Banana streak badnavirus (BSV) in South Africa: incidence, transmission and the development of an antibody based detection system

Jacolene Bee Meyer

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University of Pretoria
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LIST OF ABBREVIATIONS

A	Absorbance
µl	Microlitre
AP	Aspartic protease
ARC-PPRI	Agricultural Research Council-Plant Protection Research Institute
BanMMV	*Banana mild mosaic virus*
BBrMV	*Banana bract mosaic potyvirus*
BBTV	*Banana bunchy top babuvirus*
BC	Buffer control
BEL	BSV express locus
BEV	Banana endogenous virus
Bp	Base pairs
BSD	Banana streak disease
BSV	*Banana streak badnavirus*
BSV-Cav	BSV strain isolated from Williams (Australia)
BSV-GF	BSV strain isolated from Goldfinger (FHIA 01), Australia
BSV-IM / BSV-IRFA	BSV strain isolated from *Musa* cultivar IRFA 914
BSV-Mys	BSV strain isolated from Mysore (Australia)
BSVMysV	Banana streak Mysore virus
BSV-Onnè / BSV-OL	BSV strain isolated from Nigeria form a hybrid plantain TMP x 7002-1
BSV-RD	BSV strain isolated from Red Dacca (Australia)
C	Antibody constant region
CaMV	*Cauliflower mosaic caulimovirus*
CARBAP	Centre African de Recherches sur Bananiers et Plantains
CaVMV	*Cassava vein mosaic cavemovirus*
CaYMV	*Canna yellow mottle badnavirus*
cDNA	Complimentary deoxyribonucleic acid
CIRAD	Centre de Coopèration Internationale en Recherche Agronomique pour le Dèveloppement
CMV	*Cucumber mosaic cucumovirus*
CP	Coat protein
CSSV	*Cacao swollen shoot badnavirus*
CoYMV	*Commelina yellow mottle badnavirus*
CYMV	*Citrus yellow mosaic badnavirus*
DAS	Double antibody sandwich
DBV	*Dioscorea bacilliform badnavirus*
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
EMBRAPA	Empresa Brasiliera de Pesquisa Agropecuaria
ERPV	Endogenous pararetrovirus
FHIA	Fundacion Hondurena de Investigacion Agricola
FHIA-01	*Musa* cultivar, synonym Goldfinger
H	Antibody heavy chain
HBV	*Hepatitis B virus*
HC	Healthy Control
HeRV	*Human endogenous retrovirus*
IC	Immunocapture
Ig	Immunoglobulin

IITA	International Institute for Tropical Agriculture
INIPAB	International Network for Banana and Plantain
ISEM	Immunosorbent electron microscopy
J	Joining segment on antibody
KTSV	*Kalanchoe top-spotting badnavirus*
L	Antibody light chain
LTR	Long terminal repeat
M	Molar
Met	Methionine
mM	Millimolar
mRNA	Messenger ribonucleic acid
MWCO	Molecular weight cut off point
OD	Optical density
ORF	Open reading frame
OVI	Onderstepoort Vetenary Institute
PC	Positive control
PCR	Polymerase chain reaction
PVCV	*Petunia vein clearing petuvirus*
PYMV	*Piper yellow mottle badnavirus*
RH	RNAse H
RNA	Ribonucleic acid
RT	Reverse transcriptase
RTBV	*Rice tungro bacilliform tungrovirus*
Rubisco	*Ribulose-biphosphate carboxylase oxygenase*
SbCMV	*Soybean chlorotic mottle soymovirus*
SCBV	S*ugarcane bacilliform badnavirus*
SFV-3	*Simian foamy virus*
SRV	*Schefflera ringspot badnavirus*
TAS	Triple antibody sandwich
TC	Tissue culture
tRNA	Transcription ribonucleic acid
TVCV	*Tobacco vein clearing cavemovirus*
UP	University of Pretoria
UV	Ultraviolet
V	Antibody variable region
V_L1	One variable region on Chicken light chain
v/v	Ratio of volume added to volume
V_H	Variable region on heavy chain of antibody
VIC	Virus Indexing Centre
V_L	Variable region on light chain of antibody
w/v	Ratio of weight added to volume

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SUMMARY

Title: Banana streak badnavirus (BSV) in South Africa: incidence, transmission and the development of an antibody based detection system

Student: Jacolene Bee Meyer

Supervisor: Professor Louis H. Nel

Department: Department of Microbiology and Plant Pathology

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Various research efforts have focused on *Banana streak badnavirus* (BSV), the causal agent of banana streak disease (BSD), since the discovery of endogenous sequences of the virus in the nuclear genome of several *Musa* (banana and plantain) species. *In vitro* propagation of *Musa* was identified as one of the main activation triggers of integrated BSV sequences to cause systemic (episomal) BSD. This was especially observed in B genome-containing tetraploid hybrids. Although, the South African banana industry is based on Cavendish varieties, some plantations with tetraploid hybrids have been established. In order to investigate the occurrence of episomally expressed BSV, a survey was done in the Kiepersol area of South Africa and episomal BSV was detected in six out of seven locations sampled. No episomal BSV was detected in the Cavendish cultivars sampled in close proximity to BSV infected cultivars. To determine the risk of vector-assisted spread of endogenous BSV, which has become episomally activated after tissue culture, transmission studies with local mealybug species (*Planococcus citri* (Risso), *P. ficus* (Signoret), *Dysmicoccus brevipes* (Cockerell) and *Pseudococcus longispinus* (Targioni-Tozzetti)) were conducted under controlled conditions. Virus-free FHIA-21 was multiplied *in vitro* and resulting progeny with, putatively episomally activated BSV, served as sources for mealybug-assisted transmissions to Cavendish. Activated, episomal BSV was transmitted by three mealybug species to Cavendish. Transmission with *P. ficus* was demonstrated for the first time. Limited antiserum stocks against BSV occur worldwide and detection of the virus remains crucial for the safe movement of *Musa* germplasm between continents. Antiserum is needed in order to detect the episomal form of the virus that causes BSD. Using conventional immunization methodology, antisera against a wide spectrum of BSV isolates were produced. Twenty diverse BSV isolates were characterized by IC-PCR and selected as sources for the production of the polyclonal antiserums in two animal species. An effective triple antibody

sandwich (TAS) enzyme linked immunosorbent assay (ELISA) system; able to detect various serologically different species of BSV was developed. BSV was screened with a synthetically manufactured phage displayed antibody library; however, no satisfactory polyclonal or monoclonal antibodies were obtianed in using this approach.

CHAPTER 1

1. INTRODUCTION AND AIMS OF STUDY

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1.1 INTRODUCTION

Various nucleotide variants of endogenous *Banana streak badnavirus* (BSV) have been identified in the genome of *Musa* species (Harper *et al.*, 1999b; Ndowora *et al*., 1999, Geering *et al.,* 2005b). It is these endogenous sequences, giving rise to episomal BSV infection that poses a challenge to researchers worldwide. Not all endogenous sequences can be activated to cause Banana streak disease (BSD), while some sequences can be expressed (Harper *et al.* 1999b). Episomal infection can be triggered by various 'stress' factors on the plant, such as *in vitro* propagation of *Musa* material (Dallot e*t al.*, 2001). However, even though millions of plants are produced by *in vitro* propagation, no BSD incidences have been found in micropropagated Cavendish varieties (AAA). It is only other varieties, especially tetraploids (mainly containing the B-gene in their genome), which seems predisposed to the activation of integrated sequences to form episomal BSV infection, resulting in the development of BSD. Chapter 3 of this thesis reports the results of a survey that was done on non-Cavendish (B genome) varieties of *Musa,* established in South Africa during 1996 to 2003. Most B genome varieties are established in the Kiepersol area of Mpumalanga. The study therefore focused on collecting material from this area. Occurrence of BSV in samples, taken from seven different localities in the Kiepersol area, is described.

It is imperative to be able to detect viruses causing disease in order to formulate control strategies and conduct scientific research. Antiserum, produced by introducing an antigen to the immune response of an experimental animal, is extremely useful as it can be used in various detection methods (Sambrook *et al.*, 1989). It is vitally important that the antigen introduced is pure from any other foreign matter such as plant proteins. Very few good antisera against BSV has been produced, with that of Lockhart (Ndowora, 1998; Ndowora and Lockhart, 2000) being the most widely used because of its ability to detect a wide variety of heterogenic BSV strains and isolates. In this study (Chapter 4) the development of antibodies against a range of BSV isolates is described along with the formulation of a TAS-ELISA protocol for the detection of various BSV isolates. Chapter 5 focuses on an alternative technique used in the production of antibodies. Synthetically designed antibodies

were evaluated, instead of applying classic immunology, in order to obtain antigen specific antibodies.

Several species of mealybug have been reported to transmit BSV such as *Planococcus citri* (Risso) and *Saccharicoccus sacchari* (Cockerell) and *Pseudococcus comstiki* (Kuwana) (Lackhart and Jones, 1993; Su, 1998). In order to determine the risk of spread of endogenous BSV that has been activated to become episomal, controlled transmissions with local mealybug species were conducted, to address the need for a systematic transmission study of activated episomal BSV. Mealybug species used in the study included *P. citri* (serving as a control), *Dysmicoccus brevipes* (Cockerell) (some scientific detail in the published paper on this species is questioned), *P. ficus* and *P. longispinus* (evaluated for the first time). Very interesting results were obtained in this study and it is described in Chapter 6 of this study. The utilization of internal controls in the polymerase chain reaction (PCR) is described in Chapter 7.

Finally, the research conducted provided a clearer picture of the status of BSV in South Africa as well as providing a research-based approach in dealing with BSV strains already introduced to South Africa, mainly *via* germplasm material. The research conducted in this study also has international application as the knowledge can be applied to other localities. The TAS-ELISA detection system developed can be used against various strains of BSV while the antisera can also be used in immunocapture (IC)-PCR procedures as well as Immunosorbent electron microscopy studies (ISEM).

1.2 SUMMARY OF AIMS

1. Determine the incidence of episomal (-activated) BSV in plantations with non-Cavendish varieties in the Kiepersol area of Mpumalanga, South Africa.
2. Determine risks of episomal (-activated) BSV spreading to commercial Cavendish varieties by conducting vector-mediated transmission studies under controlled conditions.
3. Production of polyclonal antisera against a range of BSV isolates, using conventional immunization methods, in order to establish and formulate an antibody based detection method.

4. Screen BSV against a synthetic phage antibody library in order to obtain alternative monoclonal or polyclonal antibodies with a high affinity for BSV.
5. Formulate recommendations for the South African Banana Industry in handling of BSV infected plantation as well as varieties harbouring endogenous BSV sequences that are prone to become active.
6. Formulate recommendations based on results obtained, in order to inform and assist local *Musa* growers in the control and occurrence of BSV.

1.3 TECHNOLOGY TRANSFER

Technology transfer that has resulted thus far from the research conducted in this thesis:

Publication in Popular Journal

Meyer, J.B. (2004). Why all the fuss about Banana streak virus (BSV)? *Banana Talk* 18: 8-9.

Meyer, J.B., Pietersen, G., Nel, L.H. & Robinson, J. (2003). Banana streak virus (BSV) detected in Kiepersol. *Banana Talk* 16:18-20.

Poster on National Congress

Meyer, J.B., Pietersen, G., Nel, L.H., & Kasdorf, G.G.F. (2005). Characterization of BSV isolates and the development of a TAS-ELISA detection method. 43rd Congress of the Southern African Society for Plant Pathology, 23-26 January, Hartenbos Beach Resort, Mossel Bay, Western Cape, South Africa.

Oral presented at National Congress

Meyer, J.B., Pietersen, G., Nel, L.H., & Kasdorf, G.G.F. (2006). Evaluation of the efficiency of four mealybug species to transmit activated-episomal Banana streak badnavirus. 44rd Congress of the Southern African Society for Plant Pathology, 22-25 January, Magalies Park Country Club, North West Province, South Africa.

Oral to be presented at International Congress

Meyer, J.B., Robinson, J.C., Pietersen, G. & Nel, L.H. (2006). Transmission studies of activated-episomal Banana streak badnavirus from FHIA 21 (AAAB) by four mealybug species. XVII International meeting ACORBAT, October 2006, Joinville, Brazil.

CHAPTER 2

CHAPTER 2

2. LITERATURE REVIEW

2.1 GENUS: MUSA

2.1.1 Musa: a large herb

The family *Musaceae* of the order *Zingiberales* is divided into two genera namely *Musa* and *Ensete*. Banana, plantain and abaca belong to the genus *Musa*. These are large herbaceous plants consisting of an underground rhizome (or corm) with roots, a pseudostem (tightly packed leaf bases) and leaves. The leaves of the *Musa* plant are arranged in an almost concentric fashion above the pseudostem. Initiation of new leaves occurs from the apical growth point (meristem), centered at soil level within the pseudostem. At a certain stage of growth, the apical meristem can also initiate the formation of flowers instead of leaves. Fruit develop from each flower. Each fruit is called a "finger" while the clump is called a "hand" and collectively the hands are referred to as a "bunch" (Jones, 1999; Robinson 1993).

2.1.2 Propagation

Although wild species of *Musa* produces seeds, commercially cultivated *Musa* such as Cavendish varieties does not. Natural propagation occurs when a vegetative bud within the rhizome gives rise to a sucker. More than one sucker can develop from the rhizome. In a commercial plantation, selected suckers will be removed and one sucker (the spear-sucker) left for continuation of the next generation after the motherplant dies off (Jones, 1999; Robinson 1993). As more than one vegetative bud occurs on the rhizome, propagation can also be done by using "bits" (cutting the rhizome into smaller pieces before planting) (Robinson, 1993). *In vitro* propagation via tissue culture is a widely used practice in South Africa and has the advantage of a uniform plantation with uniformity in growth, flowering and harvesting (Robinson, 1993). When multiplying *Musa* material by tissue culture, large numbers of plants can be generated from a single meristem. With this practice it is important to ensure that the meristem tissue that will be be used for multiplication, are free of any diseases, especially viruses. Certified (pest and disease free) *Musa* material can be transported efficiently and safely between continents as tissue culture plantlets (Diekmann and Putter, 1996).

2.1.3 The History of Musa

Musa originated from Asia and the Pacific where a rich source of genetic diversity of *Musa* is found. The region stretches form India to Papua New Guinea and includes Malaysia and Indonesia. A high number of seeded varieties of *Musa* existed and still exist in Asia and it is speculated that natural crosses resulted in numerous hybrids of *Musa*, including some seedless, edible varieties (INIBAP, 1999). The worldwide spread of edible *Musa* is contributed solely to man. It is thought that the edible diploid (AA) and triploid (AAA) *Musa acuminata* (A-genome) cultivars were spread to monsoon areas, which include India and the Philippines, where wild *M. balbisiana* (B genome) occurred naturally. Progeny, from natural hybridization between *M. acuminata* and *M. balbisiana* cultivars, resulted in genomes with AB, AAB and ABB compositions (Simmonds, 1976).

The banana is believed to have been cultivated in South India around 500 BC. Later it was distributed from this area to Malaysia through to Madagascar. Around AD 1000 banana material was spread across the Pacific to Japan and Samoa. Later it was introduced to East Africa (AD 500) and West Africa (AD 1400) (Simmonds, 1976). However, studies on phytoliths (literally "plant stones") provide evidence that *Musa* had been cultivated on the Atlantic side of the African continent some 2500 years ago (Vrydaghs *et al.*, 2002). When the Portuguese arrived on the West Coast of Africa in the 15th century, banana and plantain were well known in Africa. It is believed that the Portuguese took banana from Africa to the Canary Islands and from there it spread to Haiti in the 16th century. Later banana was introduced to the Caribbean and tropical America (Simmonds, 1976). By the end of the eleventh century, banana occurred widely throughout the tropics.

In South Africa the sweet banana cultivars of the Cavendish group, such as Williams and Grand Nain, are mostly cultivated. Williams were imported from Australia and established in South Africa in the late 1970's while cultivars such as Grand Nain were established in the 1980's (ARC-ITSC). In 2004, percentages reflected by the different cultivars were as follow: 45 % of local plantations were represented by Williams, followed by 40 % for Grand Nain, 10 % Chinese Cavendish and 5 % Dwarf Cavendish.

2.1.4 Consumption, Application, Production and Export of Banana and Plantain

Banana, cooking banana and plantain are excellent starch sources. They are high in calories (Johnston, 1958; Sharrock and Lusty, 2000) and can be consumed in various ways e.g. fresh, cooked or fried (Dadzie, 1998). It is also a rich source of vitamin A, C, calcium and iron (FAO, 1983). Four hundred million people in tropical countries, depend on *Musa* fruit as a staple foodstuff. Ninety percent of *Musa* produced, are immediately consumed, particularly in the poorest countries of Africa, Latin America and Asia (Fourè and Tézenas du Montcel, 2000).

Musa fruit, leaves and flowers have many applications. The fruit peel can be used to feed livestock (Dadzie, 1998). *Musa* fruit can also be fermented to make wine or beer (Carreno S. and Aristizabal L., 2003). The male flower bud of *Musa* can be eaten as a vegetable (Okoro *et al.*, 2000) while leaves can be used to wrap or dish up food (FAO, 1983). Strong fibers, for various applications, can be produced from the *Musa textilis* plant (Lacuna-Richman, 2002). Overall *Musa* does not only serve as a staple food source to many tropical countries, but it also provides a valuable means of income.

More than 100 countries, located in the tropical and sub-tropical regions of the world, produce banana and plantain (INIBAP, 1998). The top five banana producing countries in the world are India, Uganda, Ecuador, Brazil and Columbia (FAO, 2001). Worldwide approximately 85-88 million metric tonnes of *Musa* are produced annually of which 30 million metric tonnes are plantain. After rice, wheat and maize, *Musa* (banana and plantain) is the world's most important food crop and produces food security for many third world countries (Fourè and Tézenas du Montcel, 2000).

According to FAO (2001), Africa was the third largest producer of banana and plantain in 2001. Eastern and Southern Africa makes up some of the highest *per capita* consumers of *Musa*, being more than 400kg per person per year. West and Central Africa produces about 10 million tonnes of banana and plantain annually and most of this is consumed locally (INIBAP, 1999). Uganda (10, 506 200 metric tonnes), Cameroon (2, 250 000 metric tones) and Ghana (1, 942 000 metric tonnes) have the highest production figures in Africa. South Africa ranks fifteenth out of the 39 African countries with a production of 268 06 metric

tonnes in 2001. Currently South African produced bananas are consumed locally and no fruit is exported (Crooks, 2004). Latin America exported the highest amount of its production of banana and plantain in 2001 (10, 041 367 metric tonnes) followed by the Asian-Pacific region (1, 710 138 metric tonnes) and Africa (418 925 Metric tonnes) (FAO, 2000).

2.1.5 Musa Cultivars

A large collection of *Musa* germplasm are kept and made available through INIBAP's Transit Centre at the Laboratory of Tropical Crop Improvement, Katholieke Universiteit Leuven, Belgium. The collection holds over 1100 accessions of *Musa*. These include wild, local, commercial and improved varieties. Each cultivar accessed, receives an ITC code for easy reference. This not only secures and conserves germplasm, but also provides valuable germplasm material to breeders, while it serves as a place from where developed *Musa* cultivars can be distributed.

M. acuminata has 2n=22 chromosomes and an AA-genome while *M. balbisiana* is also 2n=22 but with a BB genome. Cultivars, with various genome combinations *e.g*. AABB, AAAB, AAAA, ABB, AAB, AAA, AB are available.

Of the AA and AB sub-genomic groups, "Sucrier" is the most popular and mainly grown in South East Asia. Worldwide, the most widely cultivated cultivars are the AAA groups. Cavendish, Gros Michel and Lujugira-Mutika present the three subgroups from the AAA genomic group. Within these subgroups are various clones, clone sets and cultivars (Jones, 1999). Plantains, a major food crop in many African countries, occur under the AAB genome group. Other AAB subgroups include Pome, Maia Maoli-Popoulu and Iholena (Jones, 1999).

Various tetraploid hybrids (AAAA, AABB and AAAB) of *Musa* (Table 2.1) have been developed that are resistant or tolerant to Panama disease (*Fusaruim oxysporum* f. sp. *cubense*), Black Sigatoka (*Mycosphaerella fijiensis*) (Escalant *et al*, 2002) crown rot disease and burrowing nematodes (Dadzie, 1998). Breeding programmes have been developed at Fundacion Hondurena de Investigacion Agricola (FHIA) in Honduras, Centre de Coopèration Internationale en Recherche Agronomique pour le Dèveloppement (CIRAD) in France and Guandeloupe, the International Institute for Tropical Agriculture (IITA) in Nigeria and

Uganda, the Centre African de Recherches sur Bananiers et Plantains (CARBAP) in Cameroon and the Empresa Brasiliera de Pesquisa Agropecuaria (EMBRAPA) in Brazil (Escalant *et al*., 2002).

Many of these tetraploid cultivars have high yielding potential, as well as adaptability to a wide range of climates. Some have even proven to be productive in poor soil conditions (e.g. FHIA 03, AABB) (FHIA, http://www.honduras.com/fhia/banana.htm; Tezenas-du Montcel, 1996). Some triploids like IRFA 909 (AAB) have short production cycles and good ratooning properties (Tezenas-du Montcel, 1996). In recent years, sequences of *Banana streak badnavirus* (BSV), a plant pararetrovirus, belonging to the genus of *Badnaviruses*, were discovered integrated into many of these *Musa* genomes. Some integrated sequences have the ability to give rise to active, episomal BSV particles (Ndowora *et al.*, 1999; Harper *et al*., 1999b), which result in the occurrence of Banana streak disease (BSD).

Table 2.1: Improved *Musa* cultivars developed by various breeding programmes (Escalant *et al*., 2002).

PROGRAMME/ ORGANISATION	VARIETY	CULTIVAR
FHIA	Sweet	FHIA-02 (AAAB), FHIA-17 (AAAA), FHIA-23 (AAAA), SH-3640
FHIA	Sweet-acid	FHIA-01 (AAAB), FHIA-18 (AAAB)
FHIA	Cooking	FHIA-03 (AABB), FHIA-25
FHIA	Plantain	FHIA-21 (AAAB)
CIRAD	Dessert	IRFA-911, IRFA 904
IITA	Cooking	BITA-2, BITA-3
IITA	Plantain-type	PITA-16
CARBAP	Plantain-type	CRBP-39
EMBRAPA	Sweet-acid	PA-03-22, PA-12.03, PV-03-44, PV-42-320, PV-42-53, PV-42-81

2.2 GENUS: *BADNAVIRUS*

2.2.1 Badnavirus species and taxonomy

Badnavirus is one of the six genera belonging to the family *Caulimoviridae.* These are plant pararetroviruses, belonging to a class of retroviruses and transposable elements (Fig 2.2), in which members are characterized by the presence of a gene encoding for reverse transcriptase (RT). The genus *Badnavirus* has recently undergone some taxonomic changes resulting in the formation of several other genera including *Caulimovirus*, *Tungrovirus* (Species; *Rice tungro bacilliform tungrovirus* (RTBV) (Hibino *et al*., 1979), *Petuvirus, Soymovirus* and *Cavemovirus* (Mayo *et al.*, 2005). Species in the genus *Badnavirus* include, BSV (Lockhart, 1986; Harper and Hull, 1988), *Cacao swollen shoot badnavirus* (CSSV) (Patiot *et al*., 1978; Brunt, 1970), *Canna yellow mottle badnavirus* (CaYMV) (Lockhart, 1988), *Dioscorea bacilliform badnavirus* (DBV) (Harrison and Roberts, 1973; Phillips *et al.*, 1999), *Kalanchoe top-spotting badnavirus* (KTSV) (Hearon and Locke, 1984), *Piper yellow mottle badnavirus* (PYMV) (Lockhart, 1997), *Schefflera ringspot badnavirus* (SRV) (Lockhart and Olszewski, 1996) *Sugarcane bacilliform badnavirus* (SCBV) (Lockhart and Autrey, 1988) and *Citrus yellow mosaic badnavirus* (CYMV) (Huang and Hartung, 2000).

Substantial yield losses, as a result of badnavirus infection, can occur on economically important crops such as cacao (Partiot *et al.,* 1978, Ollennu, 2000), banana (Lassoudière, 1979; Dahal *et al.*, 2000, Daniells *et al.*, 2001), yam (Lockhart, 1990) and taro (Lockhart, 1990). Other commercially important crops affected by members of the genus include black pepper (Lockhart, 1997) and sugarcane (Lockhart and Autrey, 1988).

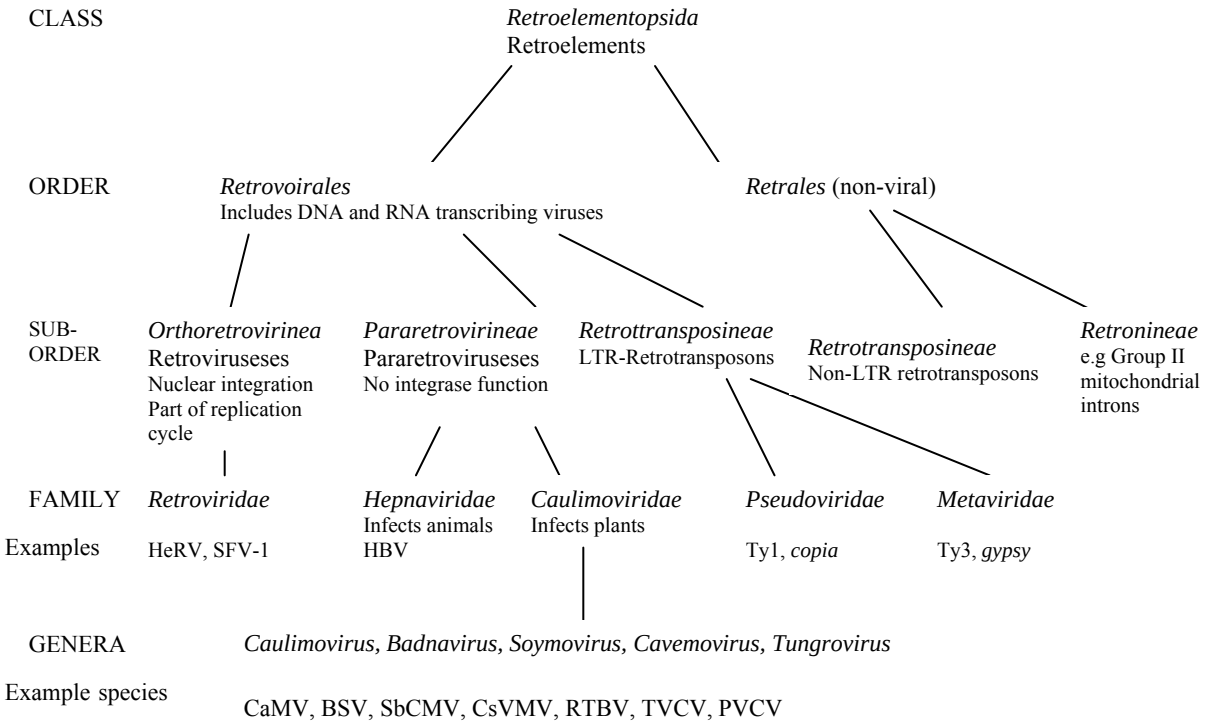

Fig 2.1: Classification of the Retroelements as published by Hansen and Heslop-Harrison (2004). Abbreviations: LTR, Long terminal repeat; CaMV, Cauliflower mosaic caulimovirus; BSV, Banana streak badnavirus; HBV, Hepatitis B virus; CaVMV, Cassava vein mosaic cavemovirus; SbCMV, Soybean chlorotic mottle soymovirus; PVCV, Petunia vein clearing petuvirus; TVCV, Tobacco vein clearing cavemovirus; HeRV, Human endogenous retrovirus; SFV-3, Simian foamy virus.

2.2.2 Transmission

Most badnaviruses are insect transmissible. BSV (Lockhart and Olszewski, 1993; Kubiriba *et al.*, 2001b; Geering *et al.*, 2005b), CSSV (Posnette, 1940), KTSV (Mayo *et al.*, 2005), SRV (Lockhart and Olszewski, 1996), CoYMV (Brunt *et al.*, 1996), PYMV (Lockhart, *et al.*, 1997) and SCBV (Frison and Putter, 1993), are transmitted by mealybugs. The citrus mealybug, *Planococcus citri,* transmits most of these viruses. CoYMV, PYMV (De Silva *et al.*, 2002), CYMV and CSSV are graft transmissible. KTSV was shown to be graft, pollen and seed transmissible (Hearon and Locke, 1984). Mechanical transmission of BSV does not appear to occur and may be attributed to the high levels of phenolic compounds and latex present in *Musa* plants (Lockhart, 1985). It has been speculated that BSV may be seed transmissible (Daniells *et al.*, 1996), however the fact that endogenous BSV sequences occur in the nuclear genome of most cultivars (Ndowora *et al.*, 1999; Harper *et al*., 1999b) make these findings questionable.

2.2.3 Genome and particle properties

All badnaviruses have a circular double stranded DNA-genome (Lockhart, 1990) of 7.4-8.0 kbp and consist of non-enveloped bacilliform particles of approximately 30 x 130-150 nm in size. The genome of BSV was first sequenced from the cultivar 'Obino l'Ewai' (BSV-OL) and is 7.389 kbp long (Harper and Hulll, 1998). Hull *et al.* (2000) describes the BSV particle as follows: "*BSV particles have an electron-dense core and the tubular portion of the particle has a structure based on an icosahedron cut across its threefold axis, with a structural repeat of 10 nm and nine rings of hexamer subunits per 130 nm length*". The complete genomes of many of these viruses such as, CoYMV (Medberry *et al*., 1990), CSSV (Hagen *et al.*, 1993; Lot *et al*., 1991), BSV (Harper and Hulll, 1998; Geering *et al.*, 2005b), SCBV (Bouhida *et al.*, 1993) and RTBV (Qu *et al.*, 1991), have been sequenced.

The positive strand of most badnavirus genomes contains 3 open reading frames (ORFs). ORFI and ORFII encode for small proteins of ~22kDa and ~14kDa respectively. For BSV the ORFI, ORFII and ORFIII regions on the positive strand, encodes for proteins of sizes 20.8 kDa, 14.5 kDa and 208 kDa respectively (Harper and Hull, 1998; Geering *et al*., 2005b). In CoYMV the protein product of ORFI was shown to be associated with virions and plant cell components. The protein encoded by ORFII was only involved with virions, strongly suggesting the role of this protein in virus assembly (Cheng *et al.*, 1996). In all badnaviruses characterized so far, ORFIII encodes for a polyprotein that is proteolytically cleaved to yield the viral coat protein (CP), aspartic protease (AP), reverse transcriptase (RT), RNAse H (RH) and cell-to-cell movement protein (Fig 2.3) (Bouhida *et al.*, 1993). By inducing mutants of the movement protein of CoYMV, it was shown that this protein is necessary for cell-to-cell movement of the virus, but not for replication (Tzafrir *et al.*, 1997). A Cysteine-rich region, unique to badnaviruses also occurs in the ORFIII region (Bouhida *et al.*, 1993; Harper and Hull, 1998). Badnavirus DNA contains two discontinuities, one in each strand (Bao and Hull, 1992). The discontinuity in the negative sense DNA is adjacent to the 3'-end of a cytosolic initiator methionine ($tRNA^{met}$) binding site (Medberry *et al.*, 1990). The genome organization of RTBV, differs from the members belonging to the badnavirus genus, as a fourth ORF occurs towards the 3' side, after ORFIII, encoding for a protein of unknown function (Qu *et al.*, 1991). CYMV has six putative ORF's on the positive strand of the genome (Huang and Hartung, 2001).

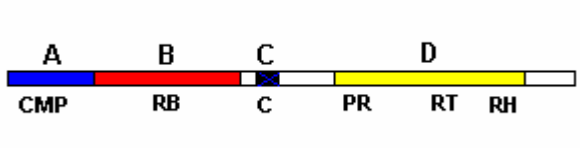

Fig 2.2: Organization of functional domains of ORF III of SCBV (Bouhida *et.al.*, 1993). Region A: contains the cell to cell movement proteins (CMP), Region B: RNA binding domain (RB), Region C: Cystiene rich region (C), Region D: Contains regions encoding for Protease (P), Reverse transcriptase (RT) and Ribonuclease H.

2.2.4 Viral replication, transcription and translation

Normal replication of badnaviruses is thought to occur as follows; after cell entry, transcription takes place, potentially resulting in sub genomic RNA, functioning as both a polycistronic mRNA and a replication template. A $tRNA^{met}$ binding site is located on the genome (Bouhhida 1993). During replication $tRNA^{met}$ may anneal to the $tRNA^{met}$ binding site of the viral transcript and so prime synthesis of the negative strand. Following synthesis of the negative sense DNA, purine rich cleavage products (formed after RH digestion of the pregenomic RNA) prime the positive sense DNA (Medberry 1990). Virally encoded RT is responsible for both the negative and positive strand synthesis. RH, also virally encoded, is co-involved with RT in the synthesis of the positive strand.

Using computer-aided analysis of the genomes of 14 pararetroviruses, Pooggin *et al.*, (1999) showed that the sub genomic RNA are structured and arranged in such a way that translation could be via a ribosome shunt mechanism. In RTBV a leaky scanning mechanism is used to express several ORFs from sub genomic RNA (Fütterer *et al.*, 1997).

The process used by Pararetroviruses, whereby DNA is synthesized through RT, is similar to that of retrotransposons. Retrotransposons are ubiquitous residents of eukaryotic genomes and are found in the genomes of plants species. Long terminal repeat (LTR) retrotransposons are members of the retroelement family, which also includes other retroviruses (Fig 2.2). LTR retrotransposons usually encode for all of the proteins required in their transposition process, including a capsid (Gag), protease, integrase, reverse transcriptase and RNase H. Most of the active plant retrotransposons that have been characterized to date, display weak activity during normal plant development. However, an increase in transcription can be induced by biotic and/or abiotic stresses, including *in vitro* propagation (*via* tissue culture)

(Hirochika *et al.*, 1996), drought stress (Kalendar *et al.*, 2000) wounding (Kimura *et al.*, 2001) and pathogen infection (Wessler *et al.*, 1995).

As with retrotransposons, certain stress factors may be involved in the activation of endogenous BSV sequences. Thus far, research has proven that hybridization of genes (Lheureux *et al.*, 2003) and tissue culture (Dahal *et al.*, 2000) causes the activation of endogenous BSV sequences in the nuclear genome of certain *Musa* species, giving rise to chronic episomal BSV.

2.2.5 Relationships based on serology and nucleotide sequences

Genomic heterogeneity seems to be featured with many badnaviruses. Serologically and genomically isolates of BSV are very diverse (Geering *et al.*, 2000; Geering and Thomas, 2002, Harper *et al.* 2005). Various variants of CCSV also exist and well known strains include Bisa, Bosumtwe, Ikiri, Kpeve, Mampong, New Juaben, Nsaba and Offa Igbo (Brunt *et al.*, 1996). Numerous strains of SCBV (Braithwaite *et al.*, 1997) and SRV (Lockhart and Olszewski, 1996) also exist.

Serologically BSV is related to SCBV (Lockhart and Autrey, 1988) while one strain of BSV reacted with an antiserum prepared against PYMV during ISEM tests (Lockhart *et al.*, 1997). PYMV reacted with antiserum prepared against SCBV but not against antiserum prepared against BSV. SRV is serologically closely related to SCBV, CSSV and RTBV (Lockhart and Olszewski, 1996).

Phylogenetic analysis of the complete sequences of specific badnaviruses revealed that SCBV is closely related to BSV-OL (Harper and Hull, 1998). CoYMV, DABV and CSSV grouped together, while RTBV grouped separately. In another study BSV-OL, SCBV, CoYMV and PYMV grouped together in a tree formation while RTBV grouped with other viruses (Lockhart *et al.*, 1997).

2.3. BSV A PARARETROVIRUS

2.3.1 Pararetroviruses

Traditionally virologists believed that only certain animal viruses could integrate themselves into a host's genome. Recently, certain plant pararetroviruses, belonging to the class

Retroelementopsida (Hansen and Heslop-Harrison, 2004) (Fig 2.2), were found as integrated sequences in the host plant's genome. Thus far, integration into the host genome has been reported only with plant viruses with DNA-genomes or those that have a DNA phase in their replication. The best described are BSV, infecting *Musa* species and causing banana leaf streak disease (banana endogenous viruses [BEV's]), Petunia vein clearing petuvirus (PVCV), infecting petunia (Richert-Pöggeler and Shepherd, 1997), Tobacco vein clearing cavemovirus (TVCV) which infects tobacco (Lockhart *et al.,* 2000) and RTBV, infecting rice (Kunii *et al.*, 2004).

