MOLECULAR DETECTION OF BANANA VIRUSES IN GERMPLASM FROM SELECTED AREAS IN KENYA

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DECLARATION AND RECOMMENDATION

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I declare that this document is my original work and has not been submitted wholly or in part to any institution for award of any degree to the best of my knowledge.

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DEDICATION

To my beloved parents Mr. and Mrs. Benjamin Wambulwa for making my dream a reality

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ABSTRACT

Banana is a popular and important crop among many communities in East Africa. It is used both as a subsistence and cash crop. Bananas provide a major source of carbohydrates for over 400 million people in tropical countries, about 25% of these being in Africa. In Uganda, Burundi, and Rwanda, banana consumption ranges from 250 to 400 kg per person per year. In Kenya, production stands at around 210,000 metric tonnes annually. However, banana production is threatened by a number of viral diseases, banana streak disease (BSD) being one of the most significant in Kenya. Others include banana bract mosaic disease (BBrMD) and cucumber mosaic disease (CMD). Banana streak disease is caused by Banana streak virus (BSV). A recent study identified seven BSV isolates in some banana growing regions of Kenya. However, the distribution of these isolates across the country is unknown. In this study, 65 symptomatic samples were used to determine the distribution pattern of BSV isolates in Kenya. The samples were sourced from five major banana growing regions of Kenya (Central Rift Valley, Eastern, Central, Nyanza and Western provinces). Detection of two *Musa* RNA viruses (*Banana bract mosaic virus* and *Cucumber mosaic virus*) was also carried out on the same symptomatic samples in order to determine any co-infection relationships. A set of 32 BSV-asymptomatic samples were used to compare the sensitivity of three BSV indexing techniques (TempliPhi, immuno-capture-PCR and direct PCR). Identification of the various BSV isolates was achieved through restriction fragment length polymorphism (RFLP) analysis. Analysis of variance and student's t-test were done using the Statistical Analysis System (SAS) software in order to compare the means of the detection techniques. The Mysore isolate was found to be the most widely distributed in the Kenyan BSV ecology (48% overall detection) with its highest incidence being recorded in Kisii-Nyanza (37.5% of all the Mysore-infected samples). The samples that were found to be infected with the Mysore isolate appeared to exhibit relatively more severe BSV symptoms. There was absolutely no detection of any RNA virus in the 65 BSV-symptomatic samples showing that there was no co-infection between BSV and the two RNA viruses. There were significant differences ($P<0.05$) among the detection means for the four BSV detection techniques. Direct PCR showed a detection of 93.8% but most of these detections were treated as false positives. TempliPhi is therefore recommended for routine indexing of *Musa* tissues for BSV due to its higher detection capacity.

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LIST OF ABBREVIATIONS

AP	Aspartic protease
BBrMV	*Banana bract mosaic virus*
BBTV	*Banana bunchy top virus*
BPYV	*Beet pseudo-yellows virus*
BSCavV	*Banana streak virus* Cavendish isolate
BSD	Banana Streak Disease
BSGfV	*Banana streak virus* Gold finger isolate
BSMysV	*Banana streak virus* Mysore isolate
BSV	*Banana streak virus*
cDNA	Complementary deoxyribonucleic acid (normally synthesized from RNA)
CIRAD	Center for International Co-operation in Agronomic Research for Development
CMV	*Cucumber mosaic virus*
CTAB	Cetyltrimethylammonium bromide
CYSDV	*Cucurbit yellow stunting disorder virus*
dNTPs	Deoxynucleoside Triphosphates
dsDNA	Double stranded DNA
ECL-PCR	Electrochemiluminescence polymerase chain reaction
EDTA	Ethylene diamine triacetic acid
ELISA	Enzyme linked immuno-sorbent assay
EPRVs	Endogenous pararetroviruses
IC-PCR	Immuno-capture polymerase chain reaction
IgG	Immunoglobulin G
ISEM	Immuno-sorbent electron microscopy
LIYV	*Lettuce infectious yellows virus*
M-IC-PCR	Multiplex IC-PCR
NARO	National Agricultural Research Organisation
ORF	Open reading frame
PBS	Phosphate buffered saline
PVP	Polyvinyl pyrolidone
QUT	Queensland University of Technology

RT	Reverse transcriptase
SAS	Statistical Analysis System
ScBV	*Sugarcane bacilliform virus*
SDW	Sterile distilled water
ssDNA	Single stranded DNA
ssRNA	Single stranded ribonucleic acid
STMS	*Musa* sequence tagged microsatellite site
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TBR	Tris bipyridine ruthenium
TNA	Total nucleic acids

CHAPTER ONE
INTRODUCTION

1.1 Background information

Cultivated bananas (*Musa spp*.) originated from two wild relatives of the domesticated banana, *Musa acuminata* (AA) and *Musa balbisiana* (BB). Different combinations of these two genomes give rise to plants of various ploidies. The A genome provides parthenocarpy, fruit quality and, in some cases, disease and pest resistance traits. The addition of the B genome within the AA or AAA combinations adds hardiness and additional disease resistance characteristics to the inter-specific hybrids (CIRAD, 2002).

Bananas, a major global food staple, are the fourth most important food in the world, after rice, wheat, and maize (INIBAP, 2002). The crop is cultivated in about 121 countries (FAO, 2001), providing a major source of carbohydrates for over 400 million people in tropical countries (Swennen *et al*., 1995), 25% of these being in Africa (INIBAP, 2002). In Uganda, Burundi and Rwanda, banana consumption ranges between 250-400 kg per person per year (3 to 11 bananas daily, depending on their sizes) (INIBAP, 2002). Bananas also provide a major source of income for farmers. In Kenya, production currently stands at around 210,000 metric tonnes annually (FAO, 2004).

Bananas and plantains are popular for a number of reasons; they are one of the cheapest foods to produce; the cost of production of one kilogram of plantain for example is less than that of most other staples, including sweet potato, rice, maize and yam (Chandler, 1995). Bananas will also grow in a range of environments and will produce fruit year-round, thus providing a source of energy when other crops are not available. They are particularly suited to inter-cropping systems and to mixed farming with livestock and are also popular as a backyard crop in urban settings (Sharrock and Frison, 1999).

Banana and plantain (*Musa* spp.) and their hybrids may however be infected by a range of viruses, including *Abaca mosaic virus* (AbMV), *Cucumber mosaic virus* (CMV), *Banana streak virus* (BSV), *Banana bract mosaic virus* (BBrMV) and *Banana bunchy top virus* (BBTV). Of these, BSV seems to be the most important in Kenya (Wangai *et al*., 2002).

Banana streak disease, caused by the banana streak virus (BSV), occurs in most banana-producing countries. The disease was first identified in Morocco in 1986 (Lockhart, 1986). The

leaf-stripping symptom earlier on observed in cultivar Mysore (AAB) in Trinidad, was for a long time considered to be a physiological disorder. Lockhart (1986) later established that this was an infection by *Banana streak badnavirus* (BSV). Before BSV and the streak disease were described, streak symptoms were also confused with those of CMV.

Banana streak badnavirus infection has been associated with yield losses of 6-15% (Daniells *et al*., 2001, Dahal *et al*., 2000) depending on the cultivar, virus species and environmental conditions. Infection also reduces plant growth and vigor, bunch weight and yield (Lockhart and Jones, 1999). In Kenya, BSV has been reported to be an important limiting factor to banana production through significant reduction in yields (Wangai *et al*., 2002). A wide range of cultivars of all genotypes are susceptible including a significant proportion of the improved tetraploid *Musa* hybrids (Lockhart *et al*., 1998; Karanja *et al*., 2008).

Plate 1: Banana leaf showing the chlorotic streak symptom caused by BSV (Lockhart and Jones, 1999)

One of the features of BSV infections is the periodic appearance and disappearance of symptoms (Lockhart, 1986) resembling the behaviour of another pararetrovirus, *Cauliflower mosaic virus* (CaMV). Symptoms can be very variable, but generally consist of chlorotic and necrotic streaks on the lamina tissue (Lockhart and Jones, 1999) (Figure 1). In some severe or extreme situations, additional symptoms including distorted bunches, altered phyllotaxis,

splitting of the pseudostem, heart rot and plant death have been reported (Lockhart, 1995). Symptoms may sometimes be mild and/or irregular, occurring on only some leaves, or sporadically during the year (Daniells *et al*., 2001; Lockhart and Jones, 1999). Symptom expression in at least some cultivars and with some strains of the virus largely depends on environmental conditions. The most seemingly well studied of these conditions is temperature. In Nigeria, symptoms were found to be more prevalent and severe at lower temperatures (22°C) than at higher temperatures (28-35°C) (Dahal *et al*., 1998). However, studies in Kenya showed that BSD symptom expression in EAH-AAA cooking cultivars is enhanced by dry conditions (Daniells *et al*., 2001, Karanja *et al*., 2010).

Banana streak disease (BSD) can be controlled by the eradication of infected plants, and the use of BSV-free planting materials (Lockhart and Jones, 1999). Tissue culture techniques may however not be useful in raising disease free plants since BSV can be carried in *in vitro* plantlets, and is therefore not eliminated by shoot-tip culture (Dallot *et al*., 2001). Virus particles can only be detected in areas of leaf tissue with symptoms. Parts of leaves with pronounced symptoms should therefore be used for serological indexing.

Banana streak virus (BSV) is a pararetrovirus and member of the *Badnavirus* genus (Family: *Caulimoviridae)* (Lockhart, 1986). Badnaviruses are bacilliform and do not contain the translational trans-activator protein found in other caulimoviruses (Lockhart and Olszewski, 1999). All members of *Caulimoviridae* have an open-circular, double-stranded DNA genome, whose replication occurs via a reverse transcription (RT) step (Hohn, 1999). In plant pararetroviruses, unlike true retroviruses, integration into the host genome is not required for replication (Hohn, 1999).

Three BSV species are currently recognised, namely; *Banana streak Mysore virus* (BSMyV), *Banana streak OL virus* (BSOLV) and *Banana streak GF virus* (BSGFV) (Hull *et al*., 2005). A fourth species, *Banana streak acuminata Vietnam virus* (BSAcVNV), has also recently been proposed based on full length sequence analyses (Lheureux *et al*., 2007). Although partial sequences have been reported for several other putative virus species including the *Banana streak Cavendish virus* (BSCavV) (Geering *et al*., 2000), *Banana streak Imové virus* (BSImV) and *Banana streak Uganda A-M viruses* (Harper *et al*., 2005), the taxonomic status of these viruses remains unresolved.

The virions of BSV were first isolated in 1985 (Lockhart, 1986) and were found to be non-enveloped bacilliform particles measuring 120-150nm x 30nm and containing a dsDNA genome of approximately 7.4kb. The genome contains three open reading frames (ORFs), encoding two small proteins of 20.8kDa (ORF I) and 14.5kDa (ORF II) and a 216kDa polyprotein (ORF III) (Harper and Hull, 1998). The first two ORFs potentially code for small proteins whose functions have not been clearly elucidated. However, studies with other badnaviruses suggest that the 14.5kDa protein is incorporated into the virus coat protein and is possibly involved in virus assembly (Stavolone *et al*., 2001). The third ORF encodes for the polyprotein whose putative components in order from N- to C- termini are movement protein, coat protein, aspartate protease and replicase (Reverse transcriptase-RT and RNase H) (Medberry *et al*., 1990; Tzafrir *et al*., 1997; Harper and Hull, 1998). The polyprotein is thought to be post-translationally cleaved by the aspartate protease (AP) but the sites of cleavage have not yet been determined.

Banana streak virus is thought to be largely transmitted by the citrus mealybug, *Planococcus citri* and possibly some other mealybug species (Kubiriba *et al*., 2001). The mealybug vector is thought to spread the disease locally, though this is thought to occur very slowly and over short distances (Lockhart and Olszewski, 1993). The use of infected planting material can also be responsible for local spread. It has been reported that the disease may be seed transmitted (Daniells *et al*., 1996). Long distance spread is primarily due to the use of infected planting material, including micro-propagated plants. Tissue culture has also been implicated in activating disease in apparently healthy plants (Harper *et al*., 1999a; Ndowora *et al*., 1999).

Inter-specific *Musa acuminata*×*Musa balbisiana* genotypes, including a number of newly created hybrids, have shown a tendency to produce BSV-infected propagules from virus-free source plants propagated by tissue culture (Ndowora *et al*., 1999). Therefore infected progeny can be repeatedly obtained following genetic crosses involving virus-free *M. acuminata* and *M. balbisiana* parents. Such infections have been correlated to the presence of BSV sequences integrated into the genome of *M. balbisiana*, a widespread progenitor of natural and created hybrid banana and plantain species (Geering *et al*., 2001b). Such integrants have been found in other plants and are collectively known as endogenous pararetroviruses (EPRVs). It was recently

shown that some EPRVs have the potential to express infectious viral genomes and ultimately viral particles (Richert-Poggeler *et al*., 2003).

A high degree of genomic and serological heterogeneity among BSV isolates has meant that indexing can be problematic. Moreover, the integration of the virus genome into the host genome (Geering *et al*., 2001a; Harper *et al*., 2002a) has been making exclusive nucleic acid-based detection of the virus frustrating, as virtually all *Musa* tissues test positive to BSV. The presence of EPRVs in the host genomes arrests the detection of cognate episomal viruses by the polymerase chain reaction (PCR), since PCR will amplify both episomal viral DNA and integrated viral sequences, leading to false positives (Harper *et al*., 1999a; Yang *et al*., 2003). Serological tools such as Enzyme-Linked Immunosorbent Assay (ELISA) and Immunosorbent Electron Microscopy (ISEM) have been shown to give false negatives due to the high antigenic variability among BSV isolates (Lockhart and Olszewski, 1993). The TempliPhi technique, also called Rolling Circle Amplification (RCA), is a highly sensitive method which selectively amplifies the circular viral DNA within a total nucleic acid extract (Lizardi *et al*., 1998). This technique has been widely used in preparation of sequencing templates with high throughput systems (Dean *et al*., 2007) and has potential for use in amplification of the circular BSV genome. The TempliPhi technique has not been used before in BSV indexing.

The banana species has other infectious viruses which include *Banana bract mosaic virus* (BBrMV), *Banana bunchy top virus* (BBTV) and *Cucumber mosaic virus* (CMV). *Banana bract mosaic potyvirus* (BBrMV) is a potyvirus with flexuous filamentous virion particles that react with a general *Potyvirus* antiserum (Bateson and Dale, 1995). The virus is a member of the genus *Potyvirus* in the family *Potyviridae* (Francki *et al.*, 1991). The *Potyvirus* genome consists of a positive-sense ssRNA molecule of approximately 10kb which is polyadenylated at the hydroxyl 3' end and is translated as a single polypeptide that is post-translationally cleaved into nine native proteins (Li *et al*., 1997). BBrMV has been reported in the Philippines and India and causes yield losses of up to 40% (Roperos and Magnaye, 1991).

The Banana bunchy top virus (BBTV) belongs to the genus *Babuvirus* (Family; *Nanoviridae*) (Vetten *et al*., 2005). The virus is characterized by small (18 to 20 nm), isometric particles with a multi-component ssDNA genome comprising at least six circular, single-stranded DNA components (referred to as DNA-R, DNA-S, DNA-C, DNA-M, DNA-N and DNA-U3) (Vetten *et al*., 2005). Each component is about 1kb and has one major protein of molecular

weight 20,100. Members of the *Nanoviridae* family are characterized by multi-component single stranded DNA circular genomes encapsidated in small isometric particles.

