Red Dacca BSVs strain was resistant to elimination using the three viral elimination methods. This could be attributed to the arrangement of the bases in the BSV strain DNA and research should be done to find out the exact reason.

It was concluded that the chemotherapy at 20mg/l together with TC be adopted in order to produce BSV-free plantlets. A cost margin analysis on the three virus elimination methods should also be done to determine the most effective method for adoption in commercial banana laboratories.

Results in this study indicated BSV infection of up to 90 % showing that the banana crop may have become tolerant to BSV; otherwise all the banana orchards in Kenya would be distinct.

CHAPTER FIVE

5.0 COST MARGIN ANALYSIS FOR PRODUCTION OF A BANANA STREAK VIRUS-FREE TISSUE CULTURE PLANTLET

Abstract

Production of a BSV-free *in vitro* plantlet is dependent on the benefits derived from producing such a plant against those of normally produced TC plantlets. First, the cost of producing one TC plantlet was calculated as well as the cost of producing one BSV-free plantlet using different elimination methods was determined at the JKUAT TC laboratory. The cost margin among the virus elimination methods used was not significant. Production of one BSV-free plantlet cost approximately Ksh 125.5 125.6 127.9, 130.4, for salicylic acid, ribavirin meristem tip culture, thermotherapy respectively. While a TC plantlet cost Ksh 85.3 therefore, there was a cost margin of Ksh 40.2, 40.3, 42.6 and 45.1 for salicylic acid, ribavirin meristem tip culture and thermotherapy, respectively. Though TC plantlet is cheaper in production, the BSV-free explant has a higher yield of approximately (90%) increase in profit compared to that of the TC plantlet. After evaluation of elimination methods in terms of cost margin analysis, chemotherapy (salicylic acid) emerged the most effective, since it is easy to implement and the cost of production is slightly lower than the other methods. Therefore, infected mother plants for TC procedure should be subjected to chemotherapy (20mg/l salicylic acid) in order to obtain BSV-free plantlets.

5.1 Introduction

The tissue culture technique ensures there are sufficient planting materials which are free from disease causing organisms. The technique also ensures use of limited number of mother plants for raising large numbers of progeny plants. These few mother plants can be maintained with required care at a limited cost (Singh *et al*., 2011).

Being a vegetative reproduction method, micropropagation results in a high degree of genotypic and phenotypic uniformity of the progeny. Using TC, the production of planting material can be achieved as per needs. Micropropagated plants exhibit uniform growth and maturity enabling one time harvesting (Singh *et al*., 2011).

The banana gross margin reflects the benefits banana producers generate by participating in the production and marketing enterprise. In the case of a trader, the difference between the sales price and the buying price will give the market margin per unit. The direct costs are the variable costs which may fluctuate as a result of the size and level of production (Several studies have been carried out to estimate the costs and returns, in order to identify the factors influencing the cost of production, and to assess the efficiency in TC banana, so as to identify the factors determining the adoption of TC banana and the risks involved (Anthony *et al.,* 2010). Though TC plantlets have more benefits as compared to conventionally produced banana, the TC procedures do not eliminate *Banana streak virus,* posing a big danger because most farmers rely on TC bananas which are believed to be free from disease. The aim of

this study was to carry out a cost-margin analysis for three different methods of BSV elimination in the production of a virus- free plantlet.

5.2 Materials and methods

Cost-margin analysis for producing one BSV-free plantlet was done by taking into account all the costs used in the production of BSV –free plantlets using the three virus elimination methods namely, chemotherapy using salicylic acid and ribavirin, thermotherapy and meristem tip culture (Chapter 4). All the materials required for production of TC and BSV virus-free plantlet using the three virus elimination methods were listed down and their prices calculated to find out which one was more effective.

5.2.1 Cost of materials required for production of one TC plantlet

All the materials required for the production of one TC banana plantlets were listed with the aim of finding out the materials required in different stages of TC such as multiplication and rooting (Table 12).

