DEVELOPMENT OF A NOVEL DNA ISOLATION PROTOCOL AND DETERMINATION OF THE TRANSMISSION MODE OF *Banana streak virus* BY PUTATIVE VECTORS IN KISII

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A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Degree of Master of Science in Biochemistry of Egerton University.

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration

I hereby declare that this research thesis is my original work and has not been submitted wholly or in part to any institution for award of any degree.

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DEDICATION

To my father’s family, and Peter Nthiga for their patience during my study.

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ABSTRACT

Banana is an important crop in East Africa. In the tropics, banana and plantains are a major source of carbohydrate for about 400 million people of whom 200 million are from East Africa. In Kenya, the banana serves both as a subsistence crop as well as a cash crop. The production of banana in Kenya is however constrained by viral diseases especially the Banana Streak Disease (BSD) which is caused by the *Banana Streak Virus* (BSV). Breeding between A and B-genomes containing banana have been significantly constrained by BSV due to activation of the integrated viral sequences in the genome of the interspecific hybrids. The episomal DNA viral particles are thought to be transmitted by several mealy-bug species but there are no reports on the transmission of the BSV by other viral insect vectors. This study aimed at elucidating the mode of transmission of BSV by mealy-bug species in Kenya and identifying any other putative vectors of BSV in samples collected from infected banana plantations. The mealy-bugs were reared on pumpkin fruits in black cages and were used in the virus transmission cycle experiments. The instars of the *Paracoccus burnerae* (mealy-bug) species were fed on BSV infected plants to acquire the virus and on the healthy plantlets for inoculation. The effect of the acquisition period on the transmission of the BSV, the latent period and the retention period of the BSV by the mealy-bug species were determined using Rolling Circle Amplification (RCA). In addition, leafhoppers and banana aphids collected from the infected banana plantations were assayed for the presence of BSV using RCA. RCA products were assayed by electrophoretic analysis. Principal characteristics of the modes of virus transmission by insects were used to conclude the mode of transmission of the BSV by mealy-bugs. Results from this study revealed that the *P. burnerae* is able to acquire the virus and transmit it after a minimum of six hours of acquisition access time. The virus had no latent period in the vector though it could be retained in the vector for four days after acquisition feeding, characteristics of semi-persistent mode of transmission. In addition, the results revealed that Banana aphids and leafhoppers are not potential vectors of BSV. Viruliferous mealy-bugs should be reduced by destroying BSV infected materials. Greater understanding of the transmission of BSV will contributed immensely to the development of control strategies for banana streak disease.

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LIST OF ABBREVIATIONS

AAP	Acquisition access period
BBTV	*Banana bunchy top virus*
BEV	*Banana endogenous virus*
BSD	Banana streak disease
BSV	*Banana streak virus*
CaMV	*Cauliflower mosaic virus*
CMV	*Cucumber mosaic virus*
CP	Capsid protein
CTAB	Cetyltrimethylammonium bromide
DAS ELISA	Double antiboby sandwitched-Enzyme linked immunosorbert assay
dNTPs-	Deoxyribonucleoside triphospate
EDTA	Ethylene diamine tri-acetic acid
IAP	Inoculation access period
IC-PCR	Immuno-capture polymerase chain reaction
NTES	Sodium chloride-Tris-EDTA-Sodium dodecylsulfate
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVP	Polyvinylpyrolidine
RCA	Rolling circle amplification
RDV	Rice dwarf virus
RFLP	Restriction fragment length polymorphism
RTBV	*Rice tugro bacillifom virus*
SCBV	*Sugarcane bacilliform virus*
SDW	Sterilized distilled water
TAS-ELISA	Triple antiboby sandwitched-Enzyme linked immunosorbert assay

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Banana is among the most vital food commodity world wide. They provide millions of people throughout the tropics and subtropics with an essential staple food and account for one of the most widely exported fruit in the world. In the tropics, bananas and plantains provide a major source of carbohydrate for about 400 million people of whom 200 million are from East Africa (Swennen *et al.,* 1995). Bananas have a complex genetic constitution in that they can be grouped into A-genome containing plants (*Musa acuminata*) and B-genome containing plants (*Musa balbiasiana*). The hybridization of these two species results in hybrids containing AAB, ABB, and AB-genomes (Simmonds, 1962).

Banana production has been constrained by BSV and the resultant BSD. BSV is found in all banana growing regions of the world, with initial symptoms of infection often being confused for other viral diseases. BSV is thought to be transmitted by several mealy-bug species (Jones and lockhart, 1993; Kubiriba *et al.,* 2001; Kubiriba, 2005; Meyer *et al.,* 2008) and through infected planting materials. Breeding programmes involving hybridization of banana containing A and B-genomes have been significantly constrained by BSV due to activation of the integrated viral sequences in the genome of the resultant interspecific hybrids (Harper *et al*., 2005). The actual mode and mechanism of BSV transmission by mealy-bugs are yet not fully understood (Harper *et al.,* 2005; Karanja *et al.,* 2008). As a result, the management of the BSD has remained a problem worldwide, and in turn the development of banana hybrids which are resistant to drought, pests and diseases, and that are high yielding has been severely contrained (Panis *et al.,* 1993).

Banana streak virus (BSV) (Family: *Caulimoviridae*; Genus: *Badnavirus*) is one of the causes of the BSD (Lockhart, 1986) which has been reported in all the countries where bananas are grown (Lockhart and Jones, 2000). The disease is recognized as a major constraint to the genetic improvement of banana and germplasm dissemination. BSV belongs to the family *Caulimoviridae* and genus *Badnavirus*. The virus has a bacilliform particle containing a non-covalently closed double stranded DNA (dsDNA) genome of 7.4 Kbp (Harper and Hull, 1998). BSV is serologically and genomically variable (Lockhart and Olszewski, 1993) and is one of the few plant viruses whose ability to cause the disease is related to the homologous viral sequences in the nuclear genome of the host (Geering *et al.,* 2001; 2005). These viral sequences are capable of causing episomal infection under stress conditions such as *in vitro* propagation

(Ndowora *et al*., 1999; Dallot *et al*., 2001). In all reported cases, these activatable sequences have been found only in the B-genome-containing *Musa* although there are other integrated *Badnavirus* sequences found in both A and B genomes that have not been associated with the disease (Geering *et al.,* 2001; 2005).

In Kenya, BSV is prevalent in all banana growing regions and affects all the popular cultivars grown by farmers (Wangai *et al*., 2002). In some of the cultivars e.g. Lisulya and Khabusi, two to three isolates were identified in a single cultivar (Karanja *et al*., 2008). Also, putative integrated DNA sequences were found in addition to the episomal viral DNA fragments (Karanja *et al*., 2008). The presence of identical or related viral sequences in the host genome might give false-positives, when standard PCR techniques are used to detect BSV. High variability of the BSV can give false-negatives when serological detection is used (Karanja *et al*., 2008). This nature of the virus makes its detection complicated. In this study, the detection of episomal BSV sequences was done using the Rolling Circle Amplification (RCA) technique also called TempliPhi; to prevent the amplification of the host genome (James *et al.,* unpublished). However, TempliPhi technique has not been used to assay BSV in nucleic acids isolated from vectors. Therefore there is need to develop a molecular tool that will isolate nucleic acids containing BSV without denaturation from putative vectors. In addition, most of the published DNA isolation protocols use deleterious carcinogenic compounds (β-Mercaptoethanol and phenol/chloroforrn solutions) in some steps of the DNA isolation procedures. The protocol developed will circumvent the use of carcinogenic solutions.

The main mode of spread of BSV is through infected suckers (Jones and Lockhart, 1993). The disease has nonetheless also been reported to be seed borne (Danielles *et al*., 1995; 2001) though pollinators have however not been implicated in BSV transmission (Kubiriba, 2005). Like most other *badnaviruses*, BSV has been thought to be transmitted semi-persistently by mealy-bugs, namely *Planococcus citri*, *Saccharicoccus sacchari* and *Dymicoccus brevipes* (Lockhart and Olszewski, 1993; Su Hong-ji, 1998; Kubiriba *et al.,* 2001). However, the mode and mechanisms of BSV transmission by mealy-bugs is poorly understood. The acquisition period, latent period and retention period determine how the virus is transmitted from infected to uninfected plantlets hence there is a need for this study. In addition, it is not clear whether the mealy-bugs are the only vectors of BSV transmission. Banana aphids, leafhoppers and whiteflies, have apparently also been implicated as plant virus vectors (Stover, 1972) and are also found in banana fields. In Kenya, the major vectors of BSV are yet to be determined.

1.2 Statement of the Problem

Banana streak virus is found in all banana growing regions of the world including Kenya. BSV has severely constrained banana production world over, with estimated crop losses ranging from 6-15%. In Kenya, the disease is distributed throughout the banana growing districts. The banana streak disease has also severely constrained banana breeding efforts due to activation of the integrated BSV sequences in the genome of developed interspecific hybrids. Currently, the only viable BSD management option is the use of clean planting materials. BSV resistant varieties have not yet been identified and the use of chemicals to control the virus might not be possible. BSD management in Kenya and elsewhere has only been based on planting virus indexed materials. The method has not been very effective due to lack of knowledge on the transmission mode of the virus. BSV is known to be transmitted by several mealy-bug species from infected to non-infected planting materials. The acquisition feeding period, latent and retention periods are not well understood. These determine how the virus is spread from infected to healthy plantlets. In addition, it is not yet known whether other sap sucking insects that feed on banana species can potentially transmit viral diseases including BSV. A better understanding of the mode of transmission of BSV by mealy-bug species and identification of other putative vectors of the virus will significantly contribute to the development of viable control strategies for this disease.

1.3 Objectives

1.3.1 General Objective

To develop a DNA isolation protocol and determine the mode of transmission of BSV by putative vectors in Kisii district of Kenya.

1.3.2 Specific Objectives

1. To develop a DNA isolation protocol for the putative vectors.
2. To determine the effect of virus acquisition period by the mealy-bug (*Paracoccus burnerae*) on successful transmission of BSV using the TempliPhi technique.
3. To determine the latent period of BSV in the mealy-bugs (*Paracoccus burnerae)* using the TempliPhi technique.
4. To determine the retention period of BSV in the mealy-bug (*P. burnerae*) using the Templiphi technique.
5. To screen banana aphids (*P.negronervosa*), leafhoppers (*Cicadulina mbila)* and whiteflies (*Aleyrodidae*) for BSV transmission in banana in Kisii using the Templiphi technique

1.4 Null Hypotheses

1. Length of virus acquisition period has no effect on the success of transmission of BSV by the *P. burnerae*.
2. There is no latent period of BSV in the *P. burnerae* during the transmission process.
3. There is no retention period of BSV in the *P. burnerae* during the transmission process.
4. Banana aphids (*Aphididae*), leafhoppers (*Cicadulina mbila*) and whiteflies (*Aleyrodidae*) are not BSV vectors.

1.5 Justification

The banana crop is being used as a staple food in Kenya and its production is being constrained by the BSV. Although the virus is thought to be transmitted by mealy-bugs and through seed, there is no evidence that horizontal transmission occurs to any significant extent in nature. Many mealy-bug species are known, but only a few species have been implicated in transmitting the disease in other countries. However, the acquisition feeding, latent and retention periods of transmission of the BSV in mealy-bug species are not well understood yet these are known to be very critical in transmission of BSV. In Kenya, no studies have been carried out to determine the mode of transmission of BSV by mealy-bugs. Similarly no studies have been carried out to determine whether there are other vectors in the Kenyan environment capable of transmitting BSV. Kenya agricultural research institute-Kisii was chosen because it is one of the KARI sub-stations that is mandated to disseminate tissue cultured banana to farmers and also had a large banana plantation with different banana germplasm (A and B-genome containing banana). Greater understanding of the transmission of BSV will contribute immensely to the development of viable control strategies of banana streak disease, thus improving banana production through breeding programmes of interspecific hybrids (A and B-genome containing *Musa* sp).

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and Distribution of Banana

Banana belong to the family *Musaceae* which comprises two genera, *Musa* and *Ensete* (Purseglove; 1985). *Musa* is the larger, more widely distributed and more diverse genus, consisting of 15 known species including the edible bananas (Simmond*s,* 1966).The edible bananas evolved from the two wild species, *M. acuminata colla* and *M. balbisiana colla*. *M. acuminata* provides the A-genome and *M. balbiasana* provide B-genome of the edible bananas (Simmonds, 1962; 1966). Hybrids of these polymorphic subspecies *M. acuminata*, led to a range of diploid cultivars designated as AAs in Simmonds classification scheme (Simmonds, 1962). Diploids give rise to AAAs triploids by chromosome restitution during meiosis. Hybridization between AAs and BBs (*M. balbisiana*) give rise to the various ABBs and AABs types.

Most cultivated *Musa sp.* are AAAs. It is thought that the domestication of a range of AAs and AAAs originally occurred in Malaysia (Simmonds, 1962). Their spread to areas where *M. balbisiana* occurred (India and Philippines) resulted in hybridization and appearance of the AAB, AB, ABB types. It is suggested that most of the bananas and plantains (AAB, ABB, AAA) grown in East Africa originated from Southern India and Malaysia (Simmonds, 1966). The cultivation of banana is limited to 40° north and south of the Equator as they cannot tolerate frost (Pursegrove, 1985). The major centers of production are central and South America, Asia and Africa (Simmonds, 1966). Bananas are sweet because the starch is converted to simple sugar (glucose) during ripening and this conversion process does not occur in plantain until they ripen to the point of rotting. This explains why plantain can only be eaten after cooking (Anonymous, 2004a). In Kenya, bananas are grown in Mt. Elgon, Kakamega, Bungoma, Kisii, Thika, Murang'a, Nyeri, Nakuru, Embu and Meru (Wangai *et al.,* 2002).

Bananas have a complex genetic constitution because of many ploidy levels (De Langhe, 1986; Ortiz and Vuylsteke, 1994). They rarely set seed by cross-pollination on their own; it is only more frequent with hand pollination, which makes it difficult for breeders (Vuylsteke *et al.*, 1993). After seed set, germination rate is low and consequently bananas have mainly been propagated vegetatively. The evolution of bananas has been slow and there is low genetic diversity in the species (Anonymous, 2004a). This has implications for the management of banana pests and diseases as it may explain why it is difficult to obtain sources of resistance (Panis *et al.,* 1993).

2.2 Importance of Banana

Bananas have played and continue to play a major role in the diets of people and the economy of Kenya. The species is an important food crop providing carbohydrate for both rural and urban households. Bananas are a source of income for majority of smallholder growers. In Kenya, they are a vital food crop grown for both subsistence and as a cash crop (K.A.R.I., 1998).

2.3 Constraints to Banana Production

Productivity of banana has been steadily declining due to the effects of pests and diseases, declining soil fertility, poor crop husbandry, socio-economic and post harvest problems (Gold *et al*., 1993). The crop is susceptible to numerous pests and pathogens including BSV, *Banana bunchy top virus,* (BBTV), *Banana mild mosaic virus* (BanMMV), *Banana bract mosaic virus* (BBrmV), *Cucumber mosaic virus* (CMV)*, Sugarcane mosaic virus* (SCMV), and *Banana virus X* (BVX). Some of these viruses such as BSV may hinder banana production in Kenya (Wangai *et al.,* 2002)

2.3.1 Integration of viral DNA in plant genome

It is now known that the DNA of some viruses can be integrated into some plants' genome (Harper *et al*., 2002). This phenomenon seems to be restricted to those viruses with DNA genomes, which include members of *Caulimoviridae.* Replication of members of the *Caulimoviridae* has two phases: 1) Transcription of RNA template from the virion DNA in the nucleus and 2) Reverse transcription of the RNA template to give dsDNA in the cytoplasm. This form of replication involves an integrase (int) gene, the products of which mediate insertion of the retrotransposons into the host chromosomes, resulting in various DNA intermediates both in the nucleus and the cytoplasm (Peterson-Burch *et al.*, 2000). However, only the integrated sequences of BSV, *Tobacco vein clearing virus* (TVCV) (family: *Caulimovridae*) and *Petunia vein clearing virus* (PVCV) (family: *Caulimoviridae;* genus: Caulimovirus*)* seem to express themselves episomally under some stresses (Ndowora *et al*., 1999).

Using specific primers for the amplification by polymerase chain reaction (PCR), it was found that the *Musa* genome contained integrated badnavirus sequences (Lafleur *et al.,* 1996). Cultivar Obino L'Ewai (AAB) for example has been shown to have a propensity to produce a large proportion of BSV infected progeny after tissue culture or cross- breeding (Ndowora *et al.,* 1999). The sequence of the episomal form of BSV DNA from a tetraploid hybrid derived

from Cv. Obino L'ewai showed that it is a typical *badnavirus* with all the features associated with episomally replicated pararetroviruses (Harper *et al.*, 1999).

2.3.2 BSV symptoms

Symptom expression due to infection by BSV varies depending on the virus isolate, host cultivars and environment (Lockhart, 1994; Gauhl and Pasberg, 1995; Dahal *et al.,* 1998; Karanja *et al.,* 2010). Some naturally occurring isolates of BSV, e.g. Rwandan isolates express more severe symptoms than others (Lockhart, 1994). In Nigeria and Kenya, the BSV symptom types and severity have been associated with changes in temperature (Dahal *et al.,* 1998; Karanja *et al.*, 2010). Chlorotic streaks at the initial stages of BSV development turn necrotic at the late stages of development and in hot conditions.

The most common foliar symptoms of BSV infection include broken or continuous chlorotic or spindle-shaped patterns (streak) (Lockhart, 1986). The streaks later turn necrotic as the disease progresses (Dahal *et al.,* 1997; 1998). Some BSV infected plants produce cigar leaf and internal pseudostem necrosis; symptoms that are characteristic of the Rwandan isolate (Lockhart, 1994). When this happens, the disease causes total yield loss since the plant does not produce bunch. Bunches or suckers may burst through the pseudostem and the plant may die pre-maturely (Gauhl and Pasberg, 1995). Heart rot strains of CMV, nematodes and fungal diseases can cause similar necrosis.

2.3.3 Characterization of BSV isolates

The taxonomy of BSV is currently under revision. Three viruses from Australia, namely: BSV-Mys (from cv. Mysore), BSV-GF (from Goldfinger) and BSV-IM (from CIRAD hybrids: IRFA 909, 910, 1914) which are significantly different from each other and from BSV-OL (from cv. Obino L'ewai, Nigeria) are now considered distinct virus species (Geering *et al.,* 2000).

An additional virus isolate from Australia namely BSV-Cav (from cv. Williams), is possibly a strain of BSV-OL (Geering *et al.,* 2000). Lockhart and Olszewski, (1993) examined five different virus isolates namely, BSV-Mad/DC (from cv. Dwarf Cavendish, Mandera), BSV-MA (from cv. Grand Nain, Morocco), BSV-MysIT (from cv. Mysore, Trinidad), BSV-DC/Mdg (from cv. Cavendish Madagascar) and BSV –RW (from Rwanda). Preliminary studies suggested that BSV-RW was sufficiently different from the previously mentioned virus isolates to be regarded as a separate species; BSV-Mys and BSV-Mys/T are synonymous (Geering *et al.*, 2005). The relationships of BSV-Mdg/DC, BSV-DC/Mdg and BSV-MA to the other virus isolates have yet to be examined.

2.4 DNA Isolation from Putative Insects

Numerous publications describe the preparation of genomic DNA from mammalian and invertebrate tissues (Sambrook *et al*., 1989; Henry *et al*., 1990; Cockburn and Fritz, 1996). These methods, often designed for cultured cells and tissues, generally require some form of cell lysis followed by deproteination procedures and subsequent DNA recovery (Sambrook *et al*., 1989). The methods vary in terms of the extent of deproteination and in the molecular weight of the DNA isolated (Strauss, 1998). The protocol described herein will promote both cell lysis and deproteination during extraction process.

Overall, DNA extraction from insects is invasive and causes damage to the specimens although efforts have been made to minimize the destruction by using small portions of the insects (Rose *et al.,* 1994; Mitchell *et al.,* 1997; De Verno *et al.,* 1998; Schneider *et al*., 1999). Fairly, non-destructive DNA extraction methods now exist (Philips and Simon, 1995; Cruickshank, 2002). In the method of Cruickshark, (2002), the specimens are cut into half, whereas in that of Philips and Simon, (1995) the abdomen is perforated several times with insect pins and submerged in the fluid to wash them. In case of the mealy-bugs, aphids and leafhoppers used in this study, these protocols may not apply since the insects are very small in size. Consequently, the protocol described in this study is the best for small insects because it salts out the DNA from all the insect's cells. Handling a whole organism of the instar of the insects is a challenge due to their size and hence, isolating DNA from their parts would be almost impossible. Indeed no protocol has been described to date that can isolate DNA from parts of aphids, mealy-bugs and leafhoppers. Therefore, there is a need to develop an appropriate DNA isolation for small these insects.

2.5 The Major Modes of Plant Viruses' Transmission

Plant viruses demonstrate a high level of specificity for the group of insects that may transmit them (Raccah and Fereres, 2009). Originally, Watson and Roberts (1939) proposed two modes of transmission: non-persistent and persistent based on the virus retention time in the vector. However, several viruses showed an intermediate retention in their vector. This led Sylvester *et al*., (1974) to assign the term semi-persistent viruses. In time, a different terminology was proposed for modes of transmission, based on the site at which the virus is carried in the insect. Thus, non-persistent viruses were termed stylet-borne, whereas persistent viruses were termed circulative (Ng. and Falk, 2006). Later on, additional parameters were attached to each of the modes of transmission. Non-persistent viruses are acquired and

inoculated during brief probing times, do not require a latent period in the vector, (Kennedy *et al.,* 1962) and are transmitted by many aphid species (Bradley, 1959).