2.3.2 Integration of BSV into the host genome

The precise mechanism by which BSV sequence integration into the host plant's genome occurs still needs to be discovered. One theory is that integration occurs by illegitimate recombination (Ndowora *et al.*, 1999). Some species of retroviruses make use of an enzyme called integrase to insert itself into the host genome. A possible integrase motif was found in ORF III of BSV, but the sequence exists at an unlikely position in the ORF when compared to that of other retroviruses. Integration into the host genome is not an essential step for BSV replication, nor that of other pararetroviruses. Lheureux *et al.* (2003) recently identified a locus on the *M. balbisiana* (BB) genome that is associated with BSV infection; it is called the BSV express locus (BEL). Evidence also suggests that different endogenous BSV have different BEL loci. According to Lockhart *et al*. (1998) and Harper *et al.* (2002), all *Musa* spp. examined so far, harbor some form of BSV sequence in their DNA. Activation of the virus, to cause episomal infection, seems to be by certain stresses on the plant and, in the case of BSV, one of the main activation triggers was shown to be vegetative propagation of *Musa* material by tissue culture (Dallot *et al.*, 2001; Ndowora *et al.*, 1999; Harper *et al.*, 1999b).

Ndowora *et al.* (1999) found two forms of integrated sequences of BSV in the *Musa*-genome through screening the genomic library of ObinoL'Ewai (*Musa* AAB group) with a probe originally designed from BSV infected (episomal BSV) tissue cultured plants originating from the ObinoL'Ewai parent. *In situ* hybridization studies with probes specifically designed from regions on the BSV genome, done by Harper *et al.* (1999b), confirmed two BSV related locations on ObinoL'Ewai chromosomes.

Of the two forms of BEV sequences, one integrant can produce active episomal infection and the other not. The BEV form, which can be activated, consists of multiple copies of BSV sequences, closely related to that of episomally expressed BSV (Harper *et al*., 1999b; Ndowora *et al.*, 1999). These activatable BEV sequences can thus be referred to as pathogenic because of their ability to cause banana leaf streak disease. In the case of ObinoL'Ewai, prediction models indicated that the genomic material of episomal BSV in propagated tissue culture progeny would be highly similar to the BEV sequences of BSV found within the motherplants genome (99.7 % identical) (Ndowora *et al.*, 1999). Most BEV sequences, which can be activated, occur in the genome of *M. balbisina* (B genome) (Geering *et al*., 2001a; Geering *et al*., 2005b), while the other integrated form, which may not be able to produce a functional viral transcript, is associated with that of *M. acuminata* (A-genome). Some *Musa* with A genes contain partial sequences of BSV with scrambled regions, which are not homologous to pathogenic episomal BSV and are not likely to become activated (Ndowora *et al*., 1999; Harper *et al*., 2002). Geering *et al.*, (2001a) demonstrated this when analyzing the genomes of *Musa* with AAA, AB and BB and finding that only those with the B genes seemed to contain the activatable BEV sequences. Recently evidence of BEV sequences integrated in *Musa* with A-genomes, which could be activated, was discovered (Geering, personal communication). Lockhart *et al.* (1998) remarked that *Musa* genotypes such as Red (AA) and Pisang lilin (AA) also contained endogenous sequences of BSV that may be expressible. Using the BSV degenerate primes (Badna 1A and Badna 4, Geering *et al*., 2005b), Geering *et al.*, (2005b) prepared, cloned and sequenced a total of 103 DNA fragments from the genomes of *M. acuminata*, *M. balbisiana*, M. *schizocarpa* (S genome), ObinoL'Ewai and Klue Tiparot (BAB). By constructing a cladeogram, the different BEV's was shown to group into distinct clades of A-genome and B genome types of BEV's.

Plants with B-genes in their genomes, with no episomal virus particles in the cells, undergoing vegetative multiplication by tissue culture, were shown to produce progeny containing systematic episomal BSV (Dallot *et al.*, 2001; Ndowora *et al.*, 1999; Harper *et al.*, 1999b). This phenomenon has never been observed with Cavendish (A-genome) cultivars that are regularly multiplied by tissue culture. Rather, progeny of tetraploid crosses seems prone to become BSV infected, once propagated by tissue culture.

As mentioned, the appearance of episomal BSV from BEV sequences was thought to occur because of homologous recombination. Two mechanisms were proposed, using the genome of ObinoL'Ewai, having both A and B genes in its genome. Firstly sequences flanking the sides of the scrambled BSV sequences would recombine, thus deleting the scrambled sequences in the centre. Recombination by direct repeats at the ends of the integrant DNA could produce a circular BSV genome that could be transcribed. Another mechanism may involve transcription of the integrated BSV sequences to produce RNA that serves as mRNA or a template to produce the viral genome (Ndowora *et al.*, 1999, Harper *et al.*, 2002). Recently genetic recombination was shown to definitely play a role in breeding tetraploid Musa hybrids (Lheureux *et al.*, 2003) with the discovery of the BEL locus on the genome of *M. balbisiana*. Researchers performed hybridization experiments by crossing episomal BSV free AAA and BB genome *Musa.* Fifty percent of the resulting progeny was positive for episomal BSV after a two-year period. The genetic mechanism of recombination may therefore be responsible in triggering BSV activation, because of the fact that an inhibiting factor (gene silencing) present with the homologous BB genome (containing BEV), is absent in recombined genes such as AAB (Lheureux *et al.*, 2003).

2.4 ISOLATES OF BSV

2.4.1 Isolate variations of BSV

In literature, the BSV-OL strain of BSV (originating from cultivar TMPx4698, a tetraploid hybrid of ObinoL'Ewai x Calcutta 4) isolated at the International Institute of Agriculture (IITA) in Onnè, Nigeria is the best described. The whole BSV-OL genome of 7389 bp has been cloned and sequenced (Harper and Hull, 1998). As previously mentioned, the genome of BSV contains 3 ORFs. Clones of a region in ORFIII and the intergenic region of the genome of BSV in cultivars from Australia (Red Dacca, BSV-RD (*Musa* AAA group), Williams, BSV-Cav (*Musa* AAA group, Cavendish subgroup), Mysore, BSV-Mys (*Musa* AAB group, Mysore subgroup and Goldfinger, BSV-GF (*Musa* AAAB group) were sequenced and compared with that of BSV-OL. BSV-RD and BSV-OL were very similar, while BSV-Cav, -Mys and -GF were different from BSV-OL in nucleotide sequence (Geering *et al.*, 2000). A Rwandan isolate of BSV was shown to be distinctly different from other BSV isolates form Mysore and Cavendish cultivars (Geering and Thomas, 2002).

Examining PCR clones from BSV isolates causing an epidemic on mainly A-genome cultivars in Uganda indicated that a vast number of different isolates of BSV were present in these plants (Harper *et al*., 2004, Harper *et al.*, 2005). IRFA 909, 910 and 914, developed by CIRAD, displayed symptoms in the areas of Australia where plants were being evaluated. Virus from the IRFA 910 clone was purified and the genome sequenced. Sequence comparison with other BSV isolates indicated the presence of a new species of BSV, designated BSV-IM (Imove). Sequence similarity between ORF I, ORFII and ORFIII of BSV-OL and BSV-IM was 60.5, 42.3 and 64.3 % respectively (Geering *et al*., 2001b). The latest genome that was completely sequenced and characterized is that of BSV-Mys (from the cultivar Mysore, AAB), it was shown to be quite distinct from BSV-OL (from cultivar Obino L'Ewai) and the species name, Banana Streak Mysore Virus (BSVMysV) was proposed (Geering *et al.,* 2005b).

2.4.2 Identified integrated isolates

Strong evidence exists that BSV-IM arose from integrated sequences. This was shown by hybridization patterns with BSV-IM, using restriction enzymes on the DNA of the two diploid parents of the IRFA hybrids (Geering *et al*., 2001b). Geering *et al*. (2001, 2005b) also sequenced the whole genome of BSV-Mys. Hybridization patterns, using restriction enzymes showed the integration of BSV-Mys into the genomes of various other *Musa*. Therefore BSV-OL does not seem to be the only strain arising from integrated sequences in the *Musa* genome (Ndowora *et al.*, 1999; Harper *et al.*, 1999b). In Australia, researchers are finding diverse integrants of BSV (Geering *et al.*, 2005a). It therefore seems that there may be a vast number of BEV awaiting discovery and characterization.

2.5 DETECTION OF BSV

2.5.1 Symptoms and experimental host range

BSV symptoms were first described by Lassoudiere (1974) on Cavendish from the Ivory Coast, while the causal agent was identified in Morocco (Lockhart, 1985). Viral leaf streak symptoms include broken or continuous streaks on the leaf lamina, which may vary from yellow, chlorotic, black or brown. Lighter symptoms include feint broken chlorotic lines or eyespots, while more severe symptoms consist of necrosis of various parts of the plant, causing plant death.

Due to the fact that the symptoms of BSV may sometimes be very similar to that of *Cucumber mosaic cucumovirus* (CMV) (Lockhart and Olszewski, 1993), symptom observation and identification is not a reliable enough identification method. Expression of symptoms can also vary with temperature changes and plants may become asymptomatic with certain changes in temperature (Dahal *et al*., 1998, Dahal *et al.*, 2000). BSV is restricted to certain *Musa* and *Ensete* hosts. Attempts to transmit BSV to other varieties in the family such as *Musa textilis*, *Heliconia* and *Strelitzia* were unsuccessful (Lockhart, 1986). Therefore, no specified indicator hosts exist for BSV.

2.5.2 Immunoelectron microscopy (ISEM)

ISEM tests on partially purified virus preparations are currently recommended by INIBAP for screening of *Musa* material for BSV (Diekmann and Putter, 1996). This technique involves the trapping of virus-specific antibodies on carbon coated or formvar grids after which the sample is added. A second layer of antibodies is then added. Virus-specific antibodies will then 'recognize' and bind to the specific virus. Bound antibodies are then observed as a dense area around the particle when examined under the microscope; this is referred to as decoration of the particle. Lockhart (1986) successfully detected BSV in ISEM tests where antiserum prepared in rabbits was used. ISEM is a very sensitive detection method. Some disadvantages of the technique are the requirement of a trained eye and the need for an expensive electron microscope; furthermore the preparation of partly purified extracts of BSV can be laborious. This technique is not ideal for the processing of a large volume of samples, as for example, in diagnostic purposes. Antiserum specific to the virus is also needed for this technique and currently limited stocks are available worldwide.

2.5.3 Enzyme linked immunosorbent assay (ELISA)

An ELISA system, the double antibody sandwich ELISA (DAS-ELISA), was first described for plant virus detection by Clark and Adams (1977). An antibody specific to the virus in question is bound to a solid phase surface. After an incubation period, the sample, possibly infected with virus, is added for 'trapping' or binding with the antibody. After incubation a second virus-specific antibody, which is conjugated with an enzyme, is added. Reaction is measured by conversion of a specific substrate by the enzyme. In the triple antibody sandwich enzyme immunoassay (TAS-ELISA) the virus-specific antibody (first antibody) to

be bound to the solid phase and the virus specific-antibody (second antibody), used for detecting virus, is prepared in different animal species. A third detection antibody is then added to detect only the second antibody. This third antibody is enzyme-conjugated IgG and specific to the animal species in which the second antibody was prepared (van Regenmortel and Burckard, 1980). Many other variations to ELISA are also routinely used in plant virus diagnostics (Barbara and Clark, 1982; Edwards and Cooper, 1985).

Isolates of SCBV, which are serologically closely related to BSV, were used by Ndowora and Lockhart (2000) to prepare a broad-spectrum polyclonal antiserum against BSV. Their seroreagents were developed against 32 SCBV-isolates from sugarcane cultivars, which represented a wide diversity of the virus (Ndowora, 1998). The BSV-Mys isolate, which is not related to SCBV, was purified separately and added to the SCBV isolates for the production of antibodies. Antiserum was prepared from chicken and rabbits. The resulting antiserum was able to detect three of five serologically unrelated BSV isolates with DAS-ELISA using the rabbit antiserum. However when testing the samples with ISEM all BSV samples tested for, had decorated bacilliform particles. For TAS-ELISA, coating was done with the rabbit IgG and virus detection with the chicken IgG. An enzyme conjugated rabbit anti-chicken antibody was used as the third detection antibody. Using this ELISA protocol, all the samples that tested positive with ISEM also were positive in ELISA, showing that TAS-ELISA was 'more sensitive' in the detection of a wider range of BSV isolates (Ndoworra and Lockhart, 2000). Thottappilly *et al*. (1998) also showed TAS-ELISA with polyclonal antibodies to detect more strains of BSV, with less steric hindrance. Agindotan *et al.* (2003), prepared polyclonal as well as monoclonal antibodies against purified virus from TMPx 7002-1 from IITA, Onnè, Nigeria that were able to detect BSV in *Musa* clones from IITA. In Chapter 4 of this thesis, the development of a TAS-ELISA system, which is able to detect a range of BSV isolates, is described.

The following variables can complicate ELISA detection of BSV:

1) The incidence of diverse strains of BSV (Lockhart and Olszewski, 1994)
2) Limited stocks of antiserum (world-wide)
3) Some antisera were only produced against a single strain of BSV e.g. those for BSV-OL by Thotappilly *et al*. (1998)

4) Uneven distribution of the virus within the *Musa* plant and temperature influences (Dahal *et al*., 1998). At high temperatures (28-35°C) BSV titre could drop to below detection limits for ISEM and TAS-ELISA tests.
5) It is also recommended to use symptomatic leaf material for BSV detection.

2.5.4 Polymerase chain reaction (PCR)

PCR is a specific and sensitive method that can be used in the detection of most viruses. Based on sequence data of the specific virus, small oligonucleotide primers (approximately 20 base pairs in length) are designed, that will 'bind' (anneal) to specific areas of the viral genome, at a specific annealing temperature. No antibodies are needed, making this a useful test when viruses are difficult to purify or if the virus is not very immunogenic. The PCR reaction is initiated by using the designed primer pair, which matches the portion of the DNA or RNA sequence to be copied. PCR is enzymatically driven, with each cycle of the reaction producing copies of the sequence flanked by the two primers. Primers can bind to the newly formed copies as well as the original sequence, resulting in the total number of copies increasing exponentially, with time (Old and Primrose, 1994). The end product is usually viewed on an agarose electrophoresis gel (stained with a chemical such as ethidium bromide or cyber green) or is quantitatively measured (fluorogenic reaction). Primers are often designed to target conserved areas on the genome of different virus strains/isolates of the same species/genus/family, to ensure that diverse strains/isolates can be detected. Targeting genomic areas that differ between isolates is useful when PCR for specific strains are developed. Various PCR detection systems, using different primers against BSV, have been developed (Lockhart and Olszewski, 1993; Harper *et al*., 1999a; Geering *et al*., 2000; Delanoy *et al*., 2003). PCR directly on crude extracts of BSV in plant sap or on purified DNA have been used to detect BSV, but has the disadvantage of detecting both integrated sequences and episomal viral particles in the *Musa*-genome. Harper *et al.* (1999a) utilized immunocapture (IC)-PCR, based on using BSV specific-antibodies to trap the episomal virus particles before performing PCR. Detection of only episomal BSV is ensured when applying this technique. Very useful primers against specific strains from Australia have been developed (Geering *et al*., 2000). Most other PCR tests are based on primers designed for the sequence of BSV-OL (Harper and Hull, 1998). Universal badnavirus primers were developed by Lockhart and Olszewski (1993) as well as Geering *et al.*, (2005b), these are especially

useful for the detection of uncharacterized strains of BSV. A real time PCR assay was also developed by Delanoy *et al*. (2003) where the sequences of BSV-OL, BSV-GF, BSV-Mys and BSV-RD were aligned and the most conserved regions selected for use in the development of the primers. When these specific primers are used in PCR, only episomal BSV is detected and not integrated sequences of the virus. Unfortunately no strains other than BSV-OL were tested in their study. The detection system in this PCR does not require gel electrophoresis as a small fluorescent probe is used to quantify the amount on amplified DNA within the PCR-tube (from there the designation "real-time"). This technique is useful when large quantities of samples needs analyzing, as for instance in diagnostic laboratories.

When seeking a method for BSV detection it is necessary to take the integration of BSV into the host genome, as well as strain diversity, into consideration. In order to ensure the widest range of probabilities in detection, antiserum used in IC-PCR must be polyclonal and prepared against the widest range of strains as possible.

2.6 EFFECTS OF BSV INFECTION ON *MUSA*

According to Lockhart (1995) three aspects makes BSV infection of *Musa* significant:

- "*Its effect on plant growth and fruit yield and quality"*
- *"It is a hindrance to germplasm exchange"*
- *"The need for certification of in vitro plantlets for international trade"*

Although BSV occurs in many countries, its effects on banana production differs: for example in Australia, the disease does not seem to have a major impact on banana production (Daniells *et al.,* 2001), whereas it was described as a factor limiting banana production in Uganda. Harper *et al.* (2004) attributed the decline in yields in various banana-growing areas of Uganda to BSV infection. Severe outbreaks was first reported in 1996 by Tushemereirwe *et al*. (1996) while farmers claim that they have been observing the disease from around 1986 in their plants. Evidence of the vector spreading the disease in Uganda was also recorded by Kubiriba *et al.* (2001a). The first time Banana streak disease was recorded, it was on a Cavendish subgroup (AAA) in the Ivory Coast (Lassoudière, 1974), showing severe symptoms and yield losses. Yield losses of 7-90 % due to BSV have been described (Lassoudiere, 1974; Dahal *et al.*, 2000; Daniells *et al.*, 2001). However, production losses

and disease symptoms seems to be influenced by various factors such as virus isolate, *Musa* genotype, crop management, vector presence and temperature conditions.

Because of strict guidelines in the exchange of germplasm (Diekmann and Putter, 1996), BSV infected germplasm may not be distributed. Most B genome containing tetraploids that have been developed in various breeding programmes (Table 2.1) show a tendency to eventually succumb to episomal BSV infection. The process of tissue culture, serves as a catalyst for BSV activation (Dallot *et al.*, 2001; Ndowora *et al.*, 1999; Harper *et al.*, 1999b). This makes it virtually impossible for tissue culture laboratories to vegetatively multiply, sell or export any of these cultivars.

CHAPTER 3

3. DETERMINING BSV INCIDENCE ON TISSUE CULTURE DERIVED NON-CAVENDISH MUSA IN MPUMALANGA

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3.1 INTRODUCTION AND MOTIVATION

Banana streak badnavirus (BSV), causing banana steak disease (BSD) occurs in most banana producing countries, including South Africa (Jones and Lockhart, 1993). The virus can occur in two main forms within the host, firstly integrated into the host genome (endogenous BSV) and secondly an episomal form (episomal BSV). Episomal infection may result due to A) the integrated form of the virus being expressed within the plant, (Ndowora *et al*., 1999), or B) introduction to the plant *via* insect vectors (Lockhart and Olszewski, 1993; Kubiriba *et al.,* 2001b; Geering *et al.,* 2005b). Only episomal BSV causes BSD. Environmental factors, such as temperature, pathogen and host, can affect BSD symptom expression (Dahal *et al*., 1998; Dahal *et al.,* 2000). Plants that are positive for episomal BSV may be asymptomatic (Lockhart, 1995; Dahal *et al*., 2000) while the most common symptoms described are yellow or chlorotic streaks on the leaf lamina, running from the leaf midrib to leaf edges as continuous or discontinues streaks. Dahal *et al.* (2000) showed that BSV could have a significant effect on bunch weight and number of fingers. Results indicated that loss of bunch weight varied from insignificant to 82 % loss, depending on the cultivar. Similarly, Daniells *et al*., (2001) showed that a 6 % loss in yield occurred in Williams (AAA), which was episomally infected with a specific strain of BSV (BSV-Cav), while fingers were 5 % shorter in length compared to that of uninfected fingers.

Recently, BSV was recorded in the Tzaneen area on 18 month old *Musa* plants of the cultivars FHIA 25 (AAAB), Goldfinger ([FHIA 01], AAAB) and High Noon (AAAB) (Meyer and Pietersen, unpublished results). Plant material for these cultivars were imported from the germplasm collection in Belgium (INIBAP) and FHIA, as virus free tissue culture (initiated meristem). Upon arrival, the material was multiplied by tissue culture and indexed for BSV three months after planting. Indexing was performed on samples of the three month old plants at the Virus indexing Centre (VIC) of the ARC-Plant Protection Institute (PPRI) in Pretoria and no BSV was detected (Robinson, personal communication). Dallot *et al*. (2001) and Ndowora *et al*. (1999) reported that BSV negative plants, undergoing vegetative

multiplication by tissue culture, could become positive when the integrated form is present in the motherplant's genome. This is especially true for tetraploid *Musa* hybrids (Ndowora *et al*., 1999; Dallot *et al.*, 2001; Lheureux, *et al.*, 2003), with a B-component in their genome (Geering *et al*., 2001a; Geering *et al*., 2005b). These findings could explain the occurrence of episomal BSV that was detected in the FHIA 25, Goldfinger and High Noon tetraploids from Tzaneen. BSV integrants occurring in Cavendish (AAA) varieties, such as Williams, seems to be inactive sequences (Geering *et al.*, 2001a). To date, tissue culture activation of endogenous BSV form Cavendish varieties have never been observed. However integrated BSV activation was also shown some cultivars, such as Red (AAA) and Pisang lilin (AA), after propagation by tissue culture (Lockhart *et al.*, 1998).

Experimental transmission of the virus was demonstrated with mealybugs (Lockhart and Olszewski, 1993; Kubiriba *et al.,* 2001b; Geering *et al.,* 2005b). The possibility of BSV infection occurring as a result of vector transmission cannot be ruled out in the above-mentioned Tzaneen material. It is however highly unlikely, as no mealybug species were reported to occur within the plantation. Additionally, no external sources of BSV existed within the plantation itself as no BSV was recorded in any of the other cultivars established within close proximity (1.5-3.0 meters), or further away from of the BSD infected plants (personal observation). Other *Musa* plantations (Cavendish varieties) are established several hundred meters away from this plantation. No research has been conducted on the distances that vectors may cover in order to transmit BSV. No evidence of plant-to-plant spread was obtained in a study where uninfected Williams and Williams infected with BSV-Cav (a strain of BSV originating from Cavendish) were planted in close proximity (1.5 x 1.7m) to each other in the same plantation (Daniells *et al.*, 2001). Some scientists argue that the mealybug vector is slow moving and reason that infection will only occur over short distances in close proximities of infected plants. Immature mealybugs are however fast moving and very efficient in transmitting *badnaviruses* (Lockhart, 1995). Furthermore, ants, which are often associated with mealybug colonies, may assist in the spread of the vector in and between plants or plantations. Other badnaviruses, such as Cacao swollen shoot virus, has been show to spread by mealybugs which were dispersed by wind (Ollennu, 2000).

Although commercial plantations in South Africa mainly consist of Cavendish varieties, other plantations have been established with tetraploid *Musa* varieties. Many of these tetraploid cultivars were imported into South Africa as virus free germplasm. Some plantations, established with tetraploids and other exotic *Musa* cultivars, have been indexed for BSV after their establishment. However, BSD development is not always immediate (probably because of a latent phase in plant growth) and may occur only as the plants become older. Furthermore, there is a lack of information and guidelines in this regard to growers. This is mainly due to the fact that the episomal activation of BSV, causing BSD in *Musa* material propagated by tissue culture, has only been reported in the last few years (Dallot *et al.*, 2001, Ndowora *et al.*, 1999) while tetraploid cultivars, resistant to Panama disease, has only been planted in South Africa within the last decade.

Panama disease (caused by *Fusarium oxysporum* f. sp. c*ubense*) has become an increasing threat to almost all the banana-cultivating areas of South Africa (Viljoen, 2002). *Musa* tetraploid varieties, showing tolerance or resistance to diseases such as Panama, have been developed and could serve as potential replacements in areas where Cavendish (very susceptible to Panama Disease) have been destroyed by Panama (Escalant *et al*, 2002). There is an increased demand from overseas markets for tissue culture plants of tetraploid varieties. However, many of these display BSD after *in vitro* multiplication, making it impossible for tissue culture laboratories to export or market such *in vitro* propagated tetraploids.

The risk of viral spread from potential BSV infected cultivars (harbouring episomal BSV as a result of integrated BSV activation) to commercial Cavendish varieties required investigation as some of the mealybug vectors, which were shown to transmit BSV, to occur in South Africa. Currently, limited knowledge exists about the epidemiology of BSD in South Africa and especially the possible role of the vector in the spread of this disease. Therefore, a study, as described in the rest of this Chapter, was conducted in plantations established in the Kiepersol area of Mpumalanga, South Africa, in order to determine the incidence of BSV. Vector transmission studies are reported in Chapter 6 of this thesis.

3.2 MATERIALS AND METHODS

3.2.1. Geographical area

The sub-tropical area of Kiepersol in the Mpumalanga province of South Africa is covered with large *Musa* plantations. Because of its subtropical climate, lower planting densities are used in this area (less than 2000 plants/ha). Such planting conditions provide the correct microclimatic conditions optimal for growth and yields (Robinson, 1993).

Various tetraploid varieties of *Musa* are established in the Kiepersol area and are always in close proximity to Cavendish plantations. Alternative cultivars, resistant to Panama disease, are needed in this area as Panama disease already has a negative impact on many of the susceptible Cavendish plantations established in this area.

The majority of *Musa* with the B-component incorporated in their genome harbours activatable integrated BSV (Geering *et al.*, 2001); therefore A-genome cultivars (Cavendish and FHIA 17) were sampled from plantations established with 'mixed' cultivars (A and B genome *Musa*). These A-genome samples were taken for two purposes (emphasis indicated in bold):

1. To act as tissue-cultured controls with no episomal BSV, in contrast with **tissue cultured** cultivars with activated episomal BSV (B genome *Musa*).
2. To indicate the presence of possible **vector transmitted episomal BSV** from B- to A-genome cultivars or selections.

In total five sites with 'mixed' A and B genome cultivars were sampled. At other sites only a single cultivar in a plantation was sampled in order to determine the BSV status of the cultivar/plantation. Random sampling was done at all sites.

3.2.2 Sampling

Approximately 15cm^2 of leaf lamina material (at the base of the youngest or second youngest leaf) were taken from each plant in the field. For nursery plants, a part of the leaf, or whole leaf of each plant was taken as a sample. Samples consisted of individual plants, which were pooled together in batches of four to fifteen (see Tables 3.1-3.7), keeping specific cultivar-plantation combinations together. A total of 33 pooled samples were taken from 19 sites at seven different locations.

At Burgershall Research station (Location 1, Appendix 2), a large number of different *Musa* cultivars have been established in order to conserve germplasm as well as to evaluate cultivars and selections for commercial cultivation. A total amount of amount of 17 samples, sampled from seven different sites (Sites A-G) could be taken from this location. Many of the plantation blocks were located next to each other. In Appendix 2 planting distances between plants are shown. Cultivars sampled from this site include FHIA 17 (AAAA) and FHIA 02 (AAAB) imported from FHIA, Honduras, Kamaramasenge (AAAB) a cultivar originating from single cells that was regenerated at Leuven, Belgium, Pisang Mas (AA), Cultivar Rose (AA) imported from INIBAP, Williams (AAA), Bluggoe (ABB), Pelipita (AAABB), Ducasse (ABB), FHIA 23 (AAAB), FHIA 25 (AAAB), Lady Finger (AAB), Red King (AAA), FHIA 01 (AAAB), High Noon (AAAB) and FHIA 18 (AAAB).

Other locations sampled were mainly from commercial plantations established on the farms of growers in this area. Location 2 (DANROC) belongs to a grower that has Panama disease on his farm and therefore alternative cultivars are being planted and evaluated at this location. Five sites (Sites H-L) were sampled (Appendix 2). Cultivars sampled include Pisang awak (ABB) a selection made from plants growing in a local rural area, PKZ (AAAB), High Noon (AAAB) plants that were planted next an older plantation of Williams (AAA) (also sampled), FHIA 25 (AAAB), FHIA 17 (AAAA), FHIA 18 (AAAB) and FHIA 01 (AAAB). At Site I, where PKZ was sampled, IRFA selections (AAAB) IRFA 909 and IRFA 910 was removed from the block because of BSV detection (October 1999, unpublished results). At Sites J and K plants were planted in a tramline formation, with a 2 meter and 6 meter tramline between the Site J and K respectively.

At an existing Cultivar trail (Site M), located on the farm of Mr. Jan Prinsloo (Location 3, Appendix 2), High Noon (AAAB) and Williams (AAA) that occurs in a mixed plantation was sampled (Fig 3.1). A 4m tramline exists between each two rows.

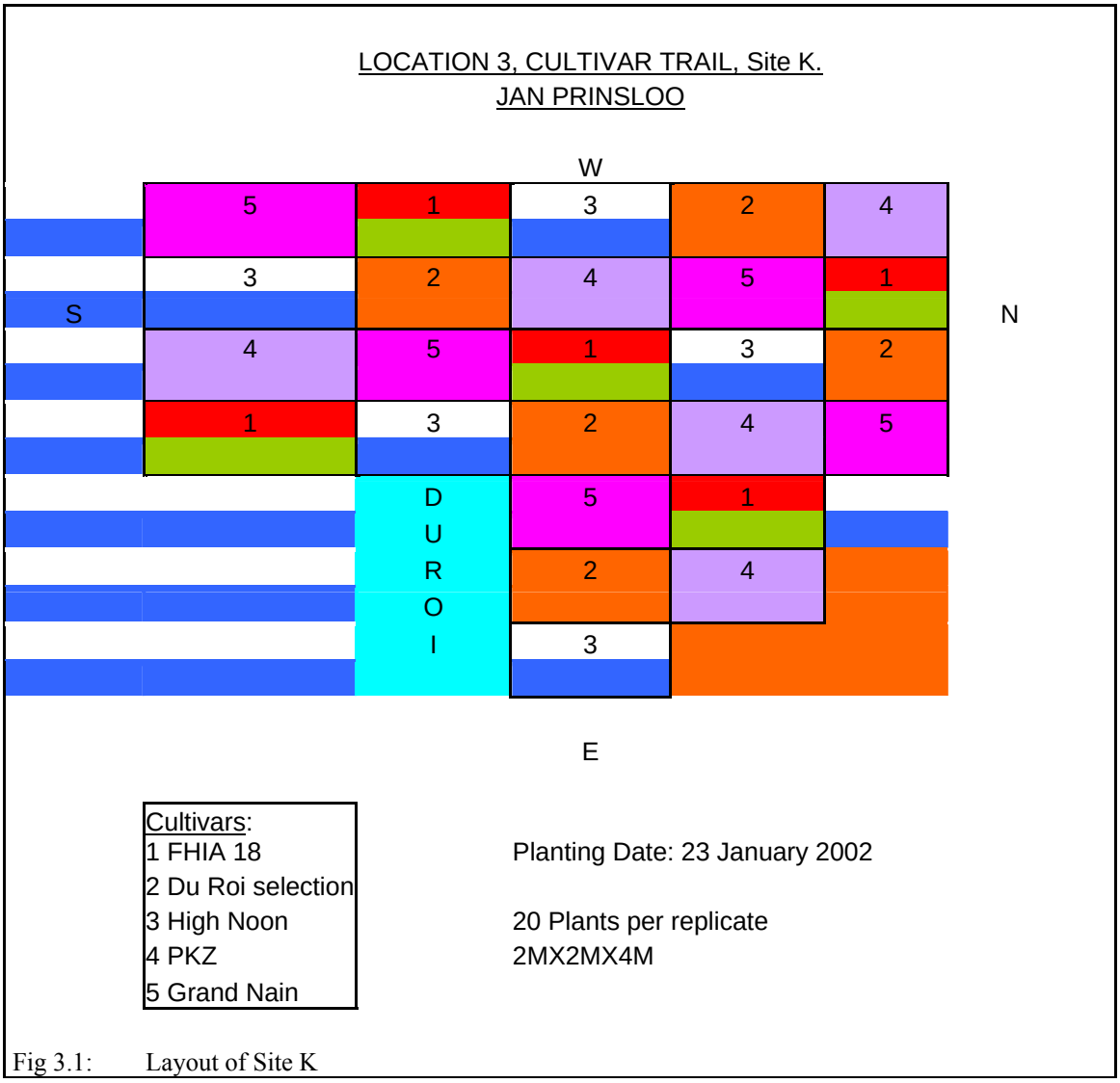

Fig 3.1: Layout of Site K

At Locations 4 and 5 single hectare plantations of FHIA 01 (AAAB) and High Noon (AAAB) were established. At Location 4 the plantations was surrounded by blocks planted with Cavendish and Avocado cultivars. Because of the large size of the plantations, fifteen plants were sampled by walking in and X formation across the plantation (Fig 3.2). Appendix 2 indicates the age and planting distances of the sites sampled (Sites N and O) at Location 4. While Appendix 2 indicates the sites (Sites P and Q) sampled at Location 5.

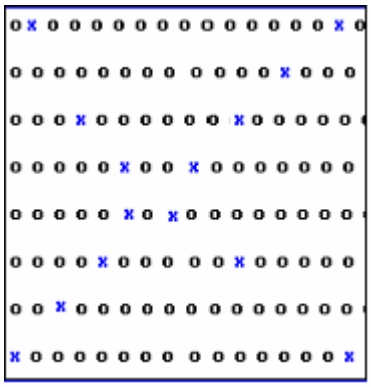

Fig 3.2: Schematic representation of a cross section mode of sampling in large plantations. Positions at which samples were taken are indicated by an 'x'. The figure is not to scale and data points may vary slightly between plantations.

Location 6 (Appendix 2) represents a farm on which the Cultivar PKZ (AAAB, a local selection from FHIA 01) was first discovered. The site (Site R) was planted with a statistical layout in order to evaluate different selections. Site S, sampled at Location 7 (Appendix 2) was also established with a statistical layout in order to evaluate different cultivar selections. Here each plot in a replicate consisted of 20 plants planted in two rows (ten plants per row), 1.8 meters apart from each other.

3.2.3 IC-PCR and ISEM detection of BSV

Approximately 1cm^2 of material was cut from each leaf in each separate sample. The material was finely ground in BSV extraction buffer, using a mortar and pestle. A sprinkling of carborundum powder was added to the mortar during the maceration process. The ratio of sample to buffer was 1:10 (w/v). Detection of BSV was conducted with immuno-capture reverse transcription polymerase chain reaction (IC-PCR). The primers and protocols for the detection of BSV-OL, BSV-RD and BSV-GF were used as described by Harper *et al.*, (1988) and Geering *et al.*, (2000), [Annexure B, sections B.1.2 & B.1.3]. A known positive (99/2722) and negative (94/1609) control [Annexure E, section E.1.1] was included. Resulting PCR-amplicons were loaded on a 1 % agarose gel, in 1x TAE buffer and submitted to a current of 100V for 30 min for evaluation [Annexure E, section E.1.3].

Only samples of cultivar PKZ were examined with ISEM. Purified IgG specific to BSV, (Ndowora, 1998; kindly provided by B.E.L. Lockhart), were diluted 1:1500 in 10 mM Tris-HCL, pH 7.4. Drops of diluted antiserum (10µl) were placed on a slab of dental wax in a moist Petri dish chamber. In each following step, where a new reagent was added, the wax

slab with reagents was incubated in a moist Petri dish chamber. Nickel EM grids (300 mesh), coated with necollodion plastic film, were carbon-coated and floated on the drops of antisera and incubated for 60 min at 37°C. After washing the grids with 20 drops of 0.1M phosphate buffer (pH 7.4), they were placed on 20µl of partially purified virus (miniprep) drops and incubated 16h at 4°C. Grids were rinsed with 20 drops of 0.1M phosphate buffer (pH 7.4) and 20 drops of double distilled water followed by negative staining. Virus was stained by dropping five drops of 2 % uranyl acetate over the grid surface and briefly draining excess fluid on filter paper. Electron microscope examination (using an ABT 002A electron microscope) was done at 10 000 to 20 000 times magnification (80 Kv) and ten random lens-fields were counted for each grid.