Table 12: Cost (Ksh) of materials required for producing one TC plantlet

Item	Specification	Unit price (Ksh)
Ms. media	litres	162.0
Sucker	pieces	40.00.0
gelrite	Kilo grams	30,000.0
consumables	assorted	assorted
Sucrose	Kilo grams	200.0
BAP	grams	7500.0
IAA	grams	7500.0
NAA	grams	7500.0
Labour	Per day	1000.0
Furadan	Kilo grams	900.0
Fertilizer	kilograms	4000.0
Miscellaneous	%	10% of total
Jam jars	pieces	100.0

5.2.2 Cost of materials required for production of one BSV-free TC plantlet using meristem tip culture

After all the materials required for the production of one TC plantlet was done, additions of DNA extraction and PCR work and those of producing a meristem tip culture were listed down (Table 13).

Table 13: Cost (Ksh) of materials required for production of one BSV-free TC plantlet using meristem tip culture

Item	Unit	Unit price (Ksh)
TC plantlet	Piece	85.3
Petridishes	Carton	4,000.0
Parafilm	Roll	6900.0
Liquid nitrogen	Kg	200.0
PCR tubes	packet	2000.0
Primers	Bases	4000.0
Taq polymerase kit	kit	30,000.0
Molecular work consumables	piece	pieces
Labour for molecular work	day	2000.0
Miscellaneous	%	10% of total

5.2.3 Cost (Ksh) of materials required for production of one BSV-free TC plantlet using thermotherapy

All the materials required in thermotherapy treatment, molecular work consumables and labour were added to the list of items required for the production of one TC plantlet (Table 14).

Table 14: Cost (Ksh) materials required for production of one BSV-free TC plantlet via thermotherapy

Item	Unit	Unit price (Ksh)
TC plantlet	Piece	85.3
Liquid nitrogen	Kg	200.0
PCR tubes	packet	2000.0
Primers	Bases	4000.0
Taq polymerase kit	kit	30,000.0
Molecular work consumables	piece	pieces
Labour for molecular work	day	2000.0
Incubator	pieces	250,000.0
Miscellaneous	%	10% of total

5.2.4 Cost (Ksh) of materials required for production of one BSV-free TC plantlet via chemotherapy

All the requirements for production of one plantlet using both salicylic acid and ribavirin were listed down in addition to molecular work requirements and that of one TC plantlet (Table 15).

Table 15: Cost (Ksh) of materials required for production of one BSV-free TC plantlet using salicylic acid and ribavirin

Item	Unit	Unit price (Ksh)
One TC plantlet	Pieces	64.9
Salicylic acid	Kilo gram	7,500.0
Ribavirin	Grams	22,500.0
Liquid nitrogen	Kilo gram	200.0
PCR tubes	Packet	2,000.0
Primers	Bases	4,000.0
Taq polymerase kit	Kit	30,000.0
Molecular work consumables	Pieces	2.0
Molecular work labour	Day	2,000.0
Miscellaneous	%	10 % of total

After listing all the items required in the production of BSV–free explant using the three virus elimination techniques, their costing verses their benefits were evaluated to see which of them is effective in terms of implementing and in increasing profits.

5.3 Results

All the required items, their costs and relative benefit for BSV- free plantlets were determined for the analysis of producing BSV-free TC banana explants. The costings were done using current prices Kenyan shillings (Ksh). The cost of producing one TC plantlet is Ksh 85.3 (Table 16). Meristem tip culture was Ksh 127.9, salicylic acid ksh 123.5, and ribavirin Ksh 123.6 as indicated in Tables 17 and 19 respectively. Thermotherapy technique had the highest cost of production ksh 130.4 (Table 18).

Table 16: Materials and cost (Ksh) for TC plantlet production as at 2012

Item	Multiplication stage	Rooting stage	Greenhouse	Total (Ksh) for one plantlet
Ms. media	7.7	1.1	0.0	8.8*
Sucker	40.0	0.00	0.0	40.0
gelrite	0.1	0.1	0.0	0.1
consumables	0.1	0.1	0.0	2.0
Sucrose	1.9	0.1	0.0	2.0
BAP	0.1	0.0	0.0	0.1
IAA	0.1	0.0	0.0	0.1
NAA	0.0	0.1	0.0	0.1
Labour	3.0	1.0	1.0	4.0
Furadan	0.0	0.0	0.5	0.5
Fertilizer	0.0	0.0	1.5	1.5
Miscellaneous	10% of total			6.0
Jam jars	5.0	5.0	0.0	10.0
Total in Ksh				85.3