Semi-persistent viruses need longer periods (hours) for acquisition and transmission than do non-persistent viruses (Pirone and Blanc, 1996; Blanc *et al.,* 2001). They have a narrower range of vector species (Blanc *et al*., 2001). However, the semi-persistent viruses do not need latent period and are lost when the vector moults. In persistent viruses, the longer the acquisition and inoculation times the higher is the rate of transmission (Duffus, 1972), they also have a narrow range of vectors; pass through moult and need a latent period. Many advanced biological, microscopical, immunological, molecular techniques as well as electronic devices have subsequently been used to determine the mechanisms of transmission in other vectors (Raccah and Fereres, 2009). As a result two principal modes of transmission have emerged which are circulative or internal mode, where the virus crosses body barriers and enters the circulatory system of the insect and accumulates inside the salivary glands (Gildow, 1985, 1987, 1989) and non-circulative or external mode, where the virus remains attached to the cuticle (cuticle-borne) of the insect and does not cross body barrier (Gildow 1989)

2.5.1 Mode of BSV transmission

Apart from Rice tungro bacilliform virus (RTBV), most of the other *badnaviruses* occur in vegetatively propagated plants and are perpetuated through infected planting materials (Anonymous, 2004a). Mealybugs (*Homoptera, pseudococcidae)* or leafhoppers (*Cicadellidae*) are thought to transmit badnaviruses semi-persistently (Lockhart and Olszeweski, 1993; Su Hong-ji, 1998; Kubiriba *et al*., 2001). These vectors are thought to acquire the virus after a minimum of 5 minutes of feeding with a maximum of 72 hours of inoculation access time for some mealy-bug species (Kubiriba, 2005). However, the transmission efficiency is thought to increase with longer acquisition feeds and all life stages can acquire and transmit the virus (Anonymous, 2004b). Jones and Lockart, (1993) suggested that BSV may be transmitted in a semi-persistent manner by the citrus mealy-bug (*P. citri*) from banana to banana under greenhouse conditions. In other greenhouse experiments, *Sugarcane bacilliform virus* (SCBV) from sugarcane was transmitted to banana by sugarcane mealy-bugs (*S. sacchari*) (Jones and Lockart, 1993). Kubiriba *et al.,* (2001) reported that *D. brevipes* and *S. sacchari* transmitted BSV from banana to banana but these mealy-bug species were obtained from sugarcane and pineapple fields and not from the banana fields. In addition, Kubiriba (2005) collected *D. brevipes, S. sacchari* and *Pseudococcus longispinus* from the bananas and successfully transmitted the BSV from banana to banana in both greenhouse and in the banana where plants

inoculated with *P. citri* had the most plants infected. One hundred and twenty hours feeding period (the peak time for BSV transmission) was observed from the time the fresh virus source plants were placed on the virus free plants (Kubiriba, 2005).

Plants which were inoculated with *P. citri, D. brevipes,* and *Pseudococcus* species first showed symptoms six, eight and ten weeks after inoculation, respectively. Cocoa swollen shoot virus (CSSV), a close relative of BSV is reported to be transmitted by a number of mealy-bug species, though some species appear to be more efficient vectors than others (Roivainen, 1980). Hence it is possible that several mealy-bug species other than those already tested can transmit BSV. Apparently, mealy-bugs are thought to retain the BSV for up to five days after acquisition (Kubiriba, *et al*., 2001; Su Hong-ji, 1998). As little as two mealy-bugs may transmit the BSV from the virus source plants to virus free plants (Meyer, 2006; Meyer *et al.,* 2008) and even if the viral load is low, mealy-bugs may be able to transmit 50% of the virus to the healthy plants (Meyer, 2006; Meyer *et al.,* 2008). Meyer *et al.,* (2008) reported that another mealy-bug species; *Pseudococcus ficus* can transmit the BSV from banana to banana. However, this mealy-bug species is found on the *Ensete ventricosum* which belong to the family *Musaceae* in Ethiopia (William and Matile-Fererro, 2000). However, effect of acquisition period, latent and retention periods are not well known with respect to transmission of BSV by mealy-bug species.

2.6. Vectors

2.6.1 The Mealy-bugs

Mealy-bugs are a highly specialized group of sucking insects that are characterized by having conspicuous surface wax (Williams, 1989). According to Vasvary (2004), a generalized life cycle for mealy-bugs is difficult to describe because of the wide diversity between the species in *Pseudococidae* family. In most cases, it consists of three stages which are: - the egg (found in the ovisac), immature (nymphs) and adults (male & females).

Some mealy-bugs are parthenogenetic while others reproduce sexually. Eggs retained in the ovisac hatch into tiny and active crawlers (first instars), which move in search of feeding sites. Once suitable feeding sites have been found, the mealy-bug nymphs then remain localized. Males are winged, but are rare and have rudimentary non-functional mouth parts. The adult females are wingless, largely immobile and have functional mouth parts (Cox, 1989). Although these features limit the mobility of some life stages, mealy-bugs are effective as virus vectors (Roivainen, 1980; Su Hong-ji, 1998).

Populations of mealy-bugs on cocoa tend to be patchily distributed and many trees are not infested or carry few mealy-bug colonies (Cornwell, 1958). Large infestations are infrequent and many mealy-bug species are associated with attendant ants. The ants maintain and defend the mealy-bug colonies, remove the honeydew produced and build protective cacoon tents of soil and organic debris. Thus, mealy-bug populations are influenced by the distribution of ant colonies with complex interactions between *Coccidophilic* species and antagonists (Bigger, 1981). The mealy-bugs can be spread through the canopy of the plantation or along the ground for CSSV and BSV (Strickland, 1951; Cornwell, 1956; Thresh, 1958; Kubiriba, 2005), or be spread by wind currents (Strickland, 1950, Kubiriba, 2005) and ants (Cornwell, 1957). Passage from tree to tree along the ground and by ants appears to be less important (Strickland, 1951). Hence, movements of mealy-bugs through the canopy and by wind currents are deemed to be responsible for most of the BSV transmission (Kubiriba, 2005). Mealy-bugs moving through the canopy seem to be responsible for the radial spread and that by wind currents for new outbreaks (Kubiriba, 2005). Mealy-bugs and virus spread between trees seem to be facilitated by close spacing and interlocking canopies (Cornwell, 1958, Kubiriba, 2005).

When composition and size of the mobile population of *P. njalensis* on Cocoa was assessed, 91.8% consisted of first instars (Cornwell, 1958). These are efficient vectors under controlled conditions and presumably in the field, but at least some nymphs may migrate before they feed to become infective (Dale, 1957). In Kenya, several mealy-bug species have been recorded on different host plants as shown in Table 1

Table 1: Host distribution of mealy bug species recorded in Kenya

Mealybug species	Host Distribution	Author
**Dysmicoccus brevipes* (Cockerell)	Pineapple	Williams & Granara de Willink (1992)
Geococcus coffeae (Green)	Coffee	Williams (1958); Williams & Watson (1988); Williams & Granara de Willink (1992)
Maconellicoccus hirstus (Green)	Hibiscus but not on Musaceae	Williams (1996)
Paracoccus burnerae (Brain)	*Ensete sp*	Ben-Dov (1994); De Lotto (1967)
Paraputo anomalus (Newstead)	*Musa sp.*	Williams (1958)
**Planococcus citri* (Risso)	Citrus and vegetable	Cox, (1989); Watson *et al*., (1995)
**Pseudococcus comstocki* (Kuwana)	Irish potato (Ghana), Coffee (Kenya)	Williams & Granara de Willink (1992)
Pseudococcus longispinus (Targioni Tozzetti)	Grapevines	Williams & Granara de Willink (1992); Williams & Watson (1988); De Lotto, (1967)
Rastrococcus iceryoides (Green)	*Musa sp.*	Williams (1989)
*[#]*Saccharicoccus sacchari* (Cockerell)	Sugarcane	Williams & Granara de Willink (1992)

**These are the mealybug species that have been reported to transmit BSV under screenhouse conditions before*

[#] Has not been identified on bananas but also reported to have transmitted BSV under screenhouse conditions

Source: (Dahal et al., 1997)

2.6.2 The Whiteflies (*Aleyrodidae*)

Spiraling whiteflies (*Aleurodicus diperces)* are sap-sucking insects that damage and discolor plant leaves and tissues. Similar to aphids and mealy-bugs, whiteflies excrete honeydew that may lead to black sooty mold. Ants feed on this honeydew and protect the

whiteflies from the natural predators. Though whiteflies are also found in banana plantations, they have not been tested for the presence of BSV and their capacity to transmit the virus. It has been reported that adult whitefly mouthparts are similar to those of other homopterans, especially aphids (Rosell *et al.*, 1995). The feeding behavior of whiteflies resembles that of aphids in being piercing and sucking and involving a salivary sheath (Janssen *et al.,* 1989).

2.6.3 The Aphids (*Aphididae*)

Aphids transmit some of the most important viruses of crop plants. More than 200 aphid species have been shown to transmit viruses and many others are as yet untested though they have this ability (Eastop, 1981). Most of the 228 species of aphids recorded as virus vectors belong to the subfamily *Aphididae,* which include the genera: *Aphis, Myzus* and *Macrosiphum* (Eastop, 1977). *A. gossypii* is the vector of about 50 non-persistently transmitted viruses including those of bananas, and seven persistently transmited viruses.

Aphis gossypii is a term applied to aphids from many hosts and may include several taxonomic entities (Eastop, 1981). The *Pentalonia nigronervosa;* belongs to the genera: *Myzus* and is a banana aphid. Reproduction is largely parthenogenetic although sexual reproduction also occurs. The distribution is almost co-existensive with the cultivation of bananas (Eastop, 1981). *Pentalonia nigronervosa* is found in the crown of the plants, at the base of pseudostems, or between outer leaf sheaths. Young suckers are the most, heavily infected. However, there is no evidence to date on the potential for BSV transmission by these vectors.

2.6.4 Leafhoppers (*Cicadellidae*)

Unlike aphids, leafhoppers have a simple life cycle in which the egg hatches to a nymph, which feeds by sucking and passes through a number of moults before becoming an adult. Mensfin *et al*., (1995) reported that the feeding behavior of the leafhoppers, *Cicadulina mbila,* was similar to that of aphids, with the mouthparts, surrounded by the salivary sheath, penetrating to the phloem in the preferred host plants (Nault, 1997). There are about sixty (60) subfamilies in the leafhopper family (*Cicadellidae*) and two of these subfamilies (*Agalliinae* and the Deltocephalinae) contain species that are plant virus vectors. The *Agalliinae* have herbaceous dicotyledonous hosts while most *Deltocephalinae* feed on monocotyledons (Nault and Ammar, 1989). There are about 15000 described species of leafhopper in about 2000 genera. Of these, only 49 species from 21 genera have been reported as being plant virus vectors (Nault and Ammar, 1989). The potential of leafhoppers to transmit BSV has not been reported.

2.7 BSV Diagnosis Techniques

2.7.1 Serological diagnosis

Due to serological heterogeneity between BSV isolates, mixtures or cocktails of antibodies raised against different isolates have been used to capture a wide range of serotypes (Ndowora *et al.,* 1999). Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) protocols to detect BSV have been used in Kenya (Karanja *et al.*, 2008).

Some BSV isolates are serologically only distantly related to others and the degree of cross-hybridization between them is low (Lockhart and Olszewski, 1993). Hence, Triple antibody sandwich-enzyme linked immunosorbent assay (TAS- ELISA) has been only marginally successful, sometimes failing to detect a number of BSV isolates (Lockhart and Olszewski, 1993). The method is also not useful in distinguishing between individual isolates detectable by the cocktail of polyclonal antibodies. More specific diagnostic techniques based on the nucleic acid analysis have been developed to ameliorate these difficulties (Ahlawat *et al.*, 1996; Harper *et al.,* 1999).

2.7.2 Nucleic acid –based diagnosis of BSV

These methods involve extraction of DNA from leaf samples and amplification of a portion of the DNA, specified by pre-designed primers to BSV, in a polymerase chain reaction (PCR). Degenerate PCR primers have been designed using conserved sequences of *badnavirus* (Bouhinda *et al.,* 1993) and used for detection of BSV (Lockhart & Olszewski, 1993; Ahlawat *et al.,* 1996). Other PCR primers were designed using nucleotide sequences of BSV from Nigeria, and tested for detection of BSV (Harper and Hull, 1996). Although the PCR technique appears to be more sensitive than the serological techniques, assessment of health status of plants indexed soley by PCR may not be appropriate due to detection of inactive BSV DNA integrated into the *Musa* genome (Harper *et al.,* 1999), and hence recommendation for use of a diagnostic method based on a combination of serological and PCR techniques. This technique is refered to as immunocapture-polymerase chain reaction (IC-PCR).

Immunocapture-PCR can detect the episomal forms of BSV because it involves trapping the virus particles using polyclonal antibodies (IgG PMxR-2C). These trapped particles are sources of template DNA for the PCR process. The high temperatures involved are able to denature the protein coat; and the viral DNA is released and then amplified by PCR (Harper *et al.,* 1999). Despite its increased efficiency in detection, there may be a need to refine the IC-PCR technique to enhance system for detection of divergent BSV isolates (Kubiriba, 2005). Due to the variability of Kenyan BSV isolates and the nature of the virus (both integrated and

episomal), it would be essential to develop a diagnostic kit which can quickly and reliably detect BSV (Karanja *et al.,* 2008). For example the primer set 1A-4, was unable to amplify some of the BSV isolates in both Ugandan and Kenyan samples (Kubiriba, 2005; Karanja *et al.,* 2008; Karanja, 2009) despite very clear BSV symptoms. Because of such limitations, another novel technique for detection of BSV based on a DNA amplification technique called RCA (Templiphi) has been developed and used in amplifying the genome of geminiviruses (Haible *et al.,* 2006). In addition, the technique has been used in amplifying the genome of the BSV (James *et al.*, unpublished).

2.7.3 TempliPhi method

This technique has been used to amplify circular dsDNAs of begomoviruses without amplifying linear dsDNAs. In nature, the replication of circular DNA such as plasmids and viruses occurs via rolling circle mechanisms (Kornberg and Baker, 1992). As a laboratory method, linear rolling circle amplification (RCA) (Fire and Xu, 1995; Liu *et al.,* 1996; Lizardi *et al.*, 1998) is the prolonged extension of an oligonucleotide primer annealed to a circular template DNA. A continuous sequence of tandem copies of the circle is synthesized. Rolling circle amplification has the advantange of not requiring a thermal cycling instrument. Two primers are used to perform exponential (hyperbranched) RCA, one for each strand (Lizardi *et al.*, 1998).

Multiply-primed RCA uses *Phi29* DNA polymerase which has a capacity to perform strand displacement DNA synthesis for more than 70,000 nucleotides without dissociating from the template (Blanco *et al.*, 1989) and its ability which allows efficient DNA synthesis to continue for many hours. Pyrophosphatase is added to the reaction to eliminate the inhibitory accumulation of pyrophosphate (Dean *et al.*, 2001).

The use of random hexamer primers with three thiophosphate-protected ends is also vital, allowing circular DNA molecules to be amplified at least 10,000 folds by protecting the primer from the 3’ exonuclease activity of the *Phi29* DNA polymerase (Dean *et al.,* 2001). Despite its novelty, this method has not been used in diagnosing BSV in Kenya. However, the method has proven to be very efficient and reliable in diagnosing BSV elsewhere since it only amplifies the circular DNA of the virus excluding the host genome (Dean *et al.*, 2001; James *et al.*, unpublished); unlike in IC-PCR and other PCR techniques. The Templiphi method can in addition help to resolve the problems of having false negative and false positive results (James *et al.*, unpublished)

CHAPTER THREE
MATERIALS AND METHODS

3.1 Sample Collection

3.1.1 Collection of mealy-bugs

A direct searching method for mealy-bugs under the pseudostem sheaths and below ground level under the roots as described by Kubiriba *et al.,* (2001) was employed in this study. This method was used because the sampling was done in commercial plantations. The vectors were collected from the infected banana fields in KARI-Kisii. This agricultural centre was chosen because it is one of the KARI sub-stations that are mandated to disseminate tissue cultured banana to farmers and also had a large banana plantation. Species of live mealy-bugs were collected using a camel hair brush and placed into a specimen container (polypropylene bowls covered with muslin cloth over the ventilation holes in the lids) alive. To ensure correct typing during identification, separate specimens of various sizes of mealy-bugs were collected. The mealy-bugs were then transported to a shaded, cool greenhouse for rearing at KARI-Njoro. The mealy-bug species were identified based primarily on adult female morphological features as shown in Appendix 1 (Kubiriba, 2005). The rearing of the collected mealy-bugs was done on pumpkin fruits placed on bowl in black cages (75 cm x 32.5 cm) as shown in Figures 1 and 2. The black cages provided a dark condition for the mealy-bugs because these insects are known reproduce and multiply in large numbers in dark conditions rather than in light conditions (Kubiriba, 2005) The live species of the mealy-bugs were reared for use in the experiments described in sections 3.1.4, 3.1.5 and 3.1.6. Individual mealy-bug species was put on a detached pumpkin fruit placed on a bowl inside the rearing cage covered with a black material to create dark conditions and then sited in a shaded area in a greenhouse at room temperature at KARI-Njoro. A camel hair brush was used to place the mealy-bugs on the pumpkin fruits. The advantages of using pumpkin fruits included the fact that the fruit is not a host of *Banana streak virus* and mealy-bugs are easy to remove from hard skinned pumpkins for inoculation experiments. To prevent cage contamination by ants or other crawling insects, the cage was placed on a pan containing soapy water. The ants' antennae are repelled by hydrophobic molecules like those in detergents (soapy water) because the antennae contain lecithin molecules which are hydrophilic. The mealy-bugs were reared for four months for good culture development due to the fact that mealy-bugs have a life cycle of 4-6 weeks. The third and fourth generations were used in transmission experiments.

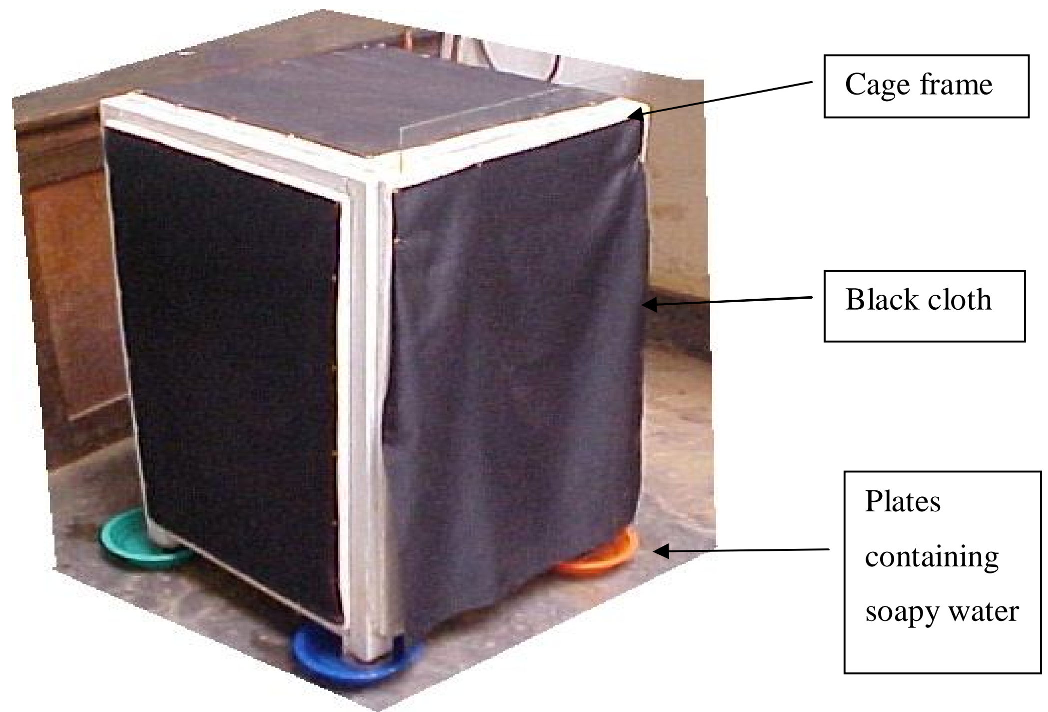


Figure 1: Rearing cage for mealy-bugs covered with black cloth and placed on plates containing soapy water

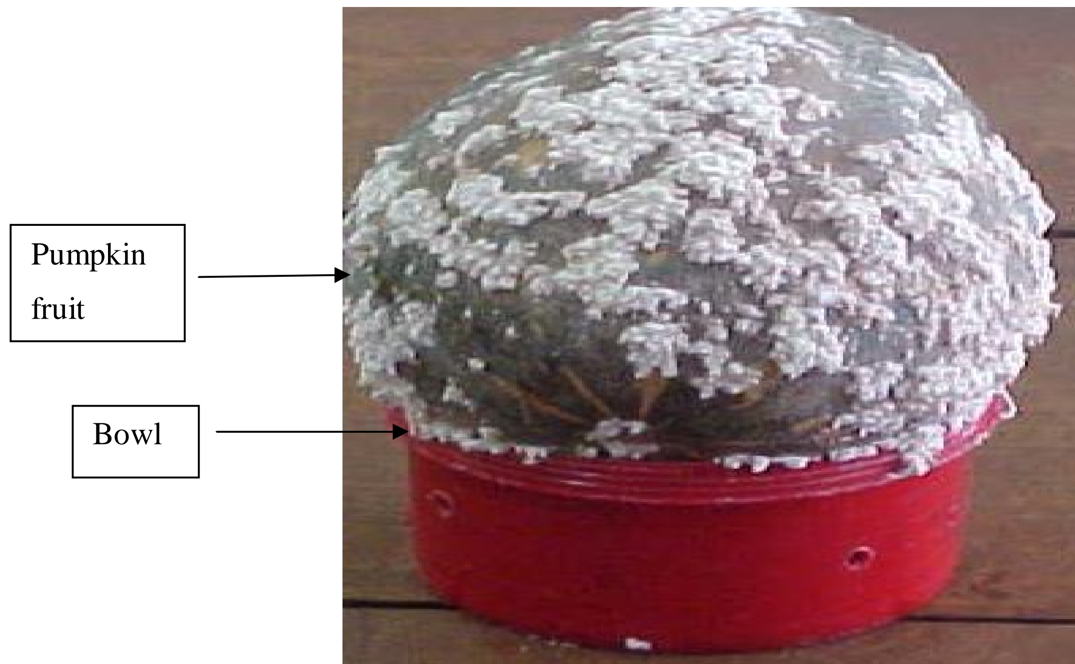


Figure 2: *P. burnerae* on a pumpkin fruit placed on a bowl

3.1.2 Collection of putative vectors for *Banana streak virus* screening

Sap-sucking insects (Banana aphids, and leafhoppers) were collected from BSV infected banana plantations in KARI-Kisii. The common species of these sap-sucking insects on the banana plantations were identified. Aphids on the banana plant pseudostem were collected using camel hair brush and were placed in vials containing 70% ethanol. Whiteflies were not found on the banana plantations at KARI-Kisii. A black trapping cage was used to trap leafhoppers found in the banana plantations. The trapped leafhoppers were put in vials containing 70% ethanol and then corked. Bottles containing the collected suspected vectors were transported to the KARI-Njoro laboratory to be screened for BSV as described in section 3.1.7.