3.3 RESULTS AND DISCUSSION

In this study, the incidence of episomal BSV, in the Kiepersol area of Mpumalanga was determined by sampling locations where 'mixed' cultivars (B genome) together with Cavendish varieties, have been established. Some sites, established with only one variety, were also sampled. Results are summarized in Tables 3.8 and 3.9.

Table 3.1, represents the results of five sites, established with A- and B genome *Musa* occurring in close proximity (1.5 to 6 meters apart) to each other. Episomal BSV-RD, BSV-GF, of both of these strains, were detected in varieties with a B-component in their genome, at four of the five sites sampled (Table 3.1). Despite being in close proximity to episomal BSV sources, no BSV was detected in any of the A-genome cultivars (FHIA 17 [AAA], Grand Nain [AAA], Williams [AAA]) sampled from Sites A, J, K and M. These findings suggests that vector mediated spread from episomally infected cultivars to AAA or AAAA cultivars has not occured at sites A, J, K or M.

Episomal BSV was detected in five of the six plantations (Sites A, B, D, E, G) sampled at Location 1 (Table 3.2). At Site C, only established with Cavendish (AAA) varieties, no BSV was detected. Despite being surrounded with plantations with episomal BSV, no BSV could be found at Site C. No episomal BSV could be detected in the samples taken at the Burgershall nursery (Site F). At the time of sampling, these plants were only six months old

and several factors, including plant age and temperature, may have influenced BSV expression. With age, these plants might still test positive for episomal BSV.

Episomal BSV was detected in B genome cultivars at all the sites (J, K, H, I and L) sampled at Location 2 (Table 3.2). This was also true for samples taken at Location 3 (Site M), Location 4 (Site N and O), Location 5 (Site P) and Location 6 (Site R). Among the strains of BSV tested for, BSV-RD occurred the most frequently, followed by BSV-GF (Fig 3.3) and lastly BSV-OL. Overall, twenty out of the thirty-three samples tested, reacted with one or more of the BSV primer-sets. These include samples from cultivars FHIA 25, FHIA 18, FHIA 01, FHIA 02, PKZ, High Noon, Kamaramasenge, Bluggoe, Pelipita and Pisang awak.

More than one BSV strain was detected in the cultivars High Noon, (Sites K, M, N and P), Pisang awak (Sites D and H) and FHIA 18 (Sites J and G). BSV-OL, BSV-RD and BSV-GF was detected in Pisang awak at both sites sampled, while differences occurred between the episomal forms of BSV found in the High Noon samples from different sites. In the sample of High Noon from Location 1, (Site G) only BSV-GF was detected, while BSV-RD was detected in High Noon at four of the five sites and the BSV-OL strain only once. Episomal BSV-GF was detected in High Noon at all five sites sampled. Strain differences also occurred between the FHIA 18; BSV-RD was detected at both sites, while BSV-OL was additionally detected at Site G.

The differences in BSV-strain combinations found for High Noon and FHIA 18 is difficult to explain. One can only speculate that strain differences may be as a result of different unknown and environmental factors that may have influenced the activation of endogenous BSV sequences. Although the plantation at Site G has existed for four and a half years, only BSV-GF was detected in the High Noon plants sampled. Being tissue cultured plants; one would expect BSV activation of all integrated strains to have occurred. However no explicit research has been done for cultivars with multiple species of BSV integrants, while a lot of research has focused on only BSV-OL, or specific cultivars such as Obino L'Ewai x Calcutta 4 (Ndowora, 1998) or only in identifying integrated sequences (Geering *et al.*, 2005a). Dahal *et al.*, (1998; 2000) showed that temperature might influence the detection of BSV and that "*BSV titre may drop below the detection limits of both TAS-ELISA and ISEM*". This may also

explain why some strains of BSV were detected or not detected with IC-PCR. The use of symptomatic tissue is recommended for BSV detection (Thottappillay *et al.*, 1998).

No BSV was detected in the PKZ sample taken at Location 7, Site S. BSV-RD was detected in PKZ from Sites I and R at very low intensity (amplicon products obtained from PCR was very weakly displayed on UV illuminated, ethidium bromide stained gel, [Annexure E, section E.1.3]). The presence of BSV in these samples was confirmed with ISEM. BSV was recently detected in High Noon, Goldfinger and FHIA 25 from Tzaneen. PKZ was tested together with these cultivars; however, no episomal BSV was detected in this cultivar. Virologists speculate that some endogenous viral sequences may have a tendency of having a lower activation rate for integrated sequences (Geering, personal communication). Although not reported, one may speculate that the host species may also influence BSV replication that could the explain the very faint PCR reactions, combined with low particle presence (roughly determined by ISEM by comparing samples to episomal BSV infected control samples), which is constantly observed for PKZ samples.

No BSV symptoms were observed at any of the locations on the plants or at the sites that were sampled. Indeed BSV symptoms are rarely, if ever, observed under South African climatic conditions. It has been reported that foliar symptom expression of BSD may be sporadic, while symptom expression is usually altogether absent in plants derived from *in vitro* multiplication (Jones and Lockhart, 1993; Lockhart, 1995). From the recently discovered episomal BSV infected plants that was located in Tzaneen, a High Noon, Goldfinger and FHIA 25 sucker (with no visible symptoms), were relocated to an insect proof tunnel at PPRI to keep as reference. The tunnel provided a constant cooler temperature (24-28°C) than that of the environment from which the plant was removed, causing the High Noon plant to develop BSD symptoms (Fig 3.4). The Goldfinger and FHIA 25 plants remained symptomless.

Table 3.1: Occurrence of BSV in plantations established with AAA/AAAA and AAAB genome cultivars.

Location	Site	Cultivar	Genome	Age (Years)	Distance	Episomal BSV
1	A	FHIA 17	AAAA	7.4	2.5 x 2	None
		FHIA 02	AAAB	7.4		BSV-GF
2	J	FHIA 17	AAAA	0.5	1.5 x 1.5 x 2	None
		FHIA 25	AAAB	0.5		BSV-RD
		FHIA 18	AAAB	1		BSV-RD
2	K	WILLIAMS	AAA	23	2 x 2 x 6	None
		HIGH NOON	AAAB	2.3		BSV-GF, BSV-RD
3	M	GRAND NAIN	AAA	1.5	2 x 2 x 4	None
		HIGH NOON	AAAB	1.5		BSV-GF, BSV-RD
7	S	WILLIAMS	AAA	1	2.6 x 1.8	None
		PKZ	AAAB	1		None

Table 3.2: Occurrence of BSV strains in different *Musa* cultivars in plantations established in the Kiepersol area of Mpumalanga.

Location	Site	Cultivar	Genome	Age (Years)	Distance	Episomal BSV[A]
1	B	Kamaramasenge	AB	3.5	3 x 1.75	*BSV-RD*
		Pisang Mas	AA			None
		Cultivar Rose	AA			None
1	C	Williams	AAA	3.6	3 x 1.75	None
1	D	Bluggoe	ABB	7.4	2.5 x 2	*BSV-OL*
		Pelipita	ABB			*BSV-RD*
		Pisang awak	ABB			**BSV-OL**, *BSV-GF, BSV-RD*
1	E	FHIA 01	AAAB	7.4	2.7 x 1.69	**BSV-GF**
1	F	FHIA 23	AAAB	0.6	n/a	None
		FHIA 25	AAAB			None
		Lady Finger	AAAB			None
		Red King	AAA			None
1	G	High Noon	AAAB	4.5	2.5 x 2	**BSV-GF**
		FHIA 18	AAAB			**BSV-RD**, BSV-OL
2	H	Pisang Awak	ABB	3.9	2.64 x 2	*BSV-OL*, **BSV-GF**, *BSV-RD*
2	I	PKZ	AAAB	1.5	2.64 x 2	*BSV-RD*
2	L	FHIA 01	AAAB	8	3.1 x 2	**BSV-GF**
4	N	High Noon	AAAB	4	3.1 x 2	**BSV-OL**, **BSV-GF**, *BSV-RD*
4	O	FHIA 01	AAAB	8	3.1 x 2	*BSV-RD*
5	P	High Noon	AAAB	4	3.1 x 2	**BSV-GF**, BSV-RD
5	Q	FHIA 01	AAAB	8	3.1 x 2	*BSV-RD*
6	R	PKZ (AAAB)	AAAB	3	3 x 1.75	*BSV-RD*

[A] = Italics indicate BSV strains for which a very weak amplicon product was obtained.

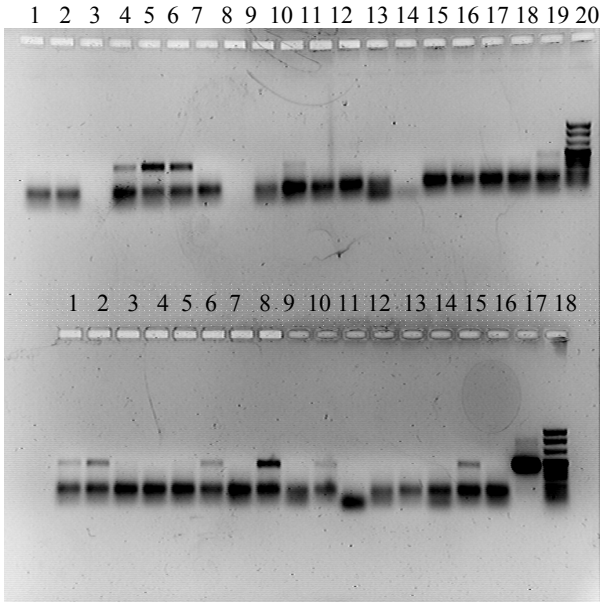

Fig 3.3: Photograph of a 1 % agarose gel, stained with ethidium bromide; a correct sized gel band of 476bp indicates the presence of BS-GF.
Top: Lane 1: Williams, Location (Loc.) 1; Lane 2: FHIA17, Loc. 1; Lane 3: FHIA18, Loc. 1; Lane 4: FHIA01, Loc. 1; Lane 5: FHIA02, Loc. 1; Lane 6: High Noon, Loc. 1; Lane 7: Kamaramasenge, Loc. 1; Lane 8: Bluggoe, Loc. 1; Lane 9: Pelipita, Loc. 1; Lane 10: P. awak, Loc. 1; Lane 11: Pisang mas, Loc. 1; Lane 12: CV Rose, Loc. 1; Lane 13: FHIA 23, Loc. 1; Lane 14: FHIA 25, Loc. 1; Lane 15: Lady Finger, Loc. 1; Lane 16: Red King, Loc. 1; Lane 17: Williams, Loc. 2; Lane 18: PKZ, Loc. 2; Lane 19: High Noon, Loc. 2; Lane 20: Molecular marker VIII.
Bottom Lane 1: P. awak, Loc. 2; Lane 2: FHIA01, Loc. 2; Lane 3: FHIA25, Loc. 2; Lane 4: FHIA17, Loc. 2; Lane 5: FHIA18, Loc. 2; Lane 6: High Noon, Loc. 3; Lane 7: Williams, Loc. 3; Lane 8: High Noon, Loc. 4; Lane 9: FHIA01, Loc. 4; Lane 10: High Noon, Loc. 5; Lane 11: FHIA01, Loc. 5; Lane 12: PKZ, Loc. 5; Lane 13: PKZ, Loc. 6; Lane 14: Williams, Loc. 6; Lane 15: Positive control, Lane 16: Healthy control, Lane 17: DNA amplicon control, Lane 18: Molecular marker VIII.

Fig 3.4: BSD symptoms observed on the leaf lamina of the cultivar High Noon. This plant was symptomless under field conditions. The plant was removed from the field and kept under a constant temperature regime of 24-28°C, under which symptom development took place.

3.5 CONCLUSION

In an attempt to determine the status of BSV in the Kiepersol area of Mpumalanga this study demonstrated the occurrence of episomal BSV in plantations established with non-Cavendish *Musa*. A further aim was to verify whether any spread of BSV has occurred from episomally infected non-Cavendish cultivars to commercially cultivated Cavendish varieties. Results indicated the presence of episomal BSV in non-Cavendish varieties at all sites with the exception of Site C (Cavendish *only* plantation), Site S (mixed plantation) and Site F (Nursery, young plants). Furthermore, episomal infection was limited to only non-Cavendish varieties having a B-chromosome in their genetic makeup. One cultivar selection, PKZ, displayed a potentially low activation rate of integrated BSV, as this BSV was not detected at all sites sampled. Furthermore, little episomal BSV particles were observed in samples from this ultivar with ISEM suggesting a possible suppression of BSV replication within the host.

It has been reported that *Musa* progeny, propagated via tissue culture from BSV-negative motherplants, may become positive for BSV after a period of time (Dallot *et al*., 2001; Nodwora *et al*., 1999). Therefore, the presence of the episomal BSV, in the non-Cavendish varieties tested, is likely as result of tissue culture propagation of the planting material. Other external factors or stresses on the plants may have played a role as well. Retro-elements, taxonomically classified together with BSV, can increase in transcription due to biotic and/or abiotic stresses, including *in vitro* propagation (Hirochika *et al.*, 1996), drought stress (Kalendar *et al.*, 2000) wounding (Kimura *et al.*, 2001) and pathogen infection (Wessler *et al.*, 1995). 'Wounding', by constantly cutting the material desired for multiplication, is part of the tissue culture procedure. As this is not a process that is natural to the plant, one may view this practice as a 'stress' that is induced on the plant material, causing the endogenous pararetroviral sequences of BSV to become activated.

In one study, various BB, AB and AAA cultivars were evaluated in order to determine which integrated sequences of BSV would be able to give rise to episomal BSV (Geering *et al*., 2001a). Their findings suggested that the integrated BSV sequences, which can be activated, were only present on the B-component of *Musa* genomes examined. In this study, none of the AA or AAA-genome cultivars tested positive for episomal BSV, even when the plants

occurred in mixed plantations. This finding suggests that spread from episomally infected plants to the A-genome varieties did not occur.

In Tzaneen, the cultivars that were positive for the BSV-OL and BSV-GF strains were removed from the orchard as a precaution against further spread of the virus. However, no evidence of spread was observed in this location or any of the any of the locations surveyed in this study. Neighboring Cavendish varieties (AAA) were constantly found to be negative for all BSV strains tested for, even if they were in close proximity to episomally infected sources. In fact, BSV was not detected in any of the A-genome varieties evaluated in this study. From a study done on Williams in Australia, (Daniells *et al.*, 2001), no evidence of BSV spread was recorded. However, the researchers did record one instance where BSV-Cav did spread in a nearby commercial plantation. This observation was recorded over a 2-year period in a plantation with 178 plants, in which 5 % of the plants were infected with BSV-Cav. The climatic and environmental factors of South Africa and Australia are highly similar. Based on this fact and the findings of this study, we can conclude that it is unlikely for BSV to cause a serious epidemic in South Africa, if introduced at any given location as integrants..

Still, the phenomenon of BSV being activated from the B genome in *Musa* remains a worldwide problem and no conclusive recommendations in the establishment of these cultivars are, as yet, offered in literature. Tissue culture laboratories still cannot risk the multiplication or selling of cultivars, prone to become infected with episomal BSV. There is a general acceptance by plant quarantine authorities that if a pathogen is present within in a country, it is no longer seen as a quarantine organism on a national level. However, certain quarantine restrictions may occur between areas within a country from which the pathogen may be excluded. Goldfinger, a B genome containing *Musa* cultivar, has been exported from an international breeding programme in Honduras (FHIA), into various countries worldwide, including South Africa and Australia (Frison and Sharrock, 1998). The BSV integrant, from which the primers for BSV-GF were developed, occurs in this cultivar. This integrant can be transcribed to form episomal BSV and was detected by IC-PCR in this study. Goldfinger was first introduced into South Africa about eight years ago and in spite of harboring expressible-integrated BSV, it was still identified as a cultivar with commercial potential. No serious BSD symptoms have ever been reported on Goldfinger and after its introduction into

Australia in the late 1990's, Goldfinger has become a commercially cultivated cultivar. This cultivar is a good replacement for Cavendish in areas affected by Panama disease, as it was shown to be tolerant to the pathogen *Fusarium oxysporum* f.sp. *cubense* causing this disease (Smith *et al*. 1998).

One of the drawbacks in the detection of BSV is that a variety of integrants with different nucleotide sequences exists. Recently, various new sequence varieties of BSV have been reported from Uganda (Harper *et al.*, 2004, Harper *et al.*, 2005). If one were to accurately control the movement of B genome cultivars between countries, one would have to identify the BSV variants occurring within the recipient country as well as the endogenous BSV sequences within the *Musa* material for export. This would require a lot of research, collaboration and funds.

In the discipline of plant pathology, one should always be aware that a pathogen that might seem harmless at first, which might 'adapt' to, or mutate, to thrive once introduced into a new environment. BSV has however not become a 'major threat' to *Musa* production in countries such as Australia (Geering, personal communication) and South Africa (Pietersen, personal communication). However, BSV infection was concluded to be a main factor in yield losses recorded from *Musa* cultivated in Uganda (Harper *et al.*, 2004). This may however be also because of different plantation management strategies, affecting plant vigor, together with more pathogenic BSV strains. Daniells *et al.* (2001) mentioned that the environmental conditions, in which their Australian trail with BSV-Cav infected Williams were done, were optimal for plant growth and development, thus compensating for the effects that BSD might have had on the plant. These scientists also remarked that stress on the plants may develop with time, causing the effects of BSD to become more pronounced, especially in follow up ratoon crops. Still, mainly Cavendish varieties, propagated from tissue culture, are cultivated in South Africa. This is because of consumer preference for Cavendish fruit, and minimizes the need for B genome containing varieties to become widely established.

In summary: Based on the findings of others and from this study a list of statements were made that may serve as guidelines for necessary recommendations to be made regarding cultivars with integrated and episomal BSV in South Africa (Appendix 1).

CHAPTER 4

4. CHARACTERISATION OF BSV ISOLATES AND THE PRODUCTION OF AN ANTISERUM

CHAPTER 4

4. CHARACTERISATION OF BSV ISOLATES AND THE PRODUCTION OF AN ANTISERUM

4.1 INTRODUCTION

Serological techniques are extremely useful for the identification, quantitative assay and routine diagnosis of plant viruses. Exploitation of the antigenic properties of plant viruses, by injection into an appropriate animal, may induce an immune response, from which desired antibodies can be obtained. Antibodies, specific to the antigen are produced in response to stimulation of the animal's immune response to the antigen and can be found in the blood serum of the immunized animal (van Regenmortel, 1982).

Banana streak badnavirus (BSV) is spread by vegetative propagation (Lockhart, 1995) as are most plant viruses. Reliable detection of BSV is necessary because of the movement of *Musa,* mainly in the form of tissue culture plants, around the globe (Diekmann and Putter, 1996). BSV is moderately immunogenic (Geering and Thomas, 2002) therefore; BSV-specific antibodies can be raised by immunization of suitable animals. Detection methods employing BSV-specific antibodies, include immunosorbent electron microscopy (ISEM) (Lockhart, 1986), enzyme linked immunosorbent assay (ELISA) (Ndowora, 1998; Thottappilly *et al*., 1998; Dahal *et al.*, 2000; Agindotan *et al.*, 2003) and immunocapture (IC)- polymerase chain reaction (PCR) (Harper *et al.,* 1999a). BSV nucleotide sequences have been found integrated into the genome of some *Musa* species, without causing banana streak disease (BSD) (Lockhart *et al*., 1998; Ndowora *et al.*, 1999; Harper *et al*., 2002; Lheureux *et al.*, 2003; Geering *et al*., 2001a; Geering *et al*., 2005b). Some of these integrants of BSV are expressible, causing systemic BSD, while other integrants are inexpressible. The absence of BSD is synonym with the absence of systemic viral particles (episomal BSV). Because BSV occurs in more than one form in nature, including the wild episomal form, detection techniques discriminating between the BSV-forms is required.

BSV-specific antiserum is essential for the detection of the episomally expressed form of the virus. Antisera for use in ISEM and triple antibody sandwich (TAS)- ELISA systems was prepared by Ndowora (1998) using BSV-Mys (Geering *et al.*, 2005b) and 32 isolates of Sugarcane bacilliform badnavirus (SCBV). SCBV has a close serological relationship with

BSV (Lockhart and Autrey, 1988) and polyclonal antiserum prepared against SCBV isolates was shown to detect most BSV-isolates, except BSV from the *Musa* cultivar "Mysore" (BSV-Mys). The antisera prepared by Ndowora (1998) were able to detect a wide range of BSV isolates including BSV-Mys. However, at present, limited amounts of BSV-specific antisera are available worldwide and with the anticipated increase of tests for episomal BSV, stocks are likely to become exhausted soon.

In order to obtain a high quality antiserum (very specific to the virus only), virus particles (for usage in immunization) needs separation from all other foreign matter such as plant proteins and cell-components. Several methods have been reported for the purification of BSV (Lockhart, 1986; Thottappilly *et al*., 1998; Nodwora, 1998; Geering *et al*., 2000; Delanoy *et al*., 2003).

Serological methods, such as ELISA systems, are useful for the screening of large quantities of samples and are especially useful for diagnostic purposes. Monoclonal antibodies, able to detect only single strains or variants of a pathogen, have limited applications. Because of the fact that numerous strains of BSV exists (Geering *et al.* 2000; Geering and Thomas 2002, Harper *et al.*, 2004, Harper *et al.*, 2005), diverse BSV strains need to be utilized in the production of polyclonal antisera, ensuring that a wide range of viral variants could be detected. The aim of the study described in this chapter, was to develop a serologically based detection system, able to detect various serologically different strains of BSV. In order to have a better understanding of the BSV sources to be used for the production of the antiserum, the BSV sources were first characterized by IC-PCR.

4.2 MATERIALS AND METHODS

4.2.1 CHARACTERISATION OF BSV-*MUSA* BY IC-PCR

4.2.1.1 History of BSV-*Musa* sources

The material used in this study (with a few exceptions) are *Musa* that was received from the International Network for the Development of Banana and Plantain (INIBAP) for virus indexing at the ARC-PPRI and in which BSV was detected by ISEM. The germplasm material, received from INIBAP (during 1999-2002) was indexed according to specified guidelines (Diekmann and Putter, 1996). Material that tested positive for BSV (during 1999 and 2002) were kept in an insect proof tunnel at temperature 24-28°C (Pietersen, unpublished

results). *Musa* sources other than those from INIBAP, collected after routine IC-PCR indexing, using the primers and protocols for the detection of BSV as described by Harper *et al.*, (1988) and Geering *et al.*, (2000), were also kept under these conditions (Table 4.2). The material was assigned accession numbers according to the system followed at the ARC-PPRI [Annexure E, section E.1.1]. During June 2003, 25 of these plants were selected as possible BSV sources.

4.2.1.2 Preparation of partly purified virus (miniprep)

In order to estimate the concentration of BSV particles, a partly purified extract (miniprep) of each BSV-source was prepared in 2003 and ISEM performed (Diekmann and Putter, 1996; also see section 4.2.1.3 of this chapter). Simultaneously, the status of other potential viruses, such as Banana mild mosaic virus (BanMMV), in the material was determined. Known healthy and positive controls were included for each round of minipreps. The protocol of (Diekmann and Putter, 1996) were followed with a few slight modifications: 3 to 5 grams of leaf tissue was extracted with 35 ml of 200 mM phosphate buffer, pH 6.0, containing 1 % Na_2SO_3. The extract was pressed trough cheesecloth and centrifuged at 8000 rpm (Beckman; JA 20 rotor) for 10 min. Resulting supernatant was filtered (Whatman, filter-paper no. 1) and mixed with 1 ml of 33 % Triton X-100 made with 200 mM phosphate buffer, pH 6.0. This mixture was layered over 5 ml of 30 % sucrose cushion in 100 mM phosphate buffer, pH 7.2 and centrifuged for 2.5 h at 35 000 rpm in a Beckman Type 50.2 rotor. The supernatant was discarded and the sides of the tube rinsed with distilled water. The pellet was resuspended by overnight shaking with stainless steel beads (2 mm radius), at 4°C in 300 µl of 10 mM phosphate buffer, pH 7.2 containing 0.85 % NaCl. Thereafter the suspension was centrifuged for 8-10 min at 12 000-15000 rpm in standard micro-centrifuge and the partially purified extract retained for ISEM examination.

4.2.1.3 ISEM examination of the BSV-*Musa* sources

Purified IgG's specific to BSV, (Ndowora, 1998; kindly provided by B.E.L. Lockhart), were diluted 1:1500 in 10 mM Tris-HCL, pH 7.4. Similarly crude antiserum specific to BanMMV (kindly provided by J. Thomas, *Ducasse* strain), was diluted 1:1000. Equal amounts of these antisera were mixed and 10µl drops placed on a slab of dental wax in a moist Petri dish chamber. For every step in the ISEM procedure a fresh slab of dental wax was used when a

new reagent was added and all reactions were incubated in a moist Petri dish chamber. Nickel EM grids (300 mesh), coated with necollodion plastic film, were carbon-coated and floated on the drops of antisera and incubated for 60 min at 37°C. After washing the grids with 20 drops of 0.1M phosphate buffer (pH 7.4), they were placed on 20µl of partially purified virus (miniprep) drops and incubated 16h at 4°C. Grids were rinsed with 20 drops of 0.1M phosphate buffer (pH 7.4) and 20 drops of double distilled water, followed by negative staining. Virus was stained by dropping five drops of 2 % uranyl acetate over the grid surface and briefly draining excess fluid on filter paper. Electron microscope examination (using an ABT 002A electron microscope) was done at 10 000 to 20 000 times magnification (80 Kv) and ten random lens-fields were counted for each grid.

4.2.1.4 Immunocapture Polymerase chain reaction (IC-PCR)

Geering *et al*. (2000), developed primers against four BSV isolates (some are now considered as strains, [Geering *et al.* 2005a; 2005b]) of BSV. These isolates originated from specific *Musa* cultivars namely Red Dacca (BSV-RD), Williams (BSV-Cav), Mysore (BSV-Mys) and Goldfinger (BSV-GF). Primers against a Nigerian isolate form a hybrid plantain TMP x 7002-1 (BSV-OL) were also developed (Harper *et al.*, 1998). BSV-IM (from cultivar IRFA 914) seems distinct from other badnavirus species (Geering *et al.*, 2001b). Primers for this isolate and all of the primers (Table 4.1) mentioned above were used to select and characterize the BSV isolates.

Table 4.1: Primers used in the characterization of BSV isolates, indicating the primer sequence and name for each primer.

Specificity of primer	Primer name	Sequence: 5'
Badnavirus degenerate primer	BADNA 1A	CTN TAY GAR TGG YTN GTN ATG CCN TTY
Badnavirus degenerate primer	BADNA 4	TCC AYT TRC ANA YNS CYC CCC ANC C
Banana streak virus IRFA 914 specific	BSV IM F1	TGC CAA CGA ATA CTA CAT CAA C
Banana streak virus IRFA 914 specific	BSV IM R1	CAC CCA GAC TTT TCT TTC TAG C
Banana streak virus Goldfinger specific	BSV GF F1	ACG AAC TAT CAC GAC TTG TTC AAG C
Banana streak virus Goldfinger specific	BSV GF R1	TCG GTG GAA TAG TCC TGA GTC TTC
Banana streak virus Mysore specific	BSV Mys F1	TAA AAG CAC AGC TCA GAA CAA ACC
Banana streak virus Mysore specific	BSV Mys R1	CTC CGT GAT TTC TTC GTG GTC
Banana streak virus Williams, Cavendish	BSV Cav F1	AGG ATT GGA TGT TAA GTT TGA GC
Banana streak virus Williams, Cavendish	BSV Cav R1	ACC AAT AAT GCA AGG GAC GC
Banana streak virus Red Dacca	BSV RD F1	ATC TGA AGG TGT GTT GAT CAA TGC
Banana streak virus Red Dacca	BSV RD R1	GCT CAC TCC GCA TCT TAT CAG TC
Banana streak virus (AP & RT region of Onne')	BSV 5317	AGT CAT TGG GTC AAC CTC TGT CCC
Banana streak virus (AP & RT region of Onne')	BSV 4673	GGA ATG AAA GAG CAG GCC

For IC, BSV specific IgG (Ndowora, 1998, kindly provided by Lockhart) was diluted 1:500 (v/v) with ELISA coating buffer [Annexure A] and 50µl added per 600µl eppendorf tube. The tubes with IgG were incubated overnight at 4°C and rinsed 2 times with PBST buffer [Annexure A] prior to the addition of each sample. Samples consisted of partially purified extracts (minipreps) each diluted with an equal volume of DEPC water of which 25µl was added to the IgG-coated tubes. Tubes were incubated at 37°C for 4 hours and rinsed 3 times with PBST buffer before adding the PCR master mixture. PCR master mixtures were prepared containing specific primer pairs (Table 4.1) and PCR performed as described by the protocols for the detection of BSV-OL, BSV-GF, BSV-RD, BSV-Mys, BSV-Cav and BSV-IM (Harper and Hull, 1988 and Geering *et al.*, 2000) [Annexure B, section B1.2, section B1.3 and B1.4]. Ten microlitres of the amplified products were mixed with loading dye [Annexure A] and loaded on a 1 % agarose gel incorporated with 8 µl of ethidium bromide (5 mg/ml solution) and viewed with a UV transilluminator [Annexure E, section E.1.3].

4.2.1.5 Detection of other viruses

To ensure the presence of *only* BSV in the plant material, specific tests for other banana viruses were performed. *Banana bunchy top babuvirus* (BBTV) was tested for by PCR according to the protocol of Thompson and Dietzgen (1995) [Annexure B, section B.1.6]. For *Banana bract mosaic potyvirus* (BBrMV) virus RT-PCR (Rodoni *et al.*, 1997, modified) was performed on crude nucleic acid extracts of samples (Pappu *et al.*, 1993) [Annexure B,

section B.1.7] and for *Cucumber mosaic cucumovirus* (CMV) immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) (Wylie *et al*., 1993) was performed on the samples [Annexure B, section B.1.5].

4.2.2 BSV PURIFICATION

4.2.2.1 BSV-*Musa* isolates

Twenty of the 25 characterized BSV isolates were selected (see explanation in results under point 4.3.1.1). Leaf material from all 20 isolates was pooled together each time a virus purification was performed (total of six separate purifications).

4.2.2.2 Purification

The purification method as described by Agindotan *et al*. (2003) (with a few minor modifications) was followed to purify BSV. Fresh BSV-infected leaf lamina were cut into pieces (excluding the midribs) and ground to a powder in liquid nitrogen. The powder was blended in 200 mM phosphate buffer, pH 6.0, containing 1 % (w/v) sodium sulphite and 2 % (w/v) polyvinyl pyrrollidone at a ratio of 1:3 (w/v). This was then filtered through two layers of cheesecloth and centrifuged at 12 000 *g* in a JA 14 rotor (Beckman) 20 min, to remove plant material. Polyethyleneglycol *(p-8000)* at 6 % w/v and 1.5 % (w/v) NaCl was added to the filtrate and stirred at 4^0C for 40 min, followed by centrifugation at 12 000 *g* in a JA 14 rotor for 20 min. The pellet was dissolved in 100 mM sodium phosphate buffer, pH 7.2, containing 1.5 % (w/w) sodium chloride. Another 4 % (w/v) of polyethyleneglycol *(p-35 000)* and 1.5 % (w/v) NaCl was added to the dissolved pellet mixture and stirred at 4^0C for 1 h. The mixture was then centrifuged at 12 000 *g* in a JA 14 rotor for 20 min. The pellet was suspended in 100-150 ml 100 mM sodium phosphate buffer, pH 7.2, containing 1.5 % (w/w) sodium chloride, after which Triton-X 100 (0.5 % (v/v)) was added. The suspension was then stirred for 1 h at 4^0C, followed by centrifugation at 12 000 *g* in a JA 14 rotor for 20 min.

The supernatant was layered over a 30 % sucrose cushion (30 g sucrose in 100 ml 100 mM sodium phosphate buffer, pH 7.2, containing 1.5 % (w/w) sodium chloride) and centrifuged at 105 000 *g* for 2 h in a Beckman Type 50.2 rotor. The pellet was resuspended in 1-2 ml of 100 mM sodium phosphate buffer, pH 7.2, containing 1.5 % (w/w) sodium chloride and stirred overnight at 4°C on shaker. An equal volume of cold chloroform was added and the

mixture was stirred for 20 min at 4^0C. After centrifugation at 12 000 *g* for 10 min, the aqueous phase was collected and centrifuged at 12 000 *g* in a JA 20 rotor (Beckman) for 5 min. The supernatant obtained was subjected to a sucrose gradient (10, 20, 30 and 40 % sucrose in 100 mM sodium phosphate buffer, pH 7.2, containing 1.5 % (w/w) sodium chloride) with centrifugation at 82 500 *g* in a SW 41 Beckman rotor for 90 min. An opaque zone, occurring close to the centre of the gradient tube, was collected; this zone was confirmed by EM to contain purified BSV particles. Sucrose gradient purified virus was concentrated into a pellet, which was resuspended in 100 mM sodium phosphate buffer, pH 7.2 at 105 000 *g* for 2 h in a Beckman Type 50.2 rotor.

The concentrated virus was examined using the EM to confirm purity before immunization of the experimental animals. Each step was monitored by EM throughout the purification process and included all supernatants and pellets that were obtained. EM examination were performed as follow: Preparations were individually blotted onto necollodion (BDH microscopical reagents, 36059) covered, carbon-coated electron microscope grids and examined, following negative staining with 2 % sodium phosphotungstate (pH 5 and 6) 2 % uranyl acetate, pH 4.3 and 2 % ammonium molybdate, pH 5.3, using an ABT 002A electron microscope at 80Kv. Grids were monitored for virus-like particles for a minimum of 10 min.

4.2.3 ANTISERUM PRODUCTION AGAINST SELECTED BSV ISOLATES

4.2.3.1 Immunization and bleeding of animals

Two New Zealand White rabbits (Rabbit 210 [R210] and Rabbit 211 [R211]) and two female goats (Goat 1F [G1F] and Goat 2F [G2F]) were selected for immunization. These animals were immunized with freshly purified virus combined with adjuvant at a ratio of 1:1 (v/v). For the first immunization (Immunization 1) the antigen were mixed with Freuds complete adjuvant. At following immunizations (Immunizations 2-4) antigen were mixed with Freuds incomplete adjuvant. Pre-immunization bleeds were also obtained for each experimental animal. After the completion of four repeated immunizations (with four-week intervals between each immunization), bleeds were obtained from the animals on a regular basis. Blood was incubated at 37°C for 1-2 hours to clot and left overnight at 4°C to separate the serum. After separation of the serum from the clot, the serum was centrifuged at 10 000 rpm

(12 000 g) for 10 min. The supernatants were mixed with an equal amount of glycerol, labeled and stored at 4°C.

4.2.4. EVALUATION OF ANTISERA AND DEVELOPMENT OF DETECTION SYSTEMS

4.2.4.1 Examination of antisera with Electron Microscopy

Determination of trapping dilution: In ISEM virus is trapped with antiserum in order to concentrate virus from solution onto EM grids. Bleeds were diluted in 0.1 M Phosphate buffer, pH 7.4 at dilutions of 1/10, 1/100, 1/1000 and 1/10000. The rest of the protocol as described in section 4.2.1.3 of this chapter was followed, with the virus sample being leaf lamina from all 20 BSV isolates macerated in BSV extraction buffer (1:10 w/v).

Determination of decoration dilution: Decoration is used to determine the identity of a virus. This technique also enables one to distinguish between mixed virus species in a sample, with a desired result showing decorated and "naked" (undecorated) particles. The decoration dilution should be high enough to ensure that particle decoration is not too dense. Bleeds were diluted in 0.1M Phosphate buffer, pH 7.4 at dilutions of 1/50, 1/100 and 1/500. Each EM grid (nickel), coated with necollodion plastic film and carbon-coated, was incubated on 20µl of a BSV preparation (leaf lamina from all 20 BSV isolates macerated 1:10 in BSV extraction buffer) on dental wax in a moist Petri dish chamber for overnight at 4°C. Grids was rinsed with 20 drops of 0.1M phosphate buffer (pH 7.4) and placed separately on 10µl droplets of diluted antisera. After incubation at 37°C for 60 min, grids were rinsed with 20 drops of 0.1M phosphate buffer (pH 7.4) and 20 drops of double distilled water followed by negative staining with 2 % uranyl acetate. Electron microscope examination (using an ABT 002A electron microscope) was done at 10 000 to 20 000 times magnification (80 Kv) and ten random lens-fields were counted for each grid.