The different strains of *Cucumber mosaic cucumovirus* (CMV) cause the banana mosaic, infectious chlorosis, and heart rot diseases (Jones and Lockhart, 1993). These diseases are present in most banana growing areas of the world. Banana mosaic disease, caused by CMV was first reported in New South Wales in 1929 and in Central America in 1957. The causal virus (CMV) has a very wide host range that serve as inoculum sources causing high frequency of infection (Jones, 1991). Numerous strains exist, varying from those inducing severe or mild symptoms to those not causing symptoms. The heart rot strain and severe leaf distortion strain found in Morocco and Taiwan are particularly destructive. The CMV with a tripartite single stranded RNA is packaged in icosahedral particles of about 28 nm in diameter. Satellite RNA, CARNA 5 has been associated with many strains of CMV, but has not been found in those strains infecting banana (Jones and Lockhart, 1993). Three strains of CMV have been identified to cause banana mosaic disease of different severity.

1.2 Statement of the problem

Banana streak disease is a wide-spread disease found infecting all members of the *Musa* genus. It has also been conclusively shown that banana streak disease poses a real challenge to the banana production industry in Kenya. An earlier study on molecular identification of Kenyan BSV isolates found seven isolates. However, the distribution pattern of BSV isolates and co-infection relationships between it and other *Musa* RNA viruses are not well understood. Since there is marked variance in the degree of virulence among the BSV isolates, the poor knowledge of their distribution pattern has significantly hindered the capacity to successfully manage banana streak disease through such strategies as quarantine measures.

The principal mode of spread of banana streak disease is by movement of diseased planting material. This stresses the importance of quarantine measures in the control of this disease. However, the success of quarantine strategies is almost entirely dependent on the availability of accurate, sensitive, low cost and simple diagnostic techniques, which enable early detection of virus infections in plant materials. Current methods of detection of BSV include visual inspection for symptoms which are highly sporadic and may be confused with those of *Cucumber mosaic virus* (CMV); immuno-sorbent electron microscopy (ISEM), which is unfortunately laborious and requires complex and expensive equipment, and the enzyme-linked

immuno-sorbent assay (ELISA) which has so far proved relatively insensitive. The serological and genomic heterogeneity among BSV isolates makes the detection of the virus difficult. Direct PCR is also limited by the integration of viral sequences in the host genome and thus it gives false positives in BSV detection.

1.3 Objectives

1.3.1 General objective

To detect disease causing viruses in *Musa* germplasm from selected areas in four major banana-growing regions of Kenya.

1.3.2 Specific objectives

1. To detect the various *Banana streak virus* isolates in samples collected from five banana-growing regions of Kenya (Nyanza, Western, Central, Rift Valley and Eastern provinces).
2. To determine the relationship between isolate type/cultivar genotype and symptom expression for banana streak disease.
3. To compare the efficacy of the TempliPhi, standard polymerase chain reaction (PCR) and immuno-capture-PCR (IC-PCR) techniques in detection of BSV.
4. To determine any co-infection relationship between the obtained BSV isolates and the *Musa* RNA viruses; BBrMV and CMV.

1.4 Hypotheses

1. There is no variation in prevalence of BSV isolates among the four banana growing regions of Nyanza, Western, Central, Eastern and Rift Valley provinces.
2. There is no relationship between isolate type/cultivar genotype and symptom expression for banana streak disease.
3. The efficiency of detection of BSV isolates in banana is the same between the TempliPhi, standard polymerase chain reaction (PCR) and immuno-capture PCR (IC-PCR) techniques.
4. There is no definite pattern of joint infection of banana by BSV isolates and other *Musa* RNA viruses; BBrMV and CMV

1.5 Justification of the study

Banana (*Musa spp*.) is one of the world's most important agricultural commodities. This tropical and subtropical crop contributes significantly to many countries' domestic and export income and in some regions is the principal source of carbohydrates. Banana streak disease, caused by the *Banana streak virus*, is a major constraint to banana cultivation, breeding programs and safe movement of *Musa* germplasm. This study aims at unveiling the distribution pattern of BSV isolates in Kenya. Knowledge of the distribution of the isolates in the Kenyan ecosystem will form the basis for development of efficient BSV management strategies. The viability of such management strategies is intimately linked to the availability of accurate, reliable and sensitive diagnostic tools. It is therefore important to establish a sensitive and accurate BSV indexing technique. Coupled with a better understanding of co-infection relationships between BSV isolates and other banana RNA viruses, the availability of a sensitive indexing tool will lead to faster detection of viruses in *Musa spp*. The findings of this study will significantly contribute to a better understanding of the epidemiology of the banana streak disease and its management in Kenya.

CHAPTER TWO
LITERATURE REVIEW

2.1 Diversity of BSV Isolates

Banana streak virus (BSV) is a plant bacilliform pararetrovirus belonging to the family *Caulimoviridae* and the genus *Badnavirus* (Hull, 1999). It is one of the five described viruses of banana (genus *Musa*) and plantain and seems to be the most widely distributed virus of these crops. This virus causes streak mosaic disease, which had until recently never been considered a serious threat to banana cultivation (Fargette *et al*., 2006). It is however now acknowledged that there is the potential for serious yield losses of up to 90% with some virus isolates (Daniells *et al*., 2001). Disease incidence varies between countries and this may be related to different virus strain, banana cultivars and vector activity (Geering *et al*., 2000).

Seven different BSV isolates with sequence identity thresholds of 90–100% have been identified in Kenya (Karanja, 2009). They include Khabusi2, BSUgAV, Lisulya, Nshule, BSImV, BSOEV and BSGfV. These were obtained after pair wise comparison of 33 sequences obtained by amplifying the RT/RNase region of the ORF 3 of the BSV genome. BSUgAV was then a known Ugandan isolate while the other six were new isolates.

Based on polymerase chain reaction (PCR) amplification with degenerate primers, followed by DNA hybridization assays, four distinct isolates of BSV were identified in a study conducted in Uganda (Lockhart, and Olszewski, 1993). Later on, 15 distinct isolates were identified in a separate study (Harper *et al*., 2005). This was after 49 samples were PCR-amplified across the conserved reverse transcriptase (RT)/RNaseH region of the genome.

These two studies in Kenya and Uganda were carried out using the Immuno-Capture Polymerase Chain Reaction (IC-PCR) and direct PCR as indexing methods. Direct PCR has the limitation of giving false positives due to the integrated BSV sequences in the *Musa* genome. IC-PCR, on the other hand, may be limited by the high serological variability of the BSV isolates (Lockhart and Olszewski, 1993). Moreover, molecular studies on *Banana streak virus* (BSV) have indicated that this virus has several unique features not found in most other plant viruses (Ndowora *et al*., 1999). It has also been shown that there are three forms of BSV, an encapsidated episomal form, an unencapsidated episomal form and an integrated form (INIBAP, 1997). The implication is that IC-PCR will only detect the encapsidated episomal form,

rendering it insensitive. This therefore calls for the development and use of alternative, potentially more sensitive detection methods.

A study of genetic variants of *Banana streak virus* in Mauritius found that some Mauritius strains were different from an earlier characterized Nigerian isolate from the banana cultivar Obino l'Ewai (BSOEV) (Jaufeerally-Fakim *et al*., 2006). This difference was on the basis of the observed size of amplicons. Both Southern blot hybridization and the nucleotide sequences of the PCR products confirmed that they were of episomal BSV origin. An isolate of *Sugarcane bacilliform virus* (SCBV) was found to be also very similar to the BSV isolated from banana samples (Jaufeerally-Fakim *et al*., 2006).

The complete genome of a Nigerian isolate of BSV (BSV-OL) has been sequenced and shown to comprise of 7389bp and to be organized in a manner characteristic of badnaviruses (Harper and Hull, 1998). Comparison of this sequence with those of other badnaviruses showed that BSV is a distinct virus (Hull, 1999). PCR with primers based on sequence data indicated that BSV sequences are present in the banana genome. Proteins encoded by BSV-Onne (currently known as BSV OL) have been shown to have the closest sequence identity to those of *Commelina yellow mottle virus* (ComYMV) (Harper and Hull, 1998).

Four isolates of BSV from Australia have been successfully cloned and sequenced. These isolates originated from banana cultivars Red Dacca, Williams, Mysore, and Gold finger, and were designated the identities BSV-RD, BSCavV-, BSMysV and BSGfV, respectively. All clones contained a sequence covering part of open reading frame III and the intergenic region of the *Badnavirus* genome. The sequence of BSV-RD showed a high identity with BSV-Onne (Geering *et al*., 2000).

2.2 Integration of badnaviruses in host genomes

Genome sequences of several pararetroviruses, including BSV (Geering *et al*., 2001b, Geering *et al*., 2005; Harper *et al*., 1999a; Ndowora *et al*., 1999), *Tobacco vein clearing virus* (Lockhart *et al*., 2000) and *Petunia vein clearing virus* (Richert-Poggeler *et al*., 1996) are found integrated in their plant host genome and are thought to be capable of activation to cause disease (Harper *et al*., 1999a). Presence of such integrated viral sequences also interferes with PCR-based detection of episomal BSV in infected banana and plantain.

The ability of BSV to cause disease is related to the presence of homologous viral sequences in the nuclear genome of the host. Stress factors such as *in vitro* propagation may

activate these viral sequences making them capable of causing episomal infection (Harper *et al*., 1999a; Ndowora *et al*., 1999; Dallot *et al*., 2001). Only *Musa balbisiana* (B genome)–containing cultivars have been known to contain these integrated, activatable sequences (Ndowora *et al*., 1999). However, there are other integrated *Badnavirus* sequences found in both *Musa* A and B genomes that have not been associated with the banana streak disease (Geering *et al*., 2001b, 2005).

2.3 Symptom expression in banana streak disease

The epidemiology of banana streak disease and its variable impact on production seems to be one of the under-researched areas. The disease has been reported to affect yield in a wide range of *Musa* genotypes in some areas while in other areas, the only cultivar affected is essentially the Mysore (Daniells *et al*., 2001). Still in some regions, banana streak disease reduces yields only slightly. It is so far not clearly understood what causes this variation. The variation in severity of BSV isolates and the consequent variation in symptom expression certainly play a role. Recent studies in Uganda suggest that heavily impacted areas might also be those in which a wide range of transmitting vectors are present (Harper *et al*., 2004).

Since BSV detection is more reliable from symptomatic than from asymptomatic samples (Lockhart, 1994), identification of factors responsible for symptom expression is important. However, all the factors that influence symptom expression are also not known (Lockhart and Jones, 1999, Ploetz, 2003). In addition to other environmental and physiological factors, temperature and genotype have been shown to influence symptom expression in BSV–infected plants. In Nigeria, greater symptom expression was observed when plants were grown at 22°C in a growth cabinet than in a screen house at 28-35°C (Dahal *et al.*, 1998).

In an earlier study in Morocco (Lockhart 1986), it had been observed that streak symptoms were more severe in leaves produced during high temperatures. In that study, it was clear that change in temperature regime rather than absolute temperature was important for expression of severe symptoms. This change in symptoms was correlated with the concentration of virus, which was higher in tissues with severe symptoms and low or absent in tissues without symptoms.

There exists a possibility that more than one isolate (normally two or three) can occur in a single cultivar. Symptom expression was found to be more severe in such situations (Karanja *et al*., 2008). Therefore, a direct correlation is possible between the number of isolates and disease

virulence. As research on the epidemiology of banana streak disease is carried out, it will be imperative to establish a precise set of conditions, both environmental and physiological, that elicit maximum symptom expression. This may incorporate, among others, factors such as water and nutrient stress, soil type, developmental stage as well as the role played by other infections.

2.4 Elimination of viruses from infected *Musa* material

In banana and plantain, meristem culture is considered to be the reference tool for virus eradication (Lockhart and Jones, 1999). Other strategies have been employed in elimination of viruses from infected plants. Cryopreservation has been used as a means to eliminate BSV and CMV from infected banana plants (Helliot *et al*., 2002). Mechanical inoculation for CMV and natural infection for BSV were carried out on banana plants of cultivar Williams (AAA Cavendish subgroup). Meristems were then excised from the infected plants and cryopreserved by vitrification. The frequency of virus eradication for CMV and BSV was 30% and 90%, respectively, following cryopreservation. Anti-retroviral and anti-hepadnavirus molecules, adefovir, tenofovir and 9-(2 phosphonomethoxyethyl)-2, 6-diaminopurine (PMEDAP), have also been efficiently used to eradicate the episomal form of *Banana streak virus* (BSV) from banana plants (Helliot *et al*., 2003). The results of these two studies were based on serological assays as the confirmation methods after treatment of the diseased plants. It would nonetheless be important to study the efficiency of cryopreservation in elimination of *Musa* viruses using a more sensitive technique such as TempliPhi.

2.5 BSV detection methods

The complexity of molecular detection of BSV is attributed to several factors among which is the presence of integrated sequences in the host genome which might give false-positives (Geering *et al*., 2001a) during PCR-based detection procedures. This is the limitation of the Polymerase Chain Reaction (PCR). Further, the high serological variability of the virus is also likely to give false-negatives during serological identification using procedures such as ELISA. To circumvent the challenge posed by the integrated sequences, detection of episomal BSV by PCR may be preceded by an immuno-capture step to prevent amplification of these sequences (Harper *et al.,* 1999b). This constitutes a technique known as immuno-capture polymerase chain reaction (IC-PCR). Despite its novelty, the IC-PCR is still deficient for BSV indexing due to the antigenic heterogeneity among the different isolates.

BSV is common and widespread in the plantain-growing area of southern Nigeria (Gauhl *et al*., 1999). Out of the 58 leaf samples taken from plants with symptoms and indexed for both BSV and *Cucumber mosaic virus* (CMV), 62% tested positive for BSV and none tested positive for CMV. In this study, Immune-Sorbent Electron Microscopy (ISEM) and Enzyme-Linked Immune-Sorbent Assay (ELISA) techniques were used as the indexing methods. Reverse transcription PCR might be a more reliable method for detection of CMV. Since ELISA has been implicated for giving false negatives in BSV detection, it is possible that the 62% was way below the true value.

As a measure to handle the indexing challenge posed by integration of BSV sequences in the host genome, a multiplex, immuno-capture polymerase chain reaction (M-IC-PCR) technique has been developed for the detection of the virus (Provost *et al*., 2006). *Musa* sequence tagged microsatellite site (STMS) primers have been selected and used in combination with BSV species-specific primers in order to monitor possible contamination by *Musa* genomic DNA, using multiplex PCR. Optimization of the immuno-capture conditions is done in order to prevent *Musa* DNA from interfering with episomal BSV DNA during the PCR step. This improved detection method successfully allows the accurate, specific and sensitive detection of episomal DNA only from distinct BSV species.

Polymerase Chain Reaction using universal or specific primers has been applied as a preceding step to the cloning of begomoviruses (Family: Geminiviridae) (Patel *et al*., 1993). Alternative to this is a method based on extraction of total DNA or impure DNA containing the replicative form of the viral genome followed by digestion with endonucleases, Southern blotting hybridization and then cloning after digestion with a single cutter enzyme (Srivastava *et al*., 1995). The low concentration of the viral genome in the preparation is however an inherent feature of this method. This translates into low efficiency during the cloning process.

Polymerase Chain Reaction-amplified DNA fragments from *Musa acuminata, M. balbisiana* and *M. schizocarpa*, as well as cultivars 'Obino l'Ewai' and 'Klue Tiparot', have been successfully cloned (Geering *et al*., 2005). About 103 sequenced clones were found to have similarity to open reading frame III in the badnavirus genome. This showed that the genomes of *M. acuminata*, *M. balbisiana* and *M. schizocarpa* contain a diverse array of endogenous badnaviruses. To ensure that no episomal sequences were present in the samples, DNA was isolated from plants that had remained free of banana streak symptoms for at least a year and had

tested negative for BSV by ISEM. The idea was to circumvent the limitation associated with PCR such that the technique may not distinguish between episomal and integrated sequences.