3.1.3 The virus-source plants and test plants for transmission

The virus source plants were obtained from the greenhouse at KARI- Njoro. These plants were screened for the presence of BSV using the Immuno-capture-PCR (IC-PCR) and Rolling circle amplification (RCA) techniques as described in sections 3.2.6 and 3.2.7. These infected plantlets were obtained from the collection of the infected germplasm materials of KARI-Njoro screenhouse. Two months old test plants (virus free) were also obtained, from KARI-Njoro tissue culture laboratory, and they were indexed for BSV using IC-PCR as described in section 3.2.6 and RCA as described in section 3.2.7 to ensure that they were not infected. These clean plantlets were planted in a 2 kg black polythene paper with soil until they were 20 cm tall with four leaves. Figures 3, and 4, show leaves of the virus source, and test plants used in the transmission experiments.

Figure 3: The infected leaves of Chirume cultivar with chlorotic streaks.

Figure 4: The virus free plants for inoculation access by *P. burnerae*

3.1.4 The effect of acquisition period on successful transmission of *Banana streak virus* in greenhouse by mealy-bugs

Two hundred second instars of *P. burnerae,* from the rearing cages were starved overnight in a bowl covered with a 50nm x 50nm size net. Before acquisition feeding, five instars of *P. burnerae* were sampled randomly and screened for transovarial transmission using RCA, (TempliPhi) technique, since their parents were collected from the infected banana plantation. The starved instars were fed on the virus source plant in the separate cage in the greenhouse. The female mealy-bug instars (6-10) were then sampled and fed on the healthy banana plant (for more than 72 h) for inoculation of the virus by the vectors. The sampling time intervals were 30 min, 1 hr, 2 hrs, 3 hrs, 4 hrs, 5 hrs, 6 hrs, and 7 hrs of acquisition access period

(Kubiriba, 2005). A portion (five *P. burnerae*) of the sampled second instars from the virus source plants, fed on the plant for a full day, were randomly picked and tested for the presence of BSV using the RCA technique to confirm the acquisition of the virus by the instars. The mealy-bugs (6-10 instars) were transferred and fed on virus free banana plants in separate cages for each time interval as shown in Figure 5. Both the acquisition and inoculation feeding were done in an improvised clip cage. In this case, each black cage was labeled in terms of time intervals. A *Banana streak virus* free plant of the same cultivar which was not fed on by viruliferous instars was used as a negative control in a separate cage. The plant leaves in each cage containing the viruliferous mealy-bug instars and the mealy-bug free cage was sampled and DNA were isolated as described in section 3.2.1.The RCA was carried out in these DNA samples to detect the BSV as described in section 3.2.7. This experiment was done in three replicates.

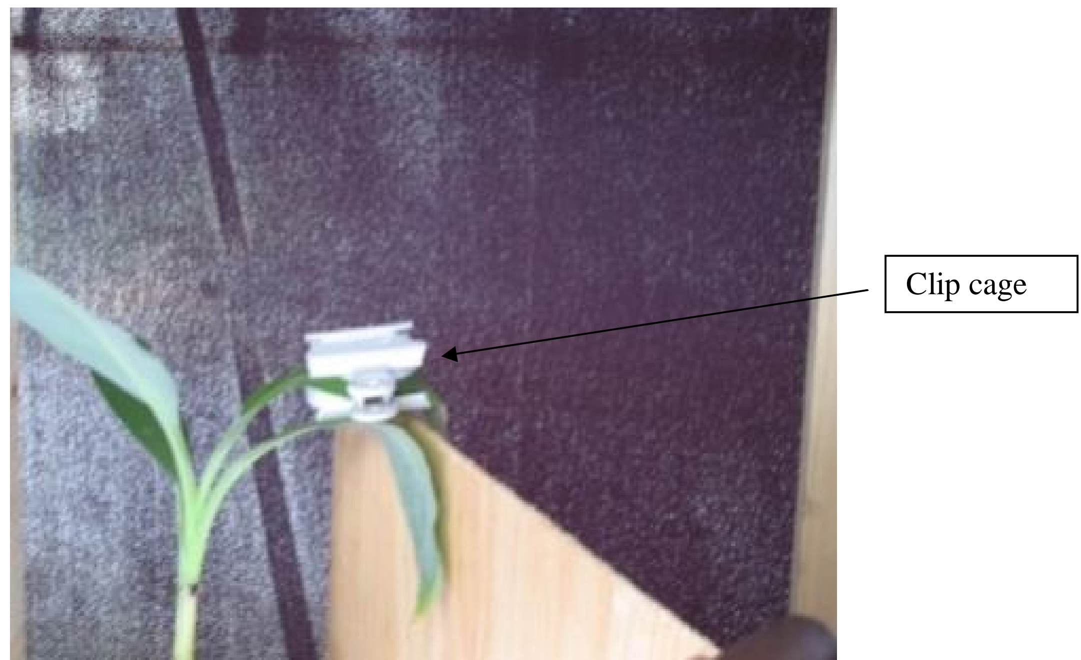


Figure 5: Showing the set up of the transmission experiments (acquisition and inoculation access)

3.1.5 Determination of latent period of *Banana streak virus* in the *P. burnerae*

Sixty second instars of the *P. burnerae,* from the rearing cages were subjected to the virus source plants in different clip cages in the greenhouse for them to acquire the virus for five days. The female mealy-bug instars were then transferred on to the pumpkin fruits (placed on the plastic bowls) using camel hair brush. These second instars of the *P. burnerae* species on the pumpkin fruit were kept in black cage where they were sampled from, during inoculation access period. Sampling was done at regular intervals of one day for a period of 3 days (i.e day 1, 2 and 3). Five to ten second instars of *P. burnerae* were picked randomly in two sets with one

set being used for assaying of BSV in mealy-bug instars (in case of inoculation failure) using the RCA, technique. The second set of mealy-bugs' instars (7-10 in numbers) was fed on virus-free plants in clip cages in the greenhouse for inoculation access time of 4 days. After four days of inoculation period, the leaf samples of the virus inoculated plants were sampled; DNA was isolated as described in section 3.2.1 and assayed for the presence of BSV using RCA technique. The cages were always placed on the plates containing soapy water to prevent ants from colonizing the mealy-bug cultures. This experiment was carried out in three replicates.

3.1.6 Estimation of the retention period of *Banana streak virus* in the *P. burnerae* in greenhouse

One hundred non-viruliferous *P. burnerae* first instars from the rearing cages were fed on the virus source plants (same cultivar) in a black cage for them to acquire the virus within the greenhouse. After five days, the mealy-bug instars from the cage were transferred to a different cage, placed on the pumpkin fruit (on a bowl) for seven days from which they were being sampled to determine the retention period. Camel hair brush was used to remove the mealy-bug instars from the infected plants on to the pumpkin fruit. Five mealy-bug instars from the virus source plant cage were sampled randomly and tested for the presence of BSV using RCA technique and thereafter, more than seven instars of the *P. burnerae* (the ones preserved for seven days) were sampled regularly at an interval of one day for a week and were fed on the clean plantlets, for inoculation access time. The leaves of the inoculated banana plants by the viruliferous instars were sampled; DNA were extracted as described in section 3.2.1 and diagnosed for the presence of the BSV using the RCA technique. This was to confirm whether the mealy-bugs had the virus and were able to transmit it in the healthy plants. Three replicates of the same experiment were set at the same time of the experimental days.

3.1.7 Screening banana aphids (Aphididae), whiteflies (Aleyrodidae), and leafhoppers (*Cicadellidae*) for *Banana streak virus* transmission

The common aphids and leafhopper species, from an infected banana plantation were collected using camel hair brush from the banana pseudostem and black trapping cage respectively. The DNA from three leafhoppers and five aphids were isolated using the DNA isolation protocol described in section 3.2.2. The DNA was quantified using biophotometer (Epperdorf, Hamburg, Germany) quality and quantity of the isolated DNA was checked using uncut unmethylated Lambda (λ) DNA and the digestibility of the DNA was determined using the restriction endonuclease (*Stu*1). Thereafter, RCA assay was carried out on the DNA samples to detect the BSV as described in section 3.2.7.

3.2 DNA Extraction and Amplification (CTAB-Based Purification)

3.2.1 DNA isolation from plant tissues

Total DNA was isolated using a modification of the cetyltrimethylammonium bromide (CTAB) protocol described by Gawel and Jarret, (1991). Fresh leaf tissue (0.4g) was ground in 300ml of extraction buffer with an aid of acid washed sand. The extraction buffer components were 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. 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 was incubated at 65 °C for 15 minutes, spanned at 15000 rpm and 750 µl transferred to a fresh tube. This was then mixed with an equal volume of chloroform/isoamylalcohol (24:1) and centrifuged for 5 minutes at 15000 rpm. Nucleic acids in the aqueous phase were then pelleted using isopropanol and centrifuged for another 5 minutes at 13000 rpm. The DNA pellet was washed with 500µl of 70% ethanol, resuspended in 100µl of nuclease-free water. The DNA was left at 4°C overnight to fully dissolve after which it was stored at -80°C.

3.2.2 DNA isolation from test insect vectors

To develop and evaluate the new protocol, several DNA isolation protocols described by Michele *et al.,* 2002 were evaluated. Samples were selected to include different haplotypes of *Pseudococcidae*, *Aphididae*, *Cicadellidae*. The insects were placed in an eppendorf tube containing absolute ethanol and stored at -80ºC pending DNA extraction. This lasted at least for overnight to inactivate the nucleases and also enhance easy destruction of the insect’s cuticle. Five mealy-bugs, three aphids and five leafhopper insects were put in each eppendorf tube separately. The insect samples were cleaned using distilled H_2O containing 2mM EDTA or Tris-EDTA (TE) by vortexing for 30 seconds, after which the dH_2O-EDTA solution was removed with a pippete. Four hundred microlitre (400µl) of extraction buffer (0.5M sodium chloride, 10mM Tris (pH 8.0), 36Mm EDTA (pH 8.0), 0.2% sodium dodecyl-sulfate (SDS), 25µl proteinase-K and 10M ammonium acetate at adjusted pH of 7.6) was added to the eppendorf tubes. The tissue/insects were ground with a sterile Teflon epperdorf grinder (Kontes, Hamburg, Germany). The mixture was incubated in a heating block at 55º C for two hours. The eppendorf tubes were centrifuged in a non-refrigerated microcentrifuge at 14000 rpm for 5 minutes to pellet the cell debris and precipitate proteins. Then 2µl of 10mg/ml *RNases* were added to the supernatant in a fresh eppendorf tube and incubated for one hour at

37º C. Two volumes of ice-cold isopropanol were added to precipitate the DNA and the mixture was mixed gently by inverting the tubes. The tubes containing the DNA were placed at -20º C for 30 minutes to allow the DNA to precipitate. The tubes were centrifuged at 14000 rpm for 15 minutes. The supernatant was removed and the same volume of cold 70% ethanol added to the pellet. The tubes were spun at 14000 rpm in a non-refrigerated microcentrifuge for 5 minutes. The ethanol from the eppendorf tube was poured off and the tubes completely air dried for at least 15 minutes. The DNA pellet was resuspended in 50 µl of distilled H_2O and incubated at 37º C for 30 minutes or at 4º C overnight to dissolve the DNA. Three samples for each insect family: mealy-bugs, aphids and leafhoppers were extracted using the protocol and purity, quality, quantity and the restrictability of the DNA was carried out as described below.

3.2.3 UV-Quantification of DNA

Fifteen hundred microlitres (1500µl) of sterilized distilled water was used as a blank. Two (2) µl of the isolated DNA from each insect family were added to 1498µl of sterilized distilled water, and the optical densities at OD260 and 280nm read from a biophotometer (Eppendorf, Hamburg, Germany).

3.2.4 DNA quantity and quality Control

The extracted DNA was run on agarose gels against uncut unmethylated λ phage DNA standards. The dilutions of various concentrations (750µl, 500µl, 250µl) of the λ DNA were made. Two microlitre of the extracted DNA were added to 4µl of loading dye. These DNA was loaded on SYBR- safe (Invitrogen) stained 1% agarose gel and ran using 1X TBE buffer alongside the three dilutions of the uncut (λ) DNA as a molecular standard. The gel was visualized using a Bench top UV transilluminator (AlphaDigiDoc, Cambridge, UK) at a wavelength 320nm.

3.2.5 DNA Digestibility Test

Two (2) µg/µl of each DNA sample were put in a 0.5 ml microfuge tube. A digestion master mix based on the recipe given in (Table 2) was prepared and kept on wet ice. Eight microlitre of this mix was added to each of the tubes containing the DNA. The mixture was gently but thoroughly mixed and the tube contents spun down using a microcentrifuge.

The digests were incubated at 37°C overnight in a heating block (water bath). The digest reaction was removed and loaded in a 1% agarose gel stained with a SYBR-safe dye. The gel was run at 100 volts for 2 hours. Lambda DNA digested with *Stu*1 was used as a molecular weight marker. The DNA was visualized in a Bench top UV-transilluminator (AlphaDigiDoc, Cambridge, UK) at a wavelength of 320nm and a photograph was taken.

Table 2: A master mix of *Stu*1 digest

Component	**X1**	**X11***
10x buffer	2.2µl	24.2µl
Stu1 [500U(10U/µl)]	0.25µl	2.75µl
H_2O	7.8µl	85.8µl
DNA	2µl	
Total	**12.25**	

* Bulk mixes for the total number of reactions +1 were prepared to allow for pipetting errors.

3.2.6 Immuno-capture Polymerase Chain Reaction (IC-PCR)

The sap from both virus source and virus free banana leaf samples were extracted and concentrated as described by Harper *et al*. (2002) and modified by Karanja *et al*., (2008; 2009). One gram of fresh leaf samples were ground in phosphate buffered saline (PBS) + polyvinylpyrrolidone (PVP) extraction buffers. Concentration was carried out by precipitation with 4% polyethylene glycol in 0.1 M sodium chloride. The sample mixture was stirred for two hours at room temperature. The virus was then sedimented at 16,000 rpm for 20 minutes in a microfuge. Thin-walled propylene microfuge tubes were coated with polyclonal antibodies generated against 32 Sugarcane Bacilliform Virus (SCBV) and BSV Mysore isolate PMX2RC (Ndowora and Lockhart, 2000). The tubes were then washed three times with 100µl of PBS-Tween-20. More than three times wash led to uncoating of the tubes with the antibodies thus only dimmers of the primers were formed. 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.

3.2.7 Rolling Circle Amplification (RCA, Templiphi)

The RCA technique was carried out using the standard protocol of Sambrook and Russell, (2001) as modified by James *et al*., (unpublished). Amplification of circular DNA of BSV was performed using a TempliPhi^{TM} Kit (GE Healthcare, formerly Amersham-UK) following the manufacturer's protocol. The Templiphi kit contained sample buffer (Random hexamers), Reaction buffer (salt and dNTPS) and Enzyme mix (*Phi29* DNA Polymerase, Random primers in 50% glycerol). Ten to twenty nanogram of total nucleic acid was dissolved in sample buffer, denatured for 3min at 95ºC and cooled down in ice for 3min. After adding 5µl of reaction

buffer and 0.2µl of enzyme mix, the reaction was run for 18–20hrs at isothermal temperature of 30ºC. The reaction was stopped for 10min at 65ºC to inactivate the *Phi29* DNA polymerase.

Aliquots corresponding to 250ng nucleic acids in 10.2µl volume of RCA product were digested using *Stu*1 for 2hrs according to the manufacturer's protocol. Restriction products were run on a 1% agarose gel, containing gel red (dye) to stain it for visualization of the bands as described. One gramme (1g) of agarose was measured using a sensitive weighing balance and suspended in 1 x TBE buffer, heated in a microwave for 3 minutes, removed and then left to cool. The SYBR-safe dye was added before pouring the gel into an electrophoresis tray or plate containing well combs for solidification. After the gel solidified, the well comb was removed and the gel placed in the tank with buffer. The DNA samples from the Templiphi reaction were mixed with 3µl loading dye and loaded in the gel wells.

A DNA molecular weight marker fragment (Hyperladder™ 1, Bioline) was loaded for comparison. The electrophoresis was carried out at 100 volts for one hour. The electrophoresed gel was visualized on a Bench top UV transilluminator (AlphaDigiDoc, Cambridge, UK) at a wavelength of 320nm and photographs taken on a gel documentation system. To estimate unknown fragment sizes, migration distances were compared to those of reference fragments from the Molecular marker (Hyperladder™ 1, Bioline).

3.3 Data Analysis and presentation

Data from the electrophoretic analysis was used to test the hypothesis and to determine the mode of transmission of BSV by the *P. burnerae*. Data was presented as tables and figures representing the detections in transmission experiments in the greenhouse for *P. burnerae*. Principal characteristics of the modes of transmission of viruses by insects were used to determine the mode of transmission of BSV by *P. burnerae*. Data generated from this study was compared with the standard characteristics as described by Raccah and Fereres, (2009) as shown in appendix 2.

CHAPTER FOUR

RESULTS

4.1 Distribution of mealy-bugs and putative vectors of BSV

Paracoccus burnerae was found on the pseudostem of the banana near the corm in their cocoon at KARI Kisii. The instars were found moving on the pseudostem. However, *P.citri*, *D. brevipes,* and *S. sacchari* were not found on the banana plantation at KARI Kisii, Chuka and at Njoro. The mealy-bug species are host specific and the three mealy-bug species were being collected from citrus, pineapple and sugarcane before as shown in appendix 6 although they use *Musa sp*. as the alternative host. The mealy-bug species was reared successfully on the pumpkin fruits. Banana aphids were also found on the pseudostem and in the sheath of the banana plants. Aphids seem to be less mobile compared to the instars of the mealy-bugs. Leafhoppers were found on the banana leaves and also on the weeds. Leafhoppers are very mobile and jumped from one plant to another. However, whiteflies were not found in banana plantations at KARI-Kisii.

4.2 DNA Extraction

4.2.1 DNA isolation from putative vectors of BSV

Various DNA extraction protocols described in Michele *et al*., (2002) for extracting DNA from formalin preserved specimens were tried but gave results that were not desirable; the DNA was degraded in case of leafhoppers as shown in Figure 6. Mealy-bugs and aphids gave little amounts of DNA that was also degraded. Due to this reason the products of these protocols were not used in downstream reactions. Most of the protocols that were tried gave DNA that had RNA contaminants.

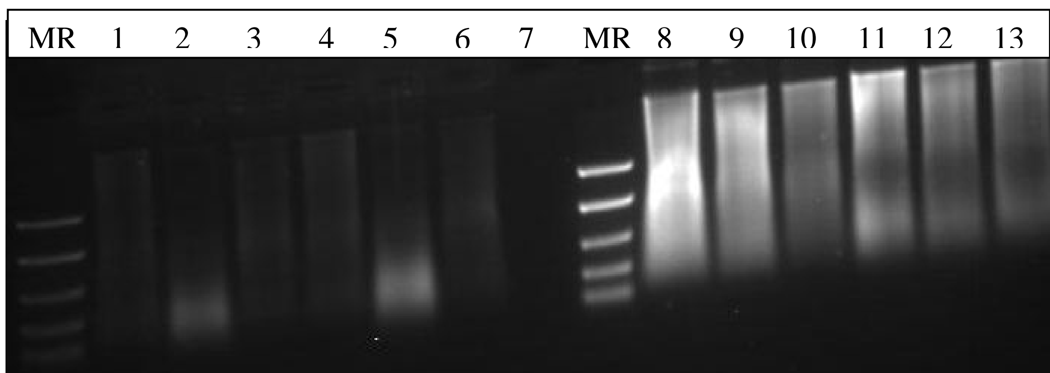


Figure 6: Quantity and quality of isolated DNA using Michele *et al*. 2002**.** Lanes 1-3 represent Aphid DNA, Lanes 4-6 represent Mealy-bug DNA, Lane 7 represent SDW, Lanes 8-13 represent Leafhopper DNA.

A novel DNA isolation protocol for mealy-bugs, aphids and leafhoppers was established. The protocol gave products that were pure (no proteins), intact (not degraded), and restrictable by the restriction enzymes. Data on purity, intactness, quality and digestability of the isolated DNA is presented in Table 3 and Figures 7 and 8. The concentration of isolated DNA ranged from 2.6-7.2ng/µl for mealy-bugs, 4.2-5.4ng/µl for aphids and 2.0-3.5ng/µl for leafhoppers (Table 3)

Consistently good preparations of DNA were obtained from mealy-bugs, aphids, and leafhoppers by use of the method described in section 3.2.2. All the DNA preparations exhibited an OD260/280nm ratio of 1.8-2.0.

Table 3: Data on UV-Quantification of DNA.

Sample no.	OD260nm	OD280nm	DNA Concentration (ng/µl)	OD260/280
Mealy-bugs				
1	0.143	0.071	7.2	2.00
2	0.144	0.077		7.2
3	0.052	0.027	2.6	1.95
Aphids				
1	0.109	0.060	5.4	1.82
2	0.106	0.056	5.3	1.89
3	0.084	0.044	4.2	1.93
Leafhoppers				
1	0.040	0.021	2.0	1.87
2	0.056	0.030	2.8	1.85
3	0.070	0.037	3.5	1.87

The optical density at 260nm was recorded and the corresponding DNA concentration (ng/µl). Mealybugs yielded the highest DNA while the leafhoppers had the lowest amount of DNA. The absorbance ratio at OD260/OD280nm of 1.8-2.0 was observed.

Both the quantity and quality of the DNA samples obtained from five aphids, five mealy-bug instars and three leafhoppers, was also quantified using uncut unmethylated λ DNA as a standard. The results indicated that the isolated DNA was high in molecular weight and was intact (Figure 7).