4.2.4.2 Determination of antigen specificity of antibodies in rabbit serum

In order to determine the antigen (BSV) specific antibody titre of each bleed for the two animal species, an indirect double antibody sandwich (DAS) ELISA also referred to as TAS-ELISA was performed.

To determine the rabbit bleed containing antibodies with the highest specificity to BSV, the following protocol was followed: Maxisorb plates (Nunc) were coated with 100µl of G1F Bleed 2 diluted in ELISA coating buffer at a dilution of 1:1000. G1F Bleed 2 was selected because the antiserum from this bleed showed good trapping and specific decoration of BSV particles in ISEM tests with the protocol described in section 4.2.4.1. Plates were incubated at 30°C for 4 hours and after discarding the contents from the plates, the plates were rinsed 3 times at 3 min intervals, using PBST in a TECAN ELISA multiwasher. This procedure was repeated after incubation of each new addition to the plates, while only the amount of washes was varied as described below.

A twenty-gram sample (comprised by the addition of one gram leaf lamina from each of the twenty BSV isolates) was prepared. The sample was ground to powder in liquid nitrogen using a mortar and pestle and blended with BSV-extraction buffer [Annexure A], to give a ratio of 1:10, sample weight: buffer volume. A BSV negative Grand Nain plant (healthy control) was also prepared separately at the same ratio. In order to standardize samples they were prepared in advance, divided into 30 ml aliquiats and frozen at -80°C in for use in follow up ELISA tests. Samples were thawed on ice and ELISA plates with rabbit antisera were rinsed 5 times at 3 min intervals. Samples were added separately to plate wells according to a planned experimental layout. The plates were incubated overnight at 4°C and washed 3 times at 3 min intervals the following day.

For each rabbit bleed, a two-fold dilution series, ranging from 1:1000 to 1: 256 000, were prepared. The dilutions were added to each well according to the experimental layout, with two replicates per treatment and incubated at 30°C for 4 hours. Thereafter the plates were washed 3 times at 3 min intervals and a goat-anti-rabbit Fc-fragment, conjugated with alkaline phosphatase enzyme (1:15000 dilution in General ELISA extraction buffer [Annexure A]) was added to each well. The plates were incubated at 4°C overnight and washed 5 times at 3 min intervals followed by the addition of ρ-nitrophenyl phosphate (PNP) substrate at 1mg PNP per milliliter of substrate buffer.

Absorbance values were measured 30 min and one hour after substrate reaction using a Labsystem multiscan MS plate reader (Amersham). After preparing a graph using the

average reading from each replicate of each treatment, the specific and non-specific titre of each bleed were determined (see Fig 4.1 for an example).

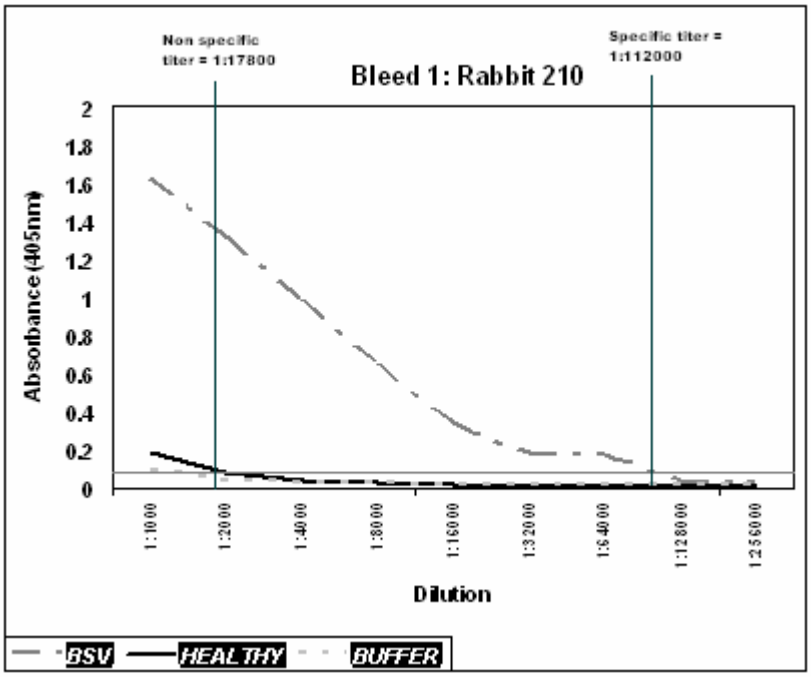

Fig 4.1: Graph of absorbance versus antiserum dilution illustrating the method used to determine specific and non-specific titres of R210 Bleed 1 against BSV infected and healthy plant material. The X-axis represents the dilutions made with R210 Bleed 1 and are plotted against absorbance values determined at each dilution (Y-axis). The dark grey dash-dot line represents the BSV-sample (20 pooled virus isolates), the solid black line represent the virus-free healthy control (Grand Nain) sample, while the buffer value (only buffer) is represented by the light-gray dashed line. The buffer threshold value of 0.1 was determined by the multiplication of the average buffer absorbance value (0.033, for all bleeds of R210 that was evaluated) three times. This threshold is represented by the grey horizontal line. Non-specific titre (1:17800) is then determined from the graph by drawing a vertical line parallel to the Y-axis, at the point (termed: "dilution end point") where the virus free healthy-control curve crosses the threshold line. The Non-specific titre value is then read from the X-axis. The specific titre (1:112000) is determined in a similar manner, at the point where the BSV sample curve crosses the threshold value.

4.2.4.3 Determination of antigen specificity of antibodies in goat serum

The same TAS-ELISA procedure as described in section 4.2.4.2 was followed for each goat serum with modifications: For each goat bleed, a two-fold serial dilution ranging from 1:500 to 1: 128000 (nine dilutions), were prepared. The dilutions were added to each well, as a coating step, according to the experimental layout, with two replicates per treatment and incubated at 30°C for 4 hours. Thereafter the plates were washed 3 times at 3 min intervals, after which a 2 % milk powder solution in PBS [Annexure A] were added to each well (150

µl per well). The plate was incubated at room temperature for 30 min. After washing, 3 times at 3 min intervals the samples were added as described above. The plates were incubated at 4°C overnight and washed 5 times at 3 min intervals followed by the addition of R211 Bleed 4 at a dilution of 1:500. This bleed was selected, as its non-specific dilution was the highest, insuring the capability to detect any non-specific reactions that any of the goat bleeds might have. Following this procedure would ensure that the goat bleed with the highest specific reaction to BSV would be selected, which would then be used in the final optimization ELISA (section 4.2.4.4). The rest of the protocol was followed as described in section 4.2.4.2.

4.2.4.4 Optimization of an indirect TAS-ELISA system

The aim of an optimization ELISA is to determine the final dilutions at which antisera may be used, in order to eliminate non-specific reactions and ensure a very specific reaction to BSV. The best bleed from each experimelnal animal specie was selected based on each bleed's antigen specificity (as determined by the above described procedures). Bleeds that showed a high difference between the BSV-specific absorbance value and non-specific absorbance value, at the first dilution used 1:1000 (rabbit, for example see Fig 4.1) or 1:500 (goat), were selected. Therefore, R210 Bleed 1 and G1F Bleed 2 were selected (see section 4.3.5.1, Table 4.6 for details).

The protocol for the TAS-ELISA described in section 4.2.4.2 was followed with modifications: For each bleed, a logarithmic dilution series was prepared, ranging from 1:1000 to 1:8000. G1F Bleed 2 antiserum was used for initial coating of the plate. Wells were loaded according to an experimental layout, which could allow each goat and rabbit dilution combination, together with different samples, to be tested. The plate was incubated at room temperature for 30 min. After washing, 3 times at 3 min intervals the samples were added. For each dilution combination three wells was replicated for the BSV sample, followed by two replicates for the healthy plant sample and one replicate for the buffer control. After washing of the plate 5 times at 3 min intervals, each dilution of the R210 Bleed 1 was added according to the experimental layout. The rest of the TAS-ELISA protocol as described in section 4.2.4.2 was followed.

4.2.4.6 Detection of individual BSV isolates with the optimized polyclonal TAS ELISA system

In the optimization TAS-ELISA it was determined that 1:8000 was the optimal dilution at which G1F Bleed 2 and R210 Bleed 1 antisera could be used to obtain absorbance values very specific to BSV (see Fig 4.2). TAS-ELISA was performed in order to evaluate the specificity of the antisera diluted at 1:8000, against the separate BSV-isolates initially used to prepare the antisera. One gram of each isolate was macerated separately in 8 ml of BSV-extraction buffer, using a mortar and pestle. The dilution was therefore 1:9. The samples were individually expressed through cheesecloth after which a two-fold dilution series ranging from 1:18 to 1:136864 were prepared for each isolate. A dilution series of an uninfected Grand Nain (94/1609) plant was included as a control. Samples were loaded onto ELISA plates with four replicates for each dilution. A buffer control was included on each plate. Absorbance readings were taken after one-hour substrate reaction time. Unfortunately no plant material was available for BSV-source 99/2773, therefore 25µl of a frozen (-80°C) mini-preparation from this isolate was diluted in 75µl of buffer and loaded to an ELISA plate, no further dilutions could be made for this isolate.

4.2.4.6 Detection of individual BSV isolates with IC-PCR

Antisera (G1F Bleed 2, G2F Bleed 1, R210 Bleed 1), displaying good specific:non-specific response ratios, were each diluted at 1:4000 and 50µl of each added separately to a number of PCR tubes. IC-PCR was performed as described earlier (section 4.2.1.5) with each isolate (diluted 1:9) only being tested with the IC-PCR system with which it tested positive previously (see Table 4.2). Isolates 00/3027, 01/0011 and 01/0017 which did not react with primers used in any of the IC-PCR-systems used previously, were included with the BSV-OL system while 02/2031 was also included in the PCR able to detect BSV-RD. An isolate 99/2775, which tested positive with EM and harbours a BSV-Mys genome-integrant (Pietersen, unpublished), was tested with IC-PCR using the BSV-Mys primer set. For each round of IC-PCR, a healthy Grand Nain (94/1609), a positive BSV control (mixture of all isolates) and a DNA-amplicon of BSV were included.

4.3 RESULTS AND DISCUSSION

4.3.1 CHARACTERISATION OF BSV-*MUSA* BY IC-PCR

4.3.1.1 ISEM detection of BSV

Bacilliform particles, characteristic of BSV, were observed in the positive control as well as in 21 of the 25 purified preparations evaluated with ISEM. No particles were observed in the healthy control. Variable amounts of BSV particles were observed in the different samples, with some particle counts being as low as 2-4 particles over all ten lens-fields. In samples 01/0011 and 01/0017 little BSV particles were observed. Isolates 99/2724, 99/0172, 00/3021 and 02/2004 had the highest concentration of decorated bacilliform particles per field (Table 4.2). The differences in particle counts may serve as an indication of the variability in particle concentration that exists between isolates of BSV from the various *Musa* hosts. It may be that the cytoplasmic conditions within the cells of certain *Musa* hosts are more favourable for BSV multiplication. Furthermore, isolates may vary in pathogenicity. Some of the BSV-sources tested, displayed severe streaking symptoms, while others only showed very faint chlorotic flecking or no symptoms at all (Table 4.9). It has been reported that *Musa* leaf tissues with low disease symptoms generally contain low virus titres (Lockhart, 1986; Dahal *et al.*, 1998; Daniells *et al.*, 2001). It was decided to include only isolates, containing bacilliform particles, in the study. Therefore, only twenty out of the twenty-five isolates tested were selected for antiserum production. An additional isolate (99/2775), which tested positive for BSV-Mys, using PCR without immunocapture, was included into the immunization schedule only once.

4.3.1.2 Detection of other viruses

No viruses other than BSV were detected with the different banana virus-specific PCR techniques. No flexuous-rod viral particles (Banana mild mosaic virus (BanMMV)) or any other virus particles, indicative of other banana viruses, were observed during ISEM examination in any of the samples tested. Furthermore, no CMV, BBTV or BBrMV was detected with specific primers against these viruses using PCR. Therefore the BSV sources were considered free from BBTV, CMV, BanMMV and BBrMV.

4.3.1.3 IC-PCR characterization of BSV

This study aimed to prepare antisera against a broad spectrum of BSV strains. Therefore IC-PCR was used in order to determine the strain-properties of the selected BSV sources. The IC-PCR results on the twenty BSV sources are shown in Table 4.2. Tests contained a known positive, negative and buffer control that responded as expected, confirming the validity of the tests performed. With primers specific to the RD and OL strains of BSV, nine and eight samples were amplified respectively. Geering *et al*., (2000) found BSV-RD and BSV-OL to be very closely related; however in three of the samples (99/2729, 900/3021 and 02/2031) BSV-RD was not detected simultaneously with BSV-OL. This indicates that the two variants may occur in different levels within the various *Musa* hosts or that some sequence variation may occur. IC-PCR with BSV-GF specific primers revealed this strain to be present in nine of the twenty isolates. With one exception, BSV-GF was always detected together with one or more other BSV strain types. Using the forward and reverse primers for BSV-IM (BSV IM F1, BSV IM R1), an amplicon of the correct size (just above 400 base pairs) was obtained for all the "IRFA" type isolates (99/0160, 99/0167, 99/0168 and 99.0169) while BSV-IM was not detected in any of the remaining BSV isolates. No amplicons were obtained for any of the isolates after IC-PCR with primers BSV Mys F1 and BSV Mys R1, were performed. An isolate that was not originally part of the twenty selected isolates, 99/2775, tested positive for BSV-Mys with PCR (without an immunocapture step). Unfortunately because of a lack of plant material, 99/2775 was only introduced once into the immunization schedule. BSV-Cav was only detected in the known positive plasmid control (courtesy of Dr. John Thomas) while this strain was not detected in any of the BSV isolates tested. This was unfortunate, as the aim of the study was to include as many BSV isolates and strains as possible. However, Geering (personal communication), found this strain to be very restricted in its occurrence as it was only found in the source from which it was originally detected and from which the primers (BSV Cav F1 and BSV Cav R1) were designed. Most of the integrated sequences of BSV found in A-genome cultivars (such as Cavendish varieties) have been found to be inexpressible (Geering *et al*., 2001a). Furthermore, vector spread of this strain seems to be almost none existent, as shown with the evaluation of Cavendish infected with BSV-Cav under natural conditions in Australia (Daniells *et al*., 2001).

In four isolates (99/2773, 00/3027, 01/0011 and 01/0017) no amplicons were obtained with any of the six PCR systems. These isolates were included for antiserum production as was speculated that the strain specific BSV primers, used in IC-PCR, could not detect these specific isolates.

Table 4.2: Summary of BSV-isolates characterized, showing IC-PCR and ISEM results obtained using Prof. Lockhart's antiserum.

BSV ISOLATES				ISEM	PCR RESULTS[y]					
	PPRI Accession	ITC Code	Accession name	[x]Particles present	BSV-IM ~400 bp	BSV-MYS 589 bp	BSV-RD 522 bp	BSV-GF 476 bp	BSV-CAV ~800 bp	BSV-OL 644 bp
1	99/2724	0041	Didiedi	+++	N	N	P	P	N	P
2	99/2726	0586	Horn Plantain	+	N	N	N	P	N	N
3	99/2729	0075	Gabon 4	+	N	N	N	N	N	P
4	99/2735	0209	Bise Egome 2	++	N	N	P	P	N	P
5	99/2736	0235	Obubit Ukom	+	N	N	N	P	N	N
6	99/2764	0667	Teeb Kum	++	N	N	P	P	N	P
7	99/2773	1431	Acuminata	+	N	N	N	N	N	N
8	99/0172	1332	FHIA 21	+++	N	N	P	P	N	P
9	99/0169		IRFA 910	+	P	N	N	N	N	N
10	99/0168		IRFA 910	+	P	N	N	N	N	N
11	99/0167		IRFA 910	+	P	N	N	P	N	N
12	99/0160		IRFA 909	+	P	N	N	P	N	N
13	00/3050	1440	CRBP 100	++	N	N	P	P	N	P
14	00/3027	1439	CRBP 14	+	N	N	N	N	N	N
15	00/3021	0495	Osakro	+++	N	N	P	N	N	N
16	01/0011	0817	Angari	+	N	N	N	N	N	N
17	01/0017	0808	Buka	+	N	N	N	N	N	N
18	02/2004	0492	Nyiretia Apantu	+++	N	N	P	N	N	P
19	02/2031	1232	Bagatow	+	N	N	N	N	N	P
20	02/2032	0808	Kahur	+	N	N	P	N	N	P

X = Intensity of particles observed by trapping with antisera in ISEM examination + = few particles, ++ = fair amount of particles, +++ = many particles. Y = Positive (P) or negative (N) result with specific PCR system the base pair size of amplicons are also indicated below each PCR system.

4.3.2 BSV PURIFICATION

4.3.2.1 Purification and observations with EM

Virus purification, based on a chloroform-extraction protocol developed by Agindotan *et al.*, 2003, were done on six occasions of which two purifications were experimental in order to asses, develop and modify the technique (Table 4.3). EM monitoring of each step in the purification process showed a gradual increase of particles in the recovered pellet/supernatant following each progressive step. In some instances some BSV particles were present in either the supernatant or pellet that was to be discarded (as prescribed in the protocol). After chloroform extraction, some debris was still present in the preparation as determined by EM. 'Purified' BSV, obtained after chloroform extraction, was utilized by Agindotan *et al*. (2003) to prepare BSV-specific polyclonal and monoclonal antibodies against BSV-OL. Although no further purification steps, such as gradient purification were performed in the preparation of purified BSV by Agindotan *et al*. (2003), these antibodies obtained, performed well in different ELISA tests. In all other previously described purifications of BSV from *Musa* a CsCl or Cs_2SO_4 gradient was used to obtain purified virus. In another protocol Agindotan *et al*. (2003) noticed that using a Cs_2SO_4 gradient decreased the amount of BSV particles dramatically (15 fold). In a purification procedure for *Cacao swollen shoot badnavirus*, Lot *et al*. (1991) included a sucrose gradient step. Therefore, after chloroform extraction, a sucrose gradient step was included for BSV purification. Instead of yielding a sharp opaque band (indicating the viral zone), viral particles were found scattered throughout the gradient after centrifugation. Scattering throughout the gradient is likely the result of BSV particles not being similar in length. Fortunately three distinct opaque zones were identified within the sucrose gradient upon illumination. Big debris did however separate to an upper zone along with lesser BSV particles, while a second zone (located beneath the upper zone) contained some finer debris and BSV particles. The lowest opaque zones (located near the middle of the tube) yielded the purest BSV preparations and were collected for immunization purposes. Final preparations appeared to contain a fairly pure preparation of BSV particles as observed with EM. Absorbance readings were taken with a spectrophotometer at 260 and 280 nm and the viral concentration (*c*) determined with $c = A_{260}/7$ (mg/ml). An average concentration of 0.02 mg/ml for the final concentrated virus was obtained when starting with 600 g of leaf lamina. Therefore: Yield = *c* (mg/ml) x volume (µl)/ 600 g. EM examination also revealed that 2 % uranyl acetate, pH 4.3 to be more effective for particle examination in comparison

to preparations made by using 2 % ammonium molybdate or and 2 % sodium phosphotungstate. More intact particles were obtained for samples stained with 2 % uranyl acetate with less background.

Table 4.3: Dates of BSV purifications done for experimental and immunization purposes.

Purification schedule	
Experimental	For Immunization
28/07/2003	19/08/2003
27/10/2003	16/09/2003
	14/10/2003
	11/11/2003

4.3.4 ANTISERUM PRODUCTION AGAINST SELECTED BSV ISOLATES

4.3.4.1 Immunization and bleeding of animals

Each experimental animal was immunized four times with the antigen, with the exception of Goat 1F which received three immunizations. The immunization schedule followed is shown in Table 4.4. Five post-immunization bleeds were taken from each of the two rabbits while three and four post-immunization bleeds were done on the two goats, respectively. All animals were also bled pre-immunization (Table 4.5).

Table 4.4: Immunization schedule followed for each experimental animal.

Animal	Dates			
	Immunization 1[A]	Immunization 2	Immunization 3	Immunization 4
Rabbit 210	21/08/2003	18/09/2003	16/10/2003	13/11/2003
Rabbit 211	21/08/2003	18/09/2003	16/10/2003	13/11/2003
Goat 1F	22/08/2003	18/09/2003	17/11/2003	Not done
Goat 2F	22/08/2003	18/09/2003	17/10/2003	17/11/2003

A: Freuds incomplete adjuvant was used with immunization 1 while Freuds incomplete adjuvant was used during immunizations 2-4.

Table 4.5: Bleeding schedule followed for each experimental animal.

Rabbits	
Rabbit 210	**Rabbit 211**
Date: Pre Immunization bleeds	
Bleed 0: 18/08/2003	Bleed 0: 18/08/2003
Date: Immunized Bleeds	
Bleed 1: 26/11/2003	Bleed 1: 26/11/2003
Bleed 2: 09/12/2003	Bleed 2: 09/12/2003
Bleed 3: 09/01/2004	Bleed 3: 09/01/2004
Bleed 4: 21/01/2004	Bleed 4: 21/01/2004
Bleed 5: 28/01/2004	Bleed 5: 28/01/2004
Goats	
Goat 1F	**Goat 2F**
Date: Pre Immunization bleeds	
Bleed 0: 22/08/2003	Bleed 0: 22/08/2003
Date: Immunized Bleeds	
Bleed 1: 17/11/2003	Bleed 1: 17/10/2003
Bleed 2: 16/01/2004	Bleed 2: 17/11/2003
Bleed 3: 05/02/2004	Bleed 3: 16/01/2004
	Bleed 4: 05/02/2004

4.3.5 EVALUATION OF ANTISERA AND DEVELOPMENT OF DETECTION SYSTEMS

4.3.5.1 Antigen specificity of the different bleeds

The values of BSV-specific and non-specific titres of each bleed are presented in Table 4.6 (rabbit) and Table 4.7 (goat). The dilution end point (buffer baseline threshold) was selected by calculating the average of all buffer readings for each bleed and thereafter arbitrary multiplying it three times. Based on the TAS-ELISA results, the best rabbit bleed was R210 Bleed 1 with a specific titre dilution of 1:112000 and a non-specific titre of 1:1780. While the best goat bleeds were 1F Bleed 2 and 2F Bleed 3 with specific-titre dilutions of 1:128000 and 1:600000. Agindotan (2003) obtained a 1:256000 titre from mice antiserum after 1 hour of substrate incubation in their TAS-ELISA system. Although BSV is described as moderately immunogenic (Geering and Thomas, 2002), the antibody titres obtained from the rabbit and goat immunizations were quite high. Unfortunately with some bleeds there was a moderate non-specific reaction to the healthy plant sample. However the two selected bleeds, R210 Bleed 1 and G1F Bleed 2, performed very well when used at a dilution of 1:8000 in the newly developed TAS-ELISA system (Fig 4.2). Overall, a good ratio between BSV/Healthy was constantly obtained at dilutions ranging from 1:2000 to 1:8000 for each bleed. The

results are presented in Fig 4.2. A dilution of 1:8000 for each bleed was sufficient to eliminate any non-specific reaction and retain a high ratio for BSV/Healthy. Very specific reactions against BSV were obtained when compared to the healthy and buffer controls. For optimal results a dilution of 1:8000 is therefore recommended.

A trapping dilution of 1/10000 (v/v) was shown as sufficient to concentrate particles on EM grids for ISEM examination. The best dilution for decoration of the BSV particles for ISEM was at a dilution of 1/50 (v/v).

Table 4.6: Absorbance values for specific and non-specific antibody dilutions from rabbit serum immunized with BSV.

Rabbit(R) and Bleed number	**Buffer Threshold**	**BSV-non-specific Titre dilution**	**BSV-specific Titre dilution**
R210 – Bleed 1	0.100	1:1780	1:112000
R210 – Bleed 2	0.093	1:2000	1:56000
R210 – Bleed 3	0.099	1:12000	1:128000
R210 – Bleed 4	0.088	1:12000	1:96000
R210 – Bleed 5	0.088	1:13000	1:100000
R211 – Bleed 1	0.083	1:7000	1:257000
R211 – Bleed 2	0.094	1:10000	1:318000
R211 – Bleed 3	0.092	1:16000	1:257000
R211 – Bleed 4	0.092	1:32000	1:435000
R211 – Bleed 5	0.083	1:16000	1:258000

Table 4.7: Absorbance values for specific and non-specific antibody dilutions from goat serum immunized with BSV.

Goat (1F/2F) and Bleed number	**Buffer Threshold**	**BSV- non-specific Titre dilution**	**BSV-specific Titre dilution**
1F – Bleed 1	0.247	1:7000	1:13000
1F – Bleed 2	0.243	1:14000	1:128000
1F – Bleed 3	0.172	1:13000	1:96000
2F – Bleed 1	0.137	1:11000	1:85000
2F – Bleed 2	0.158	1:64000	1:512000
2F – Bleed 3	0.148	1:32000	1:600000
2F – Bleed 4	0.194	1:16000	1:320000

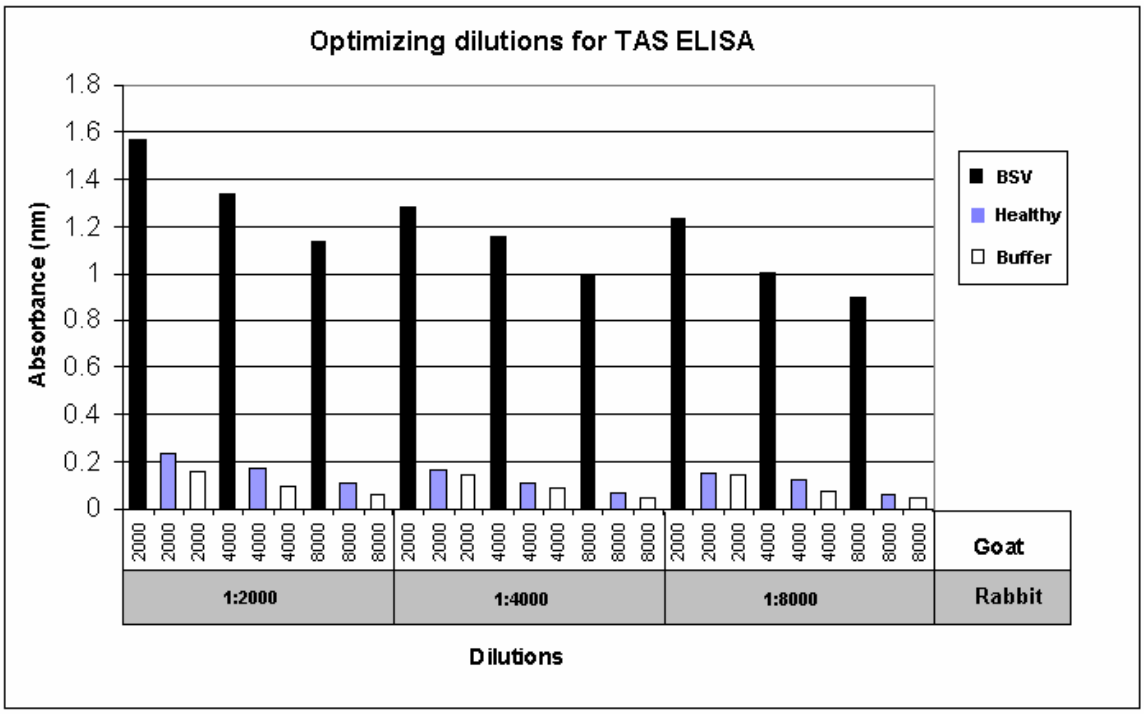

Fig 4.2: Graph of absorbance values obtained in TAS-ELISA with different dilutions for G1F Bleed 2 (goat, diluted 1:2000 to 1:8000) and R210 Bleed 1 (rabbit, diluted 1:2000 to 1:8000). Comparison of the absorbance values between each dilution combination, allows one to determine the dilution at which the highest ratio between specific and non-specific reactions can be obtained. For each dilution a BSV (black bar), virus-free healthy control (grey bar) and buffer control sample (clear bar) was tested. At a dilution of 1:8000 for both bleeds, negligibly low non-specific reactions were obtained with the healthy and buffer samples, making this dilution ideal for routine TAS-ELISA tests. A substrate reaction time of 1 h was used.

4.3.5.2 Testing of individual BSV isolates (TAS-ELISA and IC-PCR)

Each BSV isolate that has been used for immunization was tested individually with TAS-ELISA and IC-PCR in order to evaluate antibody specificity. Absorbance values (OD 405nm) obtained and IC-PCR results for each isolate, is summarised in Table 4.8. A short description of symptoms (Fig 4.3) is also included in Table 4.8. In TAS-ELISA samples with values that were more than two times that of the healthy control (0.042 x 2 = 0.084) were considered to be positive for BSV. Three of twenty-one BSV-isolates used for immunisation, could not be detected with the newly developed TAS-ELISA. BSV could readily be detected in the remainder of the isolates at sample dilutions of up to 1:288. All bleeds that were evaluated (G1F Bleed 2, G2F Bleed 1, R210 Bleed 1) gave the same results. Two isolates that previously tested negative with IC-PCR could be detected in the TAS-ELISA; isolate 99/2773 had an absorbance value of 0.800 while isolate 00/3027 had a value of 0.771. An extra isolate, 99/2775 that was included into the immunization schedule only once, could not be detected with the TAS-ELISA. Isolates 01/0011 and 01/0017 were also undetectable with

TAS-ELISA. Throughout the duration of this study, these plants displayed little or no symptoms (only occasional feint chlorotic flecking) on the leaf lamina. *Musa* tissues with low disease symptoms generally contain low virus titres (Lockhart, 1986; Dahal *et al.*, 1998; Daniells *et al.*, 2001) and could explain the difficulty to detect these isolates with TAS-ELISA. It is also possible that, limited amounts of virus particles from these two sources could have been introduced upon immunization. Virus purification was performed on a pooled sample representing sources with variable (from high to low) concentrations of virus. Usually, small amounts of virus are lost during the process of purifying virus particles. With initial ISEM examinations, very few BSV-like particles were observed in isolates 01/0017 and 01/0011. Geering and Thomas (2002) reported BSV to be moderately immunogenic and it may be that some isolates, being less immunogenic, caused a milder immunogenic response in the experimental animals which were immunized. Additionally, (for particles present at lower concentrations in the purified virus mixture during immunization), a lower immune response would be expected. Particles present at lower concentrations (with weak immunogenic properties) would have to compete with particles of strains that were present at overwhelmingly higher concentrations.

IC-PCR results complimented the TAS-ELISA findings. However, all isolates could be detected with IC-PCR, as it is a more sensitive detection method. Unfortunately isolate 99/2773 could not be examined by IC-PCR because of a shortage of fresh plant material. Additionally isolates 01/0011 and 01/0017, that tested negative with the newly developed TAS-ELISA and previously not detected with IC-PCR (using the antisera of Prof. Lockhart at 1:100) were detected with IC-PCR (IC = Goat 1F Bleed 2, diluted 1:4000) using the primers for BSV-OL. This re-enforces the idea that these two isolates may have been present within the plant at concentrations below the detection threshold during initial IC-PCR examination. The type of *Musa* cultivar from which these isolates were sampled may also play a role in regulating BSV expression, as some cultivars may be more resistant or tolerant to the virus. Similarly episomal BSV-MYS was detected in isolate 99/2775 with IC-PCR, while it was negative with TAS-ELISA. BSV-RD that was not previously detected in isolate 02/2031, was detected in this isolate with IC-PCR. This isolate had an absorbance value of 0.107, which is considered low, since it is close to the value 0.084 (the value of two times the healthy control).

Overall, the newly developed TAS-ELISA has been shown to detect most isolates included for immunization and although it seemed able to detect most BSV isolates, IC-PCR remained the more sensitive technique of the two.

Table 4.8: Sensitivity of antisera used for detecting individual BSV isolates, as reflected in absorbance values (TAS ELISA) and IC-PCR results. Symptoms of individual BSV isolates are also indicated.

PPRI Accession	**Symptoms on its original *Musa* host**	**OD 405 nm***	**IC-PCR detection**
99/2724	Chlorotic and yellow streaks	1.175	Positive BSV-RD, GF, OL
99/2726	Yellow eye spots and streaks	1.108	Positive BSV-GF
99/2729	Yellow eye spots	1.020	Positive BSV-OL
99/2735	Yellow eye spots	0.341	Positive BSV-RD, GF, OL
99/2736	Chlorotic and yellow eye spots and streaks	0.448	Positive BSV-GF
99/2764	Feint chlorotic flecking	0.512	Positive BSV-RD, GF, OL
99/2773	Symptomless	0.800	Not done
99/0172	Yellow streaks	0.837	Positive BSV-RD, GF, OL
99/0169	Chlorotic and yellow eye spots and streaks	0.705	Positive BSV-IM
99/0168	Chlorotic and yellow eye spots and streaks	0.720	Positive BSV-IM
99/0167	Chlorotic and brown streaks	0.680	Positive BSV-IM, GF
99/0160	Chlorotic and yellow eye spots and streaks	0.551	Positive BSV-IM
00/3050	Chlorotic and yellow eye spots and streaks	0.822	Positive BSV-RD, GF, OL
00/3027	Few faint chlorotic streaks	0.771	Positive BSV-OL
00/3021	Faint chlorotic flecking	0.842	Positive BSV-RD
01/0011	Symptomless	0.066	Positive BSV-OL
01/0017	Symptomless	0.047	Positive BSV-OL
02/2004	Chlorotic streaks and patches	0.761	Positive BSV-RD, OL
02/2031	Faint chlorotic flecking	0.107	Positive BSV-RD, OL
02/2032	Faint chlorotic flecking	0.388	Positive BSV-RD, OL
99/2775	Yellow streaks	0.050	Positive BSV-MYS
Buffer control	N/A	0.025	Negative
94/1609	Symptomless (Healthy control plant)	0.042	Negative

*Diluted 1:9 sample/buffer

Fig 4.3: Photographs of banana streak symptoms on the leaf lamina of plants with accession numbers 00/3050 and 99/0169, illustrating yellow and chlorotic eye spots and streaks.

4.5 CONCLUSION

The aim of this study was to prepare a serologically based detection method, able to detect a wide variety of BSV-strains. Therefore, an immune response was induced in experimental animals (two New Zealand White rabbits and two goats), by immunization with purified BSV. Twenty BSV isolates, of which the BSV strain compositions were determined using six different IC-PCR systems, were chosen as sources from which BSV particles could be purified. The primers used in the IC-PCR, (Harper *et al.*, 1998; Geering *et al.*, 2000; Geering *et al*., 2001b) were specific to known BSV strains (BSV-OL, BSV-RD, BSV-GF, BSV-Cav, BSV-Mys, BSV-IM). Some of the BSV-isolates used in the immunizations could not be detected with any of the known IC-PCR systems used. It was assumed that these BSV-isolates, could provide additional BSV-strains.

Virus purification, based on a modified method of Agindotan *et al.* (2003), yielded fairly pure virus preparations. A sucrose gradient step was included into the protocol used in this study. This sucrose gradient step was found to be essential after chloroform extraction of the virus in order to obtain a purer preparation for immunization purposes.

Following immunization, a number of bleeds, from two different animal species, were successfully obtained containing polyclonal antibodies specific to BSV. Using the two most promising bleeds, a TAS-ELISA system was developed, able to detect a range of BSV isolates in crude plant sap at dilutions of 1:9 to 1:288 (leaf lamina:buffer). Remaining bleeds may be perfectly suitable for ISEM, PCR and IC-PCR (following an optimisation of dilutions) and would be able to detect (at least) the most common strains of BSV. IC-PCR detection was successful (with G1F Bleed 2, G2F Bleed 1 and R210 Bleed 2) in detecting all the isolates used in immunization, showing the antiserum to be useful for the detection of episomal BSV. Some of the isolates, with very low absorbance values in TAS-ELISA, could easily be detected with IC-PCR, which is a more sensitive technique.