*Cost at the JKUAT –TC laboratory

Table 17: Cost of materials (Ksh) required for production of one BSV- free plantlet via meristem tip culture at the JKUAT –TC laboratory

Item	Unit	Unit price (Ksh)	Price (Ksh) for one plantlet
TC plantlet	Piece	85.3	85.3
Petridishes	Carton	4,000.0	2.0
parafilm	Roll	6900.0	0.5
Liquid nitrogen	Kg	200.0	2.0
PCR tubes	packet	2000.0	0.1
Primers	Bases	4000.0	8.0
Taq polymerase kit	kit	30,000.0	20.0
Molecular work consumables	piece	40,000.0	2.0
Labour for molecular work	day	2000.00.0	2.0
miscellaneous	%	10% of total	6.0
Total in Ksh			127.9

Table 18: Cost of materials (Ksh) required for producing of one BSV- free plantlet via thermotherapy at the JKUAT –TC laboratory

Item	**Stages**		
	multiplication	Rooting	Total (Ksh) for one plantlet
One TC plant			85.3
Primers	4.0	4.0	8.0
Liquid N2	1.0	1.0	2.0
PCR tubes	0.1	-	0.1
Taq pol kit	10.0	10.0	20.0
consumables	2.0	0.1	2.0
Molecular work labour	1.0	1.0	2.0
Miscellaneous	-	-	6.0
Incubator	5.0	0.0	5.0
Total			130.4

Table 19: Cost of materials (Ksh) for producing one BSV – free explants using salicylic acid and ribavirin at the JKUAT –TC laboratory

Item / stages	Multiplication	Rooting	Total (Ksh) Salicylic acid	Total (Ksh) ribavirin
One TC plant			85.3	85.3
Primers	4.0	4.0	8.0	8.0
Liquid N2	1.0	1.0	2.0	2.0
PCR tubes	0.1	-	0.1	0.1
Taq pol kit	10.0	10.0	20.0	20.0
Salicylic acid	0.1		0.1	-
Ribavirin	0.2	-	-	0.2
consumables	2.0	0.1	2.0	2.0
Molecular work labour	2.0	-	2.0	2.0
Miscellaneous	-	-	6.0	6.0
Total			125.5	125.6

When the total cost of production for the three virus elimination techniques were compared, salicylic acid had the lowest cost (125.5) followed by salicylic acid and ribavirin at Ksh 125.6 and Ksh 127.9, respectively (Table 19). Thermotherapy had the highest cost Ksh 130.4 this was because of the high cost of electricity and purchase of an incubator.

5.4 Discussion

The cost incurred when using the four virus elimination methods used in this work were used to conduct a cost margin analysis. Though TC has many benefits compared to conventionally produced plantlets, it still have some limitations since the technique does not eliminate BSV from explants. *Banana streak virus* is the most prevalent banana virus in Kenya causing 6- 90% yield loss (Wangai *et al*., 2002) therefore, BSV-free plantlets guarantees an increase in profit.

The cost of producing one TC plantlets was estimated at Ksh 85.3 while that of producing an explant using salicylic acid, ribavirin, meristem tip culture and thermotherapy was estimated at Ksh 125.5, 125.6, 127.9 and Ksh 130.4, respectively. Chemotherapy (salicylic acid) emerged the easiest to implement because it is cheaper and less cumbersome than the other methods. All TC plantlets should therefore be indexed for BSV in the laboratory to ensure that the materials being released to the farmers are BSV-free.

At the moment, one banana TC plantlet at the JKUAT greenhouse costs Ksh 100, this implies that when the recommendation is implemented, the banana plantlets will cost slightly higher than this because of the cost used in elimination method and cost of diagnostics. Since the cost margin between TC plantlet and BSV-free plantlet is not high it will easily be adopted by other banana TC producing laboratories.