The sequence-independent rolling circle amplification (RCA) approach has been previously used for the detection and charactererisation of many DNA-viruses infecting humans, animals and plants (Grigoras *et al*., 2009, Johne *et al*., 2009). The complete *Begomovirus* (Family: Geminiviridae) genome has nonetheless been cloned using a simple method based on the Rolling Circle Amplification (RCA) mechanism by the bacteriophage *Phi29* DNA polymerase (Inoue-Nagata *et al*., 2003). This technique uses the TempliPhi kit, a novel commercial kit that uses an isothermal method for the exponential amplification of circular DNA. The TempliPhi technique has also been used in *in vitro* random mutagenesis to alter properties of enzymes (Fujii *et al*., 2004), amplification and cloning of *Polyomavirus* genomes (Johne *et al*., 2006). By using an isothermal multiply primed rolling-circle amplification protocol, the complete genomic DNA of a novel *Papillomavirus* was amplified from a skin lesion biopsy of a Florida manatee (*Trichechus manatus latirostris*) (Rector *et al*., 2004). This is one of the most endangered marine mammals in United States coastal waters. This method can also be applied to amplify the genome of BSV, a badnavirus in the family Caulimoviridae. No work has so far been reported on BSV detection using the TempliPhi technique in Kenya.

As a molecular tool, the TempliPhi technique has proved to be invaluable particularly during preparation of sequencing templates from bacterial cultures (Reagin *et al*., 2003). The TempliPhi DNA sequencing template amplification kit eliminates the requirement for extended bacterial growth prior to sequencing. It also saves time by eliminating the centrifugation and transfer steps normally required by the classical preparatory methods.

Electrochemiluminescence polymerase chain reaction (ECL-PCR) has also been applied in the detection of plant viruses. The approach involves the introduction of a multi-purpose nucleic acid sequence that is specific to a tris (bipyridine) ruthenium (TBR)-labeled probe into the 5´ terminal of the primers. The method has been used to detect *Banana streak virus*, *Banana bunchy top virus*, and *Papaya leaf curl virus* (Tang *et al*., 2007). Although the use of this technique showed that it could reliably identify viruses in infected plant samples, the PCR limitations for *Badnavirus* indexing still persist (Harper *et al*., 2002a).

2.6 Other *Musa* viruses

Relationships among *Musa* viruses have been reported; *Banana bract mosaic virus* (BBrMV) has been detected in some banana cultivars that exhibit CMV-like symptoms and not the characteristic bract mosaic symptom (Rodoni *et al*., 1997). The report indicates that CMV may therefore mask the development of the characteristic symptoms of BBrMV in some banana cultivars, or alternatively, the CMV-like symptoms may be due to a different strain of BBrMV. BSV symptoms are also often confused with those produced by CMV. Both viruses can cause a wide range of symptoms in banana, depending on the banana cultivar, virus strain, and environmental conditions (Hu *et al*., 1995; Singh *et al*., 1995). Another interesting relationship exists between *Sugarcane bacilliform virus* (SCBV) and BSV. SCBV is serologically related to BSV (Lockhart and Autrey, 1988) and can be transmitted from sugarcane to banana by *Saccharicoccus sacchari* (Cockerell) but not from banana to sugarcane (Lockhart and Olszewski, 1993).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

The study was carried out mainly at KARI–Njoro Kenya, in the Plant Diagnostics laboratories of the Biotechnology Section. Material was collected from various parts of the country as described in section 3.2.

3.2 Sample collection

Two batches of banana leaf samples were collected. One included samples of different cultivars in banana fields expressing banana streak disease symptoms. This sampling was carried out in farmers' fields in five major banana growing regions in Kenya: Nyanza (S00º 41.020´; E034º 47.389´), Western (N00º 45.050´; E034º 36.978´), Central (S00º 46062´; E037º 24.524´), Eastern (S001º 41564´; E037º 99826´) and Rift Valley Provinces (S00º, 20.412´; E035º, 56.077´). Samples of different banana cultivars of varying genotypes were collected from each region. Samples with varying degree of banana streak disease symptom expression were collected. The first sampling was based on the assumption that the variance in the degree of symptom severity is due to the differences in the type (or number) of isolate(s). The second sampling was done from KARI-Kisii and aimed at collecting asymptomatic samples from a field prone to banana streak disease infection. These samples (32) were of varying banana cultivars.

In both sampling events, the second last leaf was sampled because it is at the optimal stage when the virus is multiplying most actively and the plant cell walls can easily be broken to release the virus.

For the symptomatic samples, BSV symptom descriptions on all the samples were recorded. To quantify symptom severity, each sample was scored on a scale of 0-5 (Karanja, 2009), where 0 is no symptoms, 1 is localised flecks, 2 is scattered discontinuous streaks, 3 is continuous streaks covering moderate portion of lamina, 4 is continuous chlorotic streaks, and 5 is necrotic streaks as shown in Figure 2.

Details of the locality and the cultivar genotype were recorded for all collected samples. The samples were then packed in silica gel bottles and transported to Kenya Agricultural Research Institute (K.A.R.I.), Njoro. Indexing was done for BSV using the TempliPhi method for all the symptomatic samples as described in Section 3.4. Other two *Musa* viruses (CMV and

BBrMV) were also indexed for the symptomatic samples as described in Section 3.6. The asymptomatic samples were indexed for BSV using both TempliPhi and IC-PCR (Section 3.6) for comparison.

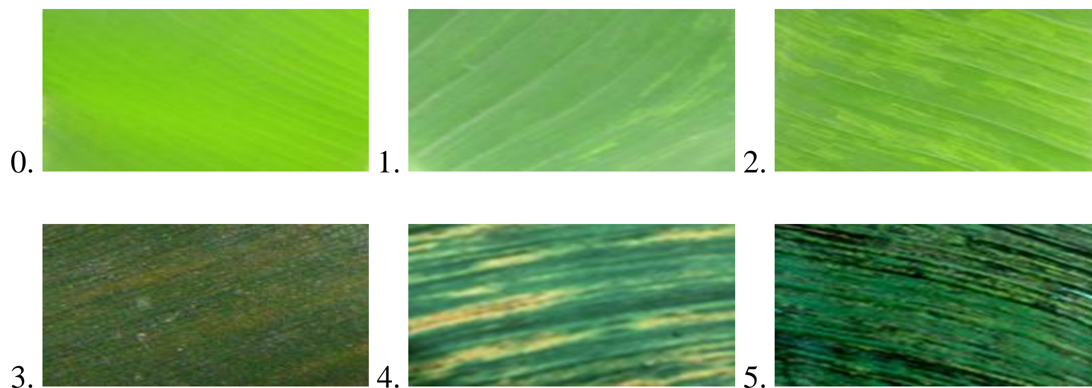

Plate 2: Banana streak disease (BSD) symptom severity scoring scale. 0 is no symptoms, 1 is localised flecks, 2 is scattered discontinuous streaks, 3 is continuous streaks covering moderate portion of lamina, 4 is continuous and conspicuous chlorotic streaks, 5 is necrotic streaks (after Karanja 2009).

3.3 Nucleic acids extraction

In an attempt to establish an optimal method for banana nucleic acid isolation, two protocols were evaluated. In the first approach (Queensland University of Technology Banana protocol), total banana nucleic acid was isolated using the Cetyltrimethylammonium bromide (CTAB) protocol described by Gawel and Jarret (1991) as modified by James *et al*. (2011). Dry leaf tissue (0.05g) was ground in 300ml of extraction buffer and liquid nitrogen. The extraction buffer components are shown in Appendix 1. During buffer mix preparation, all ingredients except CTAB were mixed and made up to 450 ml using deionised water. The 10g of CTAB was then dissolved in 50ml of water in a falcon tube and mixed gently to avoid foaming. The CTAB was then combined with the rest of the buffer solution and mixed well. The slurry of ground leaf tissue in extraction buffer was incubated at 65 °C for 15 minutes, spinned at 13000 rpm and 750 µl transferred to a fresh tube. This was then mixed with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged for 5 minutes at 13000 rpm. Nucleic acids in the aqueous phase was then pelleted by isopropanol and centrifuged for another 5 minutes at 13000 rpm. The DNA pellet was washed with 500µl of 70% ethanol, and resuspended in 100µl of nuclease-free water.

The DNA was left at 4°C overnight to fully dissolve. The total nucleic acid extracts for the 32 samples collected from Kisii (samples for comparison of techniques) were treated with 10mg/ml of ribonuclease A (RNase A) since only DNA was of interest for these samples. Two µl of the RNase A were incubated with 100µl of the total nucleic acid extract at 37°C for 2 hours. The nucleic acids were then stored at -20°C.

The second approach (NARO CTAB protocol), was also CTAB-based but with some variations with regard to centrifugation periods and the precipitation method. The spinning time for all the centrifugation steps (except the final washing step) was 10 minutes instead of the 5 minutes in the earlier approach. Precipitation of the nucleic acids was by means of 0.5M NaCl in place of isopropanol.

The quality and intactness of the nucleic acid extracts was tested by both electrophoresis on 1% agarose gels and PCR amplification for two house-keeping genes (ribulose-1,5-biphosphate carboxylase and actin) using a number of randomly selected samples (8-10). Amplification of the actin gene by PCR was carried out as described. A PCR master mix of 12.55µl was prepared comprising of 1.25µl of PCR buffer, 0.5µl dNTPs, 0.75µl of 50mM $MgCl_2$, 0.25µl of banana actin forward and reverse primers (shown in Appendix 2), 0.05µl *Taq* (5U per µl), 8.5µl SDW and 1µl of the total banana nucleic acid extract. The PCR cycling conditions for the actin gene PCR were an initial denaturation at 94ºC for 2 minutes, 35 cycles (94ºC for 20 seconds, 57ºC for 20 seconds and 72ºC for 30 seconds) and a final extension at 72ºC for 3 minutes. On the other hand, RT-PCR for the photosynthetic ribulose-1,5-biphosphate carboxylase (rubisco) gene was used to test the quality of the RNA in the total banana nucleic acid extract. The CTAB extract was first denatured at 99ºC for 1 minute and immediately placed on ice for 5 minutes prior to cDNA synthesis. The cDNA synthesis system comprised of 4µl 5X RT buffer, 3µl 50mM $MgCl_2$, 0.5µl *Avian myeloblastosis virus* Reverse Transcriptase (RT) (Qiagen), 0.25µl 10mM dNTPs and 7.25µl of SDW. The temperature regimes for cDNA synthesis were 25°C for 5 minutes; 42°C for 60 minutes; then 70°C for 15 minutes. One µl of the cDNA was used in the subsequent PCR step. The PCR master mix consisted of 1.25µl PCR buffer, 0.5µl 10mM dNTPs, 0.75µl 50mM $MgCl_2$, 0.25µl of banana rubisco RNA forward and reverse primers (10pmol/µl) (sequences shown in Appendix 2), 0.05µl *Taq* (5U per µl), 8.5µl SDW and 1.5µl of total nucleic acid extract. The cycling conditions for the rubisco PCR were an initial denaturation at 94ºC for 2 minutes, 30 cycles (94ºC for 20 seconds, 57ºC for 20 seconds

and 72ºC for 30 seconds) and a final extension at 72ºC for 3 minutes. Electrophoresis for both actin and rubisco PCR products was carried out on 1.5% agarose gels with either Tris-Acetate EDTA (TAE) or 1× Tris-Boric EDTA (TBE) as running buffers (buffer composition shown in Appendix 1). It is worth noting that rubisco RT-PCR was only carried out on the representatives of the 65 total nucleic acid extracts.

The 32 DNA extracts were subjected to spectrophotometric analysis to determine their purity based on the OD260/280nm ratio. A sample was prepared by diluting 1µl of DNA in 750µl of SDW and the absorbance read at the two wavelengths one at a time.

3.4 The Rolling Circle Amplification (TempliPhi)

The full *Banana streak virus* genome was amplified and isolated using the TempliPhi Kit (GE Healthcare, Buckinghamshire, United Kingdom) according to James *et al*. (2011). Two mixes were prepared. For master mix 1 (MM1), 5µl of TempliPhi sample buffer was mixed with 1µl of the isolated sample and 1µl of a 50 µM stock solution (4.16 pmol/ul of each primer) of TempliPhi degenerate primers (Appendix 2). The mix was then heated at 95ºC for 3 minutes to denature the DNA followed by cooling to room temperature or 4ºC. Denaturation was necessary because BSV genome is double stranded. Master Mix 2 (MM2) was prepared by mixing 5µl of TempliPhi reaction buffer and 0.2µl of TempliPhi Enzyme Mix. Five µl of the TempliPhi premix (mix 2) was transferred to the cooled, denatured sample (MM1) then incubated at 30ºC for 18 hours. After the incubation period, the enzyme (*Phi29* DNA polymerase) was heat-inactivated at 65ºC for 10 minutes. The samples were then cooled and stored at 4ºC.

3.5 Restriction enzyme digest and gel electrophoresis

Twenty five µl of the TempliPhi reaction product from each of the symptomatic samples were incubated separately with the restriction enzyme *Stu* I (Gibco BRL, Eggenstein) for 2 hours. A 20 µl aliquot of the digested TempliPhi product was mixed with 2µl of 5× gel loading dye (Biolabs) and electrophoresed for about 20 minutes at 100V on a 1% SYBR Safe-stained agarose gel using 1× TBE or TAE as the running buffers (buffer components shown in Appendix 1). The gel was visualised under ultra violet (UV) illumination with Gel Doc (BIO-RAD) Software (USA). Internal standards (positive controls) for BSV isolates generated using *Stu*I (New England BioLabs) were used to identify the isolate(s) present in each sample based on the

published isolates (James *et al*., 2011). The distribution and the relationship between isolate type/banana cultivar genotype and symptom expression were determined using this data.

3.6 Comparison of *Banana streak virus* indexing techniques

Three BSV indexing techniques (TempliPhi, IC-PCR and direct PCR) were separately carried out on 32 asymptomatic but possibly infected samples to compare the efficacy of the methods in BSV detection. Each treatment was replicated three times. TempliPhi was done as described in Section 3.4. TempliPhi products digested with *Stu* I were electrophoresed on 1% SYBR Safe-stained agarose gel as described in Section 3.5.

For IC-PCR, the sap from all the asymptomatic samples was extracted by grinding 1 g of silica gel-dried leaf sample in 5ml of the BSV extraction buffer (components shown in Appendix 1). Before carrying out the IC-PCR on all the samples, optimization was done for the antibody and antigen in concentrations of 5:1000, 10:1000 and 15:1000 in a carbonate coating buffer (components shown in Appendix 1). The optimization was carried out on infected symptomatic banana tissues. Thin-walled polypropylene microfuge tubes were coated with polyclonal antibodies generated against BSV. The tubes were then washed three times with 100µl of PBS-Tween-20 (PBS-T). Sap extract (about 100µl) of the samples was added to each tube and the tubes incubated at 37ºC for 3 hours. The tubes were again washed twice with PBS-T, once with sterile distilled water (SDW) and then dried briefly before carrying out PCR directly in the tubes (Harper *et al*, 2002b).

The PCR step was carried out using both degenerate and isolate-specific BSV (Gold finger) isolate primers in separate reactions. Two pairs of different types of BSV degenerate primers (Badna and Harper's 1A/4') were first evaluated. The Badna pair appeared to give more consistent results and was therefore used in subsequent experiments. Primer sequences and expected amplicon sizes are shown in Appendix 2. Experiments for each primer pair were also replicated thrice. A 20µl PCR mix contained 10µl of Go *Taq* Green master mix (Promega), 0.5µl of each primer at a concentration of 10pmol/µl, 8µl of SDW and 1µl of template DNA. The PCR cycle conditions were an initial denaturation at 94ºC for 5 minutes, 5 cycles (94ºC for 30 seconds, 37ºC for 30 seconds and 72ºC for 1 minute), followed by 30 cycles (94ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 1 minute) and a final extension at 72ºC for 10 minutes. The PCR products were electrophoresed on a 1.5% SYBR Safe-stained agarose gel as described in Section 3.5.