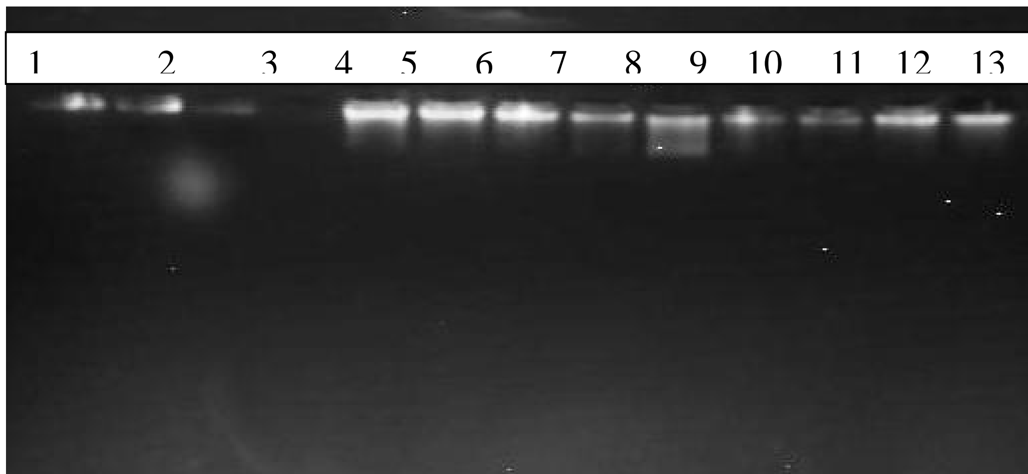


Figure 7: Quantity and quality of isolated DNA from putative vectors of BSV; Lanes 1-3-uncut unmethylated λ DNA standards (750ng, 500ng, 250ng respectively), Lane 4 represent Negative control (SDW), Lanes 5-7-Aphid DNA, Lanes 8-10-Leafhopper DNA, Lanes 11-13- Mealy-bugs DNA.

The quality of DNA samples extracted from five mealy-bugs, five aphids and three leafhoppers was further determined by assaying for their digestability using *Stu*1. The results showed that leafhopper and mealy-bug DNA were more restrictable than the Aphids DNA (Figure 8). All the isolated DNA were restrictable by *Stu*1

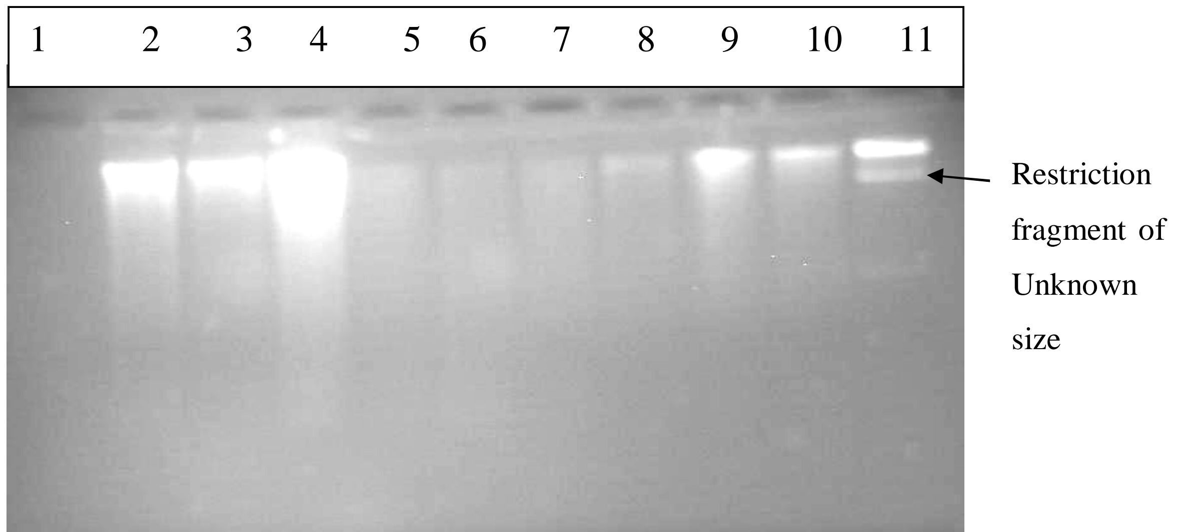


Figure 8: Digestability of DNA from aphids, mealy-bugs, leafhoppers and bacteriophage λ DNA using *Stu*1 (Biolabs): Lane 1 is negative control (SDW), Lanes 2-4 is aphid DNA, Lanes 5-7, is leafhopper DNA, Lanes 8-10 is mealy-bug DNA, Lane 11 is positive control (λ phage DNA).

4.2.2 DNA isolation from the banana plants

DNA was successfully isolated from both infected and healthy banana plants using the CTAB based DNA isolation protocol described in section 3.2.1. The electrophoretic results showed that the plant DNA was high in molecular weight and intact (Figure 9)

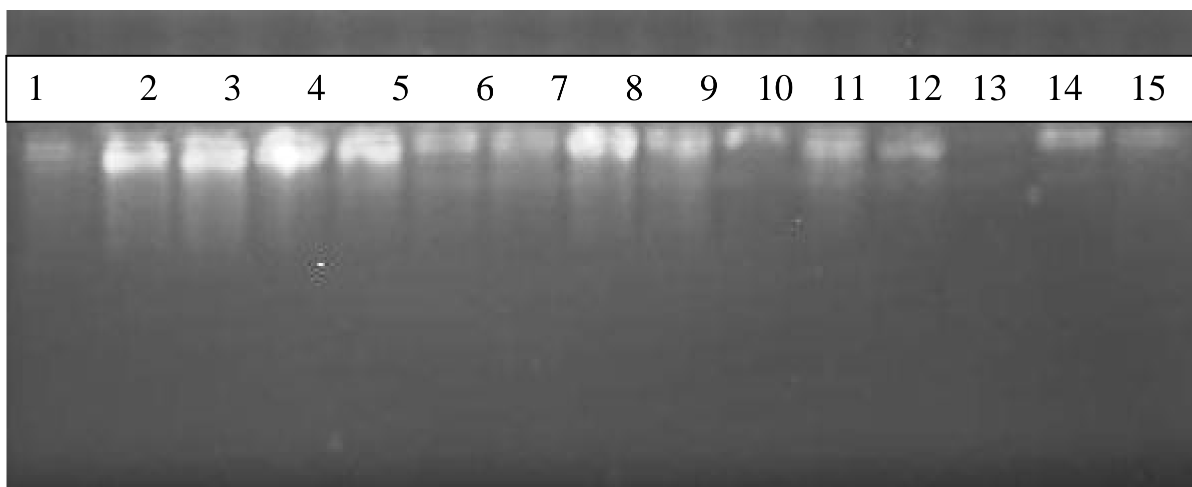


Figure 9: Quality of isolated DNA from plants. Lanes 1-15 represent DNA from banana plants

4.3 Screening of virus source plants and virus free plants

Screening of the virus source plants and virus free plants was carried out. Chirume cultivar, a triple A-genome containing banana plant tested positive for BSV but Cavendish, a triple A-genome containing cultivar tested negative with both the Immunocapture (IC)-PCR and Rolling circle amplification (TempliPhi) techniques as shown in Table 4 and Figures 10 and 11 respectively.

Table 4: Detection of BSV in source and virus free plants.

Cultivar	Immunocapture PCR			Rolling Circle Amplification		
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
Virus source plant(Chirume)	+	+	+	+	+	+
Virus free plants						
Cavendish	–	–	–	–	–	–
Cavendish	–	–	–	–	–	–
Cavendish	–	–	–	–	–	–
Cavendish	–	–	–	–	–	–
Cavendish	–	–	–	–	–	–
Cavendish	–	–	–	–	–	–
Controls						
Positive (Mysore)	+	+	+	+	+	+
Negative (SDW)	–	–	–	–	–	–

Rep-replicates; SDW-Sterilized distilled water. The virus source plants tested Positive while Cavendish tested negative with both IC-PCR and RCA as indicated in the table.

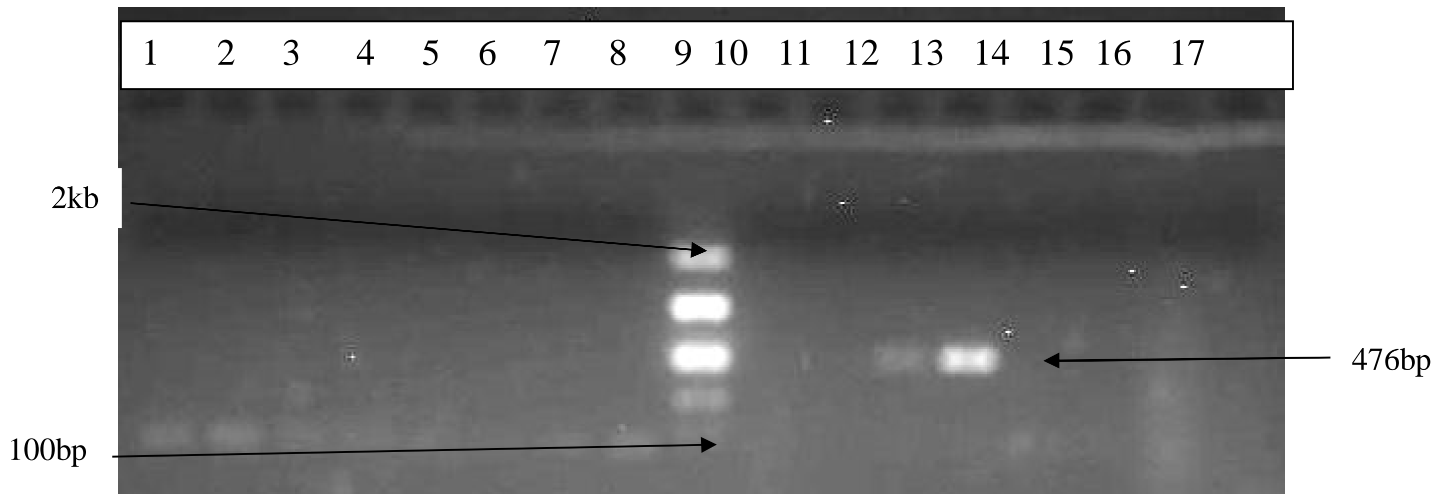


Figure 10: Immunocapture-PCR products from screening virus source and test (healthy) plant DNA. Lanes 1-8, 10, 11, 14, 16 and 17 represent healthy virus free plants (Cavendish); Lanes 12 and 13 represent Chirume cultivar a virus source plant; Lane 9 represents Molecular marker (Easyladder, Bioline).

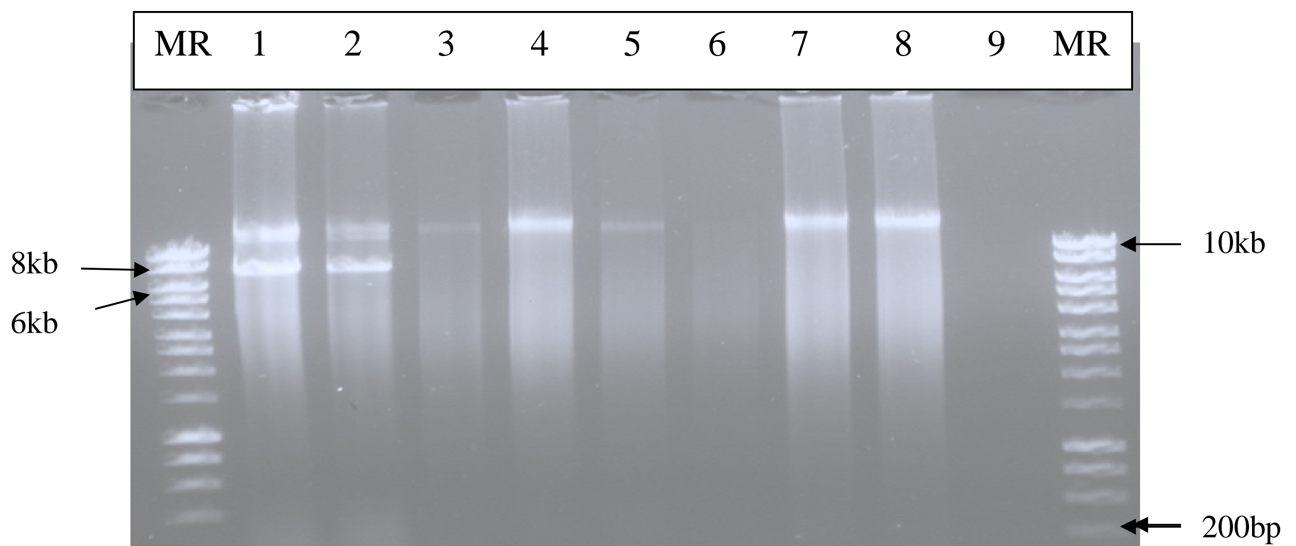


Figure 11: Rolling Circle Amplification (TempliPhi) products from DNA of infected and healthy plantlets assayed before their use in transmission experiments. Lane MR represent Molecular marker (Hyperladder^{TM} 1, Bioline), Lanes 1-2 represent infected plantlets (symptomatic), Lanes 3-8 represent healthy banana containing AAA genome (Cavendish) and Lane 9 is a negative control (SDW).

4.4 Detection of BSV in *P. burnerae*

The presence of BSV in mealy-bugs was assayed in the first generation (F_1) instars to establish whether the virus can be transmitted through transovarial infection. The viruliferous mealy-bugs were assayed for presence of BSV. The results confirmed that the virus cannot be transmitted from the parent to the offspring and though the mealy-bugs could acquire the virus through feeding (Table 5 and Figures 12 and 13). Mixed infection of the viruliferous mealy-bug was recorded.

Table 5: Detection of BSV in *P. burnerae* instars by Rolling Circle Amplification.

No. of mealy-bug instars grinded	First Generation (First instars) RCA results			Mealy-bug instars after Acquisition RCA results		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep.3
5	–	–	–	+	+	+
5	–	–	–	+	+	+
5	–	–	–	+	+	+
Controls						
Negative (SDW)	–	–	–	–	–	–
Positive (mysore)	+	+	+	+	+	+

SDW-Sterilized distilled water; Rep.- replicate: The first instars tested negative for BSV with RCA before they were fed on the infected banana plants while after acquisition feeding of one day they tested negative for BSV.

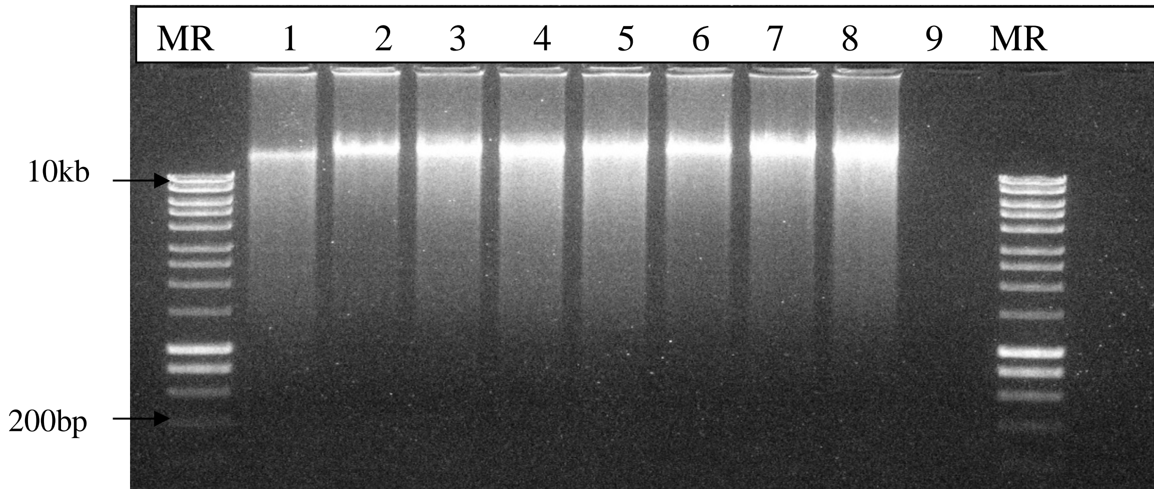


Figure 12: TempliPhi products from mealy-bug instars before acquisition access time; Lane MR- Molecular marker (Hyperladder™ 1, Bioline), Lanes 1-8 is mealy-bug instars DNA, and Lane 9 is Negative control (SDW).

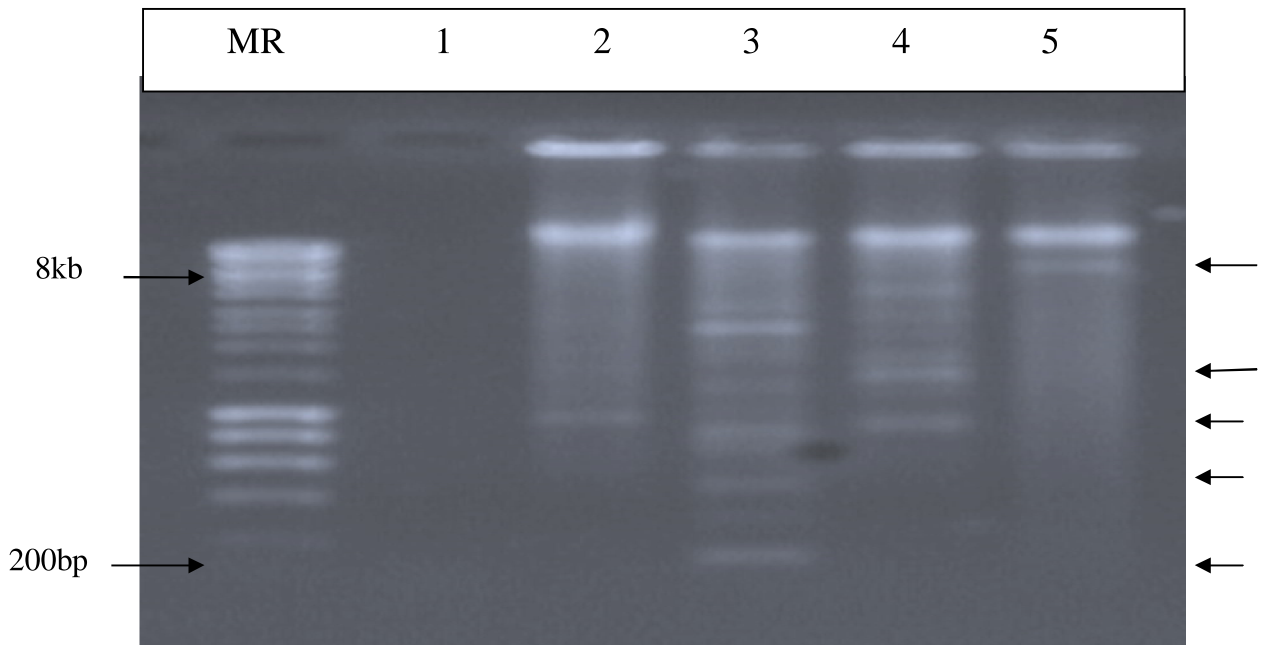


Figure 13: TempliPhi products from viruliferous Mealy-bugs; MR is Molecular marker (Hyperladder™ I, Bioline). Lane 1 is Negative control (SDW), Lanes 2-4 are DNA from viruliferous mealy-bugs and Lane 5 is positive control (Mysore). Arrows show positive RCA products from infected samples.

4.5 Effect of Acquisition Period on Successful Transmission of *Banana streak virus* by *P. burnerae*

The effect of acquisition period on transmission of BSV by *P. burnerae* was determined using the Rolling circle amplification (TempliPhi) assay. The results revealed that *P. burnerae* acquired BSV after feeding on infected plants for a minimum of six hours (Table 6). Cool conditions were also more favourable to acquisition of the virus (Figure 14, and 15).

Table 6: The data on Effect of acquisition on BSV transmission during hot and cool days

Cavendish receptor plants							
Source of transmission	No. of mealy-bugs	Acquisation period	Inoculation period	Condition of the day	Detection of BSV by RCA (Templiphi)		
					Rep.1	Rep.2	Rep. 3
Chirume	10	30 min	4 days	Hot	-	-	-
	9	30min	4 days	Cool	-	-	-
,, ,,	10	1hr	4 days	Hot	-	-	-
	7	1hr	4 days	Cool	-	-	-
,, ,,	9	2hrs	5 days	Hot	-	-	-
	10	2hrs	4 days	Cool	-	-	-
,, ,,	7	3hrs	4 days	Hot	-	-	-
	6	3hrs	4 days	Cool	-	-	-
,, ,,	7	overnight	4 days	Cool	+	+	+
,, ,,	8	4hrs	4 days	Hot	-	-	-
	9	4hrs	4 days	Cool	-	-	-
,, ,,	6	5hrs	4 days	Hot	-	-	-
	9	5hrs	4 days	Cool	-	-	-
,, ,,	8	6hrs	4 days	Hot	-	-	-
	5	6hrs	4 days	Cool	+	+	+
,, ,,	9	7hrs	4 days	Hot	-	-	-
	6	7hrs	4 days	Cool	+	+	+
Controls							
Negative (HC & SDW)	N/A	N/A	N/A	N/A	-	-	-
Positive (Mys)	N/A	N/A	N/A	N/A	+	+	+

No.- Number; Rep-Replicate; HC-Healthy plants; Mys-Mysore; SDW-Sterilized Distilled Water; N/A-Positive and Negative controls not fed on by the viruliferous mealy-bugs.