It would be interesting to evaluate antibodies from the different bleeds against unknown or new strains of BSV. Antiserum (G2F Bleed 1) has been employed for routine diagnosis in the laboratory of the ARC-PPRI (IC-PCR and ISEM) and was found to be specific for detecting individual strains of BSV such as BSV-OL, BSV-RD, BSV-IM and BSV-GF in IC-PCR. Unfortunately no BSV-Mys source has been identified in South Africa and this strain

of BSV has never been detected in routine diagnosis, therefore, the antisera could not be further evaluated against this specific strain. The antisera has not been evaluated for the detection of unknown strains of BSV. Existing antisera, such as that of Lockhart and Ndoworra (2000), that are used routinely world-wide, are also able to detect all of the common strains of BSV (BSV-OL, BSV-RD, BSV-Mys, BSV-IM and BSV-GF) while the antisera failed to detect unknow strains of BSV from oter counties such as Uganda and Kenya (Harper, unpublished results). Two of the bleeds, selected for use in the TAS-ELISA have recently been sent to Dr. Glyn Harper from the UK for evaluation on African isolates of BSV. Samples of the R210 Bleed 1 and G1F Bleed2 antiserums were also sent to Marie-Line Caruana, CIRAD in Montpellier (France), Lawrence Kenyon for the UK and Andrew Geering from Queensland, Australia.

The standardized TAS-ELISA protocol developed [Annexure C] is easy to follow and reproducible results can be obtained. The TAS-ELISA would be very useful for routine diagnostics, especially when large volumes of samples need analysis.

CHAPTER 5

5. SCREENING OF BSV WITH A SYNTHETIC PHAGE-DISPLAYED LIBRARY OF ANTIBODIES DERIVED FROM CHICKEN IMMUNOGLOBULIN GENES.

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5. SCREENING OF BSV WITH A SYNTHETIC PHAGE-DISPLAYED LIBRARY OF ANTIBODIES DERIVED FROM CHICKEN IMMUNOGLOBULIN GENES.

5.1 INTRODUCTION

With newly developed technology, antibodies can now be produced without the immunization of animals. Phage displayed antibody libraries, where an entire functionally active antibody repertoire can be displayed on the surface of filamentous bacteriophages have been constructed (McCafferty *et. al*, 1990; Clarkson *et al.*, 1991; Winter *et al*., 1994; Nissim *et al.*, 1994; Vaughan *et al*., 1996). Antibodies or active antibody fragments (Fab) including variable fragment (Fv), single chain variable fragment (scFv) and diabody fragments, originating from various mammals (humans, bovine, rabbit, mouse, sheep) and also from chicken (Davies *et al*., 1995; Yamanaka, *et al*., 1996) have been successfully displayed on phages (O'Brien *et al*., 1999). Phage antibody libraries represent different antibody clones, with affinity to a whole range of antigens and proteins. Desired monoclonal antibodies against specific antigens can easily be obtained from such libraries. An advantage of this technology is that a desired antibody clone can easily be multiplied in bacterial vectors, making the antibody source inexhaustible. Clones can also be stored at -70°C for long periods of time.

Classic production of antibodies is based on the immunization of animals with foreign antigens (Harlow and Lane, 1988), such as plant viruses. This technology is based on several steps and involves the purification of antigen, immunization of the animals as well as the taking of frequent blood samples. Although the classical method of antibody production is very effective in obtaining the required antibodies against an antigen, it has limitations. After immunization, only a certain amount of serum will be obtainable from the animal that was immunized. In time, serum stocks may become depleted and one has to repeat another cycle of virus purification and immunization. Sometimes, the purification of polyclonal antibodies from animal bleeds can be laborious and has low yields. Monoclonal antibodies are useful for the detection of specific strains or variants of virus species. In the production of monoclonal antibodies, whole organs such as the spleen of the animal is removed for use (Harlow and Lane, 1988). Therefore a limited amount of monoclonal antibodies can be raised. Furthermore, a disadvantage of classic monoclonal antibody production is that the immune

system of animals, especially mice (frequently used for monoclonal antibody production) are too weak or unresponsive to some antigens, especially against some highly conserved animal proteins.

Chickens are known to have an immunologic potency comparable to that of mammals (Pink, 1986). Although different immunoglobulin (Ig) types exist in nature, all have the same basic structure, including of two Heavy (H) and two Light (L) chains. These chains are linked together with a single disulfide bridge (van Regenmortel, 1982). IgY is unique to avian species (Warr *et al.*, 1995), reptile and lungfish while IgG and IgE are conserved in mammals. Structurally the H and L-chains of IgY and IgG molecules are similar; however there is a difference in the number of constant regions (C) in IgG and IgY chains. Immunoglobulin molecules consist of variable regions (V) on the H (V_H) and L–chains (V_L) that are linked to C with a joining segment (J). Another region, D (diversity region), occurs between the V region and the J-C region. In mammals combinational and junctional changes occurs between V-(D)-J, enabling the probability of creating a vast immunoglobulin repertoire (Sakano, *et al*, 1980). In chicken the L-chain locus has one functional variable (V_L1) gene and one J gene while twenty-five, pseudo-V-genes also occur. The pseudo-V-genes donate sequences to diversify the rearranged V_L1 gene by conversion (Reynaud *et al*.1987). It was also demonstrated that chicken light chains could be further diversified by somatic point mutations and by V_L1-J flexible joining (Parvari *et al*.1990).

As highly conserved regions occur on the chicken antibody framework, two single primers are necessary to perform reverse transcriptation polymerase chain reaction (RT-PCR) on the V gene repertoire of chicken IgY. This makes molecular manipulation and the construction of various immunoglobulins, which could differ in composition, possible. Using RT-PCR, cDNA of Heavy and Light chains of antibodies can be constructed. The fragments can be purified and linked with a special linker from which single chain antibodies (scFv) (displaying high antibody affinity) can be expressed (Yamanaka *et al*., 1996). Fusion of this scFv's with the phage coat-proteins results in a phage displayed antibody library. Selection (also referred to as "panning") for antibodies with an affinity to a desired antigen can then be performed (Zebedee *et al*., 1992; Yamanaka *et al*., 1996; Andris-Widhopf *et al*., 2000).

More than one method of phage selection is possible. Selection can be made by using solid surfaces for binding of the antigen (immunotubes [maxisorb or polysorb] or ELISA plate wells), biotinylated antigen (directly or indirectly) in solution or affinity chromatography. The high affinity of biotin for streptavidin represents one of the highest-affinity interactions between a protein and a ligand known in nature (Argaraña *et al.*, 1986; Wilchek and Bayer, 1999). Hence, making it a useful way of capturing biotiylated proteins or biotinylated-IgG on streptavidin-coated beads. The approach and choice of selection method depends on a few factors. One such factor can be the concentration of available antigen (Winter *et al*., 1994).

In this study (Chapter 4) polyclonal antibodies against BSV were obtained by using classic immunology. Although several bleeds were obtained, these will become depleted with time. Worldwide, stocks of BSV antisera are declining, as mandatory indexing for this virus is required in order to ensure that virus-free plants are supplied across continent borders. This study aimed to select desired monoclonal, antibodies against BSV. As synthetic antibody libraries are inexhaustible sources of antibodies, it was decided to screen the virus with a synthetic phage displayed antibody library. One of the main advantages of phage displayed antibodies is that individual antibodies can detect strains equally well while conventional poloclonal antibodies cannot. It is also possible to obtain phage displayed antibodies against specific strains of BSV.

Two panning methods were selected in an attempt to obtain high affinity monoclonal antibodies against BSV. Firstly, using a solid surface to "bind or trap" the virus (Panning Method 1) and secondly by biotinylating anti-BSV IgG (Panning Method 2) and capturing these IgG's on magnetic streptavidin polystyrene beads in order to trap BSV with this combination.

5.2 MATERIALS AND METHODS

5.2.1 PHAGE DISPLAYED LIBRARY (*NKUKU* LIBRARY)

In both panning approaches described in this chapter, a recombinant phage displayed antibody library, derived from chicken, was used. The library was developed by researchers at Onderstepoort Veterinary Institute (OVI) in Pretoria (van Wyngaardt *et al*. 2004) and is called the *Nkuku* (Zulu word for chicken) library. The *Nkuku* library was constructed by

using a phagemid vector and consists out of single chain variable fragments (scFv) displayed on phages. Based on chicken immunoglobulin frameworks, it represents approximately 2 x 10^9 individual clones. Selections of antibodies from this library against a plant virus *Cucumber mosaic cucumovirus* (CMV) (Pietersen, unpublished results) and animal viruses such as Bluetongue virus (van Wyngaardt *et al*. 2004) have been successful.

5.2.3 PANNING METHOD 1

5.2.3.1 Antigen preparation and properties

BSV was purified as described in Chapter 4 (section 4.2.2.2) of this manuscript. The quantities of purified virus were influenced by a few factors. Firstly, there is no alternative host for BSV; therefore the originally selected *Musa* BSV-sources were used even where viral concentrations were low (initially determined by ISEM). As a mixture of BSV isolates were desired in order to represent a wide spectrum of BSV strains, plants with less viral particles (ISEM observations) were also included because of their unique BSV composition. Loss of viral particles during the purification procedures does also occur. The virus concentration used in panning method 1 was 2.0µg/ml, which low in comparison to the optimal concentrations necessary for screening (10-100µg/ml).

5.2.3.2 Surface binding of antigen

The panning protocol and other methods used were adapted from the protocols from OVI (Van Wyngaart, *et al*. 2004) and Hoogenboom (1997) and are stipulated in Annexure D. This panning method was performed by binding the antigen to a solid surface, using a maxisorb-microplate well (Nunc).

5.2.4 SCREENING PHAGE PARTICLES BY ELISA

Using ELISA, populations of phage-clones, obtained after rounds three and four of selection, were screened for their binding properties to BSV and selected antigens. In this initial screening, “polyclonal” phage-antibodies are identified (polyclonal ELISA). Afterwards, “polyclonal” populations were again screened by ELISA, in order to identify “monoclonal” phage-antibodies (monoclonal ELISA).

5.2.4.1 Polyclonal ELISA

All reagents used in this ELISA were added to the plate-wells at a volume of 50µl per well unless indicated otherwise. Selected wells of a maxisorb microplate (Nunc) were coated with chicken anti-BSV IgY (kindly provided by Lockhart) at a dilution of 1:500 in PBS [Annexure A]. The plate was incubated at 37°C for 30 min, followed by washing the plate three times with PBS. BSV leaf lamina freshly macerated (1:10) in BSV extraction buffer [Annexure A] and 1 %Na_2SO_3) were added to selected wells. Controls included BSV-free Cavendish (HC) macerated in PBS buffer (1:10) and a 4 % milk powder solution. The plate was incubated for 2 h at 37°C and washed three times before the addition of PBS containing 2 % ELITE low fat milk powder (2 %MPBS) as a blocking reagent. Phages obtained as input for selection-round five (phages obtained after panning round four) and selection round four (phages obtained after panning round three) were added separately to selected wells. These phage-selections were separately diluted at a dilution of 1:25 by the addition of PBS containing 4%ELITE low fat milk powder (4 % MPBS) and 0.2 % Tween-20. The plate was incubated at 37°C for 2 h and washed thereafter with PBS containing 0.1 % Tween-20. From this point forward, all washes were performed in the same fashion. Mouse anti-phage antibody (B62MCA) was diluted 1:1000 in 2 % MPBS containing 0.1 % Tween-20. This buffer was used in all further dilutions described below, unless indicated otherwise. The plate was washed and a rabbit anti mouse peroxidase conjugate (P0260 [Daco]) added at 1:1000. Orthophenylenediamine dihydrochloride (OPD [Sigma, P38061]) substrate was added to 5 ml 0.1 M citrate buffer, pH 4.5, containing freshly added peroxidase (2,5 µl per 5 ml). The reaction was stopped after 30 min with concentrated sulphuric acid. Absorbance (A) values were determined at an optical density (OD) of 492 nm.

5.2.4.2 Monoclonal ELISA

A glycerol stock, prepared from the output of selection-round four, was thawed and added to 25 ml of 2xTYE agar [Annexure A] containing 100 µg/ml ampicillin (AMP) and 2 % glucose (GLU) (2xTY-AMP-GLU) [Annexure A] to give an absorbance value of 0.03-0.05 at 600 nm. The culture was incubated at 37°C with continual shaking (24000 rpm) until cells reached an A_{600} of 0.5. Cells were plated out on 2xTYE-AMP-GLU agar at dilutions of 10^{-2} to 10^{-5} and the plates incubated at 30°C overnight to allow cell colonies to develop. The next

day each well of three sterile 96 well tissue culture plates (Nunc, Cat. no. 259684) was filled with 100 µl 2xTY-AMP-GLU. Using a toothpick, single bacterial colonies were individually transferred to a plate-well. The plates prepared in this step will be referred to as the master plates. Each bacterial colony could potentially present a single clone of phage-antibody. Four wells of the plate were kept open as a control against spillage (when newly inoculated plates are shaken) of which two were utilized for the positive control; keyhole limpets hemocyanin (KLH) (Sigma, H-8283). Using a sterile transfer device (Sigma, R-2508) inoculum from each master plate was transferred two times to a new plate filled with growth medium (150 µl 2xTY-AMP-GLU). These plates were incubated at 37°C for 2,5h with continuous shaking (23 000 rpm). For storage at -70°C, 50 µl of a 60 % glycerol solution was added to each well of the master-plates. After incubation of the newly inoculated plates, 50 µl 2xTY-AMP-GLU containing 10^8 plaque-forming-units (p.f.u.) of helper phage (M13-KO7) were added to each well. The plates were incubated for 30 min at 37°C and centrifuged at 1,700 g for 10 min after which the supernatant was aspirated off by using a multi-channel pipette. The bacterial pellet was resuspended in 150 µl 2xTY-AMP- kanamycin (KANA) and cells were allowed to grow overnight at 30°C. The following day the plates were centrifuged at 1,700 g for 10 min and 25 µl of the supernatant, diluted with 25 µl of 4 % MPBS containing 0.2 % Tween-20, were used in the phage ELISA.

The ELISA protocol, as described in section 5.2.4.1, was followed with a few modifications. In this ELISA the desired monoclonal antibodies were screened against the BSV antigen, control plates for all substances with potential phage-binding properties (which were used during panning) were included. For each treatment a separate maxisorb plate were prepared. Treatments included a plate for purified BSV, BSV infected plant juice, the healthy control (virus free plant juice), chicken anti-BSV IgY (each at 20µg/ml) and milk powder. All plates were first coated with chicken anti-BSV IGY except the plates on which milk powder would be tested. Two wells on each plate were also used as control wells where KLH was trapped directly to the well surface at a dilution of 1:1000 (v/v). After addition of substrate the reaction was stopped after 35 min.

5.3 PANNING METHOD 2

5.3.1 ANTIGEN PROPERTIES

Four separate purifications of BSV was performed (as described in Chapter 4, Section 4.2.2.2) in order to have pure virus for screening with the synthetic *Nkuku* library. The absorbance of the pooled virus preparation was A_{260} = 1.674. The concentration of the virus preparation was determined as 0.4 mg/ml with 2,2 ml of virus preparation available.

5.3.2 PURIFICATION OF IMMUNOGLOBULINS (IGG) FROM GOAT AND RABBIT ANTISERUM

A volume of 500µl of rabbit (R201, Bleed 1) and 1ml of goat (1F, Bleed 2) antiserum was purified separately. The method for IgG purification described by Clark and Adams (1977) was used with a few modifications. Each separate serum was mixed with sterile water at a ratio of 1:10 and thereafter saturated ammonium persulphate (ratio 1:1, vol:vol) was added to the mixture. After an incubation period for 1h at room temperature, the mixtures were centrifuged at 11 000 rpm for 20 min. Each pellet was gently re-suspended in 1ml of half strength PBS and dialyzed overnight in half strength PBS buffer at 4°C using a dialysis cassette with a 3,500 (molecular weight cut off point [MWCO]) type membrane (Pierce). A column (pH7.4) with pre-swollen micro-granular anion exchanger (DE 52, Whatmann) in half strength PBS was prepared for each antiserum (goat and rabbit) that was purified. After centrifugation in a micro centrifuge (13000 rmp for 2 min), each dialyzed antibody solution was loaded to a prepared column and fractions of 1ml collected at the bottom. The columns were regularly topped up with half strength PBS. Ten 1ml fractions were collected for each antiserum type. Antibody concentrations for each fraction were determined at A_{280}. For each antiserum that was purified the three fractions with the highest absorbance readings were pooled and a final reading was taken for the pooled antibody fractions. For rabbit IgG an A_{280} of 1,400 equals a concentration of close to 1,0 mg/ml. Based on this, the IgG concentration (*IgC*) of each antibody was calculated as follow: $A_{280}/_{1.400}$ = *IgC* mg/ml.

5.3.3 BIOTINYLATION OF IGG AND IMMOBILIZATION ON STREPTAVIDIN BEADS

IgG was biotinylated according to a protocol developed by the manufacturer of streptavidin beads (Pierce). For biotinylation the reagents were added together according to the manufacturers instructions and incubated for 30 min at room temperature followed by overnight dialysis in PBS buffer at 4°C using a dialysis cassette with a 3,500 MWCO membrane (Pierce). Two tubes, each containing 0.2 mg of streptavidin covered polystyrene microspheres (beads), were washed 2 times at 2 min intervals in PBS, by using a magnet to draw the beads to one side of the tube. Goat and rabbit IgG were each added (final concentration of 5μg/ml) to a separate tube containing the washed streptavidin beads in 500μl PBS. Incubation was performed at room temperature for 30 min while gently rotating the tube.

5.3.3.1 ELISA with biotinylated IgG

To ensure that biotinylation of the IgG were successful ELISA was performed after incubation of the biotinylated IgG with the streptavidin beads. The ELISA was performed in a single eppendorf tube.

Figure 5.1 illustrates the ELISA procedure that was followed for the evaluation of biotinylated goat IgG. After washing the beads 3 times, at 2 min intervals, using the magnet, BSV was added at a concentration of 2 μg/ml. Incubation was for 60 min at room temperature followed by washing the beads 3 times, at 2 min intervals using the magnet. Rabbit anti BSV IgG was added at 2 μg/ml and the mixture was incubated 45 min at room temperature. Another 3 washes, with 2 min intervals (using the magnet) were performed before adding the anti-rabbit conjugate at 1:1500. Substrate was added after following the same incubation and washing procedure, as described above. For the negative control, buffer was used instead of BSV. The same procedure were followed for the biotinylated rabbit IgG, however goat IgG was used as a second detection antibody, while a anti-goat conjugate was used.

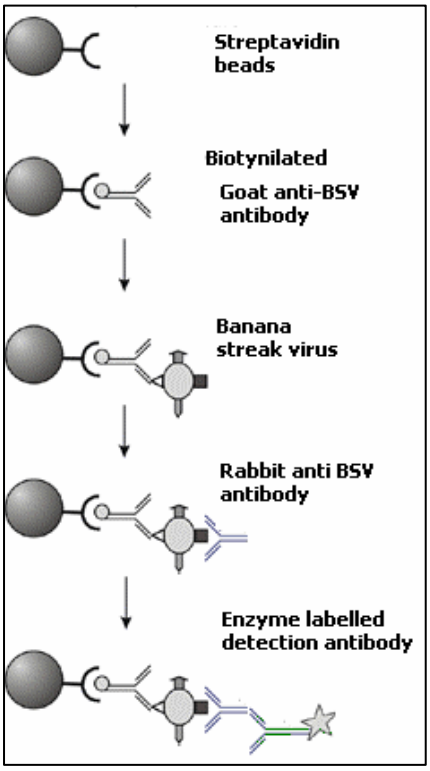

Fig 5.1: An ELISA protocol developed for the evaluation of biotinylated IgG on streptavidin beads. Direct capture of target antigen (BSV) was obtained by using biotinylated goat IgG immobilized on streptavidin beads. Rabbit anti-BSV IgG served as a second BSV-detection antibody while an enzyme labeled anti-rabbit conjugate was used in the final step before adding substrate. After a 60 min incubation period the fluid was transferred to a clean Maxisorb (Nunc) ELISA plate and absorbance values determined with a Labsystem multiscan MS plate reader (Amersham) (figure adapted from - http://www.dynalbiotech.com).

5.3.4 PANNING

5.3.4.1 Equilibration of phages and streptavidin beads

Two separate eppendorf tubes, each containing two hundred microlitres of streptavidin beads (100 µl = 1mg of beads), were washed 3 times with 2 % MPBS, at 2 min intervals between washes by application of magnets to draw beads aside. The beads were incubated in 2 % MPBS for 2 h at room temperature with continuous gentle rotation.

Phages from the *Nkuku* library were diluted with 2 % MPBS (0.1 % Tween-20 added) to yiled a concentration of 5 x 10^{12} phages. Pre-immune goat serum and rabbit serum was added (50 µl of a 1mg/ml solution of each). Fifty microlitres of the healthy control plant, macerated in PBS, was also added. Addition of these compounds was to ensure that all phages that might react with any of these components in the panning would be saturated and eliminated, leaving only virus-specific antibodies.

5.3.4.2 Capturing of biotinylated IgG and BSV on beads

Ten micrograms of bioltinylated goat and rabbit IgG, diluted in 2 % MPBS, was added separately to a tube containing equilibrated beads and incubated for 30 min at room temperature. After washing 5 times with PBS at 2 min intervals, by applying the magnet, BSV were added to each eppendorf tube at a concentration of 20 µg/ml in PBS. Overnight capturing of the antigen was performed by at 4°C, during continuous gentle rotation of the tubes.

5.3.4.3 Panning with phages

After washing the tubes 3 times with PBS, the phage library was added to the tubes containing biotinylated goat and rabbit IgG with captured BSV. Incubation at room temperature for 2h was performed with gentle rotation of the tube. Eight washes, each with 500µl of PBS containing 0.2 Tween-20, followed by seven washes with 500 µl PBS were performed. For each wash the magnet was used to draw the beads aside with 2 min intervals between washes.

5.3.4.4 Elution

Bound phages were eluted by addition of 1 ml 100 mM triethylamine, pH12 to the eppendorf tube containing the beads and rotating the tube for 10 min. The eluted phages were transferred to an eppendorf tube with 0.5 ml 1 M Tris (pH 7.4) and vortexed for 1 second for quick neutralization. Standard panning procedures, as previously described (Appendix D, sub heading: Panning with eluated phages), were followed from this point onwards.

5.3.4.5 Polyclonal ELISA

In order to establish if there were any binders selected from the last round of panning, an ELISA was performed. Selected wells of a maxisorb microplate (Nunc) were coated with 50µl of 20µg/ml purified rabbit anti-BSV IgG in PBS for three hours at 37°C. All reagents used in this ELISA were applied at 50µl per well, unless indicated otherwise. The plate was incubated at 37°C for 30 min, followed by washing three times with PBS and addition of the samples. Diluted, purified BSV was added at 20µg/ml to selected wells coated with rabbit

anti BSV. BSV-free Cavendish (HC) macerated in PBS buffer (1:10) were added to similarly coated wells. An unpanned *Nkuku* library sample (1:25 library in 4 % MPBS with 0.2 % Tween-20 added) and phages obtained after the last round of panning (input for selection-round five) were added as controls to selected uncoated plate wells. After incubation of the plate for 2 h followed by washing it three times with PBS, 200 µl of 2 % MPBS was added to each well as a blocking reagent. Phages as input for selection-round five (dilution of 1:25 4 % MPBS containing 0.2 % Tween-20) were added to the wells, to which BSV and HC samples were added previously. The plate was incubated at 37°C for 2 h and washed thereafter with PBS containing 0.1 % Tween-20; all washes from this point on were done in the same fashion. Mouse anti-phage antibody (B62MCA) was diluted 1:1000 in 2 % MPBS containing 0.1 % Tween-20. This buffer was used in all further dilutions described below, unless indicated otherwise. The plate was washed and a rabbit anti mouse peroxidase conjugate (P0260 [Daco]) added at 1:1000. OPD (Sigma, P38061) substrate was dissolved 5 ml 0.1 M citrate buffer, pH 4.5 and containing freshly added peroxidase (2,5 µl per 5 ml). The reaction was stopped after 30 min with concentrated sulphuric acid and absorbance values measured at 492 nm.

5.3.4.6 Monoclonal ELISA

The protocols for ELISA and the preparation of master plates containing potential monoclonal antibodies, as described in section 5.2.4.2 of this Chapter, were followed.

5.3.4.7 ELISA with selected phage clones

Possible clones, obtained from the monoclonal ELISA, were selected from the stored master plate. A control clone (with no affinity for BSV) was also added. From each well, cells were lifted separately with a sterile toothpick and each added to 10 ml of 2xTY-AMP-GLU. Cultures were shaken overnight at 37°C and then 10^8 p.f.u. of helper phage (M13-KO7) in 2xTY was added the next day. After incubation at 37°C for 2h the tubes were centrifuged at 3000 rpm for 15 min. The bacterial pellet was resuspended in 10 ml of 2xTY-AMP-KANA and shaken overnight at 37°C. Tubes were centrifuged and the supernatant used in ELISA at a ratio of 1:1 (phage containing supernatant: 4 % MPBS containing 0.2 % Tween). ELISA procedure as described in section 5.2.4.2 was used, with separate treatments being purified BSV at 20 µg/ml, BSV infected plant sap, the HC, a mixture of purified goat and rabbit anti-

BSV IgG (each at 20 µg/ml) and milk powder. Previously selected phage-antibodies successfully detecting Bluetongue virus particles (van Wyngaardt, *et al.*, 2004) were added as another separate control to the ELISA performed.

5.4 RESULTS AND DISCUSSION

5.4.1 PANNING METHOD 1: SURFACE BINDING OF ANTIGEN

Four rounds of enrichment were completed for this panning method by binding the antigen to a solid surface of a maxisorb-microplate well (Nunc). After each round of panning it seemed that the bacterial colonies (representing bacterial cells containing phage-displayed-antibodies) on the TYE agar plates became denser. This suggested a possible enrichment of phages selected after each consecutive round of panning, however, these cells with phage-antibodies needed further evaluation by ELISA.

ELISA with the "polyclonal" phage-antibodies from the last panning rounds (input selection-rounds four and five) also looked promising, as a slight reaction against BSV, but none of the other controls (HC and milk powder), was observed. Therefore it was decided to move on to the step where monoclonal phage-antibodies could be evaluated.

Two-hundred-and-seventy single bacterial colonies were transferred to a master plate, representing potential monoclonal phage-antibodies. However, when these monoclonal antibodies were screened with ELISA, some clones reacted with BSV, milk powder and the HC alltogether. Others did not react with any of the specific antigens showing a non-selective reaction. These results suggest that the antibodies that were selected were not specific to BSV. Therefore, no individual clones against BSV were obtained with this panning method.

5.4.2 PANNING METHOD 2: BIOTINYLATION

5.4.2.1 Purification and biotinylation of IgG

For each antiserum, ten 1ml fractions were collected from the purification column. Peak values at A_{280} were obtained in fractions 4-5 for each bleed ranging from 0.450-0.583 and 1.949-1.422 for the rabbit (R 210 Bleed 1) and goat (G 1F Bleed 2) bleeds respectively. These were pooled and the final concentrations of the purified IgG determined. Concentrations were 1.29mg/ml for the purified goat IgG and 0.57mg/ml for the rabbit IgG.

5.4.2.2. ELISA with biotinylated IgG

To ensure that biotinylation did not affect the affinity of the IgG for BSV, an ELISA with biotinylated IgG was performed. After substrate incubation of 60 min, an absorbance reading of 1.583 was obtained at 405 nm where BSV was used as antigen. For the negative control, where only buffer was added, an absorbance reading of 0.076 was obtained. The result confirmed that the biotinylated IgG were captured on the streptavidin beads and that biotinylation of the IgG took place without affecting the affinity of the antibodies for BSV.

5.4.2.3 Selection rounds

As with the panning using a solid surface, there seemed to be enrichment of antibodies as the plated-out colonies again became denser (Fig 5.2). However, this result does not guarantee that the desired antibodies, having a high affinity to BSV, are selected because of the presence of other proteins within the panning environment. Therefore phage-displayed-antibodies requires further testing with ELISA.

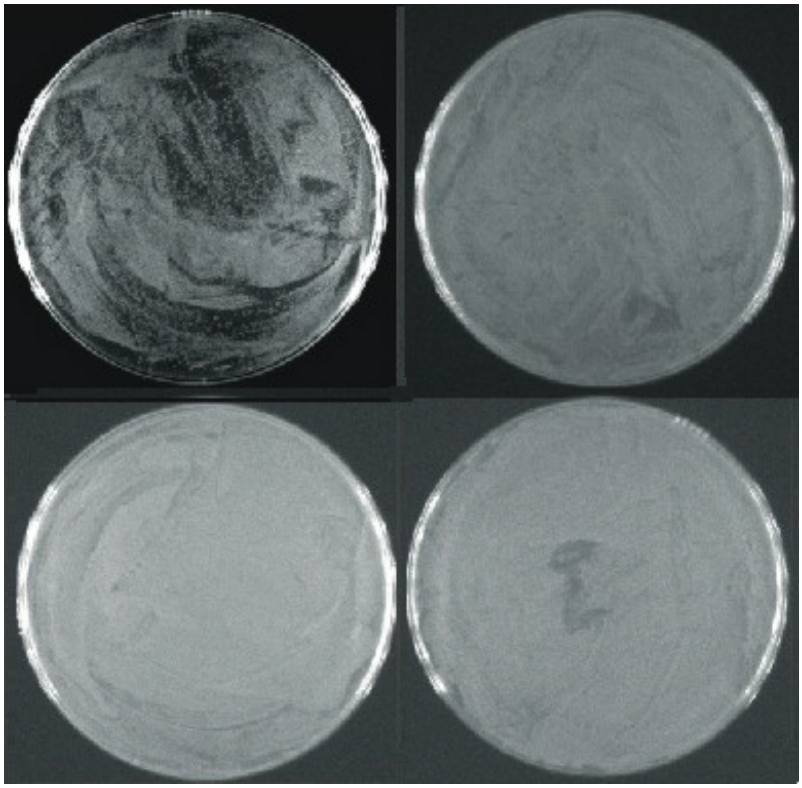

Fig 5.2: Four selection rounds presented by different agar plates, representing bacterial colonies carrying phages with antibodies. Possible enrichment serves as signs of possible antigen selection. The plate in the left top corner presents round one output, while the following rounds (2-4) are presented in a clockwise fashion.

5.4.2.4 Polyclonal ELISA

BSV was detected at an average reading of 0.286 while the HC gave a reaction of 0.250. The un-panned library gave an average reading of 0.147 while phages from input selection-round five gave a reading of 2.500. Despite the low reaction of the phages against BSV and its reaction on the HC, it was decided to continue towards testing individual clones of the library that may have been selected against BSV. It was reasoned that once the individual clones were separated out of the 'polyclonal' antibody mixture, higher specific BSV-affinity would be obtained. Once separated from the polyclonal mixture, individual clones would also be available in greater numbers; therefore higher absorbance values would be expected for clones with a high affinity to BSV.

5.4.2.5 Monoclonal ELISA

To fulfil the aim of this experiment, it was necessary to select phage binders *only* reacting to BSV and none of the other proteins, used for various reasons throughout the panning. Therefore possible monoclonal BSV-"binders" could be separated from non-specific "binders" present within the obtained polyclonal selection. In the first monoclonal ELISA, screening of 92 clones was performed. Very similar reactions were observed with the purified BSV preparation, BSV containing plant sap and HC treatment plates. Absorbance signals on the plate treated with only the milk powder control were very low. Out of the total number of clones tested, only one well (representing a clone) on the purified BSV treatment plate (position B7) seemed to have a reaction only against BSV. However, this reaction did not occur on the plate with the BSV infected plant juice treatment. All BSV strains were included in the BSV plant juice treatment (obtained from macerating BSV infected leaves with PBS 1:10). However, it was reasoned that the reaction at well B7 on the plate treated with the purified BSV preparation, was perhaps against a specific strain of BSV being more prevalent within the purified BSV preparation, and perhaps present in a lower concentration within the BSV plant juice preparation. Two other clones with a moderate reaction against BSV and a low reaction to the other treatments were also selected from wells A7 and B12 respectively.

Throughout the test, control wells for "spillage-indication" remained clear, suggesting that wells were not contaminated by spillage. The KLH control plates gave a positive OD signal of 1.815 on the BSV treatment plate. OD 492 nm readings for B7, B12 and A7 were 1.005,

0.710 and 0.740 respectively. Such absorbance values are considered to be moderate to high. Therefore these clones were tested with a final ELISA, in which these specific clones could be tested more accurately.

5.4.2.6 Phage ELISA with selected clones

By going back to the selected wells on the glycerol plate it is ensured that the originally selected nonoclonal phage-antibodies is tested, avoiding any possible contaminants. Clones were also multiplied in greater volumes, ensuring a high concentration of antibodies to be available. Clones in positions B7, B12 and A7 on the master plate were re-isolated and tested in this final ELISA reaction. Well B9 also served as a control with no significant reaction to BSV. Previously selected phage-antibodies successfully detecting Bluetongue virus particles (van Wyngaardt, *et al.*, 2004) were added as a separate control to the ELISA performed, these responded as expected. Unfortunately no enzymatic reactions, with the phage antibodies obtained, were observed in this final ELISA. Therefore, it seems that selection of phage-antibodies against BSV were unsuccessful.

5.5 CONCLUSION

Although monoclonal antibodies from the *Nkuku* library were successfully obtained against other viruses (van Wyngaardt, *et al.*, 2004), no antibodies with a significant affinity to BSV were obtained during this study. A factor that could have played a role in selection was the low concentration of purified BSV during panning. In panning method one, 2.0µg/ml of antigen was used while it was increased to 20µg/ml for method two. In previous phage selection protocols a high quality antigen of high concentration (10-100µg/ml) is needed (Hoogenboom, 1997). Although the purification of BSV from *Musa* was effective, it was not possible to obtain very high concentrations of the virus. Some of the reasons being the use of more that one BSV source, with some sources having very low viral particle concentrations as estimated by ISEM. Some sources never displayed clear BSV symptoms and it is recommended that symptomatic tissue be used for optimal BSV concentrations. BSV infected plant tissue with no symptoms, often have absorbance values that are below detection levels (Dahal *et al.*, 1998).

Neither of the two panning approaches used seemed to be effective. High affinity antibodies, produced by Lockhart, were used to 'trap' BSV on solid-surface during panning method one.

Purified IgG (prepared from antisera produced in Chapter 4 of this thesis), from two different animal species (goat and rabbit) were used in panning method two. These antibodies, used in both panning methods, were included in order to ensure the capturing and presence of BSV during panning. Furthermore, the trapping BSV with antibodies was an attempt to increase the number of BSV particles present during panning. For optimal trapping of the antigen out of purified virus preparations solutions and BSV infected plant juice, the BSV-specific antibodies were used at reasonably high concentrations.

In the approach followed for panning method two, it was hypothesized that biotinylation on streptavidin beads would be more effective, because the reaction was performed in solution, therefore allowing more effective binding of the desired antigen to the immobilization system. With this panning method we also used a higher concentration of antigen. Purified IgG from rabbit and goat performed very well in ELISA reactions and would have undoubtedly trapped BSV particles. And indeed, ELISA showed BSV to have been successfully detected with the biotinylated antibodies, showing immobilization of the biotinylated-IgG-virus complex to have been successful. Strict controls were also included throughout the panning and ELISA protocols followed.

However, after several rounds of enrichment, no BSV specific clones could be obtained from either one of the two panning approaches that was followed. Out of a total of 362 clones that was analyzed, no “binders” with a high affinity for BSV could be found. Unlikely as it may seem, it could be possible that the binder or binders against BSV was “missed” in the selecting process, or that possible binders have a low affinity to the specific proteins of BSV. Working with a pooled group of BSV strains had an influence on the BSV concentration, which is critical for panning. Another approach that could be followed could be to screen specific individual strains of BSV, with higher concentrations, with the library.

Fortunately conventionally produced polyclonal antibodies prepared against BSV (Chapter 4) worked well in ELISA for the detection of this virus. Therefore, phage-displayed monoclonal antibodies were no longer essential but would have been usefull in the detection of specific BSV strains.

CHAPTER 6

CHAPTER 6

6. TRANSMISSION OF BSV WITH FOUR MEALYBUG SPECIES.

6.1 INTRODUCTION

The discovery that plants could be cultured *in vitro* and multiplied into large numbers from a single meristem, has led to various applications in important fields, such as science and agriculture. *In vitro* propagation of *Musa* by tissue culture has many advantages such as enabling growers to establish a uniform plantation, with uniformity in growth, flowering and harvesting (Robinson, 1993). Large numbers of plants can be generated from a single meristem, ensuring that a single desired *Musa*-clone (motherplant) could be multiplied into numerous identical copies.