Direct PCR was carried out on the 32 asymptomatic samples using the Badna degenerate primers only. The PCR mix comprised of 5µl of Top *Taq* master mix (Qiagen), 0.25µl of each primer at a concentration of 10pmol/µl, 3.5µl of SDW and 1µl of template DNA. The PCR cycling regimes were an initial denaturation at 94ºC for 3 minutes, 35 cycles (94ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 1 minute) and a final extension at 72ºC for 3 minutes. The PCR products were electrophoresed on a 1.5% ethidium bromide-stained agarose gel as described earlier.

3.7 Detection of *Banana bract mosaic virus* (BBrMV) and *Cucumber mosaic virus* (CMV)

Indexing for BBrMV and CMV was carried out on the first batch of samples (the 65 symptomatic samples). Total nucleic acids were extracted from 0.05g of dry leaf tissue using the CTAB protocol as described in Section 3.3, resuspended in sterile distilled water, and analyzed spectrophotometrically for purity and concentration. RT-PCR was run using a pair of primers (forward and reverse) each for the Rubisco gene, BBrMV and CMV.

A house keeping positive control RNA (Rubisco) was used to confirm the presence of RT-PCR quality RNA in the nucleic acid extracts used for virus indexing. Reverse primers for Rubisco, CMV and BBrMV were used in a single cDNA synthesis reaction to lower the cost of indexing.

The reverse transcriptase (RT) master mix for the detection of CMV and BBrMV comprised of 4µl 5× RT-PCR buffer, 3µl 50mM $MgCl_2$, 0.5µl RT (*Avian myeloblastosis virus*), 0.25µl 10mM dNTPs, 0.25µL 40U/µl RNAsin, 7µl dH_2O. The mixture was incubated in a thermal cycler at 25ºC for 5 minutes; 42ºC for 60 minutes; then 70ºC for 15 minutes. One µL of the resulting cDNA was used in a PCR reaction using Go *Taq* green with virus specific primers shown in Appendix 2.

Ten milli molar (mM) of the RT-PCR buffer was prepared by mixing 3g Tris-HCl and 0.38g KCl in a 150ml beaker and made up to 100 ml. The pH was then adjusted to 8.3 with HCl. About 0.5ml of 1M dithiothreitol (DTT) stock solution was added to 10 ml of the solution prepared in the 150mL beaker.

The PCR reaction mix constituted of 10µl of Go *Taq* green master mix, 0.5µl 10µM primer mix, 8.5µL sterile dH_2O and 1µL of the cDNA. The mix was incubated in a thermal cycler at initial denaturation at 94ºC for 2 minutes, 30 cycles (94ºC for 20 seconds, 57ºC for 20

seconds and 72ºC for 30 seconds) and a final extension at 72ºC for 2 minutes.. The PCR products were analysed on a 1.5% SYBR Safe-stained gel alongside the 200bp Easyladder (Bioline).

3.9 Data analysis

Data for identification of isolates and geographical distribution were presented as percentages in tables. Analysis of variance (ANOVA) was done using the Statistical Package for Social Sciences (SPSS version 15) to determine the relationships among symptom expression, BSV isolate and host genotype. For comparison of detection techniques, positive and negative detections were presented as 1 and 0 respectively for all the 32 samples. Means were separated based on the least significant difference (LSD) using the Statistical Analysis System (SAS version 2003) software in order to compare the means of the detection techniques. The comparison data was also expressed as percentage detections for each technique.

CHAPTER FOUR
RESULTS

4.1 BSV detection and symptoms

Data on BSV detection and symptom expression in samples from different banana growing regions is presented in Table 1. The assayed samples covered a range of *Musa* genotypes with most being of the triploid AAB genotype. Most of these AAB bananas were of the Sukari ndizi cultivar. For symptomatic samples, symptom severity scores ranged from 0-5 with symptoms ranging from mild to severe with chlorotic and necrotic streaking being the most prevalent manifestation. The streaks varied from being fine, long and narrow to short and broad. Flecking was observed for some samples (Table 1). The most common BSV isolates in the assayed samples were BSMysV and BSGfV. The other viruses assayed for; BBrMV and CMV were not detected in all the samples.

The asymptomatic samples were collected from a banana field which had a history of banana streak disease; four months before the sampling, most of the plants in this field were expressing symptoms.

Table 1: Virus detection data for 65 symptomatic banana samples collected from various banana growing regions of Kenya

Cultivar	Genome	Symptom description	Symptom severity	Locality	BSV Isolate identified	RT-PCR	
						BBrMV	CMV
Sukari ndizi	AAB	Mild yellow streak few aphids found	2	Nyamira	BSGfV	-	-
Mysore	AAB	Yellow streaking with leaf die back	4	KARI- Kisii	BSMysV	-	-
PAZ-Giant	AAA	Mild streak	1	KARI- Kisii	uncertain	-	-
Sukari ndizi	AAB	chlorotic streaking	4	KARI- Kisii	BSOEV	-	-
Sukari ndizi	AAB	Severe streaking all stems affected	5	Ibonda	BSMysV	-	-
Yangambi	AAB	Flecks	1	KARI-Kakamega	BSGfV	-	-
Sukari ndizi	AAB	Long chlorotic streaks	4	Kakamega	BSOEV	-	-
Sukari ndizi	AAB	Mild streak	2	Mumias	BSGfV	-	-
Sukari ndizi	AAB	Necrotic streaking	5	Mumias	BSMySV	-	-
Sukari ndizi	AAB	Discontinuous chlorotic streaks	2	Maara	BSGfV	-	-
Bokoboko	AAB	Severe chlorosis	4	Mumias	BSOEV	-	-
Sukari ndizi	AAB	Chlorotic streaks	4	Busia	-ve	-	-
Sukari ndizi	AAB	Severe streaking	5	Bungoma West	BSGfV and BSMysV	-	-
Libururu	EAH-AAA	Severe chlorosis	4	KARI-Mtwapa	BSMysV	-	-
Sukari ndizi	AAB	Severe streaking	5	Maragua	BSMysV	-	-
Mysore	AAB	Severe streaking	5	Murang'a	uncertain	-	-
Kibutu	AAB	Severe streaking	5	Kirinyaga	BSMysV	-	-
Mysore	AAB	Continuous streaks	3	Kirinyaga	BSGfV	-	-
Kampala	AAA	Mild flecks	1	Kirinyaga	BSOEV	-	-
Kampala	AAA	Chlorotic and necrotic streaking	5	Kirinyaga	BSMysV	-	-

Cultivar	Genome	Symptom description	Symptom severity	Locality	BSV Isolate identified	RT-PCR	
						BBrMV	CMV
Uganda green	EAH-AAA	Continuous streaks	3	Kisii	BSGfV	-	-
Jamaga	EAH-AAA	Mosaic and chlorosis	1	Kisii	BSMysV	-	-
Gikanda	EAH-AAA	Mild chlorosis	1	Kirinyaga	uncertain	-	-
Gikanda	EAH-AAA	necrosis	5	Nyeri	-ve	-	-
Sukari ndizi	AAB	Mild streak	2	Kathiani	-ve	-	-
Cavendish	AAA	Broad discontinuous streaks	2	Embu	-ve	-	-
Uganda green	EAH-AAA	Mild flecking	1	Kisii	-ve	-	-
Uganda green	EAH-AAA	Yellow streaking	4	Kisii	-ve	-	-
Lisulya	EAH-AAA	Yellow flecking, die back	1	Kisii	-ve	-	-
Mysore	AAB	Necrosis	5	Kisii	-ve	-	-
Mysore	AAB	Severe streaking	5	KARI-Kakamega	BSMysV	-	-
Sukari ndizi	AAB	Severe streaking	5	Bumula	-ve	-	-
Mysore	AAB	chlorotic and necrotic streaks	5	KARI-Kisii	-ve	-	-
Sianamule dessert	ABB	chlorotic and necrotic streaking	5	Kakamega	BSMysV	-	-
Sukari ndizi	AAB	Localised flecks	1	Mumias	BSGfV	-	-
Lisulya	EAH-AAA	mild streak	2	Kisii	BSMysV	-	-
Uganda green	EAH-AAA	yellow streak	4	Kisii	BSGfV	-	-

Cultivar	Genome	Symptom description	Symptom severity	Locality	BSV Isolate identified	RT-PCR	
						BBrMV	CMV
Mysore	AAB	Severe streaking	5	KARI-Kisii	-ve	-	-
Cavendish	AAA	chlorotic and necrotic streaking	5	KARI-Kakamega	BSMysV	-	-
Sukari ndizi	AAB	chlorotic and necrotic streaking	5	Bungoma West	BSMysV	-	-
Mysore	AAB	Severe streaking	5	KARI-Kisii	BSMysV	-	-
Mysore	AAB	Severe streaking	5	KARI-Kisii	BSOEV	-	-
Sukari ndizi	AAB	Conspicuous chlorotic streaks	4	KARI-Kisii	BSMysV	-	-
Solio	EAH-AAA	Mild streak	2	KARI-Njoro	BSGfV	-	-
Gold Finger	AAAB	Chlorotic streaks	4	KARI-Kisii	BSOEV	-	-
Solio	EAH-AAA	Discontinuous streaks	2	KARI-Njoro	BSOEV	-	-
FHIA 18	AAAB	Continuous streaks	3	KARI-Njoro	BSMysV	-	-
UG	EAH-AAA	Mild chlorosis	2	KARI-Kisii	BSGfV	-	-
Mysore	AAB	chlorotic and necrotic streaks	5	KARI-Kisii	BSMysV	-	-
Nusu Ng'ombe	EAH-AAA	Chlorotic and necrotic streaks	5	KARI-Njoro	BSMysV	-	-
FHIA 18	AAAB	Necrotic streaks	5	KARI-Njoro	BSMysV	-	-
UG	EAH-AAA	Continuous streaks	3	KARI-Kisii	BSGfV	-	-
Solio	EAH-AAA	Mosaic and flecks	1	KARI-Njoro	BSMysV	-	-
Mysore	AAB	Continuous streaks	3	KARI-Kisii	BSGfV	-	-
FHIA 18	AAAB	Mild chlorosis	1	KARI-Kisii	BSMysV	-	-
Nshule	AAA	Severe chlorotic streaks; a few mealy bugs found at this stool	4	KARI-Kisii	BSMysV, BSGfV, BSOEV	-	-

Cultivar	Genome	Symptom description	Symptom severity	Locality	BSV Isolate identified	RT-PCR	
						BBrMV	CMV
FHIA 18	AAAB	Mild chlorosis	1	KARI-Njoro	-ve	-	-
Solio	EAH-AAA	Mild chlorosis on young leaves	1	KARI-Njoro	BSOEV	-	-
Nusu Ng'ombe	EAH-AAA	Flecks	1	KARI-Njoro	BSMysV	-	-
FHIA 18	AAAB	Streaking only on older leaves	3	KARI-Njoro	BSOEV	-	-
Musera	AAA	Chlorotic streaks	4	KARI-Kisii	Uganda L	-	-
Musera	AAA	Chlorotic streaks	4	KARI-Kisii	BSGfV	-	-
Nshule	AAA	Severe chlorosis and streaking	4	KARI-Kisii	BSMysV	-	-
Gold finger	AAAB	Mild flecks	1	KARI-Kisii	BSGfV	-	-
Solio	EAH-AAA	Discontinuous streaks	2	KARI-Njoro	BSOEV	-	-

Key:

BBrMV *Banana bract mosaic virus*

BSMysV- *Banana streak virus* Mysore isolate

BSGfV *Banana streak virus* Gold finger isolate

CMV *Cucumber mosaic virus*

BSOEV- *Banana streak virus* Obino l'Ewai isolate

UG Uganda Green

FHIA Fundacion Hondurena de Investigacion Agricola

EAH East Africa Highland

4.2 Nucleic acid and banana sap extraction

The NARO CTAB protocol was used to isolate nucleic acids from all the collected samples. Initial attempts to grind the samples in acid-washed sand gave DNA that was degraded (as shown in Figure 3A). The extent of degradation reduced significantly when the samples were ground in the extraction buffer (Figure 3B). However this modification required more time since the sample had to be soaked for about 5 minutes in the buffer before grinding. Actin gene PCR for DNA samples selected randomly gave expected 664bp amplicons as shown in Figure 3C, confirming that the DNA was intact and of PCR-quality.

The RNase treatment step associated with most DNA isolation procedures was omitted in this protocol for one good reason: the RNA viruses were of interest. The single extract was therefore used to index for both the RNA and DNA viruses, which was cost saving.

The ideal way was to purify the virion particles prior to IC-PCR. However, such procedures are usually expensive and require highly specialized labour. For this reason, a sap extract was obtained for the samples on which IC-PCR was to be carried out. This sap was used in the immuno-capture step.

The immuno-capture step appeared to be more effective by using sap from fresh samples than that from dry samples. Since all the collected samples were dry, optimization was done with regard to the antigen: antibody ratio. A ratio of 1:100 was observed to be optimal for the antigen/antibody reaction. This ratio was used for all the IC-PCR assays.

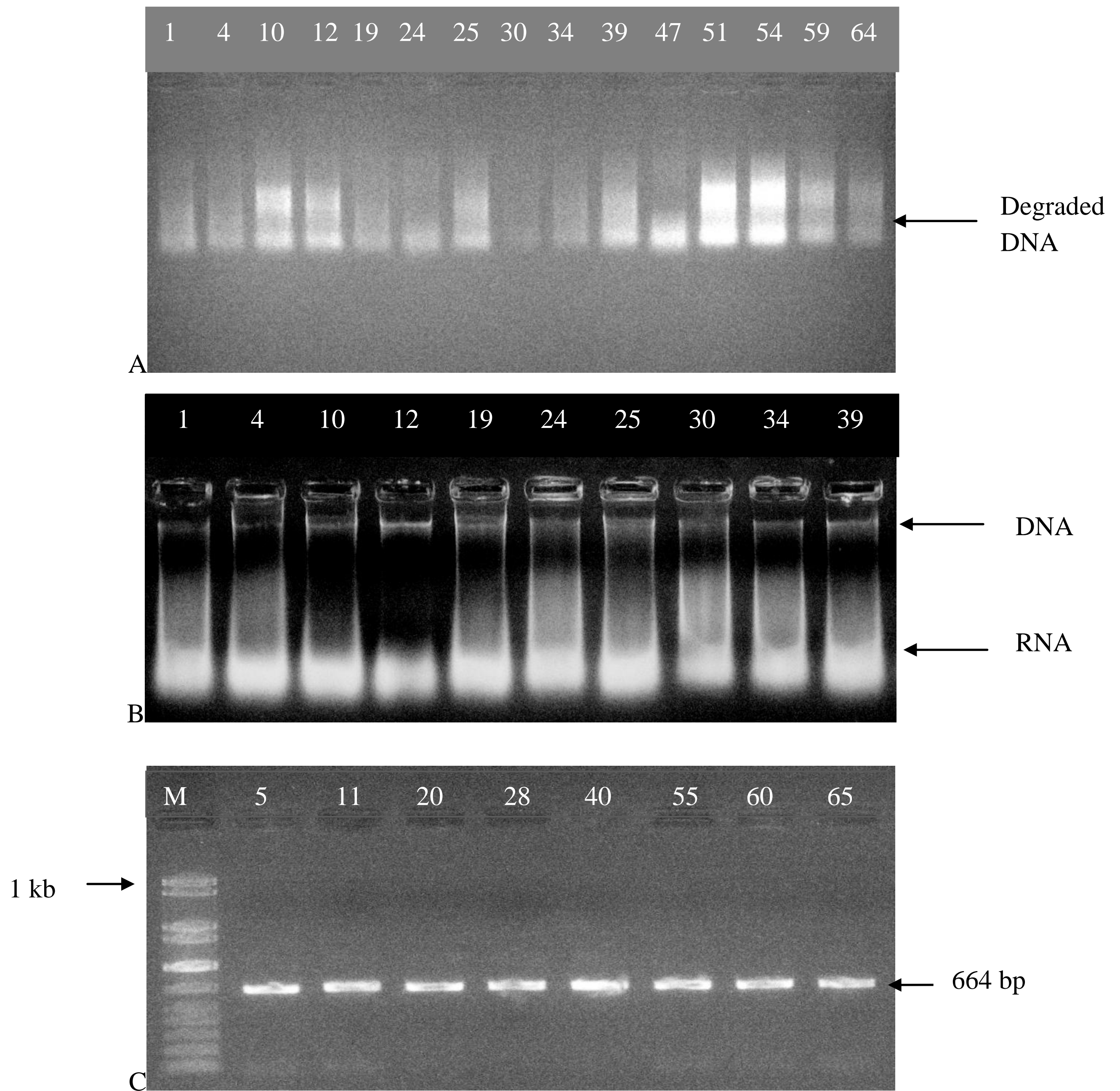

Figure 1: A- Quality check for total nucleic acids (TNA) in 15 samples isolated by the NARO CTAB protocol. B-Quality check for TNA isolated using the same protocol but with the sample being ground directly in the CTAB buffer. C-Actin gene PCR for DNA samples selected randomly. Mr is 1kb Plus molecular marker.