It is therefore recommended that chemotherapy utilizing salicylic acid be implemented at the JKUAT commercial banana laboratory to ensure that the planting materials for

sale are free from BSV infection. Virus infected material should not be distributed to avoid spreading BSV infected materials.

CHAPTER SIX

6.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

It was found that there are incidences of Banana streak virus in the initiation materials in use at the JKUAT commercial banana laboratory. It was also noted that the initiation materials have the various documented BSV strains with some having more than one BSV isolate. Some of the samples which tested BSV positive after using PCR did not show any symptoms in the field, indicating that visual observation alone should not be used for detecting BSV. Results also indicated up to 90% BSV infection, however, there are banana orchards with good yields. Leading to the conclusion that there is a possibility of BSV tolerance in banana crop. After evaluation of the various virus elimination methods, the study showed that BSV can be eliminated using chemotherapy (salicylic acid) method at 20mg/l in combination with tissue culture process which gave the cheapest BSV elimination percentage, and the highest survival.

6.2: RECOMMENDATIONS

From this stud, it is recommended that all the initiation material to be for mass propagation be indexed and the virus be eliminated using the chemotherapy (salicylic acid 20 mg/l) method since it is easy to implement and cheaper in terms of cost. Further research on combinations of the virus elimination methods (meristem and chemotherapy, chemotherapy and thermotherapy, meristem and thermotherapy)

should be done to find out whether better results can be obtained. Templiphi kit, a technique used to discriminate between integrated and episomal BSV DNA, specifically detecting the latter in several banana cultivars known to contain episomal and/or integrated sequences of Banana streak Mysore virus (BSMyV), Banana streak OL virus (BSOLV) and Banana streak GF virus (BSGFV) should be used in diagnostics should be used to reduce time spent in the screening. BSV–free orchards should be established and well maintained to ensure materials for initiation are not re-infected.

REFERENCES

Anthony, P., James, R., Geijskes, J., Dale, J., and Harding, **R**., **(2010).** Centre for Tropical Crops and Biocommodities, Queensland University of Technology, Brisbane, Qld, 4001, Australia.

Dahal, G. Hughes, J. Thottappilly, G. and Lockhart, B., (1998). Effect of temperature on symptom expression and reliability of banana streak badnavirus detection in naturally infected plantain and banana (*Musa* spp.). *Journal of Plant Disease* **82**:16-21.

Dahal, G., Ortiz R., Tenkouano A., D'A. Hughes J., Thottappilly G., Vuylsteke D., Lockhart, B., (2000). Relationship between natural occurrence of banana streak badnavirus and symptom expression, relative concentration of viral antigen, and yield characteristics of some micropropagated *Musa* spp. *Plant Pathology*. **49**: 68-79.

Dale, J., (1987). Banana bunchy top: an economically important tropical plant virus disease. Market Survey on Tissue cultured Plants. *Annals of Applied Biology volume* **128, Issue 1**, pages 55–64.

Daniells, J., Geering, A., Bryde, N., and Thomas, J., (2001). The effect of *Banana streak virus* on the growth and yield of dessert bananas in tropical Australia. *Annals of Applied Biology*. **139**, 51-60.

Diekmann, M. and Putter, J., (1995). FAO/ IPGRI. Technical guidelines for the safe movement of germplasm, no 15. FAO/ IPGRI, Rome, Italy.

Diekmann, M., Putter C., (1996). FAO/IPGRI Technical Guidelines for the Safe Movement of Germplasm. *Plant Genetic Resources* Institute, Rome.

Dijkstra, T. and Magori, T., (1994). Horticultural Production and Marketing in Kenya, Part 4: Kisii and Nyamira District. Food and Nutrition Studies Programmee, Report No 52. Ministry of Planning and National Development, Nairobi, African Studies Centre, Leiden.
DNA. *Journal of Virus Genes*, **17**: 271-278.

Doyle, J., and Doyle, J., (1990). Isolation of DNA from fresh tissue. *Focus*, **12**: 13-15

Estelitta, S., Radhakrishnan, T., and Paul, T. **(1996).** Infectious chlorosis disease of banana in Kerala. *InfoMusa* **5**: 25–26.