Usually multiplication of virus free motherplants would ensure that the tissue culture (TC) generated plants would be free of viruses such as *Cucumber mosaic cucumovirus* (CMV), *Banana streak badnavirus* (BSV) and *Banana bunchy top babuvirus* (BBTV). Certainly, when virus free Cavendish varieties such as Williams or Grand Nain are multiplied, resulting progeny are also free of CMV, BBTV and BSV. However, with other genotypes of *Musa*, especially hybrids, an interesting observation has been made. Plants derived from tissue culture were found to be infected with systemic (episomal) BSV particles, even if the original motherplant was indexed as BSV-free. In recent years, endogenous sequences for BSV, a pararetrovirus, has been confirmed in *Musa* hybrids (Lockhart *et al.*, 1998; Harper *et al.*, 1999b; Ndowora *et al.*, 1999; Geering *et al.*, 2002). Some of these integrants were proven to be expressible and cause banana streak disease (BSD) as a result of the formation of episomal BSV (Lockhart *et al.*, 1998). Geering *et al.* (2001a) examined many *Musa* genotypes and found integrated expressible sequences to be "only" associated with *Musa* having B-chromosomes. In other studies, tissue culture was proven as one of the stress factors that can trigger the episomal expression of integrated BSV sequences (Ndowora *et al.*, 1999; Dallot *et al.*, 2001).

Traditionally, *Musa* were propagated by means of suckers (corms). Such material would be true to type; however fewer plants could be propagated. Unfortunately viruses could easily spread with this method of propagation, should the vegetative material be infected. In contrast to the phenomenon of integrated BSV-expression that occurs with tissue culture

propagation, suckers derived and propagated from a virus-free motherplant source do not have a tendency to become infected.

Since 1988, multiplication of *Musa* plant material in South African banana industry was and still is, based on tissue culture propagation. Although Cavendish varieties constitute the backbone of the South African industry, alternative cultivars, resistant to destructive diseases such as Panama, are established in some banana cultivation areas. A danger exists of creating large numbers of BSV infected progeny plants from B genome initiates by tissue culture.

Mealybugs (*Hemiptera: Coccoidea: Pseudococcidae*) have been demonstrated to be vectors of various plant viruses including species of the *Closterovirus*, *Trichovirus* and *Badnavirus* genera. Nineteen species of mealybug, belonging to 13 genera, are known to occur on *Musaceae* (mainly banana and plantain) (Watson and Kubiriba, 2005). Lockhart and Jones (1993) demonstrated the transmission of BSV from banana to banana with *Planococcus citri* (Risso) and the transmission of *Sugarcane bacilliform badnavirus* (SCBV) from sugarcane to banana with *Saccharicoccus sacchari* (Cockerell). SCBV is serologically related to BSV (Lockhart and Autrey 1988; Nodwora, 1998). Experimental transmission of BSV was attempted with the pink pineapple mealybug, *Dysmicoccus brevipes* (Cockerell), (Kubiriba *et al.* 2001b); results remains inconclusive as another bacilliform virus occurs in pineapple and the viral status of both the pineapple host plants and the mealybugs collected from these plants were not confirmed. Although not common on *Musa* in Africa, *Pseudococcus comstiki* (Kuwana) also transmits BSV (Su, 1998).

It is currently not known whether the form of BSV, derived from activated integrant, is mealybug transmissible (as the natural occurring episomal forms are) and whether commercial Cavendish plantations are at risk to become infected with episomal BSV from already established B genome tissue culture propagated plants (see Chapter 3 of this thesis).

The aim of this study was to determine whether episomal BSV, probably derived from integrated BSV sequences, could be transmitted by common mealybug species to Cavendish varieties in a laboratory under controlled conditions. To achieve this, it was first necessary to obtain different vegetative plant sources. These different sources could provide the episomal-

and integrated-forms of BSV for the planned transmission studies. Dallot *et al*. (2001) used corms of the tetraploid cultivar FHIA-21 (AAAB) developed at the Fundacion Hondurena de Investigacion Agricola (FHIA) in Honduras, to prove that *in vitro* propagation with tissue culture resulted in episomal BSV positive progeny. These FHIA-21 corms, used by Dallot *et al.* (2001) were specifically used because they had no tissue culture ancestry, have never shown BSD symptoms, and tested negative for episomal BSV with immunocapture PCR (IC-PCR). Therefore, it was decided to use the same cultivar in our studies. Details of the material sourced is described in the next two sections (section 6.2.1.1 and 6.2.1.2) under materials and methods.

6.2 MATERIALS AND METHODS

6.2.1 *MUSA* PLANT SPECIES AND MATERIAL

6.2.1.1 FHIA-21 corms

A generation of corms, originating from motherplants of FHIA-21 (AAAB) with no history of TC, developed in the FHIA breeding program at Honduras, was imported following quarantine regulations (Permit No: P0009722). Corms from these sources were kindly provided by Dr. Dale Krigsvold. It was assumed that, these corms would be negative for episomal BSV (unless, stress factors, other than TC might have activated the integrated BSV sequences). The purpose of the corms was to serve as negative controls of episomally TC-activated BSV. Corms were numbered from FH-1 to FH-10. Nine months after planting, immunocapture (IC)-PCR for strains of BSV-GF, BSV-RD and BSV-OL were performed [Annexure B, sections B.1.2 and B.1.3]. For each individual plant a partial virus purification was prepared according to the prescribed method (Diekmann and Putter, 1996, Chapter 4). Each partial extract was diluted with DEPC water (1:1 v/v) and used in IC-PCR [Annexure B, section B.1.1] using GF1Bleed 2 antiserum, at dilution 1:8000, to 'trap' virus particles. For all TAS-ELISA tests performed, absorbance values were interpreted as positive if the OD 405nm were more than two times that of the virus-free Williams control. Undiluted purified extract was examined by ISEM, using the method described in Chapter 4 (Section 4.2.1.3), while GF2Bleed1 antiserum at a dilution of 1: 1 000 was used to trap the virus.

6.2.1.2 TC derived FHIA-21

INIBAP has established the world's largest *Musa* germplasm collection and has put in place a system for the safe movement of *Musa* varieties. Newly developed cultivars and *Musa* material kept at INIBAP is tested for BSV, *Banana bunchy top babuvirus* (BBTV), *Cucumber mosaic cucumovirus* (CMV) and *Banana bract mosaic potyvirus* (BBrMV) at independent virus indexing centres (VIC). Tests are standardized according to FAO/IPGRI technical guidelines for the safe movement of germplasm (Diekmann and Putter, 1996).

The improved tetraploid variety, FHIA-21 (AAAB), was chosen as an ideal virus donor plant as it is known to harbor integrant(s) of BSV that become episomal after tissue culture (Dallot *et al.*, 2001, Lockhart *et al*, 1998). "Virus-free" proliferating tissue of FHIA-21 (#68, ITC.1332) was obtained from INIBAP under license (Permit No: P0010152). A germplasm health statement, accompanying the material from INIBAP, declared the material sent to PPRI to be free of all the viruses tested for, including bacilliform viruses. Thus the FHIA-21 material received was previously declared to be virus free by various independent virus-indexing centers (VIC).

Each vial with proliferating tissue was multiplied subsequently on modified culture medium of Murashige and Skoog (1962) [Annexure A, Table A.1] followed by another three proliferating cycles, each with a four to six week interval. Shoot tips were transferred to rooting medium for root development. The pH of all the media was adjusted to 5.8 prior to autoclaving. Micro-propagated cultures were maintained in an incubation room at 28°C and a 12-hour photoperiod with 1000 lux. After 4-6 weeks plants were planted in fertilized soil medium and hardened off.

Sixty-seven tissue culture plants were derived from the proliferating tissue of FHIA-21 received from INIBAP. These were given numbers ranging from TC-1 to TC-67 and tested separately for BSV four months after planting, using the newly developed TAS-ELISA system [Annexure C]. Once again absorbance values were interpreted as positive if the OD 405nm were more than two times that of the virus-free Williams control (threshold value). Only plants with absorbance values that were considerably higher than that of the threshold

value were subsequently tested individually with IC-PCR (using the GF1Bleed2 antiserum at 1:8000 for trapping, see Chapter 3 and 4) for BSV-OL, BSV-GF and BSV-RD.

6.2.1.3 Recipient and control plants

As recipient plants for the mealybug transmissions, 200 virus-free Williams (Cavendish subgroup, AAA), tissue culture derived plants were established. Four plants were set aside to serve as control feeding sources for non-viruliferous mealybugs. All the potted plants were tested with IC-PCR (using the antiserum of GF1Bleed2 at a 1:8000 dilution, [Annexure B, section B1.1]) for BSV-OL, BSV-RD and BSV-GF prior to conducting any transmission studies. The primers and protocols for the detection of BSV-OL, BSV-RD and BSV-GF were used as described by Harper *et al.*, (1988) and Geering *et al.*, (2000), [Annexure B, sections B.1.2 & B.1.3]. These plants were also tested with the newly developed TAS-ELISA protocol [Annexure C]. For both tests, plants were pooled in batches of 10, presenting one sample.

In order to standardize experiments, all plants were kept under the same conditions: plants were planted in 5 litre growing bags filled with fertilized potting soil and kept under 12 hour light illumination at a temperature regime of 24-28°C in an insect-proof facility.

6.2.2 CONFIRMATION OF ENDOGENOUS BSV IN FHIA 21

6.2.2.1 Total DNA isolation and PCR

Total DNA of TC-1 (episomal BSV-negative) and FH-4 was extracted using a modified method of Gawel and Jarret (1991), [Appendix B, B.2.1]. After purification, PCR was performed with the primers Musa-T32 (5'-GGCTTATGATGCTGACCACAT-3') and BSV510.2 (5'-GCCATAAAAACGAACCTTGCT-3') (Ndowora, 1998). The sequence complimentary to primer BSV510.2 is located at nucleotide positions 10-30 in the BSV-OL genome. Another primer BADNAT1 (5'-GCG GMY MWI GCT CTG ATA CCA-3'), was used together with Musa T32. BADNAT1 is a degenerate primer for BSV (Geering, unpublished primer sequence). PCR conditions as described for BSV-OL were followed: The PCR master mixture for each reaction contained 50µM of each NTP, 0.4 µM of each primer, 1.5 mM $MgCl_2$, 1 x ammonium buffer, 1.25 U Biotaq^{TM} DNA Polymerase (BIOLINE, UK), in a total volume of 25µl. Samples were incubated in a Hybaid thermocycler. Thermocyler

conditions were; denaturation at 94°C for 30 seconds, 35 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 1 min, followed by a final elongation step of 72°C for 5 min. PCR products were loaded to a 1 % low melting agarose gel, stained with ethidium bromide [Annexure E, section E.1.3]. The gel was placed on an UV transilluminator and corresponding bands of approximately +1400 kb were cut from the gel using a sterile scalpel for each sample. DNA bands were purified from the agarose using the TA 050 CLN cleanmix kit (Talent-Srl, ITALIA) by following the manufacturer's instructions.

6.2.2.2 Cloning, transformation and sequencing

Only products obtained with the primers T32 and BSV510.2 were cloned. Products from T32 and BADNAT1 were sequenced directly after gel purification. Cloning was performed according to the manufacturer's instructions (pGEM-T easy vector system, Promega, A1360): An A-tailing procedure was followed before ligation into the vector; this is because of the fact that DNA obtained after PCR (using Biotaq DNA polymerase) amplification contains blunt ends. Two microlitres of purified DNA were mixed, together with 1µl Taq buffer, 0.2mM dATP, 5U Biotaq™ DNA Polymerase (BIOLINE, UK) and 10µl sterile DEPC water. The mixture was incubated at 70°C for 20 min and 2µl of product added to the following ligation reaction mixture: 5µl of rapid ligation buffer, 1µl pGEM-T easy vector, 3U T4 DNA ligase and 6µl sterile DEPC water. The reaction was incubated overnight at 4°C. Transformation of competent cells (JM 109, high efficiency, Promega, 1380) was performed as described in the pGEM-T easy vector system protocol [Annexure E, section E.1.4] and plated out on Petri dishes with suitable culture medium (LB/amp/IPTG/X-Gal, [Annexure A]). Bacterial colonies obtained were screened for transformants [Annexure E, section E.1.5]. Six colonies, representing six clones were selected. Selected pGEM-T plasmids, with inserted clones, were purified using the E.Z.N.A Plasmid Miniprep kit I (PeQlab, 12-6942-01). Sizes of the inserts were determined by digesting 2µl of the purified product with EcoR1 enzyme (0.75µl) in 1.5µl buffer-H and 10.75µl sterile DEPC water. Purified plasmids with correct clone insert sizes were sent to the DNA Sequencing Unit, University of Cape Town (Mrs. Dianne James) for sequencing. During sequencing, the primers for puc/M13 forward [5'-(CGCCAGGGTTTTCCCAGTCACGAC)-3'] and puc/M13 reverse [5'-(TCACACAGGAAACGAGCTATGAC)-3'] were used. Clones were sequenced in both

directions and final sequences were edited and complied using DNAMAN, Version 5.2.9 (Lynnon Biosoft, Canada).

In a further attempt to search for integrated BSV-OL and BSV-RD in the FH-4 corm, PCR was also performed with the primers for BSV-OL and BSV-RD on plant sap of FH-4 and TC-1.

6.2.3 REARING OF MEALYBUGS

Four mealybug species, *P. citri*, *Pseudococcus longispinus*, *P. ficus* and *D. brevipes* were used separately as vectors in the transmission studies. All colonies were kept separately in Perspex cages 0.4m x 0.4m x 0.6m (length x width x height), with mesh covered ventilation holes (Fig 6.1). Cages were placed on trays filled with oil. *P. citri* was received from a laboratory colony kept at Citrus Research International in Nelspruit, Mpumalanga, South Africa (kindly provided by Dr. B. Tate). *P. citri* successfully developed and multiplied to large numbers of nymph and adult stages on butternut. *D. brevipes* was received from the ARC-Pineapple Research Station in Hluhluwe, Kwazulu Natal, South Africa. Mealybugs were collected from a pineapple field on pineapple stalks (kindly provided by Mrs. H.A.Tustin) and placed on butternut for rearing. This colony was kept on butternut for six months before using the insects in the transmission studies. *P. ficus* were received as females with egg sacks from Dr. V. Walton, (University of Stellenbosch) and colonies were successfully reared on butternuts. *P. longispinus* was received from the University of Pretoria (UP), South Africa (kindly provided by Dr. K. Krüger). This colony was kept on a *Philodendron* sp. at UP and pieces of leaf and petioles were brought directly to ARC-PPRI for placement on individual source and control-source plants for the determined acquisition access period.

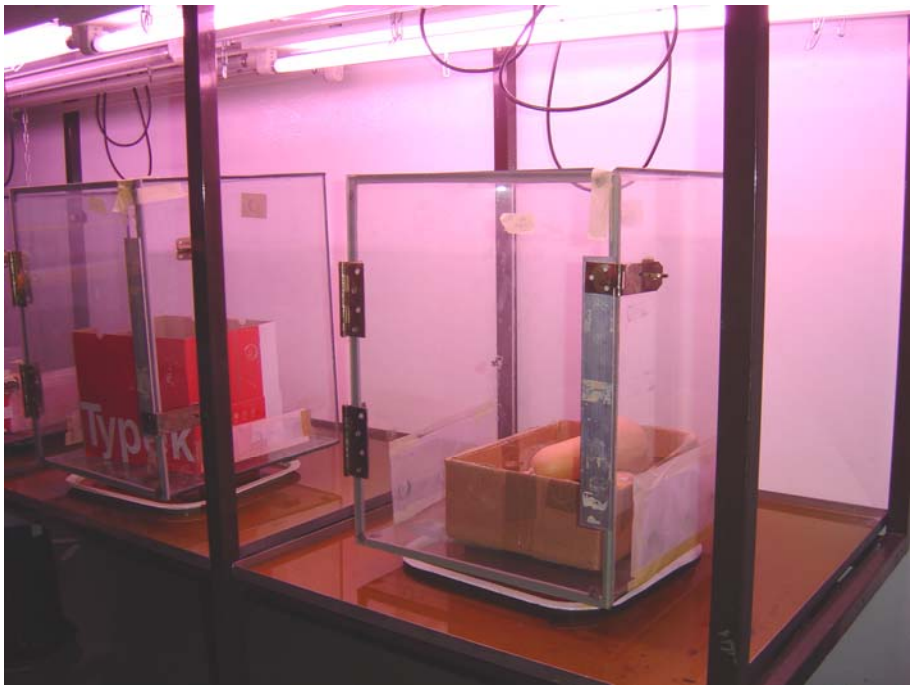

Fig 6.1: Photograph of insect cages in which mealybug colonies were reared. At PPRI mealybug colonies were established and reared on butternut. The bottom trays underneath the cages were filled with oil to ensure that no cross-contamination between colonies could occur.

6.2.3.1 Taxonomic status of mealybugs

The taxonomic status of the mealybug colonies were determined by Dr. I.M. Millar, an entomologist at ARC-PPRI, Vredehuis, Pretoria, South Africa, according to published procedures (Millar, 2002; Williams, *et. al*, 1992.; Williams and Watson, 1988). Furthermore the identities of *P. citri*, *P. longispinus* and *P. ficus* were confirmed by PCR with species-specific primers. PCR confirmation for the identity of *P. ficus* was performed at UP with primers designed by Saccaggi (MSc thesis 2005, unpublished primer sequences), UP. Primers for *P. longispinus* and *P.citri* were designed and supplied by Anita Severn-Ellis (unpublished primer sequences). Total genomic DNA of *P. longispinus* and *P.citri* were extracted according to the protocol of Epstein *et al.*, (1992). Three to four insects were macerated in HB buffer (0.1 M Tris-HCl pH 8, 10 mM EDTA pH 8, 0.35 M NaCl, 7 M urea) by using a sterile plastic pestle. An equal volume of phenol-chloroform (1:1) was added. After gentle mixing the mixture was centrifuged at 13,000 rpm for 15 min. Two volumes of cold ethanol and 0.3M (final concentration) sodium acetate, pH 5.2, were added to the supernatant. Ethanol precipitation was done overnight at -20°C. After centrifugation (13,000 rpm for 10 min), the pellet was dissolved in DEPC water. A half microlitre of the DNA was used as template in the following PCR reaction: denaturing at 94°C for 3 min, followed by

35 cycles of 94°C for 1 min, 62°C for 30 seconds (for *P.citri* annealing temperatures 65°C, 55°C and 45°C were also tested) and 72°C for 2 min. Final extension was at 72°C for 2 min.

6.2.4 TRANSMISSIONS

Each transmission existed of three treatments with 10 plants each and is summarised in Fig 6.2. The three treatments were 1) Transmission from a TC derived FHIA-21 plant (positive for episomal BSV) to 10 Williams receptor plants, 2) Transmission from FH-4 corm to 10 Williams receptor plants 3) Transmission from virus free Williams to 10 Williams receptor plants (control treatment). Transmissions with each vector species were done separately, but each transmission was performed under the exact same controlled conditions in an insect quarantine facility.

6.2.4.1 Donor plants and acquisition access period

Fig 6.2 represents a schematic outlay of the transmission procedure. Each donor plant was placed wholly or partly (Fig 6.3) in an insect proof cage. Donor plants for episomal BSV included symptomatic tissue-cultured FHIA-21 plants: TC-10 (*P. citri*), TC-26 (*P. longispinus*), TC-5 (*D. brevipes*) and TC-12 (*P. ficus*) (see Table 6.1-6.4). IC-PCR and TAS-ELISA results confirmed these sources to harbour episomal BSV (section 6.3.2). Nearly identical absorbance values and IC-PCR results were obtained for these sources. Donor plants, serving as controls, included a corm of FHIA-21 (FH-4: no BSV symptoms, integrated BSV control) and healthy Williams.

Transmissions with each mealybug species were done separately. Individual mealybug colonies were placed on each donor source by placing a colonized butternut (for P. *citri, P. ficus* and *D. brevipes*) or colonized *Philodendron*-pieces (for *P. longispinus*) with sufficient numbers of mealybugs, on the donor plants (Fig 6.3). Mealybugs were allowed to crawl unto the donor plant leaves. Butternuts (or plant-pieces) were removed one day after placement on the donor plants. A total feeding period of 72h (3 days) was allowed from the time that the mealybug colony was first placed on the donor plant. Kubiriba *et al*., (2001b), showed this to be the peak time period for virus acquisition by *D. brevipes* mealybugs, while a 120h inoculation access period was the peak time determined for virus transmission.

6.2.4.2 Receptor plants and inoculation access period

For each individual donor source plant, leaves infested with 'viruliferous' mealybugs were carefully cut into smaller pieces with a sterile scissor. Ten individual Williams plants were used as receptors in each treatment. Receptor plants, representative of each treatment were labelled and placed on plastic blocks in a separate tray, filled with soapy water. A rough estimation of mealybugs on each donor leaf-piece was made, in order to ensure that equal amounts of mealybugs could be placed on each healthy receptor plant. The next day, all donor plant leaf-pieces were removed. A 120h-feeding period was allowed from the time that the fresh donor leaf pieces were placed on the receptor plants. Following this, the number of mealybugs found feeding on each receptor plant was counted; thereafter plants were sprayed with an insecticide to kill off all mealybugs (Fig 6.2).

6.2.4.3 Evaluation of BSV transmission to receptor plants

Three and six months after transmission, receptor plants were evaluated with TAS-ELISA [Annexure C] and IC-PCR in order to evaluate the potential transmission of episomal BSV-OL, BSV-RD and BSV-GF.

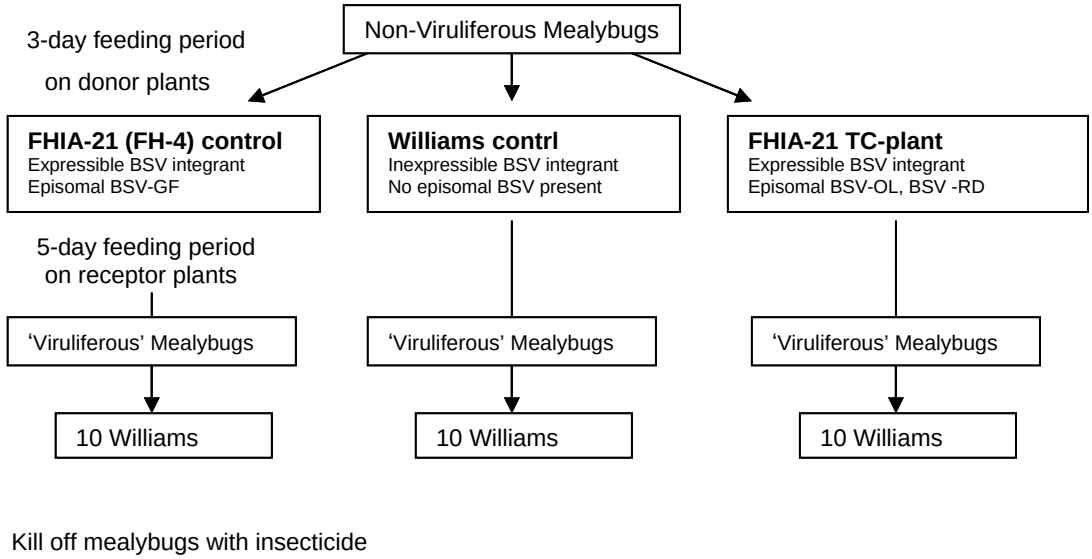

Fig 6.2: Schematic representation of the schedule utilized on individual mealybug species, in order to demonstrate the transmissibility of expressed-episomal BSV. The BSV status of each plant type is indicated in the block below the specific donor source. FH 4 = the corm with no tissue culture history, TC = FHIA-21 that was multiplied by tissue culture.

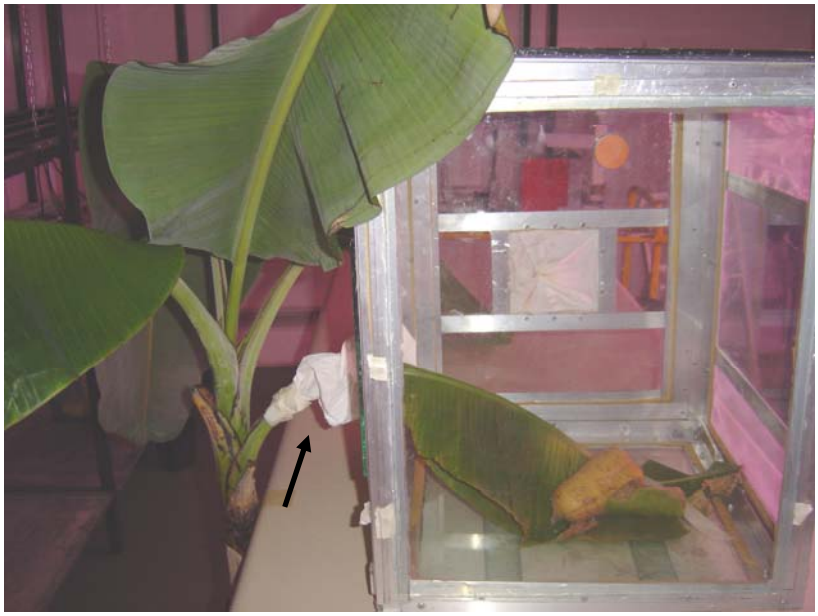

Fig 6.3: Photograph illustrating the establishement of non-viruliferous mealybugs on the FH-4 donor plant. A leaf of the plant was carefully slipped through an insect proof gauze-tunnel (arrow) built into the insect proof cage. The end of the gauze-tunnel, was closed off with masking tape on the plant petiole that was still connected to the donor plant. The figure also demonstrates how butternuts were placed on the leaf. After the acquisition access period the whole plant leaf (with feeding mealybugs) was cut off from the plant and cut to smaller pieces. Williams receptor plants onto which the leaf pieces were placed was kept in a separate quarantine chamber.

6.3 RESULTS AND DISCUSSION

6.3.1 BSV STATUS OF FHIA CORMS

Eight of the ten FHIA-21 corms were successfully established. The results of IC-PCR with the three strains of BSV are shown in Table 6.1 and Fig 6.4. Unexpectedly, one or more strain of episomal BSV was detected with IC-PCR in seven of the eight corms. IC-PCR results suggests that other factors in the field may have encouraged the activation of integrated BSV sequences, or that external sources, such as vector transmission, were responsible for BSV infection. This conclusion may be drawn from the fact that the number of different strains detected in the different corms varied, as did the absorbance values (OD 405nm).

With ISEM, bacilliform particles were only detected in two of the eight corms (FH-3 and FH-7). The absorbance values for corms FH-3 and FH-7 correlated with the ISEM results, while the value for FH-5 was also interpreted as positive. It is known that PCR is a more sensitive detection technique than ISEM or ELISA. Only one corm, FH-7 showed signs of BSD in the form of irregular yellow streaks on the leaf lamina. Dahal *et al*. (1998) recommended that symptomatic tissue be used for the detection of BSV. Because most of the FHIA-21 corm material was asymptomatic, it is possible that episomal particles occurred at levels below detection thresholds for ISEM and ELISA tests.

FH-1 tested negative for BSV-OL and GF, while FH-4 was negative for BSV-OL and BSV-RD (Table 6.1). FH-8 tested negative for BSV-RD, BSV-GF and BSV-OL, however in subsequent tests BSV-GF (Fig 6.7) and BSV-RD (Fig 6.8) was detected in this corm. Because it was determined that episomal BSV-RD and BSV-OL could be detected in the tissue cultured FHIA-21 (see results described below in section 6.3.2) it was decided to use FH4 as a control. The FH-4 corm remained negative for BSV-OL and BSV-RD during the time it was used for transmission studies and was still negative for these strains of BSV 20 months after their initial establishment.

Table 6.1: Results indicating presence of episomal BSV in FHIA-21 corms (with no history of *in vitro* propagation) with three different detection methods.

FHIA 21 Corms	**IC-PCR**			**ELISA**	**ISEM**
Corm Number	**BSV-OL**	**BSV-GF**	**BSV-RD**	**OD 405nm**	**Particles present**
FH-1	Negative	Negative	Positive	0.054	None
FH-2	Positive	Positive	Positive	0.052	None
FH-3	Positive	Positive	Positive	0.086	Yes
FH-4	Negative	Positive	Negative	0.058	None
FH-5	Positive	Positive	Positive	0.065	None
FH-6	Positive	Positive	Positive	0.063	None
FH-7	Positive	Positive	Positive	0.758	Yes
FH-8	Negative	Negative	Negative	0.058	None
CONTROLS					
Buffer	Negative	Negative	Negative	0.023	None
Healthy Williams	Negative	Negative	Negative	0.032	None
Positive control	Positive	Positive	Positive	1.092	Yes

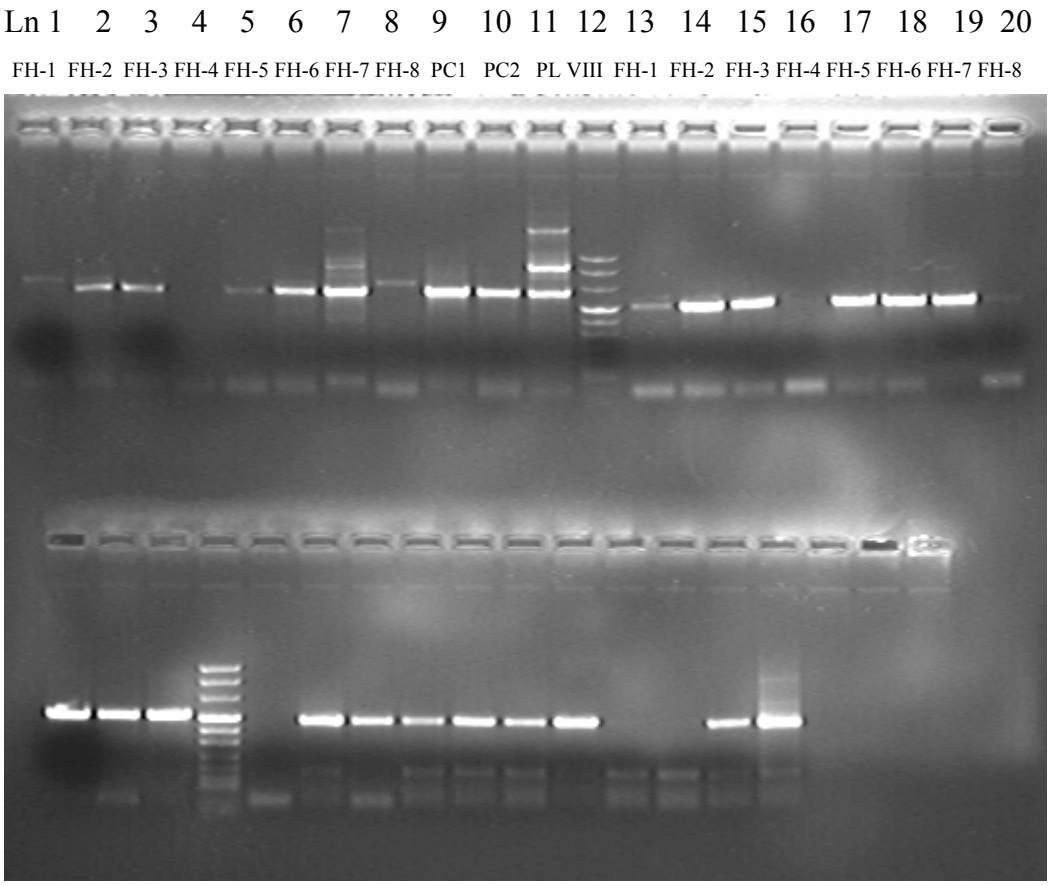

Fig 6.4: Ethidium bromide stained 1 % agarose gel showing IC-PCR products for BSV-OL. (Lanes (Ln) 1-11), BSV-RD (Ln 13-23), BSV-GF (Ln 25-35). Lanes 12 and 24 represents the marker (DNA molecular weight marker VIII, Roche Diagnostics). FH-1 to FH-8 represents the FHIA corms while PC1 and PC2 are positive controls. HC is a healthy control and PL is a DNA plasmid-control specific to the isolate.

6.3.2 BSV STATUS OF DONOR FHIA TC-DERIVED PLANTS

Sixty-seven tissue culture plants of FHIA-21 were successfully multiplied, rooted and established in pots with fertilized potting-soil. Four months later, nine out the sixty-seven rooted TC-plants had moderately to high titres for BSV (Fig 6.5) as determined by TAS-ELISA. Therefore, 13.4 % of the progeny was positively infected with episomal BSV after 3-4 subcultures. This correlates with findings of Folliot, *et al*. (2004) where 10 –20 % of progeny were infected with episomal BSV after 6-11 subcultures of tissue culture, depending on the cultivar. Cultivar sources, used for micro-propagation in their studies, were also negative for episomal BSV. In our study the BSV infected TC plants developed symptoms of BSD. This is quite unique as Lockhart (1995) states that BSD symptoms are also usually totally absent in plants derived from *in vitro* multiplication.

Samples with an absorbance value higher than that of the threshold value (0.064), was interpreted as positive. Plants number TC-5, TC-10, TC-12, TC-15, TC-26, TC-39, TC-43, TC-66 and TC-67 had an absorbance reading above the threshold value, with 0.162 (TC-67) being the lowest and 0.956 (TC-26) the highest absorbance values.

IC-PCR was performed on the nine samples that had an absorbance value that was well above that of the determined threshold value. The results confirmed the presence of episomal BSV in all the TC derived plant samples tested (Fig 6.6 and 6.8) Episomal BSV-OL (Fig 6.6) and BSV-RD (Fig 6.8) were detected by IC-PCR in all TC plants that tested positive in TAS-ELISA. One exception was TC-66 in which only BSV-OL and not BSV-RD, was detected.

The source material from INIBAP was declared to be virus free by various VIC's. Therefore both BSV-OL and BSV-RD sequences is likely to have originated from integrated BSV sequences in the FHIA-21 genome, with expression being triggered by the tissue culture process. According to Geering *et al*., (2000) BSV-RD and BSV-OL have virtually identical nucleotide sequences. Clones analyzed contained a sequence that covered part of open reading frame III and the intergenic region of the badnavirus genome. They found that the BSV-RD sequence differed by only two nucleotides from BSV-OL over a 1,292 bp range. However, throughout this work, these two strains were not always detected simultaneously in the same sample (also see Chapter 3). It may be that the BSV-RD or BSV-OL 'strain variants' in FHIA-21 and other cultivars may differ from each other in some instances, or that irregular detection with IC-PCR occurred.

BSV-GF was not detected in the TC derived plants, although extremely faint PCR products were obtained when evaluated on an ethidium bromide stained gel [Annexure E, section E.1.3], which was interpreted as negative results. BSV-GF was never detected in any of the receptor plants where a TC-derived plant was used as donor in the transmission (see section 6.3.6). The BSV-GF strain was detected in some of the FHIA-21 corms from Honduras at high intensities. It is uncertain whether the sequences found in the corms were triggered by other external stresses on the plant, or from vector transmission. The presence of a BSV-GF integrant has not been reported from FHIA-21. Some researchers believe that certain strains

of BSV have low activation rates, which may be the case for BSV-GF. Primers specific to BSV-GF was designed from BSV originating form the cultivar Goldfinger (Geering *et al.*, 2000), which probably harbours an expressible BSV integrant. This cultivar has been accepted as a commercial cultivar in Australia and does rarely display symptoms of BSD, supporting the idea that the integrant may have a low activation rate. In Kiepersol, South Africa, no BSD has been reported in plantations established with this cultivar, although episomal BSV is present (Chapter 3). Unfortunately no research has been done to show weather integrated BSV-GF occurrs in the cultivar FHIA-21.