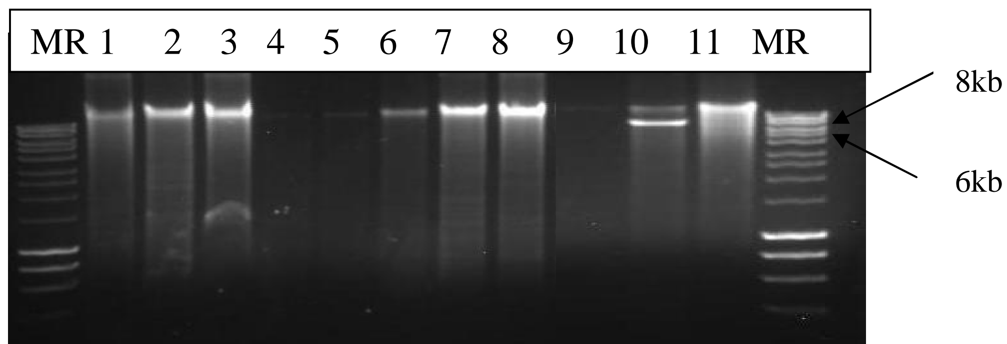


Figure 14: TempliPhi (RCA) products of DNA from Plant samples inoculated with BSV by viruliferous mealy-bugs during hot days: Lane MR- represent Molecular marker (Hyperladder ™ 1, Bioline), Lanes 1-8 represent DNA from plant samples, for acquisition access times of 30min, 1hrs, 2hrs, 3hrs, 4hrs, 5hrs, 6hrs, 7hrs, respectively; Lane 9 represents a negative control (SDW), Lane 10 represents a positive control (Mysore), and Lane 11 represents healthy plant DNA. Arrow shows an RCA product corresponding to positive control.

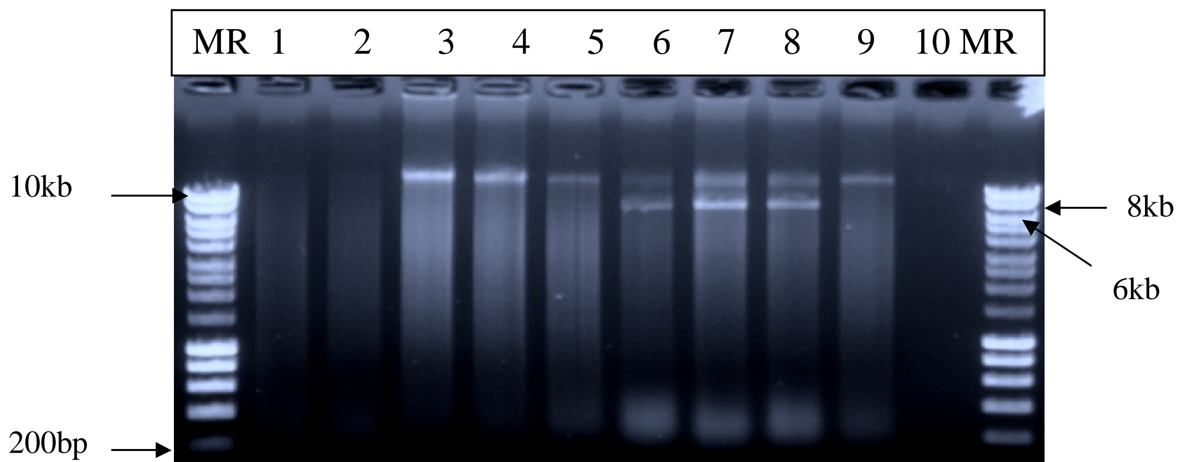


Figure 15: TempliPhi products of DNA from banana plants samples fed on by mealy-bug instars during cool days. Lane MR represent Molecular marker (Hyperladder™ 1, Bioline), Lanes 1-5 represent Plant DNA from acquisition access times of 1hr, 2hrs, 3hrs, 4hrs, 5hrs, respectively; Lanes 6-7 represent plant DNA from acquisition access time of 6hrs and 7hrs; Lane 8 represent a positive control (Mysore); Lane 9 represents a negative control (healthy plant DNA); Lane 10 is a negative control (SDW). Arrow shows an RCA product corresponding to infected material and positive control.

4.6 Latent Period of *Banana streak virus* in *P. burnerae*

The latent period of the BSV in the *P .burnerae* was determined using the RCA. The results presented in Table 7 and Figure 16 indicated that the BSV has no latent period in the

vector's gut during transmission process. The *P. burnerae* was able to transmit the BSV immediately after acquisition. This is a characteristic of non- persistent and semi-persistent modes of transmission.

Table 7: Data on latent period of BSV in *P. burnerae*.

Source of transmission	No. of mealy-bugs	Acquisition period	Days after acquisition period before inoculation	Inoculation period	Detection of BSV by RCA (TempliPhi)		
					Rep. 1	Rep. 2	Rep. 3
Chirume	7	5 days	1	4 days	+	+	+
,,	9	5 days	2	4 days	+	+	+
,,	8	5 days	3	4 days	+	+	+
Controls							
Positive (Mys)	N/A	N/A	N/A	N/A	+	+	+
Negative (HC & SDw)	N/A	N/A	N/A	N/A	-	-	-

HC-Healthy plants; Mys-Mysore; SDW-Sterilized distilled water; N/A-Positive and Negative controls not fed on by the viruliferous mealy-bugs; Rep.-Replicates. The inoculated banana tested positive with RCA for BSV after three days of acquisition feeding.

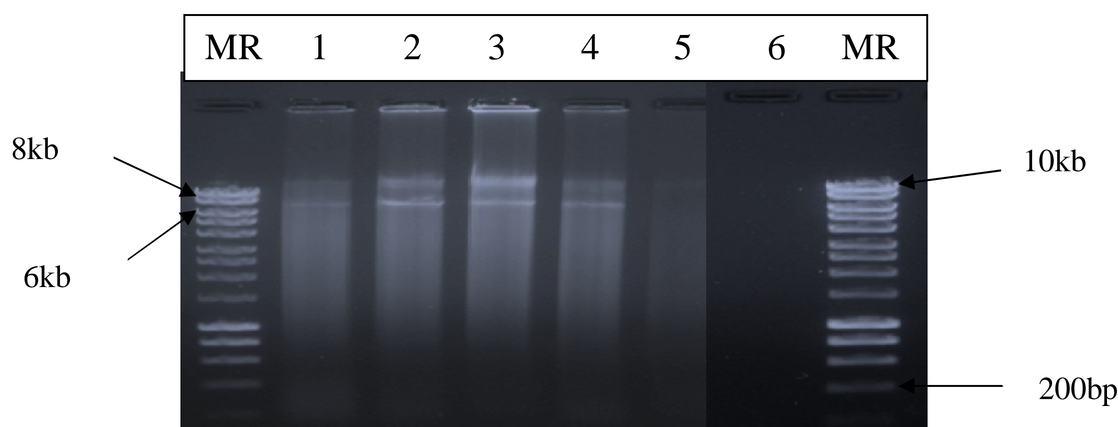


Figure 16: TempliPhi products for DNA from plants exposed to infected mealy-bug (Latent period). Lane MR- represent Molecular marker (Hyperladder™ Bioline) Lanes 1-3 Plant DNA from samples inoculated by viruliferous instars after 1-3 days of acquisition access period; Lane 4-Positive control; Lane 5- Negative control (Healthy plant DNA) and Lane 6- Negative control (SDW).

4.7 The Retention Period of *Banana streak virus* in *P. burnerae*

The retention period of BSV was successfully estimated using RCA technique. It was found that *P. burnerae* were able to retain the virus for four days (Table 8 and Figure 17). A 7.4 kb RCA products was identified in *P. burnerae* that retained the BSV virus. This means that BSV does not cross the gut membranes of the vector. This exhibited a characteristic of semi-persistent mode of transmission.

Table 8: Data on retention period of BSV in *P. burnerae*

Source of BSV transmission	No. of mealy-bugs	Acquisition period	Days after acquisition period before inoculation	Inoculation period	Detection of BSV by RCA (TempliPhi)		
					Rep.1	Rep 2	Rep. 3
Chirume	8	5 days	1	4 days	+	+	+
,,	6	5 days	2	4 days	+	+	+
,,	8	4 days	3	4 days	+	+	+
,,	10	4 days	4	4 days	+	+	+
,,	9	4 days	5	4 days	-	-	-
,,	7	4 days	6	4 days	-	-	-
,,	6	4 days	7	4 days	-	-	-
Controls							
Positive (Mysore)	N/A	N/A	N/A	N/A	+	+	+
Negative (dH_2O)	N/A	N/A	N/A	N/A	-	-	-

Rep.-Replicates; *N/A-Positive and negative controls not fed on by the viruliferous mealy-bugs*

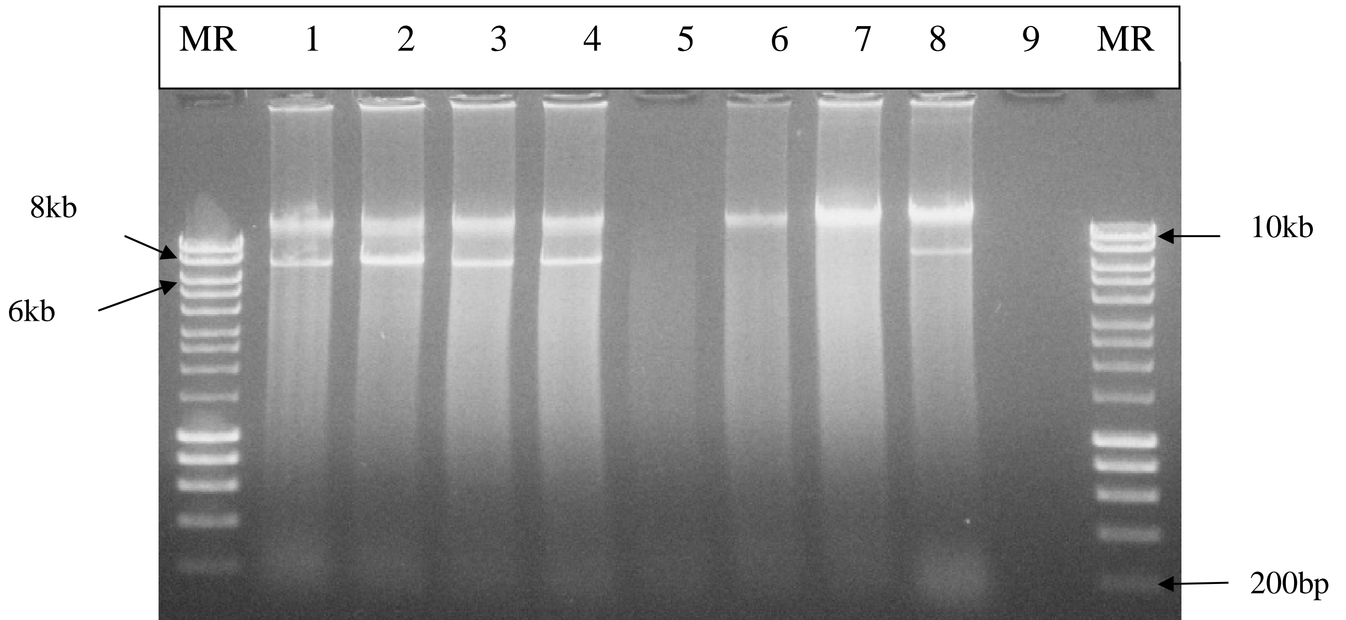


Figure 17: TempliPhi products for DNA from banana plants exposed to viruliferous *P. burnerae.* Lane MR- Molecular marker (Hyperladder^{TM} 1, Bioline); Lanes 1-7 represent DNA from plant samples inoculated by viruliferous *P. Burnerae* and allowed to hold for 1, 2, 3, 4, 5, 6 and 7 days before inoculation, respectively; Lane 8 represents Positive control (Mysore); Lane 9 is a Negative control (SDW).

4.8 Screening of Other Putative Vectors of *Banana streak virus*

Presence of BSV in both banana aphid and leafhopper DNA samples was evaluated using the RCA assay. The results obtained showed that neither banana aphids nor leafhoppers were infected with BSV as shown in Tables 9 and 10; and Figures 18 and 19 respectively. No positive RCA product was detected in DNA isolated from the banana aphids and leafhoppers.

Table 9: Screening of DNA samples from aphids sampled from infected banana plants for BSV

Sample No.	No of aphids grinded	RCA detection of BSV		
		Replicate 1	Replicate 2	Replicate 3
1	5	_	_	_
33	5	_	_	_
28	5	_	_	_
18	5	_	_	_
5	5	_	_	_
6	5	_	_	_
15	5	_	_	_
8	5	_	_	_
42	5	_	_	_
23	5	-	_	_
13	5	_	_	_
16	5	_	_	_
Controls				
Positive (Mysore)	N/A	+	+	+
Negative (Healthy plant)	N/A	_	_	_

N/A- Banana aphids DNA were not included; only viral and healthy plant DNA was assayed. All the tested samples for BSV gave negative results indicating that aphids are not potential vectors of the virus.

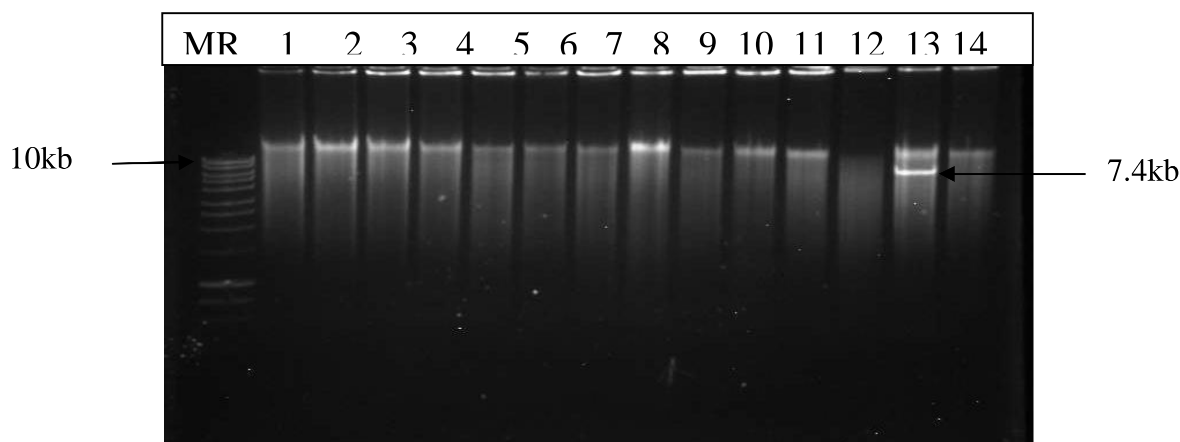


Figure 18: TempliPhi products for DNA from aphids collected from infected banana plants: Lane MR is Molecular marker (Hyperladder™ 1, Bioline). Lanes 1-12 is aphid DNA; Lane 13 is Positive control (Mysore) and Lane 14 is Negative control (healthy Plant DNA).

Table 10: Screening of DNA samples from leafhoppers sampled from infected banana plants for BSV.

Sample No.	No of leafhopper grinded	RCA detection of BSV		
		Replicate 1	Replicate 2	Replicate 3
1	3	–	–	–
3	3	–	–	–
8	3	–	–	–
10	3	–	–	–
6	3	–	–	–
16	3	–	–	–
11	3	–	–	–
4	3	–	–	–
31	3	–	–	–
26	3	–	–	–
Controls				
Positive (Mysore)	N/A	+	+	+
Negative (SDW)	N/A	–	–	–

N/A- Without leafhopper DNAs; SDW-Sterilized distilled water. All tested leafhoppers gave negative results with RCA indicating these insects are not the potential vectors of BSV.

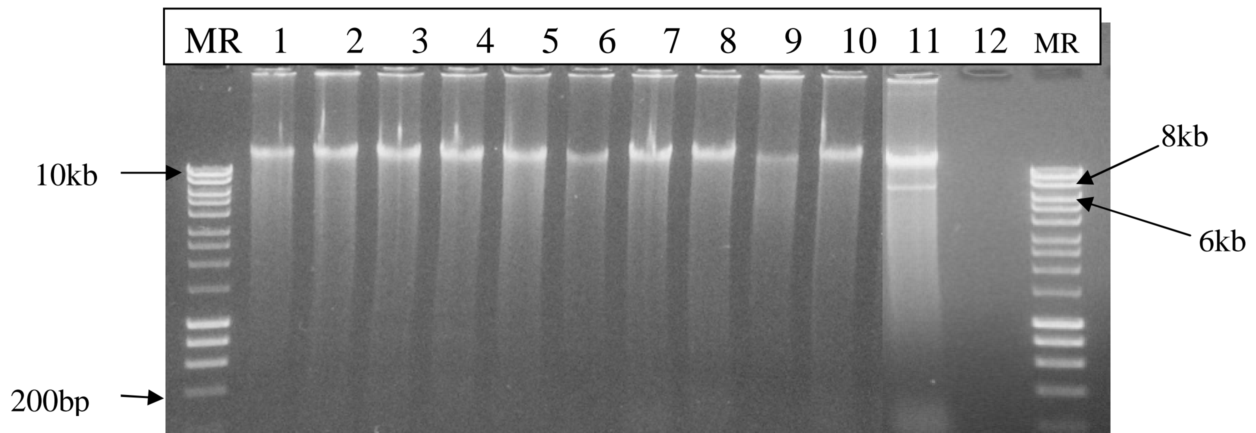


Figure 19: TempliPhi products of DNA from leafhoppers collected from infected banana plantations: Lane MR-represent a DNA Molecular marker (Hyperladder^{TM} 1); Lanes 1-10 represent leafhopper DNA; Lane 11 represents a positive control (Mysore); and Lane 12 represents a Negative control (SDW).

CHAPTER FIVE

DISCUSSION

5.1 Sampling of mealy-bugs and other putative BSV vectors

Sampling of the mealy-bug species was carried out at the KARI-Kisii banana plantation where only one mealy-bug species was identified and collected from the banana plants. Destructive sampling (Hughes, 1998) was not used because the study was undertaken on commercial fields. A similar mealy-bug species was obtained when sampling was carried out at KARI-Njoro and in Meru (Chuka and Nkubu). The mealy-bug species collected at KARI-Kisii was the *P. burnerae* (De Lotto, 1967; Ben-Dov, 1994). The work described in this thesis presented the first time that *P. bunerae* mealy-bugs were sampled from banana plantations and used to study the transmission of BSV under greenhouse conditions. This species of the mealy-bug had been reported on the *ventricosum ensete* in Kenya before and it associated with the Ensete streak disease(De Lotto, 1967; Ben-Dov, 1994). Interestingly, the same species of mealy-bug sampled in this study also was found on the *Ensete* sp plant in Egerton University (Njoro). Further evaluation revealed that the majority of the *Ensete* sp plants were infested by this mealy-bug species and the aphid *Pentalonia nigronervosa*. The finding that the sampled mealy-bug species also infested *Ensete sp*, corroborated the findings of earlier studies in Kenya (Ben-Dov, 1994). This contributed to the identification of the mealy-bug. An isolate with similar genome sequence of BSV was successfully amplified using Goldfinger primers on Ensete DNA samples on which mealy-bugs were found (Wambulwa Moses Cheloti unpublished).

All the life stages of *P. burnerae* were found and collected from the pseudostem/ corm of the banana plants inside the leaf sheath. Higher populations of *P. burnarae* were commonly noticed in a pseudostem/ corm of banana plants. Though all life stages were identified, first and second instars of mealy-bugs are known to play a major role in transmission of BSV (Su Hong-ji, 1998; Kubiriba *et al*., 2001) than the old instars and adults. Adult Males have been shown to have non-functional mouth parts and therefore are not vectors of BSV (Cox, 1989). The young instars are tiny enough to be spread by wind and accordingly, transmission over distance may occur during strong winds. More so, young instars can spread to up to seven banana plants from the original banana within a period of 7 years (Kubiriba, 2005). On the converse, adults are immobile and are not active feeders due to degeneration of their mouthparts.

Banana aphids were found on the banana sheath of the pseudostem. Aphids have been reported as a vector of many plant viruses, transmitting them in a non-persistent manner (Atreya *et al*., 1995; Raccah and Ferere, 2009). Aphids however seemed to be less mobile compared to the instars of the mealy-bugs. Leafhoppers were also found on the leaves of the banana plants and on surrounding weed.

5.2 Development of a DNA isolation protocol from vectors

The optimization process in this study was serendipitous. A DNA isolation protocol that gave good preparation when used to extract DNA from aphids, leafhoppers and mealy-bugs was successfully developed. The developed isolation protocol was superior to other published DNA isolation protocols for tissues preserved in formalin (Michele *et al*., 2002). The extraction buffer of the protocol developed in this study contained Sodium chloride-Tris-EDTA (STE), sodium dodecyl sulphate (SDS) and proteinase K (Hillis *et al*., 1996; Aljanabi and Martinez, 1997; Favret, 2005) and a salting out agent (ammonium acetate) which exerted shrinking effect to the insect tissues. In addition, the pH 7.6 of this buffer also differed from the pH of the other known DNA isolation protocols from insects. The salting out agent used in this study has also been used in aphids and other animal tissues DNA isolation protocols but at different and separate steps, which increased the time for DNA isolation.

Aljanabi and Martinez (1997) used 5M Sodium chloride (NaCl) to isolate DNA from aphids but this additive was added after 3hrs of incubation at 55º C. The protocol developed in this study, involved the addition of the salting out agent (10M ammonium acetate) in the extraction buffer to maintain the double helical structure of DNA and to argument the functions of sodium chloride as well as rupturing the cells and nuclear membranes to release the DNA by shrinking the cells. The ammonium ion (cation) of this salting out agent was thought to have attracted the phosphate group of the DNA at 5' and 3' OH end and reacted with them to form a complex that made it easier for the DNA to be separated from the protein by centrifugation. The conjugate base of the salting out agent in this protocol has a better buffering effect as compared with chloride ions used in other protocols as a salting out agent salt (Aljanabi and Martinez, 1997).

The use of 5M NaCl after incubation period in other DNA isolation protocols (Aljanabi and Martinez, 1997), imposed risks of not rupturing the cuticle and the nuclei membranes of the vectors completely. Thus, this could have resulted to low DNA yield which is also degraded by the nucleases and unrestrictable. These risks were effectively managed by addition of the 10M ammonium acetate. The addition of this hypertonic solution was thought to weaken the lateral

cell membrane junctional complexes (zonula occludens, intermediate junction and desmin filament). The solution enhanced the release of unicellular fragments from a multicellular tissue thus increasing the surface area to volume ratio for proteinase digestion. In many DNA extraction protocols, the process could last up to 24 h to complete (Rung *et al.,* 2009). The protocol described in this study shortened this by grinding the specimen in a buffer containing proteinase K, salting out and incubating at 55 ºC for 2 hours. This was clearly demonstrated by the fact that the extraction time was highly reduced when 10M of the salt was used as a component of the extraction buffer than when the salt was omitted in the buffer.