Faccioli, G., and Marani, F., (1998). Virus elimination by meristem tip culture and tip micro grafting. *Journal of Plant virus diseases control*. pp 346-380.

Faccioli, G., Colombarini, A., (1996). Correlation of potato virus S and virus M contents of potato meristem tips with the percentage of virus-free plantlets produced *in vitro*. *Potato Research* **49**:119–129. DOI 10.1007/s11540-006-9011-4

FAO, (2007). Food and Agriculture Organization of the United Nations. *Commodity market review, 2005-2007.*

FAO, (2009). Food and Agriculture Organization of the United Nations, 2009. *Commodity market review, 2008-2009*, Rome, Italy,

FAOSTAT., **(2005).** Food and Agriculture Organization of the United Nations (*faostat.fao.org*)

FAOSTAT, (2006). The FAOSTAT Prodstat module on crops contains detailed agricultural Production data [Online]. Available by Food and Agricultural Organization ***(http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567)***

FAOSTAT, **(2011**). Food and Agriculture Organization of the United Nations (*faostat.fao.org).*

Gambley, C., and Thomas, J., (2001). Molecular characterization of banana mild mosaic virus: a new filamentous virus in *Musa* spp. *Archives of Virology* **146** (7).

Geering, A., McMichael, L., Dietzgen, R., and Thomas, J., (2000). Genetic diversity among banana streak virus isolates from Australia. *Journal of Phytopathology* **90**:921-927.

Harper, G. and Hull, R., (1998). Cloning and sequence analysis of banana streak virus.

Harper, G., Dahal, G., Thottappilly, G. and Hull, R., (1999). Detection of episomal banana virus. *Journal of virology* **255**:207-213.

Harper, G., Hart, D., Moult, S. and Hull, R., (2002). Detection of banana streak virus in field samples of bananas from Uganda. *Annals of Applied Biology*. **141**: 247-257.

Harper, G., Hart, D., Moult, S., Hull1 R., Geering, A. and Thomas J., (2005). The diversity of Banana streak virus isolates in Uganda.

Hazaa, M., El- Dougdoug A., Sabah Abo El-Maaty., (2006). Eradication of banana viruses from naturally infected banana. *Journal of applied sciences research*, **2(10):** 714-722.

Helliot, B., Panis, B., Poumay, Y., Swennen, R., Lepoivre, P., and Frison, E., (2002). Cryopreservation for the elimination of *cucumber mosaic and banana streak viruses* from banana. *Plant Cell Reports*, **20**: 1117-1122

Hernandez, P., Igarza, R., González. Y., Peralta, E., Fontanella, R., Noa, C., Pichardo, T., Alonso, E., García, L. and Rodríguez, M. (1997). Nuevo method for sanitation bacteria and viruses in sugar cane (*Saccharum hybrid* sp.) *Rev. Phytopathology notebook*. **3:**153-157.

IITA, (1998). International Institute of Tropical Agriculture (IITA) annual report. IITA, IBADAN NIGERIA. *Journal of Immunoassays*. Pg 139–154.

International Network for Improvement of Banana and Plantain, (1997). Regional Network for Eastern Africa *Annual report*, Montpellier, France

International Network for Improvement of Banana and Plantain, (1998). Regional Network for Eastern Africa *Annual report*, Montpellier, France

International Network for Improvement of Banana and Plantain, (2000). Regional Network for Eastern Africa *Annual report*, Montpellier, France

ISAAA, **(1999).** International Service for the Acquisition of Agri-biotech Applications. *Annual report*. [Online] *(http:// **www.isaaa.org***)

Kahangi, E. M., Muthee A. M., Chege, B. (2006). Constraints and sustainable solutions for adoption of tissue cultured banana technology and marketing. *Proc. IC on Banana and Plantain in Africa*

Karanja, L., (2008)**.** Identification of the diversity of banana streak virus in Kenya. *Phytopathology.* Volume **156**: 11-12**,**

Karanja, L., Wangai, A., Harper, G., J. Stanley J. and Pathak, **R**., (2002). Molecular detection and variability of banana streak virus isolates in Kenya. *Journal of Phytopathology*. **156**:678-686.