The data on optical properties of the isolated nucleic acids is presented in Table 2. The values obtained for the OD260/280nm ratio for ten out of 32 randomly selected samples fell in

the range of 1.8-2.0. This implies that the DNA extracts were generally of an acceptable degree of purity (Table 2).

Table 2: Absorbance readings at both 260 and 280nm and the ratio of OD260/280nm for 10 randomly selected DNA samples using a UV spectrophotometer (Pharmaspec 1700-A11024302429).

Sample Name	Absorbance (280nm)	Absorbance (260nm)	OD260/280nm
Gold finger	0.105	0.189	1.800
Nshule	0.144	0.274	1.903
Gold finger	0.148	0.274	1.851
Sukari ndizi	0.172	0.336	1.953
FHIA 18	0.175	0.348	1.999
Solio	0.163	0.293	1.798
Mysore	0.153	0.298	1.948
Musera	0.182	0.328	1.802
Mysore	0.163	0.294	1.804
Gold finger	0.194	0.367	1.892

4.3 Identification of BSV isolates

Various restriction fragment profiles were obtained when Rolling Circle Amplification (TempliPhi) products were digested with *Stu*1 as shown in Figure 4. Most of the indexed samples (83.1%) gave observable profiles on agarose gel after *Stu*1 restriction enzyme digest. About 17% of the samples were consistently negative (Table 1). The obtained profiles were compared to the available *Stu*1 standards (James *et al*., 2011) and only 3 of the positive samples gave profiles that could not be identified with any of the available standards.

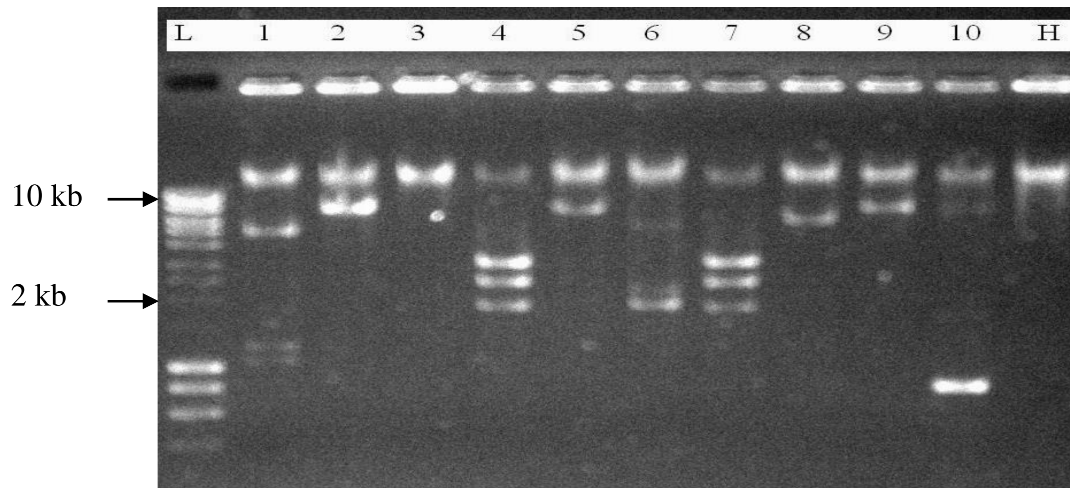

Figure 2: -TempliPhi-RFLP for samples 1-10 using *Stu*1. Lane H represents a healthy (negative) control (DNA extract of a BSD-free *Musa* tissue). L is a molecular marker (Bioline Hyperladder).

4.4 Distribution of BSV isolates

The Mysore isolate was found to be the most widely distributed in the Kenyan BSV ecology as shown in Table 3. Of all the 50 symptomatic samples that gave positive detections, 48.0% showed a profile corresponding to the Mysore isolate (BSMysV) standard profile. The percentage incidences for Gold finger, Obino l' Ewai and Uganda L isolates were 30.0%, 22.0% and 2.0%, respectively.

In terms of geographic distribution, it was observed that most of the samples collected from the Nyanza (Kisii) regions were infected with the Mysore and Gold finger isolates. A restriction fragment profile of a very rare BSV isolate namely Uganda L was observed for one of the symptomatic samples (sample 61).

Table 3: Percentage geographic distribution of three BSV isolates in 5 regions of Kenya and their percentage infection

Region	Isolate		
	BSMysV	BSGfV	BSOEV
Nyanza	1.59*	1.73	1.57
Western	1.48	1.44	1.28
Eastern	0.00	0.89	0.00
Central	1.13	0.89	1.00
Central Rift	1.34	0.89	1.57

**The percentage data was transformed using the Log (x+1) transformation.*

4.5 Relationship between genotype and symptom severity

Symptom severity of individual leaf samples were scored on a scale of 0-5; a modification of Karanja, (2009). The analysis (Table 4) was done based on the assumption that the samples were infected at almost the same time. From the results obtained, symptoms were observed to be more severe in *Musa* cultivars with AAB (with highest symptom expression mean of 3.97±0.251). Of the 28 samples with the AAA genotype, 28.6% merely expressed localised flecks and mild chlorosis (category 1). For the samples with AAB genotypes, only two out of twenty nine samples expressed category 1 symptoms. The relative sample size for the AAAB cultivars was not sufficient to make a conclusion as to how these tetraploids respond to BSV infection with regard to symptom expression. Analysis of variance (ANOVA) with regard to symptom expression showed that the three cultivar groups differed significantly ($P < 0.05$) (Appendix 3).

Table 4: Mean symptom expression degree (based on scale 0-5) for samples of three banana genotypes (AAA, AAB and AAAB)

Genotype	**N**	**Mean***	**95% Confidence Interval for mean**	
			Lower	**Upper**
AAA	28	2.61±0.283	2.03	3.19
AAB	29	3.97±0.251	3.45	4.48
AAAB	7	2.57±0.612	1.07	4.07
Total	64	3.22±0.197	2.82	3.61

**Mean symptom expression was determined based on a scale of 0-5* (Karanja, 2009).

4.6 Symptom severity and isolate type

The results presented in Table 5 suggest that the Mysore isolate generally causes more severe symptoms than the other BSV isolates (with highest mean of 4.04±0.280). The proportion of the samples infected with the Mysore isolate which expressed category 5 symptoms (necrotic streaks) was 56% compared to 18.75% and 18.2% for Gold finger and Obino l' Ewai, respectively. The mean symptom expression degree for BSOEV was 3.09±0.415 while that for BSGfV was the lowest at 2.38±0.301. Analysis of variance (ANOVA) with regard to symptom expression showed that the three BSV isolates differed significantly ($P < 0.05$) (Appendix 3).

Table 5: Mean symptom expression degree (based on scale 0-5) for banana samples infected with three common BSV isolates (BSMysV, BSGfV and BSOEV)

BSV Isolate	N	Mean	95% confidence interval for mean	
			Lower	Upper
BSMysV	25	4.04±0.280	3.46	4.62
BSGfV	16	2.38±0.301	1.73	3.02
BSOEV	11	3.09±0.415	2.17	4.01
Total	52	3.33±0.209	2.91	3.75

4.7 Comparison of Templiphi, IC-PCR and direct PCR

An evaluation of two degenerate primer pairs used for IC-PCR and direct PCR showed that the badna primers were better than 1A/4' primers. Results by the badna primers were reproducible and the amplicons on agarose gel were more distinct and clearer than those for 1A/4' (Figure 5)

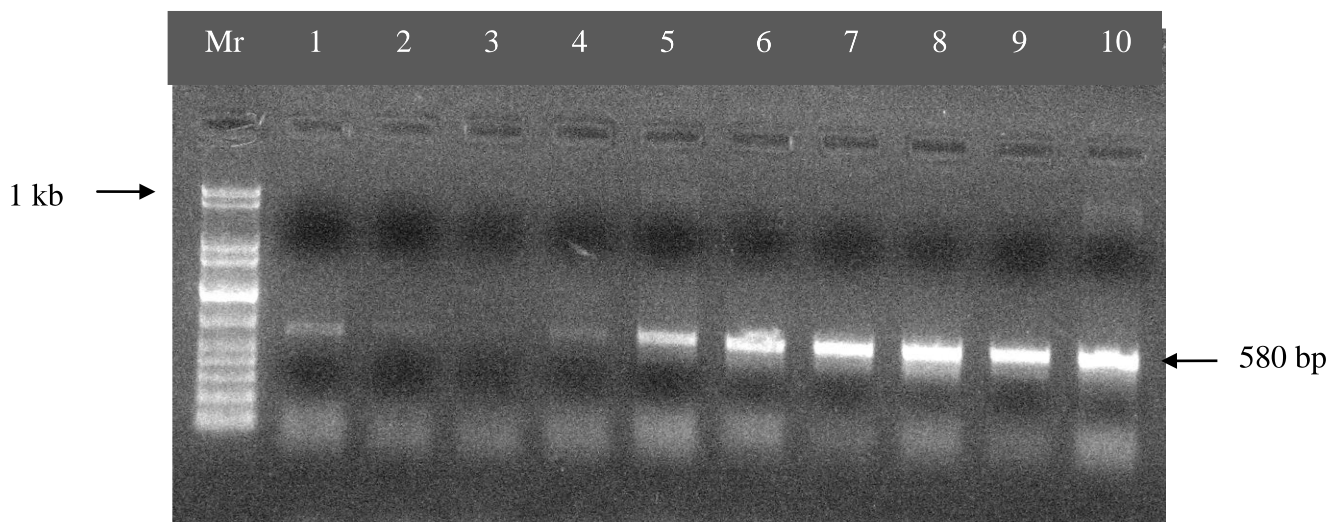

Figure 3: Gel picture showing performance of two BSV degenerate primer pairs. Five BSV-symptomatic samples were used for this evaluation. Lanes 1-5 represent 1A/4' primers while lanes 6-10 represent badna primers. Mr is a molecular marker.

Results of the thirty two asymptomatic (but possibly BSV-infected) samples indexed for BSV using IC-PCR (using both degenerate and Gold finger primers), direct PCR and TempliPhi (RCA) are shown in Figures 6-9 and Table 6. Direct PCR gave 93.8% detection with the badna degenerate primers. TempliPhi (RCA) detected the virus in 37.5% of the samples compared to the 31.3% with IC-PCR (degenerate primers) and the 14.1% detections with IC-PCR (Gold finger primers). Analysis of variance (ANOVA) for comparison of the four techniques showed that there was significant difference ($P<0.05$) among all the means (Appendix 3). The ANOVA also showed that some of the 32 asymptomatic samples differed significantly ($P<0.05$) from one another.

IC-PCR with Gold finger primers revealed a 476 bp amplicon on a 1.5% ethidium bromide-stained agarose gel for some samples (Figure 8). These positives were obtained only in samples 10, 12, 19, 20, 27 and 28. However the detections observed in samples 27 and 28 were confirmed as false positives by a repeat of the same experiment using IC-PCR with degenerate primers and by the rolling circle amplification (RCA) technique. The BSV detection results for the 32 asymptomatic samples assayed using three methods are summarized in Table 6.

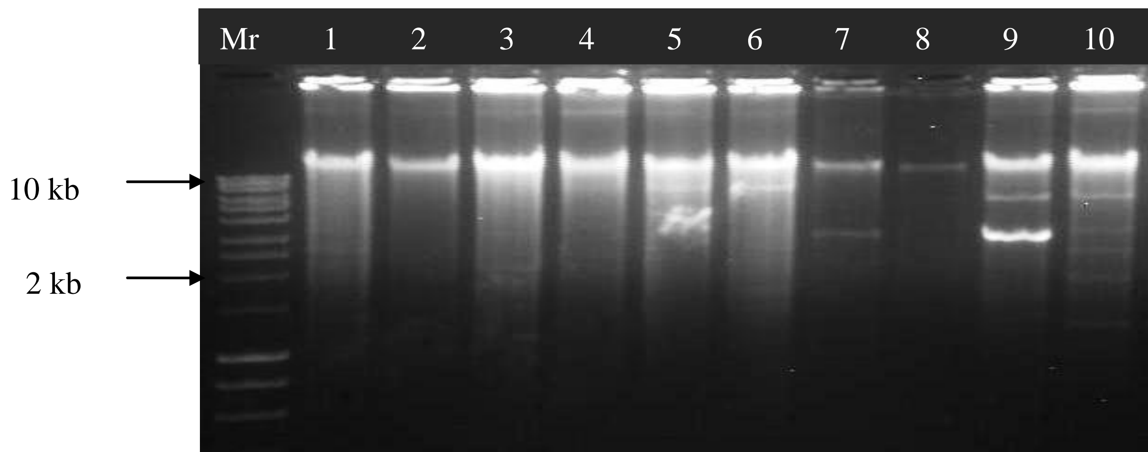

Figure 4: TempliPhi (Rolling Circle Amplification) for samples 1-10. Samples 5, 6, 7, 9 and 10 were positive, giving various isolate-specific restriction profiles. Mr is a 10 kb molecular marker (Bioline Hyperladder).

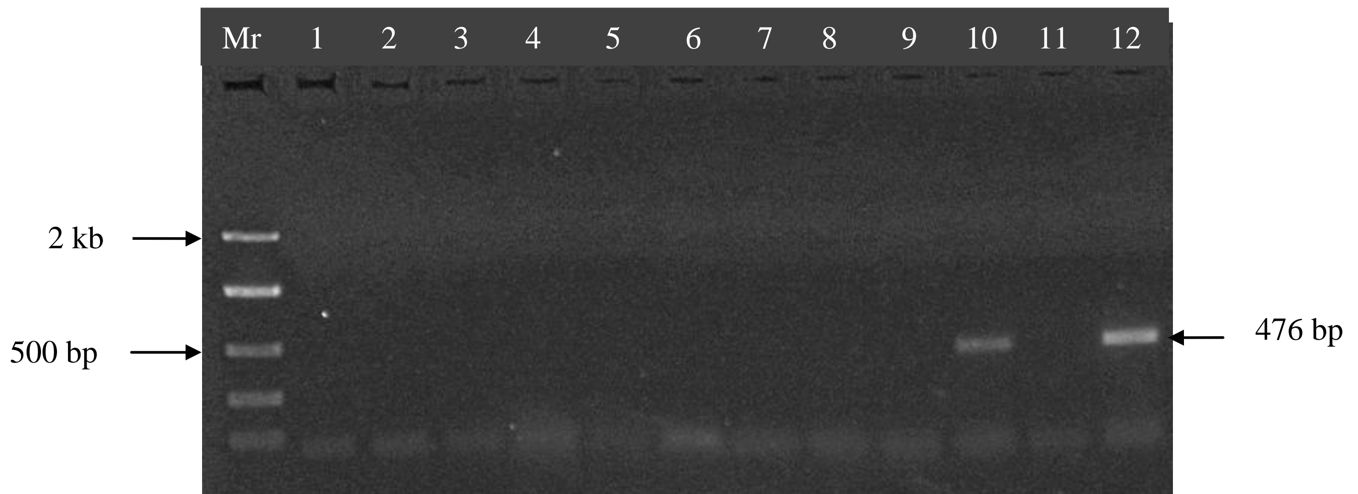

Figure 5: IC-PCR with Gold Finger primers for samples 1-12. Samples 10 and 12 revealed a 476 bp amplicon. Mr is a 2 kb molecular marker (Easy ladder).