The ammonium acetate was thought to interfere with the activity of the oxidases that catalyze the formation of the phenolic compounds during DNA isolation (Calderon *et al*., 2010). This interference could be due to the conjugate base (acetate group) that rose the pH above the physiological condition that inactivate the oxidases. Ammonium radicals provided by this salting out agent was thought to react with the 3'OH and 5'ends of the DNA to form reversible (soluble) complexes. The ammonium radicals were also thought to have imposed competitive inhibition to the negatively charged ends of the DNA molecules thus preventing the nucleic acids from being bound by phenolic compound and from being degraded by exonucleases.

Most of the known DNA extraction buffers used in insect and other animal tissues do not employ the crenation effect to lyse the cells and nuclear membranes. Most of the isolation procedures usually involve marceration of the insect tissue in a boiling Potassium hydroxide (KOH) solution (Wahl 1984, 1989; Knoike *et al.,* 2005). These methods seclerotise structures of the abdomen and genitalia, while soft tissues are discarded during preparation leading to a loss of a considerable amounts of DNA. In addition, the high temperatures used during the boiling process in KOH can lead to the denaturation and nicking of the DNA. In the protocol developed in this study, the insect's cuticle, cell and nuclear membranes junction complexes are ruptured by the highly concentrated ammonium acetate due to crenation of the cells during maceration of the insect in the buffer thus ensuring the release of a good quantity of the DNA from all the insect cells.

In most DNA isolation protocols, ethanol is used to precipitate out DNA. However, ethanol is more polar than isopropanol its alternative. Nucleic acids are polar substances and therefore tend to dissolve in polar substances (Henry *et al*., 1990). To enhance the precipitation of the DNA from the target insects (aphids, mealy-bugs and leafhopper) the protocol described in this study used the more hydrophobic isopropanol. The use of the resultant nucleic acids to

detect RNA viruses would require that the digestion step with *RNases* be omitted since these nucleic acids would be required to synthesise a complementary DNA strand through the reverse transcription process. Further, this rapid and shrinking based protocol not only isolated vector DNA but also isolated viral DNA from the vector. The protocol established in this study therefore produced high quality DNA products in a manner that was also economical time wise and cost wise besides being completely phenol/chloroform free.

5.2.1 DNA quality and quantity

The ratio of OD260/OD280 is used to determine the purity of an isolated DNA sample (Brondmann, 2008; Wachira, 1996). In this study, the ratio of the OD260/OD280 obtained for the isolated DNA samples ranged from 1.8-2.0 which was certainly due to nucleic acids. A ratio less than 1.8 could indicate the presence of proteins and/or other UV absorbers in the DNA samples, in which case it would be necessary to re-precipitate the DNA (CIMMYT, 2005; Brondmann, 2008). A DNA extract contaminated by chloroform and/or phenol normally gives a ratio higher than 2.0 in which case the extract should be re-precipitated with isopropanol (CYMMT, 2005). All the samples in this study indicated a ratio that was within the range which was attributed to relatively pure DNA. However, there is one complexity of the mealy-bug system and other vectors; this pertains to the presence of certain yeast and bacteria-like symbionts in both sexes of the insects. They are transmitted by the mother to the egg. The symbionts invade certain polyploid cells which form a small organ called the mycetome whose function is not known. When DNA is isolated from whole insects, the possibility of "contamination" of mealy-bug DNA by the symbiont DNA cannot be avoided (Deobagkar *et al.,* 1982). This may become important, particularly when one is studying differences between DNAs isolated from males and females, because the two sexes are vastly different in size. At least in the stock that was used in this study, it would have been difficult to demonstrate symbionts in adult mealy-bug females, whether virgin or gravid (Deobagkar *et al.,* 1982). However, in any kind of study, specific primers are used to amplify only the sequences of interest (BSV) and therefore solving a problem of contamination by nucleic acids from symbionts.

DNA concentration can be estimated using optical density readings at 260nm (Honeycutt *et al.,* 1992). DNA has an optical absorption at 260nm and a pure DNA sample with an OD260nm of 1.0 corresponds to 50ng/µl concentration (Wachira, 1996). Indeed it has long been known that polysaccharides like other contaminants are impossible to detect by non-degradative analytical techniques and usually they interfere with quantification of the nucleic

acids spectrophotometrically and may even cause anomalous hybridization kinetics (Draper and Scott, 1988).

Yields obtained using the OD260 readings were variable amongst the insects that the DNA was isolated from. The quantity of the DNA ranged from 2ng/µl for leafhopper DNA to 7.2ng/µl for mealy-bug DNA. These results were attributed to the variation of the hardness of the vectors cuticle; mealy-bugs had the softest cuticle while leafhoppers had the hardest cuticle. This quantity of DNA obtained was adequate for Polymerase Chain Reaction (PCR) and Rolling Circle Amplification (RCA) techniques which require about 0.3ng/µl for amplification to occur.

Running the isolated DNA against different concentrations of uncut unmethylated Lambda (λ) phage DNA, was a realistic approach used in this study to determine the intactness of the isolated DNA. Although the results obtained by this method revealed that the DNAs isolated were of good quality, relatively intact and of high molecular weight, degradation of part of the isolated DNA is always inevitable. For adequate resolution of Polymerase Chain Reaction (PCR), or Rolling Circle Amplification (RCA), Restriction Fragment Length Polymorphism (RFLPs), it is known that native DNA should migrate as a tight band of high molecular weight (CMMYT, 2005).

The quality of an isolated DNA sample can further be assayed for its quality by determining its digestability by restriction enzymes. Indeed the restrictability of the DNA is essential often before setting up large-scale digestion experiments. The *Stu*1 digest results obtained in this study with the DNAs of the developed isolation protocol suggested that the DNAs had no phenolic compounds that are known to inhibit the restriction enzymes and DNA polymerase even without addition of the antioxidant (citrate) as described by Deobagkar *et al.,* (1982). Phenolic compounds usually are formed during the isolation procedure and these bind firmly to DNA. Since many of these phenolic compounds contain methyl groups (Deobagkar *et al.,* 1982), they can end up hindering the restrictability of the isolated DNA. The production of these phenolic compounds is particularly catalyzed by the polyphenolic oxidases (Deobagkar *et al.,* 1982) in mealy-bugs. Because many factors can cause a restriction digestion to fail or succeed, a single digestion should not be the decisive factor to immediately make conclusions. Other factors such as poor reaction conditions could account for these results. Further tests need to be carried out using polymerization enzymes for example *Taq* DNA polymerase or *Phi29* DNA polymerase used in PCR and RCA reactions, respectively.

The use of the polymerase chain reaction (PCR) or Rolling Circe Amplification (Templiphi) techniques to amplify a DNA sample can also confirm whether a sample of DNA contains inhibiting compounds or not. However, the sensitivity of the two amplification techniques usually differs with RCA being more sensitive to the inhibitory compounds in the isolated DNAs (Reagin *et al.,* 2003). This is due to the nature of the enzymes (*Taq* DNA polymerase in PCR and *Phi29* DNA Polymerase in RCA) used in the two assays. *Phi29* DNA polymerase is known to recognize and amplify as little as 1 picogram of the template DNA (Reagin *et al.,* 2003). In this study, positive amplicons were obtained using RCA technique which was proof that DNAs were free from inhibitory substances such as phenolic compounds that are known to form complexes with the DNA.

5.3 Screening of virus source plants and virus free plants

The virus source plants which had very pronounced symptoms gave positive results with both the IC-PCR and RCA techniques. This indicated that these plants had episomal viral particles that were captured in the first step of the combined serological and DNA based method in IC-PCR. In the immunocapture step, only the viral particles (antigens) are bound by the polyclonal antibodies and not the viral nucleic acids. The mealy-bug species usually pick the episomal DNA particles and not naked nucleic acids (Meyer, 2006: Meyer *et al.,* 2008). Reports from other studies have shown that naked nucleic acids for viruses that are transmitted semi-persistently and persistently, can not cause any infection to the host plants if mechanically inoculated in the host plant (Raccah and Ferere, 2009). Indeed no report has been made concerning mechanical transmission of BSV. However, Karanja *et al*., (2008) and Karanja, (2009) explained that the virus could not be mechanically transmitted from diseased to non diseased plants. Consequently there was no chance of amplifying the integrated sequences of the BSV in the PCR step. The healthy test plants in this study tested negative for BSV with the IC-PCR technique indicating that the plants had no episomal viral capsid particles.

The RCA of the DNA from the virus source plants confirmed that the plants were infected with BSV whereas the DNA samples from the healthy plants were negative for BSV. The RCA technique only amplifies the double stranded circular DNA of BSV and not the linear double stranded DNA of the plants (James *et al*., 2011). However, it is plausible that the plant mitochondria and chloroplast circular DNA was amplified but their genome sizes is always larger than that of BSV. The Templiphi products of these plants orgarnellar genome would therefore be always above the hyperladder (James *et al*., 2011).

5.4 Detection of BSV in *P. burnerae*

The results obtained in this study suggest that *P. burnerae* does not pass BSV to its offspring through transovarial transmission. Indeed, no report of transmission of BSV from parent to offspring in mealy-bug has been made so far. However, it was necessary to establish whether this happens because the *P. burnerae* used to raise a population of mealy-bugs for this study was collected from infected banana plantation. This step was also vital in determining the mode of transmission of BSV by their vector. Viruses that are transmitted persistently are usually passed from parent to offspring especially when they propagate in the vector cells. The results from this study suggest that BSV can be transmitted by the *P. burnerae* species through non-circulative mode of transmission rather than the circulative (persistent) mode of transmission. In non-circulative mode of transmission, the virus is found within the gut or the stylets of the insect vector (Raccah and Ferere, 2009). This mode of transmission comprises of non persistent and semi-persistent mode of transmission. Hence in this mode, the virus does not get into the circulatory system of the insect vector, therefore, no transovarial transmission. In case of circulative transmitted viruses, the virus is carried in interior of the body of the vector and therefore passed to other generations. Some of the circulative viruses propagate in the body of the insect vector while others do not (Gray and Gildow, 2003).

Mealy-bugs (*P. burnerae*) exhibited mixed infection after an acquisition access time of a full day, though the vector species was only able to inoculate the Mysore isolate of BSV with the amplicon size of 7.4 kb. The implication of the result is that, though *P. burnerae* can acquire other BSV isolates it may not be able to transmit them. This could be due to Capsid protein changes that confer a defective phenotype map to a region (motif) as has been hypothesized by Atreya *et al*., (1995) and Harrison and Robinson, (1998), which would influence capacity for virus transmission. The same kind of results were obtained by Meyer *et al.,* (2008) who showed that BSV-isolate GF could not be transmitted by *D. brevipes* from infected to healthy plants which they attributed to either defects in the capsid motif for transmission or changes caused by enzymatic reactions (biomolecules) in the vector gut or a lack of compatible interactions between the virion and the vector receptors.

The effective transmission of a virus has been demonstrated to be a function of compatible interactions between virion particles and factors (receptors) in the vector (Ng *et al*., 2005). Without this compatibility, no acquisition or inoculation can occur. A non-glycosylated protein deeply embedded in the chitin matrix of the vector maxillary stylets has been reported to be involved in the retention of *Cauliflower mosaic virus* (CaMV) which is transmitted semi-persistently by its vector aphids (Seddas and Boissinot, 2006). This protein receptor has been

reported in three effective vector species but not in a non-vector species and is located exclusively at the stylet tips in the bottom bed of the common duct where the food and salivary canals fuse together (Uzest *et al.*, 2007). Barriers to transmission reside in regions of both the gut and/or the accessory salivary gland of the vector, with evidence suggesting that the more stringent selection is at the accessory salivary gland (Gildow and Gray, 1993; Peiffer *et al.*, 1997). However, no information has been reported on the mealy-bug protein receptors and barriers to transmission of BSV by the vector. Results obtained in this study suggest that there could be protein receptors in the mealy-bug gut that were compatible with BSV and thus acquisition after feeding on the infected banana plants.

5.5 Effect of acquisition period on successful transmission of *Banana Streak Virus*

The *Ventrocosum Ensete* mealy-bug (*P. burnarae*) has been shown to transmit Ensete streak disease in Kenya but not BSV, BBTV or CMV (Ben-Dov, 1994; Williams and Matile-Ferrero, 2000) which are *Musa* viruses. However, the acquisition and inoculation feeding time has not been reported for the same disease. In this study, it was found that the *P. burnerae* instars were able to acquire the virus and transmit it to the healthy plants after a minimum of six hours of acquisition feeding time on the infected plants. This could imply that the acquisition access time has significant effect on the successful transmission of the BSV. The successful acquisition time of BSV with other mealy-bug species has been reported elsewhere and ranges from 5min to 1 full day (Su-Hong-ji, 1998; Kubiriba, 2005). The difference in successful acquisition access time determined in this study and those determined in other studies could be associated with the species, and more so the prevailing environmental condition. Kubiriba (2005) used three species of mealy-bugs in which *P. burnerae* was excluded in his study, to conclude that mealy-bug species can acquire the BSV within a minimum of 5 min acquisition access time. However, a study carried out in Taiwan (Su-Hong-ji, 1998) suggested that, *P. citri* can acquire the BSV after an acquisition access time of one day. It was also revealed that the acquisition time of BSV by *P. citri* was greatly influenced by prevailing environmental factors. This was corroborated by findings from this study. This means that there is effect on the acquisition access time on the successful transmission of the BSV by mealy-bug species. However, this effect is likely to be influenced by the prevailing environmental conditions. In this study, the *P. burnerae* was not able to acquire the virus during hot days and acquisition was only successful during cool days. In Taiwan, it was reported that the acquisition of BSV was more successful during the winter season than during summer season (Su Hong-ji, 1998). This acquisition access time and may be inoculation access time variation from one country to

another may be due to the fact that mealy-bug species require moist conditions for their survival especially in dark conditions (in the sheath of the pseudostem of the banana plants).

The inoculation access time of BSV by mealy-bugs was reported to be at peak after 72 hours of inoculation access feeding (Kubiriba, 2005). No information on this delay of the virus in the vector gut during inoculation access time has been revealed but it can be theorized that such delay could be due to the interaction of the vector with the BSV capsid coat protein during transmission process and the time taken by the vector to make prolonged probes.

Banana streak virus can be acquired and spread by several mealy-bug species (Kubiriba *et al*., 2001; Kubiriba, 2005; Meyer, 2006 and Meyer *et al*., 2008) with *P. citri* and *Planococcus ficus* having the 100% and 80% transmission efficiency, respectively. *D. brevipes* has been noted to have the lowest transmission efficiency of 20% (Meyer, 2006). The variation in transmission efficiency amongst the mealy-bug species can be attributed to differences in receptors of the vector that interact with the capsid protein of the virus. The lack of species specificity in transmission of virus is a characteristic of non-circulative transmitted viruses in which non-persistent and semi-persistent mode of transmission fall. However, no transmission study has been carried out to determine the relationship of the known BSV isolates (7 isolates in Kenya) with the specific mealy-bug species (10 species in Kenya). Therefore, the acquisition and inoculation of the BSV associated with the six mealy-bug species may exhibit specificity. Efforts have already been initiated to collect the five species already reported to be potential BSV vectors collected from citrus, pineapples, sugarcane and *Ventrocosum ensete* plants. In this study however, only *P. burnerae* were collected from the banana plantation.

Though electronic feeding monitoring systems (EMSs) have been used (Fereres and Collar, 2001) to study the relationship between the feeding behavior of insect vectors and their ability to transmit viruses, this study was not designed to use this technique. Where such systems are used, distinct waveforms and activities relevant to virus transmission are studied. Analysis of direct current electrical penetration graph (EPG) signals during intracellular stylet punctures allow to differentiate three specific and distinct sub-phases: II-1, II-2 and II-3. These waveform patterns are associated with transmission of persistent (Prado and Tjallingii, 1994), non-persistent (Powell *et al*., 1995, Martin *et al*., 1997, Powell, 2000), and semi-persistent (Palacios *et al*., 2002) viruses by vectors. Acquisition and inoculation of typical non-persistent viruses, such as *Cucumber mosaic virus* or *Potato virus Y*, occur during specific sub-phases of brief intracellular stylet punctures (potential drop, Pd) in nonvascular leaf tissues (Martin *et al*., 1997). In contrast, the phloem ingestion phase and the phloem salivation phase are associated with the acquisition and inoculation, respectively, of persistently transmitted luteoviruses

(Prado and Tjallingii, 1994). However, the EPG signals of BSV during intracellular punctures are not known.

From the results obtained in this study, it can be concluded that the efficiency of BSV transmission by *P. burnerae* increases with prolonged feeding period i.e with a minimum of six hours acquisition access time being required. This corresponds with studies carried out on *cauliflower mosaic virus* which belong to the same family with BSV (*Caulimoviridae*). Palacios *et al.*, (2002) reported that semi-persistent transmitted viruses require more acquisition feed for effective transmission as opposed to non-persistent mode of transmission. The acquisition time in non-persistent mode of transmission takes seconds to minutes during the time of probing as the vector searches for food. Long acquisition access feeding in semi-persistent mode, is associated with the viruses that are found in the phloem and BSV has been reported to be found in the phloem of the banana plants (Walkey, 1991). Hence, brief probing of the mealy-bugs on the infected banana plants, does not lead to successful acquisition of the virus. Therefore, the rate of BSV acquisition does not depend on the number of intracellular punctures produced by the vector, but it increases sharply after phloem ingestion (Palacios *et al.*, 2002). These findings are consistent with the model of a "sequential acquisition" of the various components of the *Cauliflower mosaic virus* transmissible complex (Drucker *et al.*, 2002), where certain proteins produced by the host or another virus in case of co-infections are acquired in specific inclusion bodies before virion complexes acquisition.

In this study, prevailing environmental factors largely affected the acquisition and inoculation feeding of the *P. burnerae*. It was revealed that during the hot days; the transmission was not successful, as compared to cool days. Su-Hong-ji (1998) reported that two *P. citri* biotypes had higher ability to transmit the BSV during cool winter than hot summer seasons. The findings of this study may imply that the mealy-bug require cool and moist places for their survival, thus affecting both acquisition and inoculation feeding. Hence, acquisition and inoculation access time for successful transmission of BSV by the mealy-bugs is dependent on the environmental conditions. It is well known that first instars of mealy-bug species are highly mobile as compared to adults and it is this stage in the life cycle that probes most during sampling of their favorite food (Su-Hong-ji, 1998).

Temperature can affect both acquisition and inoculation feed of the mealy-bugs. In Kenya, *P. burnerae* was able to acquire and transmit the BSV from infected to healthy plantlets during cool days or at night. This could be related to the feeding behavior of these insects at different environmental conditions. The results obtained in this study imply that the mealy-bugs could make short or brief probes during the hot conditions (unfavorable) which lead to a failure

of acquisition of the virus by the vector. This could be attributed to the failure of the mealy-bugs stylets to reach the phloem where the BSV is found. Indeed, it can be concluded that prolonged probing period during mealy-bug feeding is required for BSV acquisition. The prolonged feeding period can only occur when the environmental conditions are favorable for vector feeding. These facts rule out the non-persistent transmission of BSV by the mealy-bugs.

5.6 Determination of latent period of *Banana streak virus* in the *P. burnerae*

Results from this study revealed that *P. burnerae* had no latent period. The vector could therefore transmit the BSV immediately it acquired the virus. Virus-vector specificity generally involves an interaction between virus capsid proteins and membranes of the vector salivary membranes (Gray, 2004) and this interaction determines the latent period of the virus in its vector. During this period, the viruses replicate and become large in numbers in the vector. No data exists on the latent period of BSV even in other known mealy-bug species vectors. The latent period allows the virus to acquire the ability to infect the host. In other vector transmitted viruses, for example the Potyvirus, a helper component proteinase (HC-Pro) encoded by viral DNA has been shown to be a key protein during transmission process (Plisson *et al*., 2005)

The data collected in this study suggested that BSV is transmitted by the *P. burnerae* in a non-circulative mode of transmission which can be classified into two; - non-persistent and semi-persistent modes of transmission. The two modes of transmission do not require a latent period for transmissibility of the virus as is shown in appendix 7. The viruses transmitted through these two modes of transmission are found along the gut of the vector and do not cross the vectors gut membranes into the heamolyph and/or cells (Raccah and Ferere, 2009).

5.7 Estimation of the retention period of *Banana streak virus* in the *P. burnerae*

Results from this study revealed that the *P. burnerae* instars were able to retain the virus for four days after acquisition feeding time. Thereafter, the vector cannot cause any infection during inoculation access period to a healthy banana plant. This implied that though the *P. burnerae* do acquire the BSV, the virus never crosses the cell membranes of the gut of the vector. In addition, this can also imply that the BSV does not replicate in the vector's gut. However, little has been known about the retention period of BSV by mealy-bugs. Kubiriba, (2005) reported that the vector can retain the virus for five days which was within the range of the semi-persistent mode of transmission. The results from Kubiriba, (2005) contrast with those from this study, which could be due to differences in the viral loads in the viruliferous instars depending on the factors prevailing during acquisition access period. Kubiriba *et al.,* (2001) and Harper *et al., (*2002) proposed that the mode of BSV transmission could be semi-persistent. The

data from this study based on retention time of BSV in mealy-bug does agree with their suggestion. In the semi-persistent mode of transmission, the virus is retained in the vector for a minimum of five minutes and a maximum of less than a week (Palacios *et al.*, 2002; Raccah and Ferere, 2009). The results of this study do also suggest that the BSV does not replicate in the gut of the vector because the viral load in vectoring insect can not go beyond four days in this vector. If the virus replicates in the vector, it could be transmitted for a longer period than five days due to the increase in the number of the virus particles in the vector and thus this would result in persistent mode of transmission.

The pineapple mealy-bug and sugarcane mealy-bug are known to retain the virus for up to 5 and 6 days, respectively, after transfer from the virus sources (Kubiriba *et al.*, 2001). Walkey (1991) reported a retention period of viruses of 3-4 days by mealy-bugs which agree with the results obtained in this study. The results obtained in this study and those from elsewhere seem to rule out non-persistent and propagative transmission of BSV by the studied mealy-bug species.