Kartha, K., (**1986**)**.** "Production and indexing of disease-free plants", *Plant Tissue Culture and its Agricultural Application*, 219-238.

Khalid, S., Soomro, M., and Stover, R., (1993). First report of banana bunchy top virus in Pakistan. *Journal of Plant Disease* **77**: 101.

Lockhart B., Jones, D., (2000). Banana mosaic and banana streak, diseases caused by viruses. *Journal of Diseases of banana*, pp 241–293

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

Lockhart, B., (1995). Development of detection methods for banana streak virus (BSV). *Journal of Phytopathology* **89**: 346-441.

Lockhart, B., Olszewski, N., (1993). Serological and genomic heterogeneity of *Banana streak badnavirus*: implications for virus detection in *Musa* germplasm. *In breeding banana and plantain for resistance to diseases and pests journal,* pp. 105-113.

Lozoya-Saldana, H., *(*1996). Electrotherapy and shoot tip culture eliminate Potato Virus X in potatoes, *Am. Potato J.* 73: 149-154.

Magnaye, L., (1994). Virus diseases of banana and current studies to eliminate the virus by tissue culture. *The Philippine Agriculturist* **73**: 38–43

Moald, (1997). Ministry of Agriculture and Livestock Development. *Horticultural Division animal production division Annual report* 16

Morel, G., (1948). Plant Tissue Culture: Theory and Practice, *Annals Epiphytes,* **14**: 123-234.

Murashige, T., Skoog F., (1962). A revised medium for rapid growth and bioassays with tobacco tissues cultures. *Physiol Plant* **15**: 473–497.

Ndowora, T., and Lockhart B., (1997). Improved serological method for detecting banana streak virus. *Musae Africa* **11**:15

Ndowora, T., Dahal, G., Lafleur, D., Harper, G., Hull, R., Olszewski, N., Lockhart, B., (1999). Evidence that Badnavirus infection in *Musa* can originate from integrated pararetroviral sequences. *Virology* **255**:214–220

Ndowora, T., Lockhart B., (2000). Development of a serological assay for detecting serologically diverse banana Streak virus isolates. *Acta Hortic* **540**:377–388

Nguthi, F., Onyango M., Muniu F., Muthamia J., Njuguna M., (1999). Biotechnology to Benefit Small-Scale Banana Producers in Kenya. *Annual report*, KARI. Nairobi, Kenya.

Nguthi, F., (2001). Tissue culture techniques and their application in Agriculture: Case Study-Banana Priority Setting for Horticultural Crops. *Research priorities of the KARI horticultural programme.*

Niblett, C., Pappu, S., Bird, J., and Lastra, R., (1994). Infectious chlorosis, mosaic, and heart rot. *Compendiums of tropical fruit diseases*, Pages 18–19.

Palukaitis, P., Roossinck, M., and Francki, R., (1992). Cucumber mosaic virus. *Journal of Advances in Virus Research* **41**: 281–348.

Qaim, M., (1999). Assessing the Impact of Banana Biotechnology in Kenya. The International Service for the Acquisition of Agri-biotech Applications *(*ISAAA*)* Briefs No. 10

Qiaochunand, W., and Jari, P., Valkonen, T., (2009). Therapy of shoot tips and novel pathogen eradication method science directs.

Rodoni, B., Dale, J., and Harding, R., (1999). Characterization and expression of the coat protein-coding region of banana bract mosaic Potyvirus, development of diagnostic assays and detection of the virus in banana plants from five countries in Southeast Asia. Archives *of Virology.***144**: 1725–1737.

Singh, H., Uma, S., Selvarajan, R., and Karihaloo, J., (2011). Micropropagation for Production of Quality Banana Planting Material in Asia- Pacific. *Asia-Pacific Consortium on Agricultural Biotechnology* **(**APCoAB), New Delhi, India. Pg 92.

Soomro, M., Khalid, S., and Aslam, M., (1992). Outbreak of banana bunchy top virus in Sindh, Pakistan. FAO Plant Protection Bulletin 40: 95–99. Streak badnavirus by IC-PCR. *Journal of Virological Methods,* **79**: 1-8.