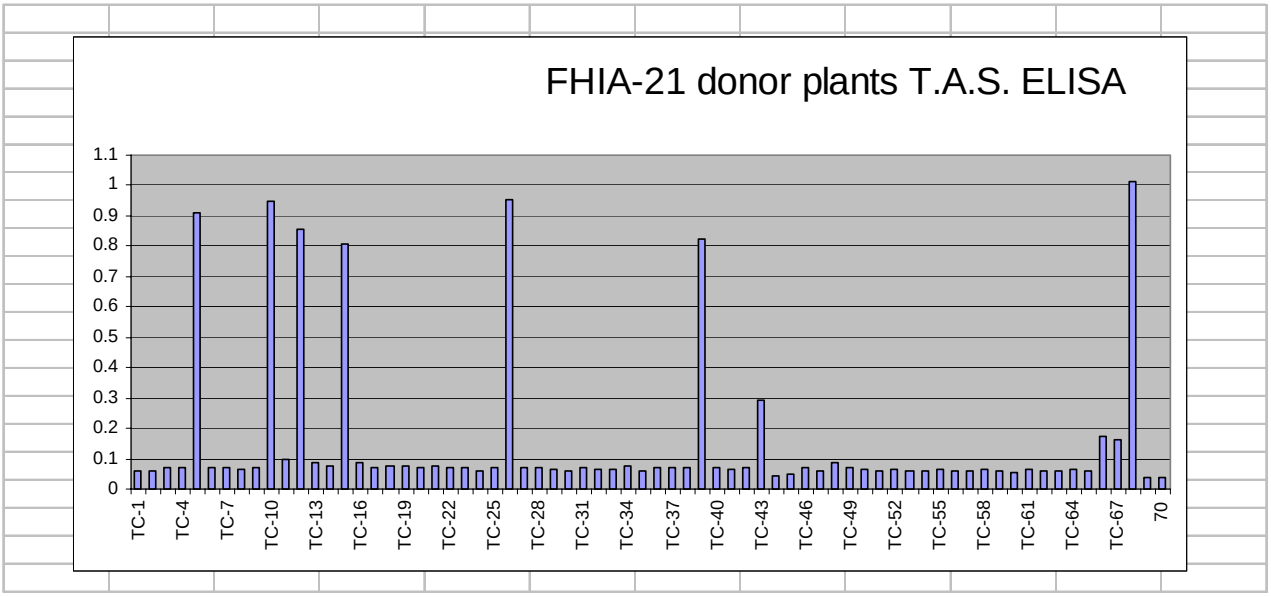

Fig 6.5: Absorbance values (OD-405nm) from TAS-ELISA on TC derived FHIA-21 plants measured four months after propagation. These values are presented by the Y-axis while plant samples are indicated on the X-axis. Plants number TC-5, TC-10, TC-12, TC-15, TC-26, TC-39, TC-43, TC-66 and TC-67 were interpreted as positive, with values more than two times that of the virus-free Williams control. The positive control is represented by Bar no 68 (1.015). Bar no 69 (0.040) represents the Healthy control and Bar no 70 (0.036) the buffer control.

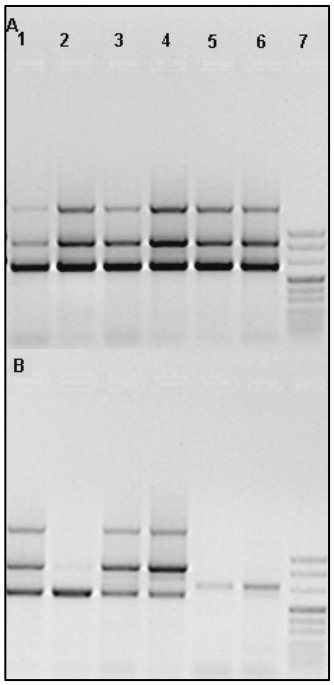

Fig 6.6: (Left) Photograph of an ethidium bromide stained agarose gel (1 %) showing IC-PCR amplicons of BSV-OL from TC derived FHIA-21 plants. The lowest band is representative of BSV-OL and is not present in the healthy control. Lane A1: TC-5, Lane A2: TC-10, Lane A3: TC-12, Lane A4: TC-15, Lane A5: TC-26, Lane A6: TC-39, Lane A7: Marker VIII, Lane B1: TC-43, Lane B2: TC-66, Lane B3: TC-67, Lane B4: episomal positive FHIA-21, Lane B5: virus-free Grand Nain, Lane B6: FH-8, Lane B7: Marker VIII.

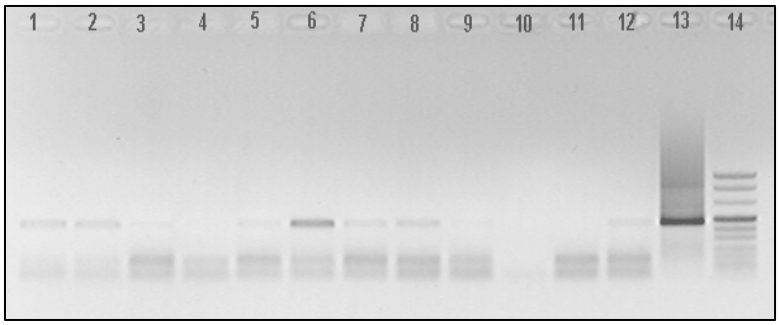

Fig 6.7: (Above) Photograph of an ethidium bromide stained agarose gel (1 %) showing IC-PCR products of BSV-GF. Lane 1: TC-5, Lane 2: TC-10, Lane 3: TC-12, Lane 4: TC-15, Lane 5: TC-26, Lane 6: TC-39, Lane 7: TC-43, Lane 8: TC-66, Lane 9: TC-67, Lane 10: Buffer control, Lane 11: virus-free Grand Nain, Lane 12: FH-8, Lane 13: Plasmid control, Lane 14: Marker VIII.

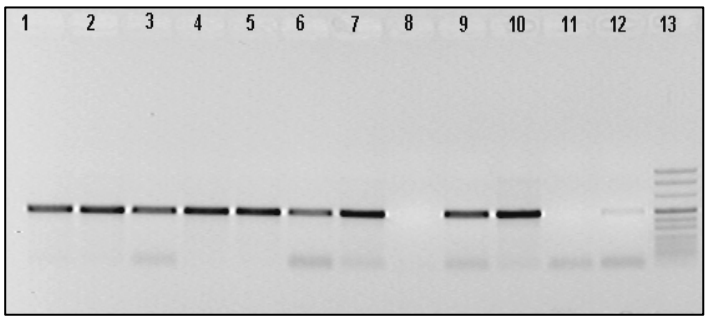

Fig 6.8: (Left) Photograph of an ethidium bromide stained agarose gel (1%) showing IC-PCR amplicons of BSV-RD in TC derived FHIA-21 plants. Lane 1: TC-5, Lane 2: TC-10, Lane 3: TC-12, Lane 4: TC-15, Lane 5: TC-26, Lane 6: TC-39, Lane 7: TC-43, Lane 8: TC-66, Lane 9: TC-67, Lane 10: Buffer control, Lane 11: virus-free Grand Nain, Lane 12: FH-8, Lane 13: Marker VIII.

6.3.3 VIRUS STATUS OF WILLIAMS RECEPTOR AND CONTROL PLANTS

With TAS-ELISA no BSV was detected in any of the batches (10 plants pooled) of Williams plants to be used as recipient and control plants (Fig 6.9). Tests were performed prior to transmission studies. Furthermore, no amplicons were obtained with IC-PCR, using the primer sets for BSV-OL, BSV-RD and BSV-GF.

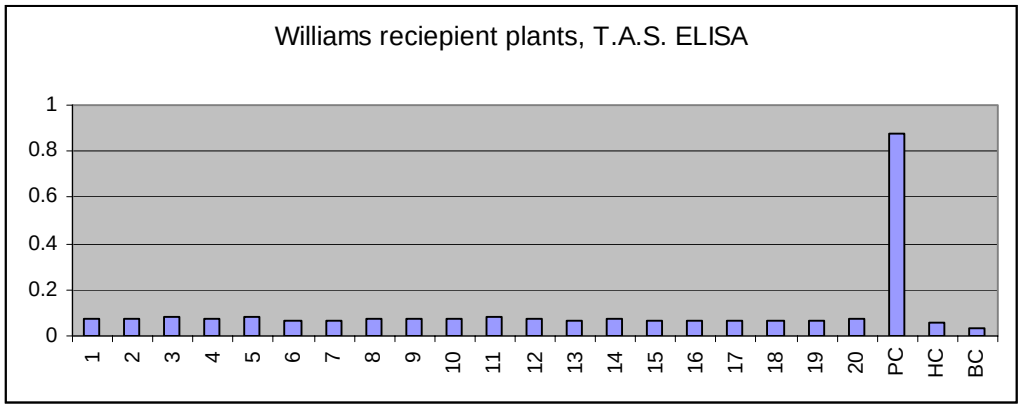

Fig 6.9: TAS-ELISA absorbance values (OD-405nm) of Williams plants to be used as recipient or control plants. PC = Positive control, HC = Virus free Grand Nain, BC = Buffer control.

6.3.4 CONFIRMATION OF ENDOGENOUS BSV IN FHIA 21

According to Dallot *et al.* (2001), FHIA-21 harbours an endogenous form of BSV. These researchers reported a band of the correct size from FHIA-21 material, after PCR amplification with the primers T32 and BSV510.2 (Ndoworra, 1998). Unfortunately Dallot *et al.* (2001) did not provide any sequence data for the amplicons obtained by using these primers, while Ndowora (1998) investigated a hybrid cross from Obino L'Ewai and Calcutta-4. Therefore, no sequence data for endogenous BSV in FHIA-21 is available. Lockhart *et al.* (1998) reported some cultivars to harbour expressible integrants from analysis of enzyme digested products of the DNA from endogenous and episomal BSV in the same cultivar. Results for digested products of endogenous BSV and purified episomal BSV DNA form FHIA-21, showed episomal BSV to be virtually identical to the integrated BSV (Lockhart *et al.*, 1998). Although the work done in this study was a slight repeat of previously reported data, it was decided to verify the identity of the endogenous BSV-strain in FHIA-21. This was done by cloning of PCR products and sequencing thereof. PCR, bands of the correct size, were obtained from FHIA-21 (TC plant with undetectable episomal BSV) total DNA

with the primers T32 and BSV510.2. After transformation, white bacterial colonies (transformed) were obtained, together with blue colonies (untransformed cells). Quick screening of six individual clones revealed that only two of the clones (Clone 1-3 and 1-6) harboured transformants. Because it was a large insert (+1400bp), transformation occurs at a lower rate than with smaller inserts. EcoR1 digests of purified plasmid revealed that the two transformants had different band sizes (Fig 6.11) and it was decided to sequence both. Blasting of the sequences revealed that Clone 1-3 had a significant alignment with the "*Musa* x *paradise* clone *Musa* 6 Banana streak virus" (AF106946.1) and "Banana streak virus OL" (AJ002234.1). All other clones and PCR products sequenced and analyzed in this study (with a molecular weight lower than 1400 bp) did not have significant alignments. After compiling a consensus sequence from the forward and reverse sequences, obtained for Clone 1-3, and removing sequences corresponding to the pGEM-T plasmid and primers, a sequence alignment was made using the software DNAMAN, Version 5.2.9 (Lynnon Boost, Canada) and is presented in Fig 6.13. From the alignment, it is evident that an endogenous sequence, corresponding to clone *Musa* x *paradise* clone *Musa* 6 Banana streak virus, sequenced by Ndowora (1998) form the cultivar Obino'LEwai, was found in the FHIA-21 genome. *Musa* sequences, which does not correspond to the sequence of BSV-OL, is found in Clone 1-3 because the first primer (T32) (Ndowora *et al*., 1999) was designed to target integrated BSV within the *Musa* genome. The second primer (BSV 510.2) specifically targets BSV-OL. Therefore, the sequence of Clone 1-3 aligns with BSV-OL where the sequence is found occurring within the genome of FHIA-21 (Fig 6.13).

PCR products were also obtained from DNA of FH-4 and TC-1 with the primers T32 and BADNAT1 (Fig 6.12). A band close to +1400bp was obtained for both; unfortunately these bands were not sequenced. PCR with the primers for BSV-OL and BSV-RD on plant sap (for integrated BSV) of FH-4 and TC-1 (with no episomal BSV-OL and BSV-RD), did however give high concentration amplicons for BSV-RD (Fig 6.10) and weaker amplicons for BSV-OL.

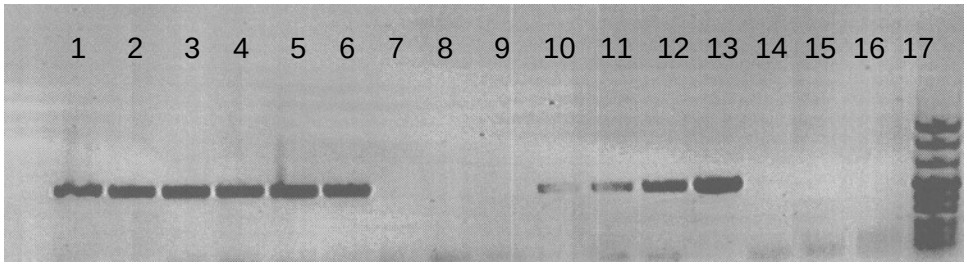

Fig 6.10: (Above) Gel photograph of a 1% agarose gel stained with ethiduim bromide showing amplicons for BSV-RD after performing PCR on plant sap samples. Lanes 1-3: TC-5, Lanes 4-6: TC-1, Lane 7-9: Virus free Williams, Lanes 10-12: FH-4 corm, Lane 13: Plasmid contol, Lane 14-15: HC, Lane 16: Buffer control, Lane 17: Marker VIII.

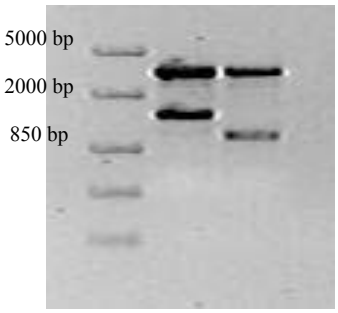

Fig 6.11: (Left) Gel photograph of purified pGEM-T plasmids digested with EcoR1 to reveal the sizes of DNA inserts. Lane 1: Molecular marker (Fermentas, SM 1113), Lane 2: Clone 1-3, Lane 3: Clone 1-6. Clone 1-3 was the correct size insert (+1400bp).

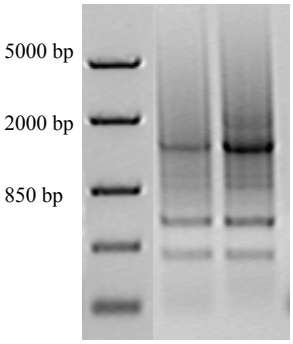

Fig 6.12: (Left) Gel photograph of PCR products obtained by using primers T32 and BADNAT1. Lane 1: Molecular marker (Fermentas, SM 1113), Lane 2: FH-4 DNA, Lane 3: FHIA-21 TC1 plant DNA.

```
TC1-3      ............................TGGCTTATGA     10
AF106946   cttctaaactcctctccacctacttgcatca---------    363
BSV-OL     nanngnnananananatnatgnagnnnnnnnnn--nnnnnnn  1880

TC1-3      TGCTGACCACAT.ACCCCAACTGCATAGTTGAACAAGGGC     49
AF106946   ------------.--------------------------    402
BSV-OL     nnnnnnnn-nnnngannnnnnnnnnnnnnnnnnnnnnnnt   1920

TC1-3      ATGCCACTGAATAAATGAGGCTAACAATAACAAAAGCATA     89
AF106946   -----------------------------------------    442
BSV-OL     nnnnnnnannnnnnnnnnn-nnnnnnnnnnnnnnnt-nnn-n  1960

TC1-3      AGGTTACTCGTCTCTACTTTTTCAAGTTCCTCTCCGAGAT    129
AF106946   -----------------------------------------    482
BSV-OL     nanangnngtnnnnnnnnnnnnnnnnnnngt-tnnna-nn-   2000

TC1-3      GCTAGCCAAGATTTAATTTAACTGCATAGTTGAACAAGGG     169
AF106946   -----------------------------------------    522
BSV-OL     nnnnn-tnntn-annnnnnnnnnnn-nt-nan-nnnnnnn   2040

TC1-3      CATGCCACTGAATAAATGAGGCTAACAATAACAGAAGCAT     209
AF106946   -----------------------------------------    562
BSV-OL     nn-nnnngn-nnnnn-nnnnnnnntngn--nntntnnnnn    2080

TC1-3      AAGGGTACTCGTCTCTACTCTTTCAAGTCCCTCTCCAGGA     249
AF106946   -----------------------------------------    602
BSV-OL     nncnnn-nnnnngnnnn-nn-nnngnnnnnnnnng-nnn     2120

TC1-3      CGCTGTCCAAGATTTAATTTTCTTTCTTATACTAGGTGAC     289
AF106946   -----------------------------------------    642
BSV-OL     tnnn-nnnnnnnnngnnnnnnnnnncncn--gnn-nn-n     2160

TC1-3      TGAACTGGACTTCAGTTGATTCCATATCTTTTATGAATTT     329
AF106946   -----------------------------------------    682
BSV-OL     ncnnnnnnnnnnnnnnnnnnnngnnnnnngnnnnnnnnnn    2200

TC1-3      TATCAATATATTCTTCTGAAGAAATACCAAGCAATTGATG     369
AF106946   -----------------------------------------    722
BSV-OL     nnnnn-nnnnn-nnnnn-nnnnnnnnnnnnnnnnnnnnn     2240

TC1-3      TTCCTGTTCCTAAATATTTGGATTCCAGGATGCTGCCCAA     409
AF106946   -----------------------------------------    762
BSV-OL     gnnnnnnnnnnnnnnnnncnnnnnnnntnn-n-na-gnn-    2280

TC1-3      GATTCAATTTATCTTTCTTATGCTAGGTGATTCACCTGGA     449
AF106946   -----------------------------------------    802
BSV-OL     annnnnncnn..-nn-ncnncnnnnnnnn-nnnnn-nnn     2318

TC1-3      TTTCAGTTGACTTGATTCCATATCTTTTAGGAGTTTTATC     489
AF106946   -----------------------------------------    842
BSV-OL     nnnnnnnnnnanannnnnnntnnannnnnnnnnnnnnnnn    2358

TC1-3      AATATACCCTTCTCAAGAAATACCAAGCAACTAATGTTCC     529
AF106946   -----------------------------------------    882
BSV-OL     nnnnnnnnnnnnntnnt-nnnnnnnnnnnnnnnnnnnnn     2398

TC1-3      TCTTCCTAAATATTTGGATTCCAAGCATTCCAAGATTTAT     569
AF106946   -----------------------------------------    922
BSV-OL     nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn     2438

TC1-3      CTATATACCCTTCTCAAGAAATACCAAGCAAGTAATTCTA     609
AF106946   -----------------------------------------    962
BSV-OL     nn-nnnnnnnnnn-nnnnnnnnnnnnannn.-g--n-n-    2477

TC1-3      GGGATGAAGGCTTTGTAACTCAGCATGTTTCACACATGAA     649
AF106946   -----------------------------------------   1002
BSV-OL     nnnnnnngnnnnannnnnnngnngn-nnnnntn-gnnnng    2517

TC1-3      CGATGGAATTGCAGTATTCGAAAATTAANATGTGACCTTA     689
AF106946   ----------------------------a-----------   1042
BSV-OL     nnnnnnnn-nnnnnn-ngnnnnngnnnn-tn-nnnnngnn    2557

TC1-3      CATTATTCACATATGATGTGAAAAGG.......ACTCATT     722
AF106946   ------------------------a-gacttca-t--c--   1082
BSV-OL     tnnnnn-tnn-nnnnnnnnnnnnnnnnnnnnnnn-aannn    2597

TC1-3      CGTCATATTGATTTATACTACAA.CTTTTTCTCTCTTTTT     761
AF106946   -a-t--t-gat------------a----------------   1122
BSV-OL     n-nnnnnnnnnnnngnnnnngnn...gnnnnnnnnnnnnn    2634

TC1-3      TAAAGACTCCAAGTACAAGAAAAACATGAAATACAAAAAT     801
AF106946   -----------------------------------------   1162
BSV-OL     cnnnnnnnnnnnnnnnnnnntntnnn-n-nnnnnnnnnn    2674

TC1-3      AAGAAAGGATCACAATTACATCAAAGTGAATCAATTTTAT     841
AF106946   -----------------------------------------   1202
BSV-OL     nnnnnnnnn-t-nnnnngtnnnnnt-nnnngnnnnnnnnn    2714

TC1-3      ACTCTTCCTTCTTCGAATAAAATAGATTTCTATATCTGAT     881
AF106946   -----------------------------------------   1242
```

Fig 6.13: Diagram of the sequence alignment of Clone 1-3 (TC1-3), "*Musa* x *paradise* clone *Musa* 6 Banana streak virus" (AF106946) and BSV-OL (BSV-OL). The alignment shows Clone 1-3 to be an endogenous clone of BSV as it stretches from within the *Musa* genome into the endogenous region of BSV-OL (in Bold, next page).

```
BSV-OL      nnnnn-nnnnnnnn-nn-nnnnngn-nnnnnnnnnn.-nn   2753

TC1-3       TTGTAAAATGTTTCTCCTTCTTTATGATATATTTAGTCGA    921
AF106946    ----------------------------------------   1282
BSV-OL      nnannnnnnnn-annanngnnnnnnnnngnnnan-nnnnn   2793

TC1-3       TAGATTTCCATCTCTCTAGTATAATGAAACTTCAATCTTC    961
AF106946    ----------------------------------------   1322
BSV-OL      nnncnngtnnnnannnnnnnnnnn-nnnnnnnnnnn-n-    2833

TC1-3       TATAACTTTCAATCGANCCAAGTTCTGATTTTCTTTTTCT   1001
AF106946    ----------------a-----------------------   1362
BSV-OL      nnngnnnnntnnnn-t-nnnnngatn-nnnnnnnnnnnnn   2873

TC1-3       TCGTTGTCTTCTTTTTTCTTCTTTTTGGATAATCTAGAAA   1041
AF106946    ----------------------------------------   1402
BSV-OL      nnnnnnnnnnnnngnnnnngnnnnannnnnnngnnn-nnn   2913

TC1-3       TTAGATGTTGCCAAGTGGCAACTTATGATATTTACAAAAT   1081
AF106946    ----------------------------------------   1442
BSV-OL      antnnn-nnnnntnnnnnnnnnnnnnannnnnnnnnnn-n   2953

TC1-3       ACCTCTTAACTAAACAAATTTATTCAAACTAGAAACAAAG   1121
AF106946    ----------------------------------------   1482
BSV-OL      nnnntngcnngnggncnnn-nnnnnnnnnnc-n-ngnnt   2993

TC1-3       ATATTTTATTTCTGAAATATCTTGCGTAAATCTTATTATT   1161
AF106946    ----------------------------------------   1522
BSV-OL      nnnn-nn-nnnannnnnnnnnnnnnnnnnnnnn-nnn-nn   3033

TC1-3       ACATATAGTTGTAGGAATCTTTTAAGGAGATAGATAAATT   1201
AF106946    ----------------------------------------   1562
BSV-OL      nnnnnnnnnnnnnnnnnan-nnntgt-nnnnnnnnngnnn   3073

TC1-3       GCATTAGATGGTCTGGGAAATAAAGGTACTTCATCCTTTT   1241
AF106946    ----------------------------------------   1602
BSV-OL      nn-n-nnnnnnnnnnnnnnnnngnnnn-nnnnnnngn-na   3113

TC1-3       CCTACAGGTTGCACCACAACGCGAGTTTACTCCTGATTTG   1281
AF106946    ----------------------------------------   1642
BSV-OL      nnnnnnnnnnnnnnnnngnnnnnnnn-nnnnnnncgcn     3153

TC1-3       AGAAATAAAAACTTCTGTGCTTGAAACACACTTTGTGCGA   1321
AF106946    ----------------------------------------   1682
BSV-OL      nn-nnnnnntnnnnnnnnncntntcntnnnnnnnnnnn     3193

TC1-3       GTTCACTTTGTGCGAGTAGAGCGCAAGATCCTAGTTCCGC   1361
AF106946    ----------------------------------------   1722
BSV-OL      nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnannnnnnnnan  3233

TC1-3       GAGCGTAGACCCGTCTGGTATCAGAGCAAGGTTCGTTTTT   1401
AF106946    ----------------------------------------   1762
BSV-OL      nnnnnnnnnnnn-nn-------------------------   3273

TC1-3       ATGGC.....................................   1406
AF106946    -----tttcatggggtaattcctttagataggagccgaag   1802
BSV-OL      -----tttcatggggtaattcctttagataggagccgaag   3313
```

6.3.5 TAXONOMIC STATUS OF MEALYBUGS BY PCR

Species-specific amplicons of 456bp (Fig 6.14) and 474bp (Fig 6.15) were obtained for *P. longispinus* and *P. citri* respectively. An amplicon for *P. citri* was only obtained at an annealing temperature of 55°C. Because of intellectual property rights, PCR confirmation of *P. ficus* was performed at UP using primers of undisclosed sequence. Correct size PCR amplicons confirmed the molecular status of the colony.

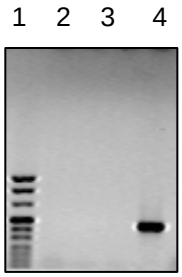

Fig 6.14: Agarose gel showing a correct size amplicon (456bp), from PCR with *P. longispinus*-specific primers. Lane 1: Molecular marker VIII, Lane 2: Buffer control, Lane 3: Isolated S. *sacchari* DNA, Lane 4: Isolated *P. longispinus* DNA.

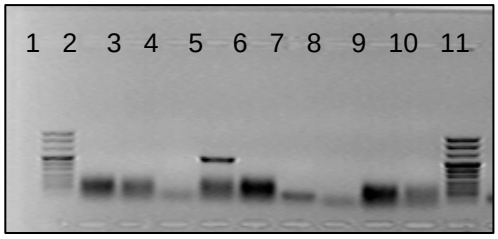

Fig 6.15: Agarose gel showing a correct size amplicon from PCR with *P. citri*-specific primers. Lane 1 and 11: Molecular marker VIII, Lane 2: Isolated *P. citri* DNA at annealing temperature 65°C, Lane 3: Isolated *P. ficus* DNA at annealing temperature 65°C, Lane 4: Buffer control at annealing temperature 55°C, Lane 5: Isolated *P. citri* DNA at annealing temperature 55°C, Lane 6: Isolated *P. ficus* DNA at annealing temperature 55°C, Lane 7: Buffer control at annealing temperature 55°C, Lane 8: Isolated *P. citri* DNA at annealing temperature 45°C, Lane 6: Isolated *P. ficus* DNA at annealing temperature 45°C, Lane 7: Buffer control at annealing temperature 45°C.

6.3.6 EVALUATION OF BSV TRANSMISSION TO RECEPTOR PLANTS

For all transmissions TAS-ELISA and IC-PCR tests were performed on the 10 Williams receptor plants in each treatment three - and six months after the inoculation access period. Only data obtained from tests performed three months after transmission is presented here, as results obtained for the the six months period dit not vary from the three months results. The necessary controls were included for both tests.

6.3.6.1 Transmission with *P. citri*

The results for the transmission of episomal BSV with *P. citri* are summarized in Table 6.2.

TC-10 donor to Williams receptor plants: In the 72-hour vector acquisition access period, TC-10 served as the donor source plant for activated-episomal BSV. Successful transmission of episomal BSV with *P. citri* was proven with TAS-ELISA and IC-PCR. Results showed that this vector was very efficient in transmitting episomal BSV as each of the ten Williams receptor plants, to which transmission was made from the TC-10 donor, tested positive with TAS-ELISA (Table 6.2). Moreover, infection was obtained even when a few mealybugs were counted on the receptor plants at the end of the inoculation access period. As little as two mealybugs were efficient in transmitting the virus from the donor plant to the receptor plant. All the TAS-ELISA absorbance values of infected Williams receptor plants were very similar (Table 6.2). Being rather constant, this absorbance value does not seem to correlate with the amount of mealybugs counted on each plant at the end of the inoculation access

period. One can speculate that the virus must have rapidly multiplied in the Williams tissue, even when there were possibly less episomal BSV particles present during the initial vector inoculation. For instance, after three months the absorbance value of the receptor plant, which was infected by only two mealybugs, was very similar to the receptor plant on which 25 mealybugs fed. The absorbance value for the plant infected by 2 mealybugs was 1.824 and that of the plant on which 25 mealybugs fed 1.733. Interestingly, the Williams receptor plants developed BSD symptoms already in the second month after sucsessful transmission. The donor plant TC-10 from which transmission was done not only had a high absorbance value (Fig 6.5), but also showed symptoms of BSD. This probably provided that mealybugs aquired a high virus concentration during feeding on the symptomatic TC-10 plant.

IC-PCR confirmed the TAS-ELISA results as all ten Williams receptor plants tested positive for BSV-OL and BSV-RD (Table 6.2). These BSV-strains were also present in the original TC-10 source. BSV-GF was not transmitted to the receptor plants, as episomal infection of this strain was not present in the TC-10 donor.

FH-4 corm control donor to Williams receptor plants: The FH-4 corm had a very low absorbance value for episomal BSV as determined by TAS-ELISA. In fact, in all TAS-ELISA tests that this corm was tested, absorbance values never reached a value above that of the threshold value (twice that of the healthy plant control). As stated previously, episomal BSV-GF was present in the FH-4 corm, while no episomal BSV-OL of BSV-RD was present. Therefore, no transmission of BSV-OL or BSV-RD was obtained. However, successful transmission from this donor to five of the ten receptor Williams plants was demonstrated. This means that even when the episomal BSV was present at levels below detection (measured by ISEM and ELISA) the vector was still efficient enough to transmit the virus to 50 % of the receptor plants. IC-PCR results confirmed a 50 % transmission rate from the FH-4 donor to the Williams receptor plants. As with the TC-10 transmission, no apparent correlation could be made between the amounts of mealybugs used in the transmission and absorbance values. In one instance where eight mealybugs fed on a particular receptor plant to transmission of the virus was obtained, while ten mealybugs failed to infect anotoher receptor plant. However, higher mealybugs counts did seemed to slightly correlate with higher absorbance values obtained in receptor plants. Here, the uneven distribution of the virus within the donor plant may have played a definite role in the infection process. It may be that some mealybugs used in the transmission process, failed to

obtain any episomal BSV, when feeding on parts of the FH-4 donor with low virus titres. TAS-ELISA values obtained for the infected Williams receptor plants remained low three (Table 6.2) and six months (repetitive data, not shown) after testing. Low absorbance values may indicate that BSV-GF does not multiply in plants as rapidly as BSV-OL and BSV-RD does. This will explain why cultivars, such as Goldfinger does not readily display BSD symptoms. However, BSD symptoms were observed on the infected Williams receptor plants in the first three months after the transmission was performed. Therefore host species may also have an influence on BSV-strain type expression.

Results further indicate that no integrated sequences of could be BSV transferred. This means that the meaybugs probably does not transmit any plant DNA between plants, or that the plant sap acquired during feeding is present in insignificant amounts. Enzymatic reactions may play a role and the insect feeding process is probably not ideal for the preservation of any plant DNA, hence integrated BSV.

Williams control donor to Williams receptor plants: No expressible integrant of BSV and no episomal BSV existed in the Williams control donor. Therefore, no transmissions were expected and none was obtained. This control together with the FH-4 corm control (no BSV-OL and BSV-RD) confirms that no cross-contamination of melaybugs between donor sources occurred.

Table 6.2: Summary of TAS-ELISA absorbance values and IC-PCR results obtained in Cavendish receptor plants, three months after the transmission of episomal BSV with *P. citri.*

Cavendish (Williams) receptor plants					
[A] Source of Transmission	Number of mealybug	TAS-ELISA OD 405nm	PCR BSV-GF	PCR BSV-OL	PCR BSV-RD
FHIA CORM 4	15	0.429	Positive	Negative	Negative
FHIA CORM 4	10	0.102	Negative	Negative	Negative
FHIA CORM 4	4	0.169	Negative	Negative	Negative
FHIA CORM 4	8	0.310	Positive	Negative	Negative
FHIA CORM 4	8	0.379	Positive	Negative	Negative
FHIA CORM 4	10	0.162	Negative	Negative	Negative
FHIA CORM 4	8	0.138	Negative	Negative	Negative
FHIA CORM 4	6	0.133	Negative	Negative	Negative
FHIA CORM 4	30	0.511	Positive	Negative	Negative
FHIA CORM 4	8	0.398	Positive	Negative	Negative
Williams VF	10	0.153	Negative	Negative	Negative
Williams VF	20	0.142	Negative	Negative	Negative
Williams VF	20	0.137	Negative	Negative	Negative
Williams VF	20	0.141	Negative	Negative	Negative
Williams VF	20	0.146	Negative	Negative	Negative
Williams VF	30	0.119	Negative	Negative	Negative
Williams VF	15	0.142	Negative	Negative	Negative
Williams VF	15	0.144	Negative	Negative	Negative
Williams VF	25	0.137	Negative	Negative	Negative
Williams VF	30	0.134	Negative	Negative	Negative
FHIA TC 10	5	1.854	Negative	Positive	Positive
FHIA TC 10	8	1.817	Negative	Positive	Positive
FHIA TC 10	3	1.963	Negative	Positive	Positive
FHIA TC 10	8	1.153	Negative	Positive	Positive
FHIA TC 10	2	1.824	Negative	Positive	Positive
FHIA TC 10	4	1.890	Negative	Positive	Positive
FHIA TC 10	4	1.756	Negative	Positive	Positive
FHIA TC 10	15	1.780	Negative	Positive	Positive
FHIA TC 10	25	1.733	Negative	Positive	Positive
FHIA TC 10	10	1.623	Negative	Positive	Positive
Controls/ Donor plants					
PC 99/2722	N/A	1.742	Positive	Positive	Positive
HC 94/1609	N/A	0.141	Negative	Negative	Negative
FHIA TC VF	N/A	0.139	Negative	Negative	Negative
FHIA CORM 4	N/A	0.169	Positive	Negative	Negative
FHIA TC 5	N/A	1.882	Negative	Positive	Positive
THRESHOLD	N/A	0.278	N/A	N/A	N/A

A) FHIA CORM 4 = FHIA-21 corm that has never been through tissue culture, received from Honduras, Williams VF = Virus free Williams tissue culture plant, FHIA TC 10 & TC 5 = FHIA-21 tissue culture derived plant from virus free germplasm collection, Belgium (INIBAP). PC 99/2722 is a BSV infected plant (infected with strains of OL, GF and RD). HC 94/1609 = Williams Healthy virus-free plant, FHIA TC VF = A tissue culture derived plant of FHIA-21 originating from virus free germplasm, Belgium (INIBAP).

6.3.6.2 Transmission with *P. longispinus*

The results for the transmission of episomal BSV with *P. longispinus* are summarized in Table 6.3. TAS-ELISA and IC-PCR tests were performed on the receptor plants in each treatment three (Table 6.3) and six months (data not shown) after the inoculation access period, with the appropriate controls included. Although TC-26 (donor), which was used in the acquisition access period, had a high absorbance value for episomal BSV (Fig 6.5), no transmission from this source was obtained. Similarly, no virus transmission was obtained from the FH-4 corm. Even with meaybug counts as high as 12 to 35 no virus transmission was obtained. None of the TAS-ELISA values, determined for the Williams receptor plants used in each treatment, were above the threshold value (two times that of the healthy-plant control). IC-PCR also confirmed that no episomal BSV-RD, BSV-OL (from the TC-plant) and BSV-GF (FH-4 corm) were transferred to the Williams control plants.

Table 6.3: Summary of TAS-ELISA absorbance values and IC-PCR results obtained in Cavendish receptor plants, three months after the transmission of episomal BSV with *P. longspinus*.