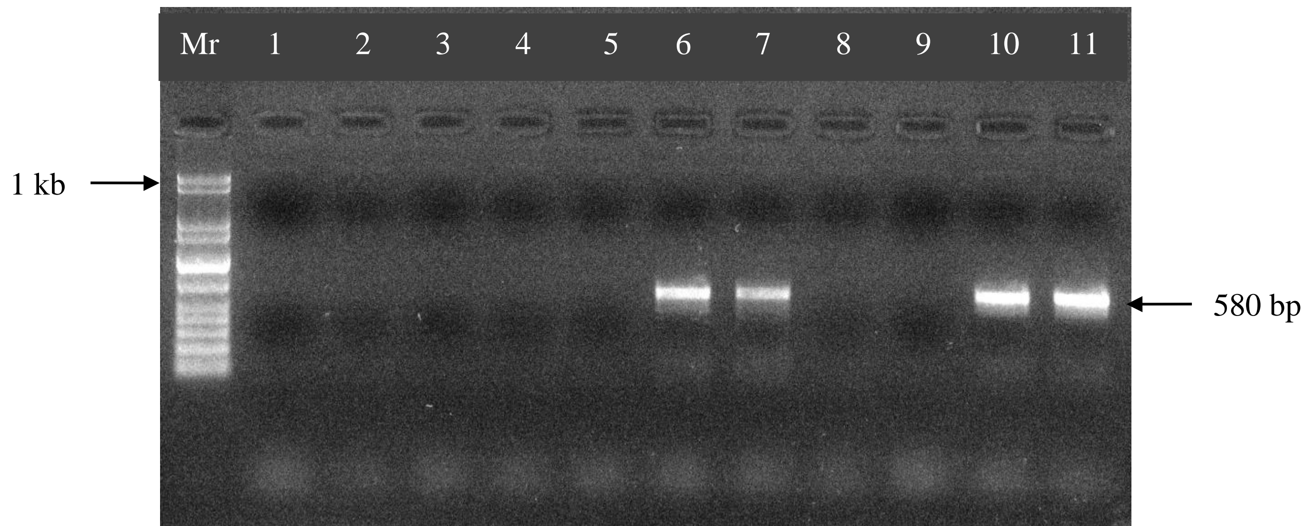

Figure 6: IC-PCR with Badna degenerate primers for samples 1-10. Samples 6, 7 and 10 revealed amplicons of size 580 bp. Lane 11 represents a positive control (Banana CTAB DNA extracted from *Musa* tissue infected with Gold finger isolate). Mr is a 1 kb molecular marker.

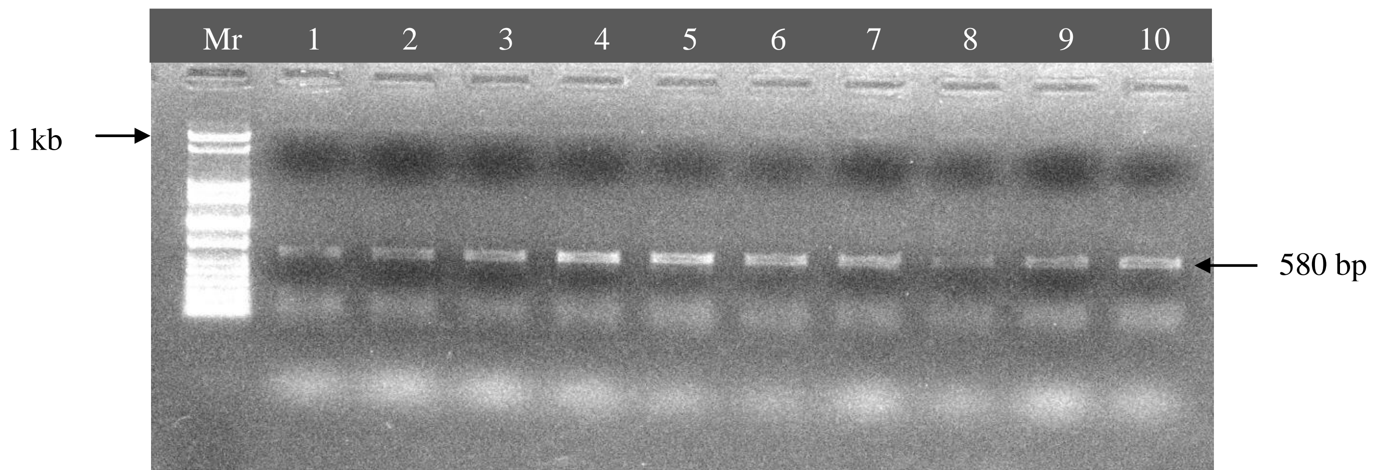

Figure 7: Direct PCR with Badna degenerate primers for samples 1-10. All the 10 samples revealed a 580 bp amplicon. Mr is 1kb Plus molecular marker.

Table 6: Details of 32 asymptomatic banana samples assayed for BSV using 3 virus indexing methods each replicated three times per sample

Cultivar	Genotype	Direct PCR (degenerate primers)	IC-PCR (degenerate primers)	IC-PCR (Gold finger primers)	TempliPhi (RCA)
Mysore	AAB	+++	---	---	---
Mysore	AAB	+++	---	---	---
Gold finger	AAAB	+++	---	---	---
Mysore	AAB	+++	---	---	---
Nshule	EAH-AAA	+++	-+-	---	+++
Nshule	EAH-AAA	+++	+++	---	+++
Musera	EAH-AAA	+++	+++	---	+++
Mysore	AAB	+++	---	---	---
Gold finger	AAAB	+++	---	---	---
Gold finger	AAAB	+++	+++	++-	+++
Gold finger	AAAB	+++	---	---	+++
Nshule	EAH-AAA	+++	+++	-++	+++
FHIA 18	AAAB	+++	---	---	---
FHIA 18	AAAB	+++	---	---	---
Solio	EAH-AAA	+++	+++	---	+++
Solio	EAH-AAA	+++	+++	---	+++
Sukari ndizi	AAB	+++	---	---	---
Musera	EAH-AAA	+++	---	---	+++
Solio	EAH-AAA	+++	+++	+++	+++
Nusu Ng'ombe	EAH-AAA	+++	+++	+++	---
FHIA 18	AAAB	--+	+++	---	+++
Solio	EAH-AAA	+++	+++	---	++-

Cultivar	Genotype	Direct PCR (degenerate primers)	IC-PCR (degenerate	IC-PCR (Gold finger primers)	TempliPhi (RCA)
Sukari ndizi	AAB	+++	---	---	---
FHIA 18	AAAB	+++	---	---	---
Cavendish	EAH-AAA	---	---	-+-	---
FHIA 18	AAAB	+++	---	-++	---
FHIA 18	AAAB	+++	---	---	---
FHIA 18	AAAB	+++	---	---	---
FHIA 18	AAAB	+++	---	---	---
Solio	EAH-AAA	+++	--+	---	---

Key:

+ presence of virus

- absence of virus

The analysis presented in Table 7 points out clearly that direct PCR was the best treatment (mean=0.94±0.00). TempliPhi exhibited a higher mean than IC-PCR. IC-PCR with Gold finger specific primers gave the lowest mean at 0.14±0.001.

Table 7: Comparison based on Least Significant Difference (LSD) test for the four detection techniques

Technique	N	Means*
Direct PCR	96	0.94±0.00
TempliPhi	96	0.38±0.019
IC-PCR (degenerate)	96	0.33±0.042
IC-PCR (Gold finger)	96	0.14±0.001

$LSD_{0.05}$ = 0.0354

*Any two means are significantly different if their difference is the greater than LSD value.

4.8 Detection of RNA viruses

A total nucleic acid extract was used for the detection of *Cucumber mosaic virus* (CMV) and *Banana Bract mosaic virus* (BBrMV) Initial attempts to detect these viruses were unsuccessful probably due to longer storage periods (> 1 week) of the nucleic acids extract samples. Working with fresh nucleic acid extracts increased the efficiency of the RT-PCR. Resuspension of the nucleic acid pellet in Tris-EDTA than RNase-free water also appeared to increase the capacity of the RT-PCR system. Before detecting the two RNA viruses (CMV and BBrMV), PCR for a house-keeping gene, ribulose-1, 5-bisphosphate carboxylase/oxygenase (rubisco) was carried out to confirm that the RNA in the total nucleic acid extract was of RT-PCR-quality (Figure 10). The house-keeping gene RT-PCR for 9 randomly selected samples specifically amplified a 370bp product showing that the RNA was standard for the RT-PCR system (Figure 10).

There was no detection of both CMV and BBrMV in all the 56 samples tested as shown in Figure 11 and 12. The likely implication is that there was no co-infection between BSV and the other RNA viruses.

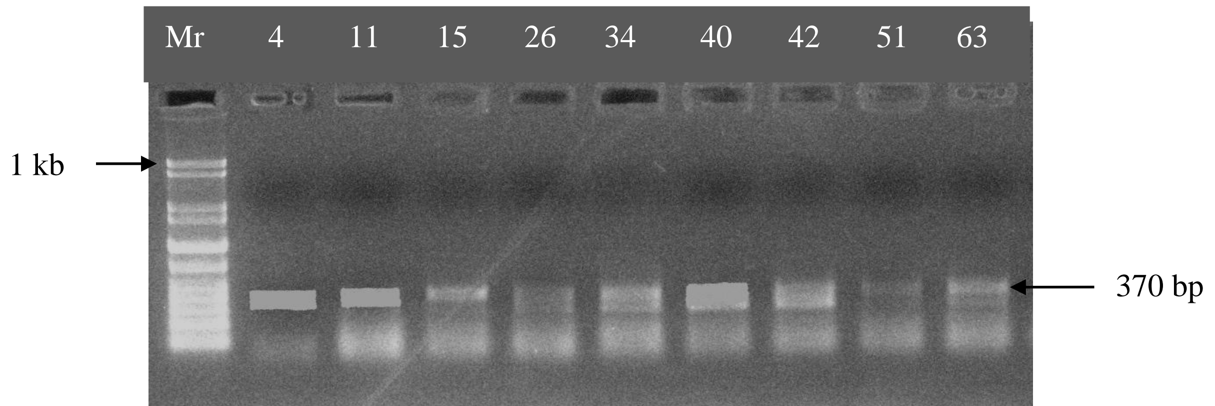

Figure 8: RT-PCR for the rubisco house-keeping gene for 9 randomly selected total nucleic acids samples. Mr is 1kb Plus molecular marker.

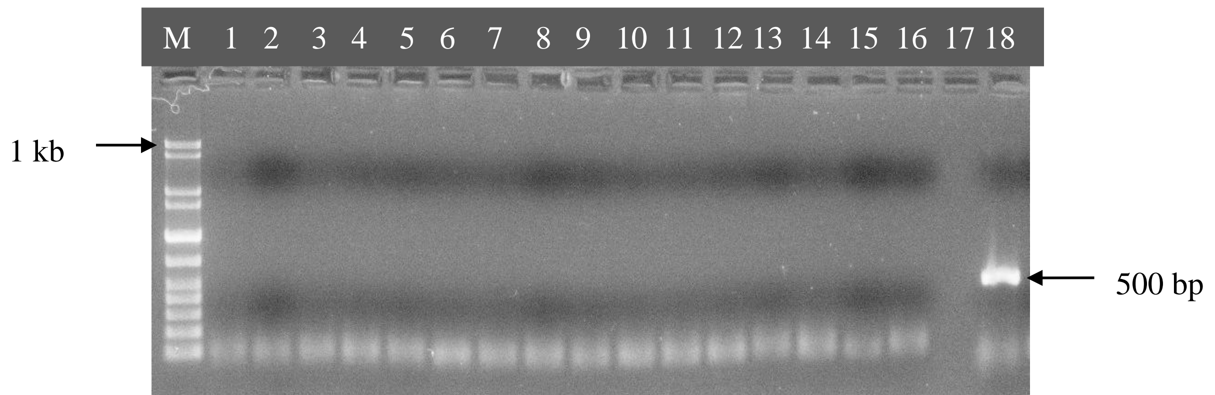

Figure 9: RT-PCR for *Cucumber mosaic virus* with samples 1-16 using the primer set CMV3/5. Lane 17 is a negative control (RNase-free water) while lane 18 is a positive control (CMV plasmid). Mr is 1kb Plus molecular marker.

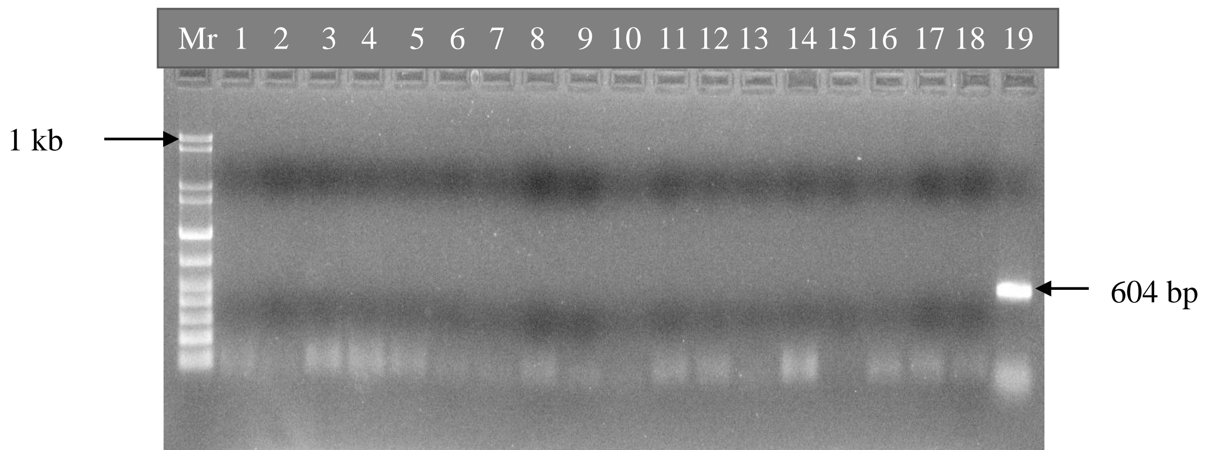

Figure 10: RT-PCR for *Banana Bract mosaic virus* for samples 1-18 with primer set Bract1/2. Lane 19 is a positive control (BBrMV plasmid). Mr is 1kb Plus molecular marker.

CHAPTER FIVE
DISCUSSION

5.1 BSV incidence

The study confirms earlier reports of the widespread occurrence of BSV in Kenya by use of TempliPhi, a novel molecular technique. BSV was detected in the five major banana growing regions sampled; Nyanza, Western, Central, Eastern and Rift Valley. Some of the symptomatic samples tested negative for BSV. The occurrence of this virus might be more frequent and more widespread in Kenya than the results presented here indicate. Dahal *et al*. (1999) reported that in an experimental field in Nigeria established with suckers from symptomatic plantain plants, only 60% of the mats expressed virus symptoms, the other 40% were asymptomatic. However, indexing of leaf samples from both symptomatic and asymptomatic mats resulted in positive tests for 70% of the symptomatic samples and 15% for the asymptomatic samples. In addition, expression and distribution of symptoms was erratic within individual leaves as well as between different leaves of the same plant. This points out clearly that the virus is not always detected in symptomatic plants. The symptoms observed could also be due to other physiological disorders such as nutrient deficiency and dehydration and not necessarily due to BSV.