Su, H., Wu, R., and Tsao L., (1993). Ecology of banana bunchy-top virus disease. Pages 308–312 *in* Proceedings of the International Symposium on Recent Developments in banana Cultivation Technology, edited by R.V. Valmayor, S.C. Hwang, R. Ploetz, S.C. Lee, and N.V. Roa. Taiwan banana Research Institute, 14–18 December 1992, Chiuju, Pingtung, Taiwan, INIBAP/ASPNET, Los Baños, the Philippines.

Thomas, J., and Iskra-Caruana, M., (2000). Bunchy top. Pages 241–253 *in* diseases of banana, Abaca and Ensete, edited by D.R. Jones. CAB International, Wallingford, UK.

Thomas, J., and Magnaye, L., (1996). Banana bract mosaic disease. *Musa* disease fact sheet no. 7. International Network for the Improvement of banana and Plantain (INIBAP), Montpellier, France.

Thomas, J., Geering, A., Gambley, C., Kessling, A., and White, M., (1997). Purification, properties, and diagnosis of banana bract mosaic Potyvirus and its distinction from Abaca mosaic *Potyvirus. Phytopathology* **87**: 698–705.

Thomas, J., Lockhart, B., and Iskra-Caruana, M., (2000). Banana mild mosaic virus. Pages 275–279 *in* Diseases of banana, Abaca and Ensete, edited by D.R. Jones. CAB International, Wallingford, UK.

Thomas, J., M. L. Iskra-Caruana, M., and Jones, D., (1994). Banana bunchy top disease *Musa* disease fact sheet no. 4. *International Network for the Improvement of banana and Plantain (INIBAP)*, Montpélliér, France. 1p.

Thottappilly, G., Dahal, G., Lockhart B., (1998). Studies on a Nigerian isolate of 1. Bouhida, M. and B. E. L. Lockhart. 1990. Increase in importance of cucumber mosaic virus infection in greenhouse grown bananas in Morocco. *Phytopathology* **80**: 981.

Tsao, L., (1998). Biological and molecular characterization of banana bunchy top virus strains and their ecology. Unpub. PhD. Thesis, *Graduate Institute of Plant Pathology and Entomology*, National Taiwan University, Taiwan, ROC. 132pp.

Tushmereirwe, W., Karamura, K., and Karyeija, **R**., **(1996).** Banana streak virus (BSV) and an associated filamentous virus (unidentified) disease complex of highland bananas in Uganda. *InfoMusa* **5**: 9–12.

Van Regenmortel, M., and Dubs, M., (1993). Serological procedures. Pages 159–214 *in* Diagnosis of plant virus diseases, edited by R.E.F. Matthews. CRC Press, Boca Raton, Florida, USA.

Wang, Q., Mawassi, M., Li, P., Gafny, R., Sela, I., and Tanne, E., (2003). Elimination of grapevine virus A (GVA) by cryopreservation of *in vitro* grown shoot tips of Vitis vinifera L. Plant Sci. 165, 321–327.

Wangai, A, Karanja, L., Ndung'u, J., Kimani, E., Kilonzo, S., and Nguthi, F., (2002). Preliminary results on surveys of banana streak virus (BSV) in Kenya, *2nd Annual symposium* KARI –NPBRC, Njoro Kenya, and Egerton University.

Wu, R., and Su, H., (1990). Production of monoclonal antibodies against banana bunchy top virus and their use in enzyme-linked immunosorbent assay, *Journal of Phytopathology* **128**: 203–208.

APPENDICES

Appendix 1

Table 3.0 CTAB extraction buffer preparation (500ml)

Chemical	**Amount**
1 M Tris –HCL (Ph8.0)	50ml
0.5M EDTA (Ph 8.0)	50ml
Sodium chloride	41g
Sodium sulphite	5g
PVP 10	10g
CTAB	10g

Appendix 2

PCR mixes of (20 μL) contained 10 μl 2x GoTaq Green Master Mix (Promega Corp, Madison, WI), 5 ρmol of each primer (table 4), 1 μl of nucleic acid extract and water to final volume. PCR cycling conditions were an initial denaturation of 94°C for 2 min followed by 35 cycles at 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 2 min. Reactions products were analyzed by agarose gel electrophoresis and amplicons visualized as previously described except that 1.5% gels were used.