Studies with other virus vectors reveals that viruses destined for inoculation are retained at sites within the stylet and food canal or foregut depending on the mode of transmission. They indicate that virions retained at the distal tip of the stylet bundle are most likely to play a determining role in transmission (Martin *et al.*, 1997; Wang *et al.*, 1996). However, one of the conundrums in transmission is that the binding of virions within the vector must be readily reversible. Considering that the food and salivary canals merge at the tip of the vector's stylet, salivation may function to enhance the release of bound virions and their delivery into plant cells (Martin *et al.*, 1997; Harris and Harris 2001). This could be true in transmission of BSV by their vector. In other vector transmitted viruses, a primary determinant of both vector transmissibility and specificity is the viral capsid protein in a non-persistent and helper component protein in semi-persistent mode of transmission. These proteins contribute immensely to the retention time of vector transmitted virus. However, no information on the capsid protein of BSV that is involved in the transmission of the virus by mealy-bug species has been reported. It can be hypothesized that the ORF I and II gene products of BSV could be involved during virus-vector interaction. Within the insects feeding apparatus, the retention sites for semi-persistent viruses have been determined only in the leafhopper-transmitted viruses (Childress and Harris 1989) and they are located in the foregut of the leafhopper. Similar data on semi-persistent mealy-bugs-transmitted viruses have not been reported; thus, information on the precise location of the mealy-bug receptor(s) recognized by the BSV Capsid protein is not known.

A distinguishing feature of semi-persistent transmission lies in the retention period of hours to days. The best characterized among the semi-persistent, vector transmitted viruses are the *Cauliflower mosaic virus*, the type member of the family *Caulimoviridae*, to which BSV belongs. The C*auliflower mosaic virus* has adopted a helper-dependent transmission strategy which determine the retention time of the virus in the vector, but with the added twist of requiring two viral-encoded, nonstructural proteins, P2 and P3 (Blanc *et al*., 2001). To date, no information on the helper dependent transmission strategy has been reported on the mealy-bug transmitted viruses such as *Badnavirus Trichovirus* and *Closterovirus*. More so, no information is available on BSV transmission strategy. However, a helper-dependent transmission strategy rather than Capsid mediated strategy could be involved during BSV transmission by the mealy-bugs, due to the fact that the vector was able to retain the virus for four days.

5.8 Screening of putative vectors for BSV transmission

The detection of the BSV by Rolling circle amplification (RCA) technique in DNA samples isolated from aphids (*P. negronervosa*) and leafhoppers gave negative results implying that these two insects may not be serious potential vectors for BSV. This could be due to the fact that virus transmission by vectors exhibit high degree of specificity. Transmission specificity can be broad or narrow but it is a prominent feature for numerous viruses and vectors especially for semi-persistently transmitted viruses. Specificity of transmission can be defined as the specific relationship between a plant virus and one or a few vector species but not others. For both viral transmissibility and transmission specificity, viral determinants are involved (Ng *et al*., 2005). The specificity of transmission is explained by several characteristics, including a recognition event between the virion, or a viral protein motif, and a site of retention in the vector (Brown and Weischer, 1998). For instance, transmission of carlaviruses involves the capsid proteins (CP) encoded by a 3 kb sub-genomic RNA but apparently no helper component (HC) (Brunt *et al.*, 1996). For CMV, the capsid protein (CP) is the only virus-encoded protein involved in transmission (Chen and Francki, 1990). Distinct capsid protein (CP) motifs are known to be responsible for the transmission of this virus by different vector species (Aphids). The amino acids in position 25, 129, 162, 168, and 214 of the capsid protein (CP) are critical for efficient transmission by *Myzus persicae*, whereas those in position 129, 162, and 168 are critical for efficient transmission by *Aphis gossypii* (Perry *et al.*, 1998). No report of the amino acid motif of the BSV has been reported which could be attributed to the failure of being vectored by the *P. nigronervosa.*

Leafhoppers transmit *Rice dwarf virus* (RDV) for which the P2 and P8 components of the outer capsid protein (CPs) are likely receptor binding sites on the virion surface, as they are crucial for vector infection and transmission (Omura and Yan, 1999). Leafhoppers have also been reported to be a vector of *Badnaviruses* but not BSV. However, no information is available on the molecular determinants of transmissibility or of transmission specificity of the vector to the transmitted viruses. The receptors compatible to the leafhoppers feeding systems could be absent on the virion surface of the BSV.

However, the results obtained in this study may not entirely rule out the possibility that the banana aphid and leafhoppers transmit BSV non-persistently because they are characterized by brief feeding probes, on leaf epidermal cells where virus particles may both be acquired and transmitted. However, it is also important to note that this feeding mechanism is unlikely to be compatible with BSV acquisition, since BSV, like other *Badnaviruses*, is reported to be largely restricted to the phloem cells (Walkey, 1991). The aphids and leafhoppers may therefore not possess the receptor cells for attachment of BSV, which may be present in the mealy-bugs.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The following conclusions are drawn from this study;

A salting out agent based protocol is adequate for DNA isolation from small insects that may contain viruses. The detection of the BSV in the isolated DNA from the mealy-bug vector can be sufficiently and effectively carried out using the RCA (TempliPhi) technique.

The *P. burnerae* is one of the vectors of banana streak disease. A minimum of six hours of acquisition access feeding time is required by this vector to acquire and transmit the *Banana streak virus* during cool days. Longer acquisition access time seems to affect the transmission positively than shorter feeding periods, which is characteristic of the semi-persistent mode of transmission. However, the virus can not be transmitted from the mealy-bug parent to its offspring.

The *P. burnerae* can transmit the BSV immediately they acquire the virus with inoculation access period (IAP) of three days. BSV does not need time in the vector to replicate and become infective, thus it is the episomal virions that are picked by the vector (*P. burnerae*) and not nucleic acids of the BSV. However, there is need for the determination of proteins both for BSV and its vector that are involved in the transmission process. These findings may lead in the future to the use of viral genes that encode for proteins that are defective as a management strategy to prevent vector inoculation and successful transmission. Further screening for, plants encoding for molecules (e.g. peptides) able to bind to cuticle protein receptors in the vector mouthparts may provide innovative virus management strategies by interfering with the process of virus retention.

The BSV can be retained by the vector for only about four days. This suggested that the virus retention site is not at the distal tips of the vector mouth parts but in the foregut. This retention time suggests that there is virus-vector interaction; otherwise the virus could be regurgitated within a shorter time. This is a characteristic of a semi-persistent mode of transmission. However, proteins mediating specificity and transmissibilty of this virus have not been identified. Hence, it is not known whether the transmission is through capsid strategy or helper component strategy.

Aphids and leafhoppers are not vectors of BSV although they have been known to be vectors of other plant viruses. This means that transmission mechanisms are remarkably different between plant viruses; with no correlation with genome type; particle morphology; or

strategy of viral protein expression. Transmission is often characterized by some degree of specificity and numerous findings indicate that there is possible involvement of a specific ligand/receptor interaction. The Capsid protein (CP), and its derivatives, e.g. reverse transcriptase (RT) product and nonstructural proteins, including a helper component (HC) or a transmission factor, have been clearly shown to be involved in transmission specificity.

6.2 Recommendations

From the results of this study the following recommendations are made:

1. The salting out DNA isolation protocol described herein and the RCA technique should be adopted to enhance efficiency and effectiveness of diagnosis of BSV in vectors.

2. Many viruses are introduced into the banana crop by the visiting insect vectors. Mealy-bugs are vectors of BSV transmission. Because of their relative immobility (take a long time to move from one plant to another), these vectors should be controlled using insecticides to effectively prevent the virus from spreading within and between banana plantations.

3. As a management option, virus source plants should be reduced. For example, the use of virus free seedlings and /or propagative organs can result in minimal primary infection. This can be implemented by the removal of sources of infection in and around the banana plants, removal of plant remains from a former seasons and creation of the time gap and/ or space gap between banana plants and plots. In this case, diagnosis should be done using the RCA technique. This will reduce the number of the viruliferous mealy-bugs that reach the virus free banana plant.

4. Modulation of the transmission process can be adopted as a measure of managing BSD. Mineral oils are hydrophobic substances that interfere with virus acquisition and retention by their vectors. Mineral oils of the appropriate viscosity and unsulfonated residues will be effective to reduce the efficiency of virus transmission by the mealy-bugs. The mode of action seems to be by interference with virus binding by their vectors. The pseudostem and corm of the banana plant should be fully sprayed with mineral oils.

5. In case of the intercropping of the banana with other mealy-bug host plants, the banana can be inoculated with insecticides that will kill the mealy-bugs the moment they infest and probe the banana crop, although avoidance of intercropping system is the best option.

6. Given the limitations of the current control strategies against BSV, there is a need for efficient and environmentally sound alternatives for sustainable banana production in Kenya.

Understanding the transmission process and the relationship between BSV and its vector can facilitate the development of novel control strategies against the virus, including the genetic manipulation of vectors and the expression of recombinant proteins in transgenic banana plants to neutralize the transmission process.

6.3 Way forward

The following interventions provide a way forward for the management of banana streak disease.

1. The viable acquisition time of BSV by mealy-bugs needs to be further confirmed by use of other modern and sensitive techniques including the electrical penetration graphs. This technique produces waveform patterns that exhibit certain characteristics associated with a particular mode of transmission. Since acquisition period is likely to vary with weather factors, such variations should be tracked using the electrical penetration graphs.

2. The location of the BSV in mealy-bugs gut needs to be identified using Immunosorbet Electron microscopy and immunofluoresent labeling of the virus. This will contribute immensely to fully determining the applicable mode of transmission.

3. The ORF I and II gene products of BSV need to be sequenced in order to establish or identify the conserved regions if any that may be involved in virus vector-interaction and that may be targeted for development of viable management options of the virus.

4. The capsid proteins of episomal BSV need to be isolated and studied for their morphological polarity that may be involved in transmission and their other characteristics that could be targeted for the development of viable management options of the virus.

5. Species specific PCR primers for *P. burnerae* need to be designed for easy identification of this vector and its biotypes. Morphological characteristics and host specificity identification is tedious, and not reliable. There could be biotypes that are specific to banana as their host rather than *Ensete sp.*

REFERENCES

Ahlawat Y., Pant R., Lockhart B., Srivastava M., Chackraborty N. and Varma A. (1996.) Association of a badnavirus with Citrus mosaic disease in India. *Plant disease*, **80:** 590-592.

Aljanabi S. and Martinez I. (1997). Universal and rapid salt extraction of high quality genomic DNA for PCR based techniques. Nucleic acids Research, **25**: 4692-4693.

Anonymous, (2004a). Banana in Asia and the Pacific. www.inibap.org/publications/inibap.factsheet-eng-/asp.pdf. (Accessed September, 2011)

Anonymous, (2004b). The Hibiscus or pink mealy-bug http://aphis.usda.gov/Ipa/pubs/fsheet fag notice/fs phphmea. Accessed September, 2011.

Atreya P., Lopez-Moya J., Chu M., Atreya C., Pirone T. (1995). Mutational analysis of the coat protein N-terminal amino acids involved in potyvirus transmission by aphids. *Journal of General Virology*, **76**: 265-70.

Ben-Dov Y. (1994). *A systematic cataloque of the mealy-bugs of the world*. Intercept Ltd., Andover, UK. 686 pp.

Bigger M. (1981). The relative abundance of the mealy-bug vectors (*Hemiptera, Coccidae* and *pseudococcidae*) of cocoa swollen Shoot disease in Ghana. *Bulletin of Entomological Research*, ***71*****:** 435-448.

Blanc S., Hebrard E., Drucker M. and Froissart R. (2001). Molecular basis of vector transmission: *Caulimovirus*. *In:* K. Harris, J.E Duffus and O.P Smith (eds) *Virus –Insect Plant Interactions.* Academic press, San Diego: *Academic press.*

Blanco L., Bernad A., Lazaro J., Martin G., Garmendia C. and Salas, M. (1989). Highly efficient DNA synthesis by the phage *Phi29* DNA polymerase. Symmentrical mode of DNA replication. *Journal of Biological Chemistry*, **264:** 8935-8940.

Bouhinda M., Lockhart B. and Olszewski N. (1993). Analysis of the complete sequence of a sugarcane bacilliform virus Genome infectious to banana and rice. *Journal of General Virology*, **74**: 15-22.

Bradley R. (1959). Loss of virus from the stylets of the aphids. *Virology*, ***8:*** 308-318.

Brondmann P. (2008). DNA extraction from different matrices. *Molecular biology methods for traceability purposes*, BFR, Berlin, Germany.

Brown D. and Weischer B. (1998). Specificity and complementarity in the transmission of plant viruses by plant parasitic nematodes: an annotated terminology. *Fundamental Applied Nematology*, ***I*****21**: 1-11.

Brunt A., Crabtree K., Dallwitz M., Gibbs A. and Watson L. (1996). *Viruses of plants*. CAB International, Wallingford, United Kingdom.

Calderon C., Quesanda M., Cano C., and Zavula P. (2010). A simple and rapid method for DNA isolation from xylophagous insects. *International Journal of Molecular Sciences*, **11**: 5056-5064.

Chen B. and Francki R. (1990). Cucumovirus transmission by aphid *Myzus persicae* is determined soley by the viral coat protein. *Journal of General Virology*, **71**: 939-944.

Childress S. and Harris K. (1989). Localization of virus-like particles in the foreguts of viruliferous *Graminella nigrifrons* leafhoppers carrying the semi-persistent maize chlorotic dwarf virus. *Journal of General Virology*, **70**: 247-251.

CIMMYT (2005). Laboratory protocols: CIMMYT Applied Molecular Genetics Laboratory. Third Edition. Mexico, D.F.: CIMMYT.

Cockburn A. and Fritz G. (1996). Isolation and purification of insect DNA. *Methods in Molecular Biology*, Vol. 50, Species Diagnostic Protocols: PCR and Other Nucleic Acid Methods ed. by J. P. Clapp), pp. 15–23. *Humana Press*, Totowa, N. J.

Cornwell P. (1958). Movement of the vectors of virus diseases in cacao in Ghana. I. Canopy movement in and between trees. *Bulletin of Entomological Research*, **49:** 613-630.

Cornwell P. (1957). An investigation into the effect of cultural conditions on populations of the vectors of virus diseases of cacao in the Gold Coast with an evaluation of seasonal population trends. *Bulletin of Entomological Research*, **48**: 375-396.

Cornwell P. (1956). Some aspects of mealy-bug behaviour in relation to the efficiency of measures for the control of virus diseases of cacao in the Gold Coast. *Bulletin of Entomological Research*, **47**: 137-166.

Cox J. (1989). The mealy-bug genus planococcus *(Homoptera*: *pseudococcidae). Bulletin of the British Museum (*Natura*l* history*) (Entomology)*, **58**:1-78.

Cruickshank R. (2002). http://taxonomy.zoology.gla.ac.uk/%7Ercruicks/rcruickshark.html (Accessed December, 2010).

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

Dahal G., Hughes J. and Lockhart B. (1997). Status of banana streak disease in Africa: problems and future research needs. *Integrated Pest Management Reviews*, ***3:*** 85-97.

Dale W.T. (1957). Studies on resistance and tolerance to cocoa virus. *Proceedings of the Cacao Breeding Conference,* West African Cocoa Research Institute 1156, 3.

Dallot S., Acuna P., Rivera C., Ramirez P., Cote F., Lockart B., and Caruana M. (2001). Evidence that the proliferation stage of micropropagation procedure is determinant in the expression of B*anana streak virus* integrated into the genome of the FHIA 21 hybrid (*Musa* AAAB). *Archives of Virology*, **146:** 2179-2190.

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

Danielles J., Thomas J. and Smith B. (1995). Seed transmission of banana streak badnavirus confirmed. *Infomusa* **4**:7-9.

De Langhe (1986). A Preliminary study of the needs of banana Research in Eastern Africa. NIBAP-EA OOLE REV. France. 58pp.

De Lotto (1967). The mealy-bugs of South Africa (Homoptera: *pseudococcidae*). *Department of Agricultural Technical Services Entomology Memoirs*, **12**: 1-28.

De Verno L., Smith C. and Harrison K. (1998). Randomly amplified polymorphic DNA evidence of introgression in two closely related sympatric species of coniferophagous *choristoneura* (Lepidoptera: *tortricidae*) in atlantic Canada. *Annauls of the Entomological Society of America*, **91**: 248-259.

Dean F., Nelson J., Giesler T. and Lasken R. (2001). Rapid Amplification of Plasmid and Phage DNA Using Phi29 DNA Polymerase and Multiply-Primed Rolling Circle Amplification. *Genome Research*, **11:** 1095-109.

Deobagkar D., Muralidharan K. Devare S., Kalghatgi K. and Chandra H. (1982). The mealy-bug chromosome system I: Unusual methylated bases and dinucleotides in DNA of a *Planococcus* species. *Journal of Bioscience*, **4:** 513-526.

Draper J. and Scott R. (1988). The isolation of the plant nucleic acids. In: Draper J., Scott R., Armitage P. and Walden R. (eds) Plant Genetic transformation and gene expression. A laboratory manual. *Blackwell Scientific Publications*, Oxford, UK, P. 201-226.

Drucker M., Froissart R., Herbrard E., Uzest M., Ravallec M., Esperandieu P., Mani J-C., Pugniere M., Roquet., Fereres A. and Blanc S. (2002). Intracellular distribution of viral gene products regulates a complex mechanism of C*auliflower mosaic virus* acquisition

by its aphid vector. *Proceedings of the National Academy of Sciences of the USA* **99:** 2422-2427.

Duffus J. (1972). Beet yellow stunt, a potentially destructive virus disease of sugarbeet and lettuce. *Phytopathology*, **62**: 161-165.

Eastop V. (1981). The wild hosts of aphid pests. *Pests, Pathogens and Vegetation* (*Ed. by* J. M. Thresh), pp. 285-309. Pitman, London.

Eastop V. (1977). Worldwide importance of aphids as virus vectors. *Aphids as Virus Vectors* (Ed. by K.F. Harris and K. Maramorosch), pp. 3-62. Academic press, New York.

Favret C. (2005). A new Nondestructive DNA Extraction and specimen clearing technique for aphids (Hemiptera) **107**: 469-470.

Fereres A. and Collar J. (2001). Analysis of noncirculative transmission by electrical penetration graphs. *In Virus-Insect-Plant Interactions*. Edited by K. F. Harris, O. P. Smith and J. E. Duffus. San Diego, CA: Academic Press.

Fire A. and Xu S. (1995). Rolling replication of short DNA circles. *Proceedings of the National Academy of Sciences of the USA*, **92:** 4641-4645.

Gauhl F. and Pasberg G. (1995). Temporal dynamics of banana streak *badnavirus* (BSV) symptoms in Musa clones in southern Nigeria. *Phytomedizin* **25:** 25-27.

Gawel N. and Jarret R. (1991). A Modified CTAB extraction procedure for *Musa* and *Ipomoea. Plant Molecular Biology Reporter* **9**: 262-266.

Geering A., Pooggin M., Olszewski N., Lockart B. and Thomas J. (2005). Characterisation of *Banana streak Mysore virus* and evidence that it's DNA is integrated in the B-genome of cultivated *Musa. Archives of Virology*, **150:** 787-796.

Geering A., Parry J., Zhang L., Olszewski N., Lockart B.and Thomas J. (2001). Is *Banana streak virus* strain OL the only activatable virus integrant in the *Musa* genome? *Proceedings of 2nd International Conference on Molecular Cellular Biology of Banana. Info Musa* **10,** P V.

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

Gildow F. and Gray S. (1993). The aphid salivary gland basal lamina as a selective barrier associated with vector-specific transmission of Barley yellow dwarf luteoviruses. *Phytopathology*, **83**: 1293-1302.

Gildow F. (1989). Luteovirus transmission and mechanisms regulating vector specificity. In: H.G. Smith and H. Barker (ed). *The luteoviridae,* pp. 88-112. CAB International, Wallingford, UK.

Gildow F. (1987). Virus-membrane interactions involved in circulative transmission of luteoviruses by aphids. *Currents Top Vector Research*, **4:** 93-120.

Gildow F. (1985). Transcellular transport of barley yellow dwarf virus into the hemocoel of the aphid vector, *Rhopalosiphum padi. Phytopathology* **75:** 292-297.

Gold C., Ogenga-Latigo M., Tushmereirwe W., Kashaija I. and Nankinga C. (1993). Farmer perceptions of banana pest constraints in Uganda. Pp.3-24. In: *Proceedings of a research Co-ordination Meeting for Biological and Integrated Control of highland Banana Pests and diseases in Africa,* Cotonou, 12-14 November 1991. Gold, C. S. and Gemmil, B. (Eds.) International Institute of Tropical Agriculture, Ibadan.

Gray S. (2004). Plant virology. www.ppru.cornell.edu/virology/plant virology.htm-5k (accessed October, 2010).

Gray S. and Gildow F. (2003). Luteovirus-aphid interactions. *Annual review of phytopathology and plant pretection*, **41**: 539-566.

Haible D., Kober S. and Jeske H. (2006). Rolling Circle Amplification revolutionizes diagnosis and genomics of geminiviruses. *Journal of Virological Methods*, **135:** 9–16

Harper G., Hart D., Moult S., Hull R., Geering A. and Thomas J. (2005). The diversity of *Banana streak virus* isolates in Uganda. *Archives of Virology*, **150:** 2407-2420.

Harper G., Hull R., Lockhart B. and Olszewski N. (2002). Viral sequences integrated into plant genomes. *Annual Review of Phytopathology*, **40:**119-136.

Harper G., Dahal G., Thottappily G., and Hull R. (1999). Detection of episomal *Banana streak badnavirus* by IC-PCR. *Journal of Virological Methods*, **79**:1-8

Harper G. and Hull R. (1998). Cloning and sequence analysis of banana streak virus DNA. *Virus Genes*, **17:**271-278.

Harper G. and Hull R. (1996). Sequence analysis of banana streak virus. *MusAfrica, **10**:128.*

Harris K. and Harris L. (2001). Ingestion–egestion theory of cuticula-borne virus transmission. In Virus–Insect–Plant Interactions, pp. 111–132. Edited by K. F. Harris, O. P. Smith & J. E. Duffus. New York: *Academic Press.*

Harrison B. and Robinson D. (1998). Molecular variation in vector borne plant viruses: Epidemiological Significance. *Philosophy of transmission Research Society*, **B321**: 447-462.