5.2 Nucleic acid extraction and quality analysis

Initial attempts to obtain intact, high molecular weight DNA using the Banana CTAB DNA isolation protocol were unyielding. However, adoption of the NARO CTAB protocol appeared to yield more intact DNA. The observation is interesting since the difference between the approaches is very minor; centrifugation durations and use of 0.5M NaCl instead of isopropanol for nucleic acid precipitation. According to other experiments carried out on the sidelines (data not shown), it was established that it is the centrifugation periods rather than the precipitation method that is likely to influence the results. The fact was validated by using the Banana protocol with the longer centrifugation periods recommended in the NARO CTAB protocol. With this approach, better DNA yields were obtained. Longer centrifugation periods tend to adequately separate nucleic acids from other cellular components (Gawel and Jarret, 1991). These cellular components include proteins which nucleases are part of. It is these nucleases (DNases and RNases) that degrade nucleic acids. There was nonetheless some degradation observed for DNA extracted using the NARO CTAB protocol and this may be

attributed to use of a non-refrigerated centrifuge during the centrifugation steps of the isolation procedure.

OD260/280nm values obtained in this study were in the range 1.8-2.0 which implied that the purity of the DNA is sufficient enough for downstream applications such as PCR and TempliPhi. Lower values indicate presence of protein contaminants while values greater than 2.0 imply that the DNA extract is contaminated with polyphenols (Honeycutt *et al*., 1992). Sample 24 (one of the 32 asymptomatic samples), gave a value of 1.798. Although this value falls on the lower side of the acceptable range, implying the presence of protein contaminants, the degree of contamination can be safely regarded as negligible.

Use of acid purified sand to crash the banana samples also appeared to give degraded DNA. For this reason, the banana leaf samples were crashed directly in the extraction buffer. Crashing the samples outside the buffer may have exposed the DNA to nucleases hence to degradation (Tassy *et al*., 2006; Lolis and Petsko, 1990). The EDTA component in the buffer chelates the divalent cations notably Ca^{2+} and Mg^{2+} which are key to nuclease activity (Sam and Perona, 1999).

Banana actin house-keeping gene was used to check the quality of DNA in the total nucleic acid extract. Actin, a ubiquitous protein in all eukaryotes, is the major constituent of thin filaments. The 42kD protein can be a monomer or polymer depending on the ionic strength of the solution. The polymeric actin (F-actin) is usually fibrous and therefore ensures mechanical support to the plant. Positive actin amplicons were obtained in this study indicating that the total nucleic acid isolated from the banana tissue was suitable for downstream procedures including PCR amplification.

Reverse transcription PCR for the ribulose-1,5-biphosphate carboxylase (rubisco) gene was also used to test the quality of the RNA in the nucleic acid extract. Ribulose-1,5-biphosphate carboxylase/oxygenase (rubisco) is an enzyme found in all photosynthetic plants, *Musa spp*. included (Lorimer, 1981). Rubisco, an enzyme usually located on the stromal surface of the thylakoid membranes, catalyses the first step in photosynthesis; condensation of a carbon (IV) oxide molecule with ribulose-1, 5-biphosphate (Hartman and Harpel, 1994). Since photosynthesis is a perpetual biochemical process in a plant tissue, the rubisco gene must be expressed all the time and therefore its mRNA can be used as a control for RT-PCR systems (Caemmerer *et al*., 1996). The rubisco enzyme consists of 8 large subunits each of size 56 kD

and 8 small ones each of size 14 kD (Hartman and Harpel, 1994; Portis, 1992). Each large subunit contains a catalytic site and a regulatory site. The role of the small subunit chain is as yet unknown (Lorimer *et al*., 1977). The enzyme is very abundant in the chloroplasts, comprising of more than 16% of their total protein (Eichelmann and Laisk, 1999). In fact, studies have shown that rubisco is probably the most abundant protein in the biosphere (Staswick, 1994). Reverse transcription (RT) PCR using the rubisco primers resulted in amplification of a 370 bp product which indicated that the RNA in the total nucleic acid extract was suitable for RT-PCR and could be used in assays to detect any RNA viruses in the banana samples

In this study, a single extract was utilized for detection of both DNA and RNA viruses. It was however noted that the indexing capacity of the reverse transcription PCR system appeared to decrease with increasing storage lengths of the total nucleic acid extract. In perspective, fresh extracts were therefore ideal for the RT-PCR system. This may be attributed to the relatively high instability of RNA particularly at temperatures above -80ºC, the range within which all the nucleic acid extracts were stored in this study.

5.3 Geographic distribution of BSV isolates

From the results obtained in this study, Kisii region had the highest incidence of BSV. This may be attributed to the high occurrence of the natural vector of this virus, the citrus mealy bug, (*Planococcus citri*) which is common in open fields in this region. Although this study forms a strong base for subsequent research, it does not give an accurate picture of the distribution of the isolates because there was no uniformity in sample sizes for the collections made in the various regions. More so, some of the samples consistently tested negative results, hence reducing the number of samples to be included in the subsequent analysis. Nevertheless, this study unveils a very important aspect of banana streak disease epidemiology; the fact that the Mysore isolate appears to be the most prevalent in the Kenyan banana germplasm.

In a genetic study conducted in Uganda, there was no obvious correlation between a particular BSV clade, species or sequence and a specific *Musa* genotype or degree of symptom severity (Harper *et al*., 2005). With regard to geography however, there was one clear relationship; one isolate was only found in the Mbale region of Eastern Uganda.

Isolate Uganda L may have been found in Kisii due to proximity to Uganda. A similar finding was observed in a Kenyan diversity study (Karanja, 2009) where the BSV isolate, Uganda A (BSUgAV), which had previously been in Uganda was found to be geographically

restricted to the Mt. Elgon region, a region neighbouring Uganda. Such scenarios occur due to the unsafe movement and/or informal exchange of *Musa* germplasm.

In a previous study on the geographic distribution of three whitefly borne closteroviruses; lettuce infectious yellows virus (LIYV), cucurbit yellow stunting disorder virus (CYSDV), and beet pseudo-yellows virus (BPYV), 201 cucurbit plant samples from the Middle East and Europe and 297 samples from California were analyzed using dot-blot hybridization with probes specific for LIYV, CYSDV, and BPYV (Rubio *et al.*, 1999). No LIYV was detected in any of the samples. CYSDV and BPYV were detected in 69 and 12 samples, respectively, but only from samples from the Middle East and Europe. CYSDV was detected only from cucurbit samples collected in Spain, Turkey, Jordan, and Saudi Arabia. BPYV was found only in cucurbits from Crete and Italy.

Some of the samples that tested positive for BSV by TempliPhi had initially appeared negative. TempliPhi, both as a BSV indexing tool and as a sequencing template preparation strategy, operates within a very narrow range of conditions. Most notable among such conditions is the amount of template included in the TempliPhi reaction system. This reaction system requires utmost 1µl of DNA. Too much input material is believed to affect the kinetics of the Phi29 DNA polymerase enzyme thus reducing its capacity to amplify the template. High template volumes may also lead to carry-over of potential inhibitors into the reaction system. These inhibitors are majorly the reagents of the nucleic acid extraction protocol such as salts and chloroform among others. Inefficient denaturation of the template may also lead to failed reactions. Since BSV has a genome with dsDNA, it is a prerequisite that the template be adequately denatured in order to allow for efficient primer annealing. For members of the *Geminivirus* genus (with ssDNA genomes), the denaturation step is unnecessary.

5.4 Relationship between genotype, isolate and symptom expression

Results of this study show that some correlation exists between genotype of the sample/isolate and the degree of symptoms observed. However, each site was only visited once, and since many factors appear to influence BSV symptom expression including environmental conditions and plant growth stage (Mobambo *et al.*, 1996; Dahal *et al.*, 1998; Daniells *et al.*, 2001), it is unlikely that a complete picture was obtained. The results obtained in a study in Nigeria (Dahal *et al.*, 1998) showed that symptom expression of BSV-infected plants at two temperature regimes (in a cool room and screen house) varied by genotype. Plantain hybrids

(genome AAB×AA) generally expressed more severe symptoms, whereas most cooking bananas did not express symptoms under either temperature regime. The most likely reason for greater expression of banana streak disease symptoms in cultivars with a *balbisiana* genome component is that the endogenous pararetroviruses (integrated sequences) in these cultivars contain all of the genetic information needed for "reconstruction" of functional BSV genome very similar to that of the infectious BSV virus. This phenomenon was particularly true for the Gold finger isolate (Gayral *et al*., 2008). The study demonstrated that BSGfV sequences are integrated only in the genome of *M. balbisiana* cultivar cv. PKW and are absent from two other common cultivars of *M. acuminata* tested. Other studies have also shown that species of the BSV clade *sensu stricto*, to which BSGfV belongs, are integrated mainly in the B genome (Geering *et al*., 2001b, 2005), whereas a minority of BSV species such as *Banana streak Cavendish virus* (BSCavV) (Iskra-Caruana *et al*., 2009) and *Banana streak acuminate Vietnam virus* (BSAcVNV) (Lheureux *et al*., 2007) are thus far reported as being specific to the A genome.

Generally, the Mysore isolate appeared to elicit more severe BSV symptoms in this study. In an earlier study to determine the infection dynamics of the Mysore isolate (Daniells *et al*., 2001), it was observed that symptoms of BSMysV infection in cv. 'Williams' were very severe, and infection would probably substantially reduce yield of the plant. In comparison to BSMysV, BSV-Cav is comparatively benign in the same cultivar. A BSV-infected plant of the Mysore cultivar was used as a source of inoculum on healthy plants. Only one out of eight plants became infected as determined by IC-PCR. In a subsequent test, using infected 'Williams' cultivar plant as a source of inoculum, all six cv. 'Williams' test plants became infected. Symptoms of infection were very severe with chlorotic streaks covering a large proportion of the leaf.

Other than genotype, the variance in symptom severity can also be attributed to other factors. Mixed infections, which have been reported in Kenya (Karanja *et al*., 2008), could be one of the causes of the variance in degree of symptoms observed. Karanja *et al*. (2008) observed that symptoms were more severe in samples which were infected with more than one isolate; the more the number of isolates, the more severe the symptoms. Previous studies have also shown that environmental and nutritional factors can influence banana streak disease symptom expression. The best studied of such factors is temperature (Dahal *et al*., 1998). This Nigerian-

based study found out that symptoms are more severe at lower (22-28ºC) than at higher temperatures (35ºC).

5.5 Comparison of BSV indexing techniques

The superiority of badna primers over the 1A/4' type in detection of BSV can be explained on the basis of the high genomic variability inherent with the BSV species. Both badna and 1A/4' primers are designed to target the replicase (reverse transcriptase/RNase H) region of the BSV ORF III (Geering *et al*., 2000). It is possible that natural molecular rearrangements have occurred over time in BSV within this region of the genome, hence lowering the capacity of the 1A/4' primers. This may have required touchdown PCR to determine a suitable annealing temperature. In this study, the classical annealing temperature of 50ºC was used for all the BSV PCRs.

The IC-PCR technique (both with specific and degenerate primers) amplified BSV sequences from banana leaf sap for some of the samples, though with minimal reproducibility. IC-PCR with the Gold finger virus specific primers gave a 476bp fragment while a 580bp amplicon was observed with the badna degenerate primers. The direct implication is that some of the samples had been infected by BSV isolates other than the gold finger isolate. This confirmed earlier reports of high genomic heterogeneity among BSV isolates (Lockhart and Olszewski, 1993). The lower percentage detection with gold finger primers points out that this primer pair could not amplify the target sequences of all the BSV isolates. The badna primers are designed to degenerately target the replicase (RNaseH/RT) region of all members of the *Badnavirus* genus. Other than *Banana streak virus*, the other members of the *Badnavirus* genus are shown in Table 8. Since these viruses are not hosted by banana, it is unlikely that they were amplified by the badna degenerate primers. To reinforce this further, the antisera used during the immuno-capture step of IC-PCR were specific to BSV.

Table 8: Members of Genus *Badnavirus* and their host ranges (Bousalem *et al*., 2008)

Species	Acronym	Host range (Family, Genus)
Cacao swollen shoot virus	CSSV	*Malvaceae, Theobroma*
Commelina yellow mottle virus	ComYMV	*Commelinaceae, Commelina*
Dioscorea bacilliform virus	DaBV	*Dioscoraceae, Dioscorea*
Sugarcane bacilliform IM virus	SCBIMV	*Poaceae, Saccharum*
Sugarcane bacilliform Mor virus	SCBMV	*Poaceae, Saccharum*
Taro bacilliform virus	TaBV	*Araceae, Colocasia*
Kalanchoe top-spotting virus	KTSV	*Crassulaceae, Kalanchoe*
Rubus yellow net virus	RYNV	*Rosaceae, Rubus*
Gooseberry vein banding associated virus	GVBAV	*Grossulariaceae, Ribes*
Spiraea yellow leaf spot virus	SYLSV	*Rosaceae, Spiraea*

Although all the 32 samples used in one of the experiments in this study were asymptomatic, it was possible to detect BSV in some samples. This is because the 32 samples were collected from a field which two months before sampling, was highly endemic with banana streak disease. The implication is that most of the 32 samples were actually infected with BSV but had masked the symptoms probably due to changes in environmental conditions.

Results of this study generally show that the TempliPhi technique has a greater capacity to reliably detect BSV under some conditions than the other assayed techniques. This is expected because the TempliPhi technique allows for selective amplification of all circular DNAs within the tissue being indexed (Blanco and Salas, 1996). The high sensitivity, fidelity and processivity of *Phi29* DNA polymerase; the enzyme used in the TempliPhi amplification (Blanco *et al*., 1989), ensures accurate and reliable diagnosis by means of this method. A number of factors may explain the lower percentage detection by IC-PCR; the low reproducibility always inherent with IC-PCR, the high serological and genomic heterogeneity known to exist among BSV isolates (Geering *et al*., 2000; Lockhart and Olszewski, 1993) and probably the inactivity and/or

non-specificity of the antisera (Harper *et al*., 2002b). Moreover, there appears to be a link between genetic and serological diversity; the former leads to the latter (Agindotan *et al*., 2003). Since genetic diversity of *Banana streak virus* has been shown to exist in Kenya (Karanja *et al*., 2008), it is likely that serological variability among BSV isolates exists with almost the same measure as the genetic diversity. Apart from the increased sensitivity and reliability of TempliPhi compared to IC-PCR and standard PCR, TempliPhi has the advantage of allowing the identity of the isolate infecting the sample. This can be an avenue for carrying out more accurate distribution studies for other viruses with circular DNA genomes.

Results for direct PCR and IC-PCR using degenerate primers were not in agreement. Direct PCR gave a 93.8% detection level compared to the 31.3% with IC-PCR for the 32 asymptomatic samples. This finding confirms earlier reports of possible integration of badnavirus sequences in their host genomes (Geering *et al*., 2001a). Much as most of the detections by direct PCR can therefore be safely classified as false positives (Iskra-Caruana *et al*., 2009), recent studies have tentatively shown that direct PCR for detection of BSV can be reliable for tissues without the balbisiana (B) components in their genomes (James *et al*., 2011). Banana cultivars of AAA and EAH-AAA genomes can therefore be reliably indexed for BSV by means of direct PCR. Data for the direct PCR for sample 27 (AAA Cavendish) was indeed a confirmation of this finding. There was no detection of BSV for this sample using all the 3 techniques (direct PCR, IC-PCR and TempliPhi). More so, the positive detections observed with direct PCR for tissues with AAA genomes were confirmed by the other techniques. Therefore direct PCR in this case can be relied upon. However, the negative result observed for sample 21 was certainly erroneous since it was not in agreement with the results of the other techniques. The likely explanation is that the DNA for this sample was not of PCR quality.