Appendix 3

Incidence of BSV in mother orchards by visual and PCR detection method

Orchard	Variety	Sample no.	BSV detection by use of symptoms	BSV detection using PCR
JKUAT	Kampala	1J	+	+
		2J	+	+
		3J	+	+
		4J	+	+
		5J	+	+
		6J	-	+
		7J	-	-
		8J	-	-
		9J	-	+
		10J	-	+
KARI Thika	Fhia 18	T1	+	-
		T2	+	-
		T3	-	-
		T4	+	+
		T5	+	+
		T6	-	-
		T7	-	+
		T8	-	+
		T9	+	+
		T10	-	-
KARI Kisii	N/NG	51k	+	+
		52k	-	+
		53k	-	+
		54k	+	+
		55k	+	+
		56k	-	-
		57k	+	+
		58k	-	+
		59k	+	+
		60k	-	+

Appendix 4

Incidence of BSV in JKUAT greenhouse by visual and PCR detection methods

Field	Variety	Sample no.	BSV detection by visual means	BSV detection by PCR
Jkuat green house	Nusu Ng'ombe	11K	-	+
		12K	-	+
		13K	-	+
		14K	-	+
		15K	-	+
		16K	-	+
		17K	-	-
		18K	-	+
		19K	-	-
		20K	-	-
	Kampala	21J	-	+
		22J	-	-
		23J	-	-
		24J	-	-
		25J	-	-
		26J	-	+
		27J	-	+
		28J	-	+
		29J	-	-
	Fhia 18	30J	-	-
		31T	-	-
		32T	-	-
		33T	-	+
		34T	-	-
		35T	-	-
		36T	-	-
		37T	-	-
		38T	-	+
		39T	-	+
		40T	-	-

Key : + positive , - means negative

Appendix 5

MS Medium composition

Constituents	**Basal**	**Multiplication**	**Rooting**
Ms salt g/l	4.2	4.2	4.2
Sucrose g/l	30	30	30
BAP	-	9	-
NAA	-	-	10
IAA	-	1.8	-
Myo-inositol	0.1	0.1	0.1
pH	5.7 - 5.8	5.7 - 5.8	5.7 - 5.8

Appendix 6

Table 11: Murashige and Skoog's Media (1962)

Stock solution 1: mineral salts (x10)

NH_4NO_3	33g	
KNO_3	38g	
$CaCl_2.2H_2O$	8.8g	Dissolve these compounds in
$MgSO_4.7H_2O$	7.4g	1.8l distilled water. Top up to
KH_2PO_4	3.4g	2 liters. Store in the dark.
H_3BO_3	124mg	
$MnSO_4.4H_2O$	446mg	
$ZnSO_47H_2O$	214mg	
$Na_2MoO_4\,2H_2O$	50mg	
$CUSO_4.5H_2O$	5mg	
$COCL_2.5H_2O$	5mg	Dissolve in 100ml distilled
KI	16.6mg	water. Take 10mls and add to
		the solution above.

<u>Stock Solution 11: Vitamins (X100</u>)

GLYCINE	40mg	Dissolve in distilled H_2O
&		
MYOINOSITOL	2g	make up to 100ml store in
NICOTINIC ACID	10mg	refrigerator.
PYRIDOXINE HCL (B6)	10mg	
THIAMINE HCL (B1)	2mg x5 =10mg	Dissolve in 20 ml of
distilled		
		H_2O. Take 4ml add to the
		Solution above

Stock Solution I11: Fe (X200)

NA_2EDTA	745mg	First dissolve Na_2EDTA
in		
$FESO_4.7H_2O$	557mg	warm distilled water
		Then fe_2SO_4 $.7H_2O$ and
top		
		up to 100ml

Stock Solution Iv: Hormones

Ms Media preparation (1000ml)

	Multiplication Media	**Rooting Media**
Bap	10ml/l	-
IAA	1ml/l	-
NAA	-	10ml/l

Materials used in production of a tissue culture plantlets