Henry J., Raina A. and Ridgway R. (1990) Isolation of high-molecular-weight DNA from insects. *Analytical Biochemistry*, **185**: 147–150.

Hillis D., Mable B., Larson A., Davis S. and Zimmer E. (1996). Nucleic acids IV: Sequencing and cloning. In Hillis, D.M., Moritz and Mable B.K. eds. *Molecular systematic*. Sinnauer Associates, Sunderland, M.A.

Honeycutt R., Sobral B., Keim P. and 1rvine J. (1992). A rapid DNA extraction method for Hughes J. (1998). *Banana streak badnavirus* research at the International Institute of Agriculture. In: *Banana streak virus:* a unique virus . *Musa* interaction*? Proceedings of a workshop of the PROMUSA Virology Working Group*. Montpellier, France*, 19-21 January 1998*. pp. 29-33.

sugarcane and its relatives. *Plant molecular biology*, **10**: 66-72.

Hull, R. (2002). *Mathews' plant virology.* 4th edition. New York. USA. *Acadermic press.*

James A., Geijskes R., Dale J., and Hardling R. (2011). Development of a novel Rolling Circle Amplification technique to detect *Banana Streak Virus* that also discriminate between integrated and episomal virus sequences. *Plant disease* **95**: 57-62.

Janssen J., Tjallingii W. and Van Lenteren J. (1989). Electrical reconding and ultrastructures of stylets penetration by the greenhouse whitefly. *Entomological Experience and Applied* biology**, 52:** 62-81.

Jones D. and Lockhart B. (1993). *Banana streak virus*. Fact sheet No.1. Montpellier, France International Network Improvement of Bananas and plantains.

Karanja L., Wangai A., Pathak R. and Harper G. (2010). Effect of environment on the expression of banana streak virus symptoms in banana cultivars in Kenya. *African Journal of Biotechnology*, **9:** 19.

Karanja L. (2009). Diversity of *Banana streak virus* in Kenya. PhD Thesis, Egerton University, Kenya.

Karanja L., Wangai A., Harper G. and Pathak R. (2008). Molecular identification of *Banana streak virus* isolates in Kenya. *Journal of Phytopathology*, **156:** 678-686.

Kennedy J., Day M. and Eastop V. (1962). *A conspectus of Aphids as Vectors of Plants Viruses.* Commonwealth Institute of Entomology, London.

Kenya Agricultural Research Institute. (K.A.R.I) (1998). *Biotechnology to Benefit Small Scale Banana Producers in Kenya. Annual Progress* Report 1997.Nairobi, Kenya.

Knoike S., Erlachier S., Hausmann A., Miller M. and Segerer A. (2005). A procedure for combined genetalia dissection and DNA extraction in Lepidoptera. *Insects Systematic Evolution*, **35**: 401-409.

Kornberg A. and Barker T. A. (1992). DNA replication. W.H. Freeman and company, San Francisco: *Academic press.*

Kubiriba J., Legg J., Tushemereirwe W. and Adipala E. (2001). Vector transmission of banana streak virus in the greenhouse in Uganda. *Annuals of Applied Biology*, **139:**37-49.

Kubiriba, (2005). Epidemiology of Banana streak virus (BSV) in East African highland Banana. PhD, Thesis, University of Greenwich, UK. .

Lafleur D., Lockhart B.and Olszewski N. (1996). Portions of the *Banana streak badnavirus* genome are integrated in the host *musa spp. Phytopathology* **86:** 100-101.

Liu D., Daubendiek S., Zillman M., Ryan K. and Kool E. (1996). Rolling circle DNA synthesis: Small circular oligonucleotides as efficient templates for DNA polymerase. *Journal of American Chemistry Society*, **118***:* 1587-1994

Lizardi P., Huang X., Zhu Z., Bray-Ward P., Thomas D. and Ward D. (1998). Mutation detection and single-molecule counting using isothermal rolling circle amplification. *Natural Genetics*, **19:** 225-232.

Lockhart B. and Jones D. (2000). Banana streak. In: Jones D.R. (eds), *Diseases of Banana, Abaca* and Enset, Willingford, UK, CAB International, pp.263-264

Lockhart B. (1995). Banana streak virus infection: Epidemiology, diagnosis and control. Technical Bulletin No. 143. Taipei, Taiwan; Food and Fertility Technology Centre for the Asian and the Pacific Region (ASPAC).

Lockhart B. (1994). *Banana streak virus.* Pp. 19-20. In: *Comperdium of Tropical Plant Disease.* Ploetz, R.C., Gentmyer, G.A., Nishijima, N.T., Rogrbach, K.G., Ohr, H.D. (eds.). St. Paul, MN: American Phytopathological Society Press.

Lockhart B. and Olszewski N. (1993). Serological and genomic heterogeneity of the banana streak *badnavirus*: Implications for virus detection in *Musa* germplasm. Pp. 105-113. In: *Breeding Banana and Plantain for Resistance to Diseases* and *Pests, j. Genry (Ed.).* Montpellier, France: INIBAP.

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

Martin B., Collar J., Tjallingii W. and Fereres A. (1997). Intracellular ingestion and salivation by aphids may cause the acquisition and inoculation of non-persistently transmitted plant viruses. *Journal of General Virology*, **78**: 2701-2705.

Mensfin T., Den Hollander J. and Markham P. (1995). Feeding activities of *Cicadulina mbila (*Hemiptera: Cicadellidae) on different host plants. *Bullutin of Entomological. Research*, **85:** 386-396

Meyer J. (2006). *Banana streak badnavirus* in South Africa: Incidence, transmission and development of an antibody based detection system. MSC, Thesis, University of Pretoria etd.

Meyer J., Kasdorf G., Nel L., and Pietersen G. (2008). Transmission of activated-episomal *Banana streak OL(badna) virus* (BSOLV) to cv. Williams banana (*musa spp*) by three mealy-bug species. *Journal of Plant Disease*, **92**: 1158-1163.

Michele K. Nishigushi P. Egan M. Kiziran D. Aloysius P. Lorenzo P. Horward C., Rosenbaum E., Yael W., Desalle R., and Gonzalo G. (2002). *DNA isolation procedures*. In: *Techniques in Molecular System and Evolution editors Rob DeSelle et al.@ 2002 Birkhauser Verlag Basel/Switzerland*, **2002**.

Mitchell A., Cho S., Regier J. C., Miller C., Poole R. and Mathews M. (1997). Phylogenetic utility of elongation factor -1α in *Noctuoidea* (insecta: *Lepidoptera*): the limits synonymous substitution. *Molecular Biology and Evolution*, **14**: 381-390.

Nault L. (1997). Arthropod transmission of plant viruses: a new synthesis. *Annual of Entomological Society of America*, **90:** 521-541.

Nault L. and Ammar E. (1989). Leafhopper and planthopper transmission of plant viruses. *Annuals Review of Entomology*, **34:** 503-529.

Ndowora T. and lockart B. (2000). Development of a serological assay for detecting serologically diverse banana streak virus isolates. *Acta horticulturae*, **540**: 377-388.

Ndowora T., Dahal G., La Fleur D., Harper G., Olszewski N. and Lockhart B. (1999). Evidence that badnavirus infection in *Musa* can originate from integrated Pararetroviral sequences. *Virology*, **255**: 214-220.

Ng J. and Falk B. (2006) Virus-vector interactions mediating non-persistent and semi-persistent transmission of plant viruses. *Annual Review of Phytopathology* **44**: 183–212.

Ng J., Josefsson C., Clark Q., Franz A. and Perry K. (2005). Virion stability and aphid vector transmissibility of *Cucumber mosaic virus* mutants. *Virology*, **332**: 397-405.

Omura T. and Yan J. (1999). Role of outer capsid proteins in transmission of *phytoreovirus* by insect vectors. *Advances in Virus research* **54**: 15-43.

Ortiz R. and Vuylsteke D. (1994). Plantain breeding at IITA.Pp 130-156. In: *The Improvement* and *testing of Musa: A global partnership Jones. D.R* (Ed.). Montpellier,France, International Network for the improvement of bananas and plantain.

Palacios I., Drunker M., Blanc S., Leite S., Moreno A. and Fereres A. (2002). *Cauliflower mosaic virus* is preferentially acquired from the phloem by its aphid vectors. *Journal of General virology*, **83**: 3163-71.

Panis B., Van Wauwe D. and Swennen R. (1993). Plant regeneration through direct somatic Embryogenesis from protoplasts of banana (*Musa spp.). Plant Cell Reports* **12:** 403-407.

Peiffer M., Gildow F. and Gray S. (1997). Two distinct mechanisms regulate luteovirus transmission efficiency and specificity at the aphid salivary gland. *Journal of General Virology*,**78**: 495-503.

Perry K., zhang L. and Palukaitis P. (1998). Amino acid changes in the coat protein of C*ucumber mosaic virus* differentially affect transmission by the aphids *Myzus persicae* and *Aphids gossypii Virology*, **242**: 204-210.

Peterson-Burch B., Wright D. and Voytas D. (2000). Retroviruses in plants. *Trends Genetics*, **16**:151-152.

Philips A. and Simon C. (1995) Simple, efficient, and non-destructive DNA extraction protocol for arthrophods. *Annauls of Entomological Society of America*, **88**, 281-283.

Pirone T. and Blanc S. (1996). Helper dependent vector transmission of plant viruses. *Annual of Review of Phytopathology*, **34:** 227-247.

Plisson C., Uzest M., Drucker M., Froissart R. and Dumas C. (2005). Structure of the mature p3-virus particle complex of *Cauliflower mosaic virus* revealed by cry-electron microscopy. *Journal of Molecular Biology*, **346**: 267-77.

Powell A. (2000). Insect transmission of plant viruses: a constraint on virus variability. *Current Opinion Plant Biology*, **3:**336-340.

Powell G., Pirone T. and Hardie J. (1995). Aphid stylet activities during potyvirus acquisition from plants and an in vitro system that correlate with subsequent transmission. *European Journal of Plant Pathology*, **101**: 411–420.

Prado E. and Tjallingii W. (1994**).** Aphid activities during sieve element punctures. *Entomologia Experimentalis et Applicata*, **72**: 157-166.

Purseglove J. (1985). Tropical crops. Monocotyledons (Revised). Longman Group, Harlow, Essex 568pp.

Reagin M., Giesler T., Merla A., Resetar-Gerke J., Kapolka K. and Mamome A. (2003). TempliPhi: A Sequencing Template Preparation Procedure That Eliminates Overnight Cultures and DNA Purification. *Journal of Biomolecular Techniques,* **14:** 143-148.

Roivainen O. (1980). Transmission of cocoa swollen shoot virus by mealybugs (Homoptera: *Pseudococcidae*). *Journal of the Scientific Agricultural Society of Finland*, **49:** 203-304.

Rose O., Brookes M. and Mallet J. (1994). A quick and simple nonlethal method for extracting DNA from bitterfly wings. *Molecular Ecology*, **3**: 275.

Rosell R., Litchty J. and Brown J. (1995). Ultrastructue of the mouthparts of adult sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: *Aleyrodidae*). *International Journal of Insect Morphology and Embryology*, **24:** 297-306.

Rung A, Miller D. and sceffer S. (2009). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism method to distinguish three mealy-bug groups within the *Planococcus citri–P. minor* species complex (Hemiptera: Coccoidea: *Pseudococcidae*). *Journal of General Entomology*,**102**(1): 8-12

Sambrook J. and Russell D. (2001). *Molecular Cloning*: A laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Sambrook J., Fritsch, E. and Maniatis T. (1989). *Molecular Cloning*: a Laboratory Manual. Cold Spring Harbor LaboratoryPress, Cold Spring Harbor, NY.

Seddas P. and Boissinot S. (2006). Glycosylation of beet western yellows virus proteins is implicated in the aphid transmission of the virus. *Archives of Virology*, **151**: 967–984.

Schneider D., Legal L., Dierl W. and Wink M. (1999). Androconial hairbrushes of the Syntomis (Amata) Phegea (L.) group (Lepidoptra, *Ctenuchidae*): A Synapomorphic Character Supported by sequence data of the mitochondria 16S rRNA gene. *Zeitschrift fur Naturfarschung*, **54**C: 1119-1139.

Simmonds N. (1966). *Bananas.* Second Edition. Longmans,London. 256Pp.

Simmonds N. (1962). The evolution of the bananas. Longmans, 170pp.

Stover R. (1972). *Banana, Plantain* and *Abaca diseases.* Common Wealth Mycological Institute, Kew Surrey, UK. 316pp.

Strauss, W. (1998) Preparation of genomic DNA from mammalian tissue. *Current Protocols in Molecular Biology* (ed. by F. M. Ausubel) Supplementary 42, section 2.2.1–2.2.3. John Wiley, USA.

Strickland A. (1950). The dispersal of *Pseudococcidae* (Hemiptera-Homoptera) by air currents in the Gold Coast. In: *Proceedings of the Royal Entomological Society London*, **25:** 1-9.

Strickland A. (1951). The entomology of swollen shoot of cacao. I. *The insect species Involved* with notes on their biology. *Bulletin of Entomological Research*, **42:** 63-105.

Su Hong-Ji (1998). First occurrence of the banana streak badnavirus and studies on vectorship in Taiwan. Pp. 20-25. In: *Banana streak virus: a unique virus-Musa interaction?* Proceedings of a workshop of the PROMUSA Virology Working Group. Montpellier, France, January 19-21.

Swennen R., Vuylsteke D. and Ortiz R., (1995). Phenotypic diversity and patterns of variation in West and Central African Plantains (*Musa spp.,* AAB Group *Musaceae*). *Economic Botany*, ***49:*** 320-327.

Sylvester E., Richardson J. and Frazier N. (1974). Serial passage of strawberry crickle virus in the aphids *Chaelosiphon Jacobi* with isolates of Sowsthistle yellow vein virus and strawberry crickle virus. *Phytopathology*, **74:** 598-602.

Thresh J. (1958). The spread of virus disease in cacao. *Technical Bulletin West African Cocoa Research Institute,* 36pp.

Uzest M., Gargani D. and Drucker M. (2007). A protein key to plant virus transmission at the tip of the insect vector stylet. *Proceedings of the National Academy of Sciences of the USA*, **104:** 17959–17964.

Vasvary L. (2004). Mealy-bugs, Order Homoptera; Family: *Pseudococcidae.* http://aesop.rutgers.edu/~ floriculture /grower/mealy-bug.htm. (Accessed October, 2010)

Vuylsteke D., Swennen R. and Ortiz R. (1993). Registration of 14 improved tropical *Musa* plantain hybrids with resistance to black sigatoka. *Hortscience*, **28:** 957-958.

Wachira F. (1996). Development of molecular markers in Camellia. PhD Thesis, Dundee University.

Wahl D. (1984). An improved method for preparing exuviae of parasitic Hymenoptera. *Entomological News* **95**: 227-228.

Wahl D. (1989). Further notes on preparation of exuviae of parasitic Hymenoptera. *Entomological News*, **100**: 181-182.

Walkey D. 1991. *Applied Plant Virology*. 2nd Edition. London:Chapman and Hall. 338 pp.

Wang R., Ammar E, Thornbury D., Lopez-Moya J. and Pirone T. (1996). Loss of potyvirus transmissibility and helper-component activity correlate with non-retention of virions in aphid stylets. *Journal of General Virology*, **77**: 861–67.

Wangai A., Karanja L., Ndung'u J., Kimani E., Kilonzo S., and Nguthi F.(2002). *Preliminary results on surveys of Banana streak virus* (BSV) *in Kenya, 2nd Annual Symposium* K.A.R.I-NPBRC, Njoro, Kenya, Egerton University, 2002.

Watson G., Ooi P. and Girling D. (1995). *Insects on plants in the Maldives and their management*. International Institute of Biological control, Ascot, UK. 129 PP.

Watson M. and Roberts F. (1939). A comparative study of the transmission of *Hyocyamus* virus 3, Potato virus Y and cucumber mosaic virus by the vector *Myzus persicae* (Sulz), *M. circumflexus* (Buckton) and *Macrosiphum gei* (Koch). *Proceedings of Research of Society London B*, **127:** 543-576.

Williams D. and Matile-Ferrero D. (2000). A new species of the mealy-bug genus *Cataenococcus* Ferris from Ethiopia on *Ensete ventricosum,* a plant infected by virus. [Hemiptera, *Pseudococcidae; Musaceae*]. *Revue Francaise d'Entomologie* (N.S.) **21:** 145-149.

Williams D. (1996). A brief account of the hibiscus mealy-bug *Maconellicoccus hirsutus* (Hemiptera: *Pseudococcidae*), a pest of agriculture and horticulture, with descriptions of two related species from southern Asia. *Bulletin of Entomological Research* **86**: 617-628.

Williams D. J. and Granara De Willink M. C. (1992). *Mealy-bugs of central and South America*. CAB International, Willingford, UK. 635 PP.

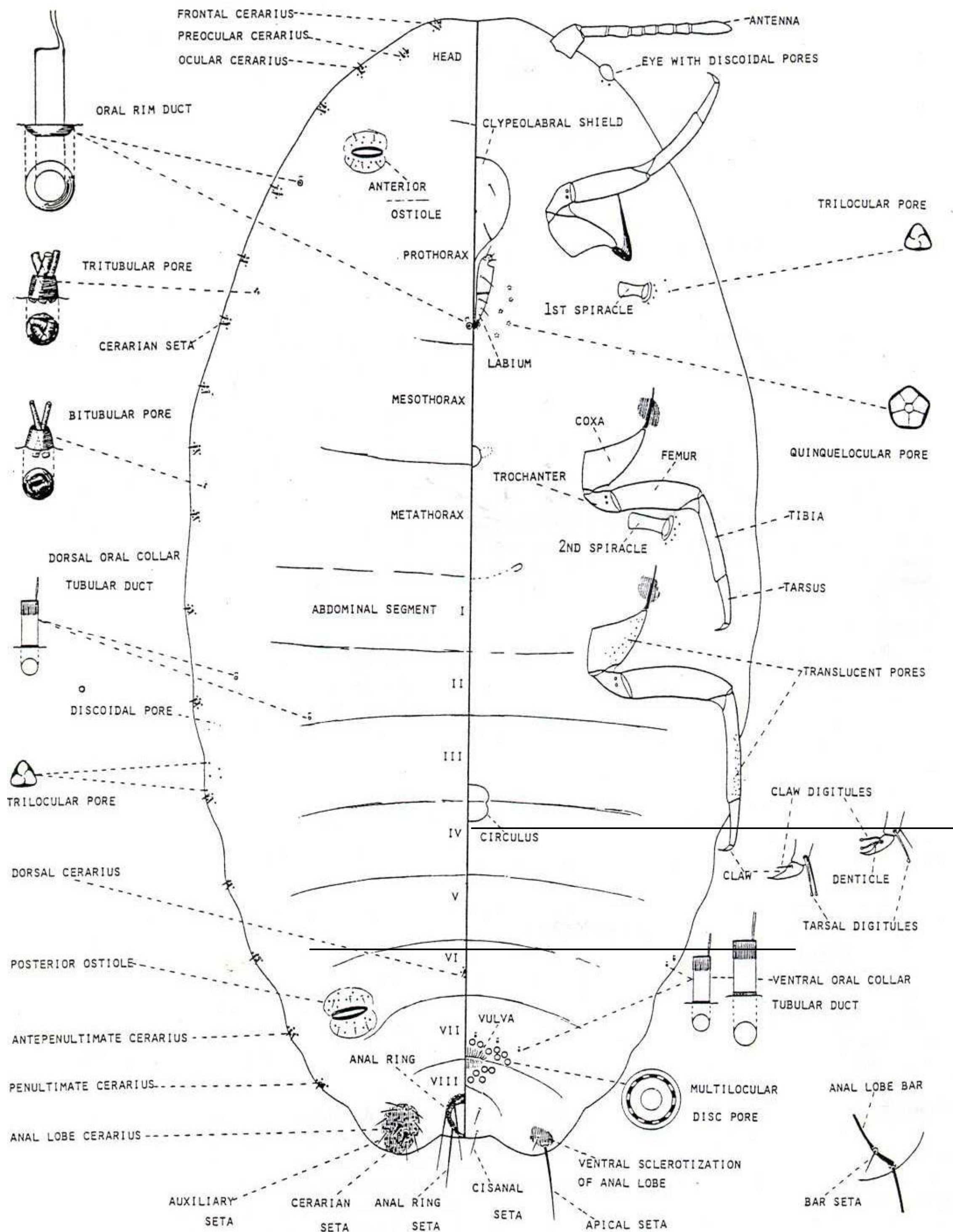
Williams D. (1989). The mealy-bug genus *Rastrococcus* (Homoptera: *Pseudococcidae*). *Systematic Entomology*, **14**: 433-486.

Williams D. and Watson G. (1988). The scale insects of the tropical South Pacific region. Part 2. The mealy-bugs (*Pseudococcidae*). CAB International, Wallingford, UK. 260 PP.

Williams D. (1958). The mealy-bugs (*Pseudococcidae*: Homoptera) described by M.W. Maskell, R. Newstead, T.D.A. Conkerell and E.E Green from Ethiopian region. *Bulletin of the British Museum (Natural History) (Entomology*), **6**: 205-236

APPENDICES

APPENDIX 1: General morphology of an adult female mealy-bug



(Williams, 1985), © The Natural History Museum, London).

APPENDIX 2: Principal characteristics of the modes of virus transmission by insects

Feature	External (noncirculative)		Internal (circulative)
	Nonpersistent	Semipersistent	Persistent
Duration of retention	Brief (few hours)	Intermediate (few days)	Long (days to months)
Duration of acquisition and transmission	Brief (seconds)	Intermediate (hours)	Long (hours to days)
Latent period	Not required	Not required	Required
Tissue where virus is acquired and inoculated	Epidermis and parenchyma	Epidermis, parenchyma and phloem	Mostly parenchyma and phloem
Pre-acquisition fasting	Increase transmission	No effects	No effect
Passage through moult	Negative	Negative	Positive
Insect species specificity	Low	Intermediate	High
Sequential inoculation	Poor	Intermediate	Good
Transovarial transmission	Negative	Negative	Positive
Effect of acquisition time	No effect	No effect	Increase transmission

Source:Hull, 2002; Raccah and Ferere, 2009