As yet, it is not clear why the integrated sequences are never amplified by direct PCR in tissues exclusively containing acuminata (A) genome, but earlier studies had established that the numerous infectious BSV endogenous pararetroviruses (integrated sequences) of different BSV species are restricted to the B genome (Dahal *et al*., 1999; Geering *et al*., 2001b). According to these studies, the integrated sequences in A genomes are usually non-infectious; they cannot be activated by the tissue culture stress. This suggests that the non-detectability of these endogenous pararetroviruses in A genomes is linked to the position of these sequences in the genome.

Modified antibody concentration of 1:100 rather than the recommended 1:1000 (Lockhart 1986) was used to trap the BSV particles in this study. The non-reproducibility of results with this technique may be attributed to a number of factors; the antibody concentration used initially was too low for detection of Kenya BSV isolates, the virus titre in the samples might have been lower than the detection limits or the antibody might have overstayed before use.

5.6 Detection of *Cucumber mosaic virus* and *Banana bract mosaic virus*

Cucumber mosaic virus and *Banana bract mosaic virus* were not detected in the samples. The implication is that there was no co-infection between BSV and any of the two RNA viruses. Similar findings were observed in Nigeria (Gauhl *et al*., 1999) where samples exhibiting BSD symptoms were indexed both for BSV and CMV. Most of the samples tested positive for BSV while no CMV was detected. However, a sample size of 65 (used in this study) may not have been large enough to give a clear picture of co-infection patterns between BSV and the two RNA viruses (CMV and BBrMV) in all banana germplasm.

It is a common phenomenon for plants to be infected by two or more viruses in natural conditions (Falk and Bruening, 1994). Different interactions among viruses have been observed, such as synergism, helper-dependence, cross-protection, replacement, mutual suppression, and a mixture of antagonistic and synergistic interactions (Falk and Bruening, 1994). Certain pairs of virus co-infection have been used to induce symptoms more severe and to present higher virus accumulation than expected when they interact in an additive manner. In a study to investigate the co-infection relationship between *Cucumber mosaic virus* (CMV) and *Zucchini yellow mosaic virus* (ZYMV) (Zeng *et al*., 2007), it was conclusively shown that these two viruses were synergic on the *Cucurbitaceae* crops. This was established by observation of increased severity of the symptoms produced following inoculation.

The synergic interaction between CMV and ZYMV has been reported in the last 20 years (Poolpol and Inouye, 1986a; Poolpol and Inouye, 1986b; Wang *et al*., 2002; Wang *et al*., 2004; Fattouh, 2003). Fattouh, 2003 detected the mean ratio of coat protein of CMV and ZYMV from complexly inoculated zucchini squash (*Cucurbita pepo*) by enzyme-linked immunosorbent assay, and found that coat protein of CMV was higher than that of ZYMV. Using the molecular hybridization method, Wang *et al.* (2002 and 2004) detected that the amounts of CMV RNAs and its coat protein increased in cases of complex infection.

CHAPTER SIX
CONCLUSIONS, RECOMMENDATIONS, CHALLENGES AND FUTURE PROSPECTS

6.1 Conclusions

The Mysore and gold finger isolates of Banana streak virus are the most prevalent in Kenya. The Mysore isolate showed high incidence in Kisii region.

Musa cultivars containing a balbisiana genome component exhibit more severe banana streak disease symptoms. Such cultivars include the FHIAs (FHIA 17 and FHIA 18), plantains, Sukari ndizi, Mysore, gold finger among many others.

The Mysore isolate elicits relatively more severe symptoms compared to other BSV isolates.

There is no co-infection relationship between Banana streak virus and either of the two RNA viruses (CMV and BBrMV).

The rolling circle amplification-based TempliPhi technique is the most sensitive BSV indexing technique. Immuno-Capture polymerase chain reaction (IC-PCR) using BSV degenerate primers also shows a high sensitivity but unlike the TempliPhi technique, IC-PCR does not allow for isolate identity.

6.2 Recommendations

Arising from the findings of this study, the following recommendations are made:

1. It would be important for the concerned organizations to put in place strict quarantine measures on movement of *Musa* germplasm from Kisii region. This is because the Mysore isolate, which appears to elicit the most severe symptoms, is the most prevalent in this region. It will be important to put in place effective indexing strategies for all *Musa* germplasm moving outside this region.
2. Since increased symptom expression was observed in *Musa* cultivars containing a balbisiana component in their genomes, it would be important to make use of such cultivars in experiments aimed at studying BSV symptom expression in *Musa*.
3. All screening facilities for *Musa* germplasm should embrace TempliPhi as the principal indexing tool. Immuno-Capture polymerase chain reaction (IC-PCR) is also fairly sensitive but it may leave out some isolates due to the high serological variability usually exhibited by BSV isolates. Reproducibility is also an issue of concern with IC-PCR.

6.3 Challenges and Future Prospects

It is an undisputed fact that rapid and reliable detection of *Banana streak virus* has been an enormous challenge over the last two decades. Although the RCA-based TempliPhi technique offers a remedy to this problem, the solution seems to be only partial. The long incubation period, need for incubation and electrophoresis apparatus required for the TempliPhi method have meant that this technique cannot be used in the field for rapid indexing of banana samples, a need which has remained unaddressed for a long time. It would be worthwhile for future research to focus on establishing a method based on the rolling circle amplification principle that will circumvent the long incubation periods and the electrophoretic procedures.

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APPENDICES

APPENDIX 1: RECIPES FOR BUFFERS AND STOCK SOLUTIONS

CTAB buffer

50 ml of 1M Tris–HCl pH 8.0

50ml 0.5M EDTA pH 8.0

41g NaCl

5g $NaSO_3$

10g polyvinylpyrrolidone (PVP)

10g of 3.5% CTAB

BSV extraction buffer (1 litre)

1 litre Phosphate buffered saline

20g Polyvinylpyrrolidone (PVP)

10g Na_2SO_3 (to be added just before use)

Carbonate coating buffer (pH 9.6, 1 liter)

1.59g Na_2CO_3

2.93g $NaHCO_3$

0.20g NaN_3

10X TAE buffer (pH 8.0, 1 litre)

48.4g Tris

11.4ml Glacial acetate

20ml EDTA

10X TBE buffer (pH 8.0, 1 litre)

108g Tris

55g borate

40ml 0.5M EDTA (pH 8.0)

10 mg/ml RNAse A

100 mg of RNAse (Sigma # R4875) were dissolved in 10 ml of 10 mM Tris (pH 7.5), and 15 mM NaCl followed by heating in boiling water for 15 minutes and allowed to cool slowly to room temperature. Working stock was stored at 4°C.

10 mg/ml ethidium bromide stock

100 mg of ethidium bromide were dissolved in 10 ml sterile double distilled H_2O. The tube wrapped in aluminum foil and stored at 4°C.

CAUTION: Ethidium bromide is extremely mutagenic.

0.5M EDTA (pH 8.0)

186.12g of hydrated disodium EDTA (MW=372.24) were dissolved in approximately 750 ml of SDW. NaOH pellets were added to bring the pH to 8.0. After EDTA is in solution, it was brought to 1000 ml with SDW then autoclaved.

APPENDIX 2: PRIMER SEQUENCES AND THEIR PRODUCT SIZES

a) Primers for banana (Ban)house-keeping genes ribulose-1,5-biphosphate carboxylase/RubisCo (Rub) and Actin (Act)

Primer name	Sequence (5'-3')	Product size
BanRub f	GGCTTCACCGGCTCAATCCA	370bp
BanRub RNAr	CCACACGAACCCCTCGTGG	370bp
BanAct f	CTGGTGATGGTGTGAGCCAC	664bp
BanAct DNAr	CATGAAATAGCTGCGAAACG	664bp

b) BSV degenerate primers

Name	sequence	product size
BadnaFP	ATGCCITTYGGIAARAAYGCICC	580bp
BadnaRP	CCAYTTCRAIACISCICCCCAICC	
1A	CTNTAYGARTGGYTNGTNATGCCNTTYGG	580bp
4'	TCCAYTTRCANAYNSCNCCCCANCC	

c) TempliPhi degenerate primers

Primer name	Primer sequence (5'-3')	Reference
1A	CTNTAYGARTGGYTNGTNATGCCNTTYGG	Harper *et al*., 2002b
4'	TCCAYTTRCANAYNSCNCCCCANCC	"
BadnaFP	ATGCCITTYGGIITIAARAAYGCICC	Yang *et al*., 2003
BadnaRP	CCAYTTRCAIACISCICCCCAICC	"
BSV2292	ATGARYTAHATWAGRTGYTMSCC	James *et al*., 2011
BSV2826	TYYWGAAARCATGGTGGGRGARGA	"
BSV3298	YTCCCAYCTTTCRAAKACYTT	"
BSV3517	KRATMTTYTWTYTDGAARATCC	"
BSV3700	KTGGBAGTTTKGTRAAGARYTC	"
BSV4030	TGCARRTGYTWYGCYTGYGGAGA	"
BSV6652	GAAAARRTMTGYGCNTAYGCVAG	"

Definition of DNA degenerate bases

I	A or T or C	N	any DNA base
B	C or G or T	R	A or G
D	A or G or T	S	C or G
K	G or T	W	A or T
M	A or C	Y	C or T

d) Non-degenerate BSV primers

Primer code	Primer identity	Sequence (5'-3')	Product size
BSV-1	RD-F1	ATCTGAAGGTGTGTTGATCAATGC-	522
BSV-2	RD-R1	GCTCACTCCGCATCTTATCAGTC	
BSV-3	Cav-F1	AGGATTGGATGTGAAGTTTGAGC	782
BSV-4	Cav-F2	ACCAATAATGCAAGGGACGC	
BSV-5	Mys-F1	TAAAAGCACAGCTCAGAACAAACC	589
BSV-6	Mys-R1	CTCCGTGATTTCTTCGTGGTC	
BSV-7	GF-F1	ACGAACTATCACGACTTGTTCAAGC	476
BSV-8	GF-R1	TCGGTGGAATAGTCCTGAGTCTTC	
BSV-9	BSV4673-F1	GGAATGAAAGAGCAGGCC	644
BSV-10	BSV5317-R1	AGTCATTGGGTCAACCTCTCTGTC	

e) Reverse Transcription-PCR primers

Primer name	Sequence (5'-3')	Product size
Bract1	GACATCACCAAATTTGAATGGCACATGG	**604bp**
Bract2	CCATTATCACTCGATCAATCCTCACAG	
CMV3	TTTTAGCCGTAAGCTGGATGGACAACCC	**500bp**
CMV5	TATGATAAGAAGCTTGTTTCGCGCA	

APPENDIX 3: STATISTICAL ANALYSIS TABLES

SAS output table for comparison of BSV indexing techniques

Sample	Technique	N	Mean*
1	1	3	1.00
1	2	3	0.00
1	3	3	0.00
1	4	3	0.00
2	1	3	1.00
2	2	3	0.00
2	3	3	0.00
2	4	3	0.00
3	1	3	1.00
3	2	3	0.00
3	3	3	0.00
3	4	3	0.00
4	1	3	1.00
4	2	3	0.00
4	3	3	0.00
4	4	3	0.00
5	1	3	1.00
5	2	3	0.33
5	3	3	0.00
5	4	3	1.00
6	1	3	1.00
6	2	3	1.00
6	3	3	0.00
6	4	3	1.00

Sample	Technique	N	Mean*
7	2	3	1.00
7	3	3	0.00
7	4	3	1.00
8	1	3	1.00
8	2	3	0.00
8	3	3	0.00
8	4	3	0.00
9	1	3	1.00
9	2	3	0.00
9	3	3	0.00
9	4	3	0.00
10	1	3	1.00
10	2	3	1.00
10	3	3	0.66
10	4	3	1.00
11	1	3	1.00
11	2	3	0.00
11	3	3	0.00
11	4	3	1.00
12	1	3	1.00
12	2	3	1.00
12	3	3	0.66
12	4	3	1.00
13	1	3	1.00
13	2	3	0.00
13	3	3	0.00
13	4	3	0.00
14	1	3	1.00

Sample	Technique	N	Mean*
14	3	3	0.00
14	4	3	0.00
15	1	3	1.00
15	2	3	1.00
15	3	3	0.00
15	4	3	1.00
16	1	3	1.00
16	2	3	1.00
16	3	3	0.00
16	4	3	1.00
17	1	3	1.00
17	2	3	0.00
17	3	3	0.00
17	4	3	0.00
18	1	3	1.00
18	2	3	0.00
18	3	3	0.00
18	4	3	1.00
19	1	3	1.00
19	2	3	1.00
19	3	3	1.00
19	4	3	1.00
20	1	3	1.00
20	2	3	1.00
20	3	3	1.00
20	4	3	0.00
21	1	3	0.00
21	2	3	1.00

Sample	Technique	N	Mean*
21	4	3	1.00
22	1	3	1.00
22	2	3	1.00
22	3	3	0.00
22	4	3	1.00
23	1	3	1.00
23	2	3	0.00
23	3	3	0.00
23	4	3	0.00
24	1	3	1.00
24	2	3	0.00
24	3	3	0.00
24	4	3	0.00
25	1	3	1.00
25	2	3	0.00
25	3	3	0.00
25	4	3	0.00
26	1	3	1.00
26	2	3	0.00
26	3	3	0.00
26	4	3	0.00
27	1	3	0.00
27	2	3	0.00
27	3	3	0.33
27	4	3	0.00
28	1	3	1.00
28	2	3	0.00
28	3	3	0.66

Sample	Technique	N	Mean*
29	1	3	1.00
29	2	3	0.00
29	3	3	0.00
29	4	3	0.00
30	1	3	1.00
30	2	3	0.00
30	3	3	0.00
30	4	3	0.00
31	1	3	1.00
31	2	3	0.00
31	3	3	0.00
31	4	3	0.00
32	1	3	1.00
32	2	3	0.33
32	3	3	0.00
32	4	3	0.00

$LSD_{0.05}$ = 0.0354

*Any two means are significantly different if their difference is the greater than LSD value.

Key (technique):

1 Direct PCR

2 IC-PCR (degenerate primers)

3 IC-PCR (Gold finger primers)

4 TempliPhi (RCA)

ANALYSIS OF VARIANCE (ANOVA) FOR COMPARISON OF BSV INDEXING TECHNIQUES

Source	Degrees of freedom	Sum of squares	Mean square	F value	Pr > F
Sample	31	25.1015625	0.80972782	52.23	0.0001*
Treatment	3	34.15364583	11.38454861	734.39	0.0001*
Replicate	2	0.0625	0.03125	2.02	0.1353^{NS}
Sample*treatment	93	31.59635417	0.33974574	21.92	0.0001*
Error	254	3.93750000	0.01550197		

*highly significant at 0.01

^{NS}Not significant

ONEWAY ANALYSIS OF VARIANCE (ANOVA) FOR COMPARISON OF SYMPTOM EXPRESSION MEANS FOR THREE BANANA GENOTYPES (AAA, AAB AND AAAB)

	Sum of squares	df	Mean square	F	Significance
Between groups	29.579	2	14.79	7.08	0.002
Within groups	127.358	61	2.088		
Total	156.938	63			

ONEWAY ANALYSIS OF VARIANCE (ANOVA) FOR COMPARISON OF SYMPTOM EXPRESSION MEANS FOR THREE BSV ISOLATES (BSMysV, BSGfV AND BSOEV)

	Sum of squares	df	Mean square	F	Significance
Between groups	27.823	2	13.912	7.78	0.001
Within groups	87.619	49	1.788		
Total	115.442	51			