Cavendish (Williams) receptor plants					
[A] Source of Transmission	Number of mealybug	TAS-ELISA OD 405nm	PCR BSV-GF	PCR BSV-OL	PCR BSV-RD
FHIA CORM 4	18	0.092	Negative	Negative	Negative
FHIA CORM 4	15	0.125	Negative	Negative	Negative
FHIA CORM 4	30	0.125	Negative	Negative	Negative
FHIA CORM 4	5	0.097	Negative	Negative	Negative
FHIA CORM 4	18	0.087	Negative	Negative	Negative
FHIA CORM 4	30	0.111	Negative	Negative	Negative
FHIA CORM 4	30	0.108	Negative	Negative	Negative
FHIA CORM 4	8	0.098	Negative	Negative	Negative
FHIA CORM 4	20	0.104	Negative	Negative	Negative
FHIA CORM 4	7	0.097	Negative	Negative	Negative
Williams VF	10	0.092	Negative	Negative	Negative
Williams VF	15	0.100	Negative	Negative	Negative
Williams VF	25	0.089	Negative	Negative	Negative
Williams VF	15	0.092	Negative	Negative	Negative
Williams VF	10	0.117	Negative	Negative	Negative
Williams VF	35	0.084	Negative	Negative	Negative
Williams VF	15	0.091	Negative	Negative	Negative
Williams VF	15	0.113	Negative	Negative	Negative
Williams VF	15	0.075	Negative	Negative	Negative
Williams VF	4	0.082	Negative	Negative	Negative
FHIA TC 26	10	0.158	Negative	Negative	Negative
FHIA TC 26	10	0.170	Negative	Negative	Negative
FHIA TC 26	7	0.178	Negative	Negative	Negative
FHIA TC 26	1	0.150	Negative	Negative	Negative
FHIA TC 26	6	0.164	Negative	Negative	Negative
FHIA TC 26	12	0.176	Negative	Negative	Negative
FHIA TC 26	1	0.145	Negative	Negative	Negative
FHIA TC 26	12	0.153	Negative	Negative	Negative
FHIA TC 26	5	0.169	Negative	Negative	Negative
FHIA TC 26	5	0.136	Negative	Negative	Negative
Controls/ Donor plants					
PC 99/2722	N/A	1.743	Positive	Positive	Positive
HC 94/1609	N/A	0.121	Negative	Negative	Negative
FHIA TC VF	N/A	0.138	Negative	Negative	Negative
FHIA CORM 4	N/A	0.140	Positive	Negative	Negative
FHIA TC 12	N/A	1.714	Negative	Positive	Positive
THRESHOLD	N/A	0.276	N/A	N/A	N/A

A) FHIA CORM 4 = FHIA-21 corm that has never been through tissue culture, received from Honduras, Williams VF = Virus free Williams tissue culture plant, FHIA TC 26 & TC 12 = FHIA-21 tissue culture derived plant from virus free germplasm collection, Belgium (INIBAP). PC 99/2722 is a BSV infected plant (infected with strains of OL, GF and RD). HC 94/1609 = Williams Healthy virus-free plant, FHIA TC VF = A tissue culture derived plant of FHIA-21 originating from virus free germplasm, Belgium (INIBAP).

6.3.5.3 Transmission with *D. brevipes*

The results for the transmission of episomal BSV with *D. brevipes* are summarized in Table 6.4.

TC-5 donor to Williams receptor plants: TC-5 was used as the donor source for this transmission. Although the young mealyubug crawlers were very mobile, adults of the colony used in this study, moved extremely slowly. The mealybugs appeared to more adverse to light than other species used in this study. The mealybugs were slow to climb off from the pieces of donor plant that were placed on the receptor plants after the acquisition access period. Therefore the experiment was repeated and mealybugs were rather carefully placed on the leaves by using a soft paintbrush. Due to the immobility of the mealybugs, lower numbers were found to survive and actually feed during the inoculation access period. TAS-ELISA and IC-PCR revealed that the virus was transmitted to two out of the ten Williams receptor plants. Positive transmissions were obtained with four and seven mealybugs. A higher absorbance value at OD 405 nm was obtained for the transmission with the four mealybugs (1.044) than with the seven mealybugs (0.791). The BSV-OL strain was not detected in the Williams receptor plant on which four mealybugs were counted, while both BSV-RD and BSV-OL was transmitted with the seven meaybugs. Again this indicates that the concentration of the BSV-OL and –RD variants may differ from each other in FHIA-21.

FH-4 corm control donor to Williams receptor plants: No episomal BSV was transmitted from this donor to the receptor plants. Although seven mealybugs transferred the virus from TC-5 to Williams, they failed to transmit the virus from the FH-4 donor. A much lower absorbance value was determined for FH-4 (Table 6.1) than TC-5 (Fig 6.5). Therefore chances of BSV-GF transmission from the FH-4 donor were probably lower.

Williams control donor to Williams receptor plants: As expected, no transmissions were obtained.

Table 6.4: Summary of TAS-ELISA absorbance values and IC-PCR results obtained in Cavendish receptor plants, three months after the transmission of episomal BSV with *D. brevipes.*

Cavendish (Williams) receptor plants					
[A] Source of Transmission	Number of mealybug	TAS-ELISA OD 405nm	PCR BSV-GF	PCR BSV-OL	PCR BSV-RD
FHIA CORM 4	3	0.049	Negative	Negative	Negative
FHIA CORM 4	7	0.022	Negative	Negative	Negative
FHIA CORM 4	2	0.043	Negative	Negative	Negative
FHIA CORM 4	2	0.044	Negative	Negative	Negative
FHIA CORM 4	2	0.043	Negative	Negative	Negative
FHIA CORM 4	5	0.043	Negative	Negative	Negative
FHIA CORM 4	4	0.044	Negative	Negative	Negative
FHIA CORM 4	2	0.046	Negative	Negative	Negative
FHIA CORM 4	3	0.022	Negative	Negative	Negative
FHIA CORM 4	4	0.052	Negative	Negative	Negative
Williams VF	4	0.044	Negative	Negative	Negative
Williams VF	2	0.041	Negative	Negative	Negative
Williams VF	2	0.049	Negative	Negative	Negative
Williams VF	3	0.049	Negative	Negative	Negative
Williams VF	3	0.043	Negative	Negative	Negative
Williams VF	2	0.021	Negative	Negative	Negative
Williams VF	2	0.055	Negative	Negative	Negative
Williams VF	2	0.041	Negative	Negative	Negative
Williams VF	2	0.041	Negative	Negative	Negative
Williams VF	2	0.045	Negative	Negative	Negative
FHIA TC 5	4	0.038	Negative	Negative	Negative
FHIA TC 5	3	0.038	Negative	Negative	Negative
FHIA TC 5	2	0.037	Negative	Negative	Negative
FHIA TC 5	3	0.043	Negative	Negative	Negative
FHIA TC 5	8	0.036	Negative	Negative	Negative
FHIA TC 5	3	0.015	Negative	Negative	Negative
FHIA TC 5	3	0.038	Negative	Negative	Negative
FHIA TC 5	2	0.034	Negative	Negative	Negative
FHIA TC 5	7	0.791	Negative	Positive	Positive
FHIA TC 5	4	1.044	Negative	Negative	Positive
Controls/ Donor plants					
PC 99/2722	N/A	0.269	Positive	Positive	Positive
HC 94/1609	N/A	0.045	Negative	Negative	Negative
FHIA TC 10	N/A	0.836	Negative	Positive	Positive
FHIA CORM 4	N/A	0.204	Positive	Negative	Negative
FHIA TC 39	N/A	1.041	Negative	Positive	Positive
THRESHOLD	N/A	0.090	N/A	N/A	N/A

A) FHIA CORM 4 = FHIA-21 corm that has never been through tissue culture, received from Honduras, Williams VF = Virus free Williams tissue culture plant, FHIA TC 5 & TC 39 = FHIA-21 tissue culture derived plant from virus free germplasm collection, Belgium (INIBAP). PC 99/2722 is a BSV infected plant (infected with strains of OL, GF and RD). HC 94/1609 = Williams Healthy virus-free plant, FHIA TC VF = A tissue culture derived plant of FHIA-21 originating from virus free germplasm, Belgium (INIBAP).

6.3.6.4 Transmission with *P. ficus*

Results obtained for TAS-ELISA and IC-PCR are summarized in Table 6.5.

TC-12 donor to Williams receptor plants:

The TC-12 donor plant was used in the acquisition access period. In this treatment *P. ficus* transmitted episomal BSV to eight of the ten receptor plants. Very high numbers of mealybugs were used for transmission and counts of 7 to 60 transmitted the virus (Table 6.5). No apparent correlation seems to exist in the relationship between the mealybug counts and absorbance values recorded, as fairly high absorbance values was obtained for all the infected receptor plants. However, 3 and 4 mealybugs failed to transmit the virus, indicating a possible correlation between numbers of mealybugs used and actual viral transmission. This is the first time that transmission of BSV was demonstrated with this mealybug vector.

IC-PCR supported the TAS-ELISA results. BSV-OL and BSV-RD was successfully transmitted to eight and seven of the receptor plants respectively. BSD symptoms were observed in the second month after vector transmission (Fig 6.16).

FH-4 corm control donor to Williams receptor plants: BSV-GF was transmitted from the FH-4 corm to three of the ten receptor plants. Eighteen, 11 and 5 mealybugs were counted on these plants at the end of the inoculation access period. TAS-ELISA results revealed absorbance values more than three times that of the healthy-plant control. Absorbance values recorded form the receptor plants of the FH-4 transmission, were much lower than that of the absorbance values obtained from receptor plants to which BSV form TC-12 were transmitted. The same tendency was observed in the *P. citri* transmissions.

Williams control donor to Williams receptor plants: No expressible integrant of BSV and no episomal BSV existed in the Williams control donor. Therefore, no transmissions were expected and none was determined with both TAS-ELISA and IC-PCR.

Table 6.5: Summary of TAS-ELISA absorbance values and IC-PCR results obtained in Cavendish receptor plants, three months after the transmission of episomal BSV with *P. ficus*.

Cavendish receptor plants					
[A] Source of Transmission	**Number of melaybugs**	**TAS-ELISA OD 405nm**	**PCR BSV-GF**	**PCR BSV-OL**	**PCR BSV-RD**
FHIA CORM 4	23	0.048	Negative	Negative	Negative
FHIA CORM 4	10	0.039	Negative	Negative	Negative
FHIA CORM 4	19	0.036	Negative	Negative	Negative
FHIA CORM 4	26	0.036	Negative	Negative	Negative
FHIA CORM 4	11	0.134	Positive	Negative	Negative
FHIA CORM 4	18	0.038	Negative	Negative	Negative
FHIA CORM 4	18	0.163	Positive	Negative	Negative
FHIA CORM 4	5	0.189	Positive	Negative	Negative
FHIA CORM 4	11	0.031	Negative	Negative	Negative
FHIA CORM 4	6	0.037	Negative	Negative	Negative
Williams VF	4	0.040	Negative	Negative	Negative
Williams VF	26	0.042	Negative	Negative	Negative
Williams VF	27	0.038	Negative	Negative	Negative
Williams VF	20	0.034	Negative	Negative	Negative
Williams VF	12	0.032	Negative	Negative	Negative
Williams VF	4	0.038	Negative	Negative	Negative
Williams VF	24	0.036	Negative	Negative	Negative
Williams VF	24	0.030	Negative	Negative	Negative
Williams VF	5	0.034	Negative	Negative	Negative
Williams VF	10	0.045	Negative	Negative	Negative
FHIA TC 12	45	0.977	Negative	Positive	Positive
FHIA TC 12	7	0.860	Negative	Positive	Positive
FHIA TC 12	60	0.509	Negative	Positive	Positive
FHIA TC 12	4	0.033	Negative	Negative	Negative
FHIA TC 12	19	0.837	Negative	Positive	Positive
FHIA TC 12	3	0.040	Negative	Negative	Negative
FHIA TC 12	35	1.015	Negative	Positive	Negative
FHIA TC 12	51	0.831	Negative	Positive	Positive
FHIA TC 12	44	1.059	Negative	Positive	Positive
FHIA TC 12	38	0.807	Negative	Positive	Positive
Controls/ Donor plants					
PC 99/2722		0.269	Positive	Positive	Positive
HC 94/1609		0.039	Negative	Negative	Negative
FHIA TC 10		0.877	Negative	Positive	Positive
FHIA CORM 4		0.215	Positive	Negative	Negative
FHIA TC 39		1.016	Negative	Positive	Positive
THRESHOLD	N/A	0.078	N/A	N/A	N/A

A) FHIA CORM 4 = FHIA-21 corm that has never been through tissue culture, received from Honduras, Williams VF = Virus free Williams tissue culture plant, FHIA TC 12, TC 10 & TC 39 = FHIA-21 tissue culture derived plant from virus free germplasm collection, Belgium (INIBAP). PC 99/2722 is a BSV infected plant (infected with strains of OL, GF and RD). HC 94/1609 = Williams Healthy virus-free plant.

Fig 6.16: Photographs showing BSD symptoms on Williams receptor plants to which episomal BSV were transmitted by *P ficus*. Left: FHIA 4 donor (Plant no. 8; BSV-GF), Middle: TC 5/TC 12 donor (Plant no. 1; BSV-OL and BSV-RD), Right: Williams donor (Plant no. 8; no BSV present).

6.4 CONCLUSIONS

The aim of this study was to show whether episomal BSV, originating from expressed integrated BSV sequences, could be transmitted by common mealybug species. Therefore, tissue culture stress was used to activate expressible-integrated sequences of BSV, resulting in 13.4 % of episomally infected progeny. Although the virus sources (TC plants used as donors of episomal BSV in the transmissions) were different, they originated from the same germplasm accession while absorbance values were very similar. Furthermore, the conditions in which transmissions were performed were artificially manipulated to standardize the procedure. In hindsight, it probably would have been better to have used there sources in combination, in stead of as single plants, for transmissions. Nonetheless, some valuable conclusions could be drawn from this study. Three out of the four mealybugs species (of which the identities were confirmed taxonomically as well as molecularly), was proven to transmit BSV. Transmission of episomal BSV was demonstrated for the first time with the vector *P. ficus*, while the transmission of episomal BSV with *D.* brevipes was confirmed. *P. ficus* does not readily occur on *Musa* however, it is one of the species that has been recorded on *Ensete ventricosum* (Family: *Musaceae*) in Ethiopia (Williams and Matile-Ferrero, 2000). *P. ficus* also occurs in some other counties in Africa, including Angola, Egypt, Libiya, Sudan and South Africa (Watson and Kubiriba, 2005). *P. citri* and *P. ficus* were proven to be very efficient vectors transmitting episomally activated BSV-OL and BSV-RD from TC derived plants to Cavendish. *P. citri* was able to transmit BSV to 100 % of the receptor plants while transmission by *P. ficus* was to 80 % of the receptor plants. As little as two *P. citri* mealybugs were sufficient to transmit the 'activated' episomal BSV to Williams, while the

BSV reached high titres after only 3 months in the hosts (as seen in the TAS-ELISA absorbance values, Tables 6.2 – 6.5). High absorbance values for episomal BSV transmitted from TC derived plants to Williams were obtained with all three vectors that were able to transmit BSV. Banana streak disease symptoms were observed on all the Williams receptor plants in which transmitted episomal BSV was detected.

Although *D. brevipes* transmitted BSV to only 20 % of the progeny, results of Kubiriba *et al.* (2001b) are now confirmed. *P. longispinus* was the only vector shown not to transmit BSV. The efficiency of *P. ficus* and *P. citri* to transmit episomal BSV was highlighted by the fact that successful transmission of BSV-GF from the FH-4 corm was obtained. Episomal BSV-GF was present in this corm at levels under the detection level thresholds for TAS-ELISA and ISEM but and yet these vectors were able to transmit the virus, with rates as high as 30 % (*P. ficus*) and 50 % (*P. citri*). Furthermore, this corm does not show any physical symptoms of BSD. This is an important finding as the BSV-GF strain was found in localities sampled in the Kiepersol area of Mpumalanga (Chapter 3 of this thesis) on symptomless tetraploid *Musa* varieties. Low absorbance values obtained for this strain of BSV may indicate that this strain does not multiply as readily in *Musa* or that the antisera used in the TAS ELISA does not detect BSV-GF at the same levels as BSV-OL and BSV-RD. However, when the ELISA was developed, a high absorbance reading of 1.108 was obtained for a *Musa* host (99/2726) in which BSV-GF alone was detected (Chapter 4, Table 4.9). However, it is possible that strains other than BSV-OL and BSV-RD occurred in 99/2726, thus causing the high value.

Dallot *et al*. (2001) showed that FHIA-21 harbours an integrant of BSV by using one set of primers that was designed by Ndowora (1998). These primers were designed against the BSV-OL type strain of BSV derived from a hybrid between Obino L'Ewai and Calcutta 4. Using the same primers a PCR fragment of +1400bp was obtained. Sequencing of the cloned fragment showed BSV-OL to be an integrant that is present in the genome of FHIA-21. Other cloned fragments cloned and sequenced (data not presented), did not correspond to any other BSV strain types. One cannot exclude the possibility that integated BSV-GF does occur in the FHIA-21 genome, however activation of this BSV-strain from TC may be extremely low. To test for integrated BSV-OL and BSV-RD in the FH-4 corm, PCR was performed with the primers for BSV-OL and BSV-RD on plant sap of FH-4. High concentration amplicons for BSV-RD and weaker amplicons for BSV-OL was obtained. This finding

supports the possibility that integranted BSV-OL occurs in the genome of FHIA-21, especially because BSV-RD is considered as a strain variant of BSV-OL. The fact that none of the four mealybug species were able to transmit integrated BSV-OL from the FH-4 corm to Williams, proves that the integrated form of BSV is not likely to be transmitted by mealybugs. Even highly efficient mealybugs such as *P. citri* were unable to transfer any integrated BSV to the receptor plants.

From the results obtained in this study, one has to highlight the fact that episomal BSV in tissue cultured derived tetraploids, is highly transmissible by very efficient mealybug vectors to Cavendish varieties. However, South Africa is in a fortunate position, as our climatic conditions do not seem to favour Banana streak disease expression. Furthermore, no evidence of spread of BSV from B genome plantations to commercial Cavendish varieties has been recorded (Chapter 3). It is also fortunate that no aggressive strains of BSV have been reported in South Africa thus far. In conclusion, transmission of episomally activated BSV by mealybugs remains very effective, therefore, growers need to monitor these insects in their plantations carefully and proceed planting new varieties with endogenous BSV sequences that have high BSV activation rates, with caution. Further recommendations are listed in Appendix 1 of this thesis.

CHAPTER 7

7. INCORPORATION OF AN INTERNAL CONTROL FOR BSV IC-PCR

CHAPTER 7

7. INCORPORATION OF AN INTERNAL CONTROL FOR BSV IC-PCR

7.1 INTRODUCTION

The presence of interfering substances in extraction buffers used for viruses may sometimes lead to an inhibitory reaction in certain detection methods such as the polymerase chain reaction (PCR). The addition of an internal control system to a PCR reaction, may serve as an indication that the sample was actually added to the PCR reaction. Nassuth *et al.* (2000), reported on the use of several internal plant controls in the development of multiplex RT-PCR reactions for certain grapevine viruses. One of the plant controls reported in their study involves a molecule called *ribulose-biphosphate carboxylase oxygenase* (Rubisco). Rubisco is a key enzyme in plant photosynthesis (Calvin Cycle) and accounts for 16 % of the protein content of chloroplast cells. Sequences, encoding for Rubisco, of various plant species are available on the Genbank Database. Primers based on the conserved region of the large subunit of Rubisco, derived from sequnces of several plant species, were designed by Nassuth *et al.* (2000) (Table 7.1).

As BSV endogenous sequences are found in the genome of most *Musa* hosts, it remains important to distinguish between episomal and integrated forms of BSV. Immunocapture (IC)-PCR, of episomal BSV is a detection method in which actual viral particles are 'trapped' with specific antibodies. This is a useful technique for determining the presence of Banana streak disease (BSD), caused by episomal BSV. IC-PCR is usually performed in a single PCR tube, to which antibodies are bound on the inner tube surface. However, several steps, such as washing the tubes with buffer between antiserum and sample incubation steps, are needed before PCR is performed. These washing steps are employed in order to eliminate all traces of plant material. BSV-specific antiserum is also crucial in this procedure. The use of antiserum serves as a measure by which plant debris (with may harbour integrated BSV) is furthermore eliminated from the PCR reaction. Although these steps (antiserum addition and washing) are employed, a slight chance always exists that traces of plant material may end up in the PCR reaction, especially when large amounts of samples are being processed. PCR is a fairly sensitive method and if primers recognize a sequence (even if just a trace amount is present) numerous copies of the recognized sequence can be multiplied (Old and Primrose, 1994).

As mentioned, a multiplex PCR is useful to indicate that a "plant" sample has been added to a PCR reaction. In this study, we wished to incorporate such a control for the opposite reason – ensuring that no plant material was present in the reaction. Such a control would therefore serve as a measure of validating that no integrated BSV sequences (present in plant material) were detected in IC-PCR. In their studies Nassuth *et al.* (2000) tested the Rubisco primers on other plant species for which the sequence information of the Rubisco gene-region was unknown. A correct size fragment was obtained for several of these other plant species such as raspberry, strawberry, wheat ext. It was decided to incorporate this control into the existing BSV IC-PCR systems (Table 7.2) available.

7.2 MATERIALS AND METHODS

7.2.1 Multiplex PCR conditions

Two detection systems were performed; one without the presence of antibodies (only PCR) and another with BSV-specific antibodies employed (IC-PCR). Rubisco primers RBCL-H535 and RBCL-C705 (Table 7.1) were selected because of the small sized amplicon that were obtained from PCR amplification with these primers (Nassuth *et al*., 2000). Expected sizes of amplicons for BSV detection (Table 7.2) range from between 476 and 644 bp, depending on the BSV strain to be detected. BADNA 1A and BADNA 4 are degenerate primers of BSV reported by Geering *et al*. (2005b). Other primers were able to detect specific strains or variants of BSV such as BSV-OL (BSV 5317 and BSV 4673), BSV GF (BSV GF F1 and BSV GF R1) and BSV RD (BSV RDF1 and BSV RD R1) (Geering *et al.*, 2000). Thus, an unmistakable distinction between plant (Rubisco) and BSV amplicons was expected.

Table 7.1 Primers designed against a conserved region of the large subunit of Rubisco.

Published primer name	Sequence: 5'	Tm	Product size (bp)
RBCL-H535	CTTTCCAAGGCCCGCCTCA	61.17	171
RBCL-C705	CATCATCTTTGGTAAAATCAAGTCCA	55.62	
RBCL-H680	TGGACTTGATTTTACCAAAGATGATG	55.62	642
RBCL-C1321	TGTCCTAAAGTTCCTCCACC	54.83	

Table 7.2: BSV-specific primers used in combination with Rubisco primers.

Primer name	Sequence: 5'	Product size (bp)
BADNA 1A	CTN TAY GAR TGG YTN GTN ATG CCN TTY	+/- 600
BADNA 4	TCC AYT TRC ANA YNS CYC CCC ANC C	
BSV GF F1	ACG AAC TAT CAC GAC TTG TTC AAG C	476
BSV GF R1	TCG GTG GAA TAG TCC TGA GTC TTC	
BSV RD F1	ATC TGA AGG TGT GTT GAT CAA TGC	522
BSV RD R1	GCT CAC TCC GCA TCT TAT CAG TC	
BSV 5317	AGT CAT TGG GTC AAC CTC TGT CCC	644
BSV 4673	GGA ATG AAA GAG CAG GCC	

A *Musa* sample containing several species of BSV (Acc: 99/2722 containing BSV-GF, BSV-OL and BSV-RD, Meyer and Pietersen, unpublished results) were macerated in BSV-extraction buffer [Annexure A] using a mortar and pestle. Carborundum powder was added to the mortar, in order to ensure a fine consistency of the macerated sample. The ratio of sample to buffer was 1:10 (w/v) and 0.5µl of the mixture was directly used in tubes containing 25µl of a PCR master mixture was added (see following paragraph). The principle of IC-PCR, as reported by Harper *et al.*, (1999a) was followed, while the PCR reaction was modified. In IC-PCR, tubes to which 50µl of Goat 1F Bleed 2 (diluted 1:8000) [Annexure B, section B.1.1] antiserum were added, was incubated for a period of four hours at 30°C. Tubes with antiserum were washed 3 times with PBST [Annexure A]. Fifty microlitres of the macerated BSV sample were added to each BSV-antibody coated tube, followed by another incubation of 4 hours at 30°C. After 3 washes with PBST, the PCR master mixture was added. A known negative control was included in each PCR system tested.

For each BSV-specific primer pair, a separate PCR master mixture was prepared, while the same PCR conditions were followed. The reaction mixture for each 25 µl reaction contained 2.5 µl 10 x NH_4 Buffer, 2 µl of 50 mM $MgCl_2$, 175 µM of each dNTP, 6 µl of each BSV specific primer (10pmol), 0.2µM of each Rubisco specific primer (10pmol) and 0,15 µl of

0.5 U Biotaq^{TM} DNA Polymerase (BIOLINE, UK). PCR was performed in a PCR-Express thermoclycler (Hybaid). Thermocycler conditions were the following: denaturation at 94°C for 2 min, 40 cycles of denaturation at 94^{o}C for 45 seconds, annealing at 54^{o}C for 60 seconds and elongation at 72^{o}C for 60 seconds. The reaction was ended with a final elongation step at 72^{o}C for 5 min. Resulting PCR-amplicons were viewed on a 1 % agarose gel stained with ethidium bromide [Annexure E, section E.1.3].

7.3 RESULTS AND DISCUSSION

For all PCR reactions, amplicons of the correct sizes were obtained (Fig 7.1 to 7.4) with a clear distinction between those that indicate the presence of a BSV strain and the plant material (Rubisco). A Rubisco amplicon of 171bp was obtained in all reactions performed. Amplicons obtained for BSV-OL, BSV-RD and BSV-GF was 644bp, 522bp and 476bp respectively. The amplicon obtained with the degenerate primers of BSV was 600bp. An annealing temperature of 54°C was sufficient for amplification of all BSV strains, as well as the accompanying Rubisco gene. The Rubisco primers, that was designed from of the Rubisco large subunit in plant cells (Nassuth *et al.*, 2000), seems to react to all plant species tested so far. In our study these primers (RBCL-H535 and RBCL-C705) were tested for the first time on *Musa*, showing that the Rubisco conserved region to be included in the genomic makeup of this host.

In their study Nassuth *et al.* (2000) recommended that the viral primers be five times more concentrated than the Rubisco primers (5:1). These concentrations were however not sufficient for our reactions (data not shown) and the concentration ratio of BSV: Rubisco primers were slightly increased to 5:1.67. This ratio could be increased for the multiplex PCR reaction with the BSV-degenerate primer pair (BADNA 1A and BADNA 4) as very slight amplicons of the correct size (Fig 7.4) were obtained for the Rubisco primers. This may also be done for BSV-RD (primers BSV RD F1 and BSV RD R1) although a slightly stronger reaction was obtained for the Rubisco amplicon (Fig 7.1). For BSV-OL and BSV-GF the applied Rubisco primer concentration gave satisfactory results (Fig 7.2 and 7.3).

With IC-PCR, where no plant material should be present as it is washed away, no amplicons for the Rubisco primers were obtained. The DNA amplicon control from a previous PCR reaction with the BSV-specific primers also contained no Rubisco bands because of the absence of plant material. These results indicate that the Rubisco primers could serve as

useful indicators for the presence of plant material in PCR. The internal control furthermore indicates the absence of plant material in IC-PCR reactions, proving that only desired episomal BSV were amplified. The same thermocycler conditions were proven to be sufficient in the detection for more than one strain of BSV. Therefore, this is a very useful technique as more than one strain of BSV could be screened for, using the exact same thermocycler conditions.

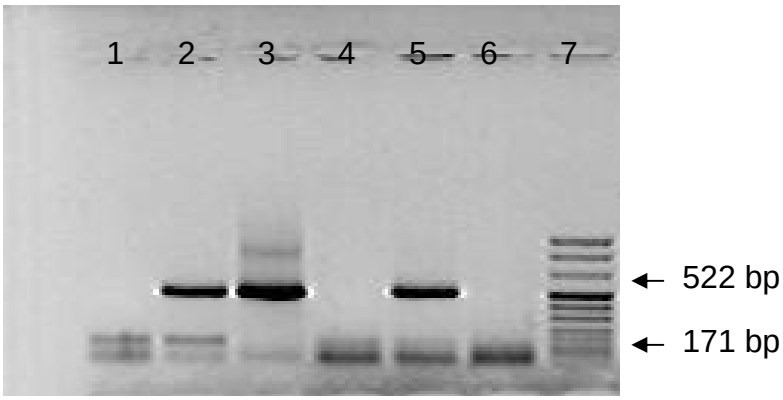

Fig 7.1: Gel photograph of amplicons obtained from a multiplex PCR with Rubisco and BSV-RD primers. Lane 1: Virus free control, Lane 2: BSV sample, Lane 3: DNA amplicon control, Lane 4: IC-Virus free control, Lane 5: IC-BSV sample, Lane 6: Buffer control, Lane 7: Molecular Marker VIII.

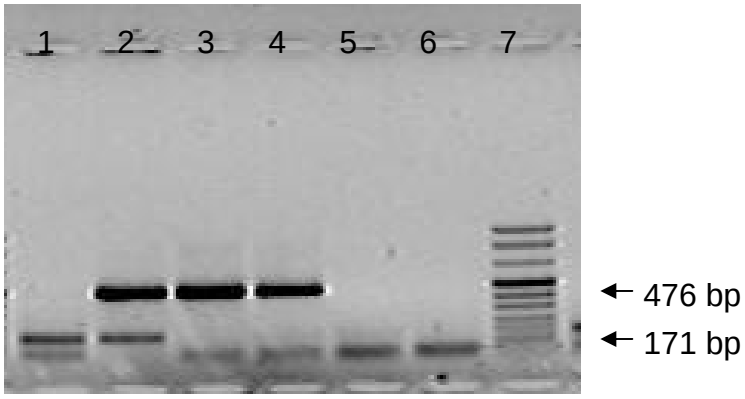

Fig 7.2: Gel photograph of amplicons obtained from a multiplex PCR with Rubisco and BSV-GF primers. Lane 1: Virus free control, Lane 2: BSV sample, Lane 3: DNA amplicon control, Lane 4: IC-BSV sample, Lane 5: IC-Virus free control, Lane 6: Buffer control, Lane 7: Molecular Marker VIII.

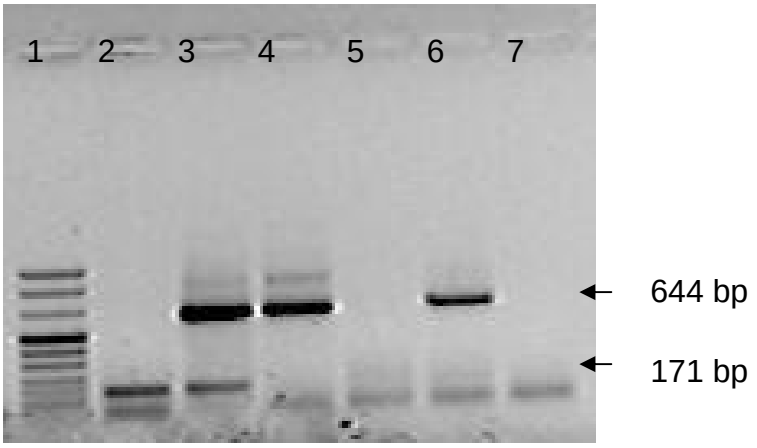

Fig 7.3: Gel photograph of amplicons obtained from a multiplex PCR with Rubisco and BSV-OL primers. Lane 1: Molecular Marker VIII, Lane 2: Virus free control, Lane 3: BSV sample, Lane 4: DNA amplicon control, Lane 5: IC-Virus free control, Lane 6: IC-BSV sample, Lane 7: Buffer control.

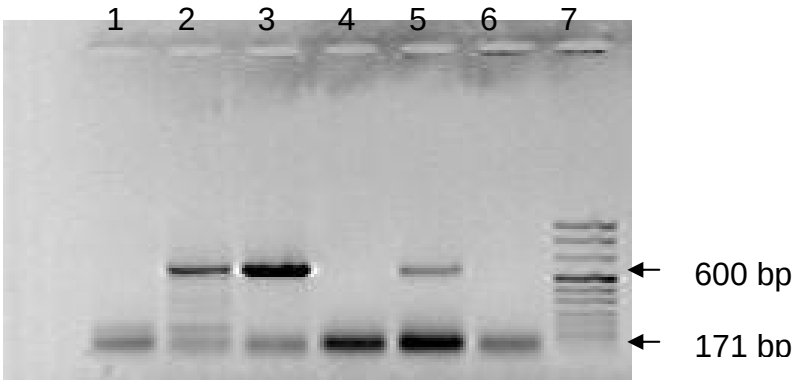

Fig 7.4 Gel photograph of amplicons obtained from a multiplex PCR with Rubisco and degenerate BSV primers. Lane 1: Molecular Marker VIII, Lane 2: Virus free control, Lane 3: BSV sample, Lane 4: DNA amplicon control, Lane 5: IC-Virus free control, Lane 6: IC-BSV sample, Lane 7: Buffer control.

7.4 CONCLUSION

Nassuth *et al*. (2000) showed plant internal controls to be useful for the indication of plant material to be present within a PCR reaction. In this study, Rubisco primers were used for the opposite reason. By using Rubisco primers one could clearly distinguish between PCR samples to which plant material was added and those where no plant material were present. This PCR would therefore be useful in order to ensure that only episomal BSV is detected in IC-PCR reactions, with no endogenous BSV (plant material) present. However, the IC-PCR step remains essential for trapping episomal BSV particles, while rinsing of the tubes with PBST is also needed. The Rubisco primers could easily detect the Rubisco large subunit gene in *Musa* and could therefore serve as a control in other *Musa* virus detection systems in order to indicate the absence or presence of plant material in PCR reactions.

APPENDIX 1

LIST OF FINDINGS FROM WHICH SPECIFIC GUIDELINES FOR BSV CONTROL CAN BE FORMULATED

1. The following strains/variants of BSV occur in plantations as integrated and/or episomal forms in South Africa – BSV-OL, BSV-RD and BSV-GF.
2. Unidentified strains of BSV may occur, however it seems highly unlikely, based on the enforcement of existing quarantine measures as well as a lack of BSD disease in local plantations. All imported material has been virus indexed, while existing tissue culture laboratories makes use of virus indexing practices. Furthermore, ISEM, wich would pick up other BSV variants, is one of the methods used in the of indexing imported material. PCR with degenerate Badnavirus primers can be used to determine if uncharacterized strains of BSV does occur.
3. However, there is little doubt that existing BSV strains have entered the country by means of imported material. The occurrence of episomal BSV is thought to have its origin from integrated (endogenous) sequences of BSV found in the genome of *Musa* cultivars, rather than from external sources. One of the stress factors that contribute to the activation of endogenous sequences of BSV is tissue culture (Dallot *et al.*, 2001). Other environmental factors and plant stresses may also play a role.
4. It is likely that indexing authorities might not have detected BSV in very young propagation or tissue culture material as a six-month period in quarantine is employed before explants are generated by tissue culture.
5. Tetraploid and B genome *Musa* were identified as material to harbor endogenous sequences that may become episomal (Geering *et al.*, 2001a; Leureux, *et al.,* 2003).
6. In this study episomal BSV was only detected in B genome *Musa*, while all A-genome *Musa* tested negative.
7. From observations, Banana streak disease symptoms do not occur under normal field conditions in South Africa. This may be because of environmental factors, such as temperature, as well as good management practices. Lockhart (1995) also states: "*Symptom expression is also usually totally lacking in plants derived from in vitro multiplication*".
8. BSV is restricted to hosts in the *Musa* and *Ensete* families. Experimental transmission of BSV to *Musa textilis* and genera related to *Heliconia* and *Strelitzia* has been

unsuccessful (Lockhart, 1995). Therefore control strategies such as removal of infected plants, would easily be feasible if an epidemic (highly unlikely) might occur.

9. Mealybugs have been found to be very efficient vectors of episomal BSV experimentally (Chapter 6 of this thesis); with three out of four vectors tested, being able to transmit BSV.
10. Episomal BSV was transmitted from plants showing no BSD symptoms and low absorbance values (determinate by TAS-ELISA and IC-PCR) by *P. ficus* and *P. citri*.
11. Fortunately, no evidence of BSV spread could be found in any of the plantations surveyed in this study.
12. According to the observations of growers in the sub-tropical areas such as Nelspruit, Kiepersol, Tzaneen and Burgershall, mealybugs are not a regular insect-pest of banana. Mealybug infestations have been observed near Stanger in Kwa-Zulu Natal (personal observation). And on banana plants kept in tunnels at the ARC-ITSC in Nelspruit (Mieke Daneel, personal communication).
13. Furthermore, no evidence of plant-to-plant spreading of BSV was observed in a trail done by Daniells *et al.* (2001) in Australia, although one incidence of the spread of episomal BSV-Cav was found from a nearby commercial plantation. In this case it was 5 % infection in a plantation of 179 plants, monitored over a two-year period.
14. In their conclusions Daniells *et al.* (2001) stated that rouging of infected plants would seem appropriate in controlling the occasional and limited outbreaks of BSV-Cav. They furthermore concluded that it would not be necessary to destroy a plantation in which a large outbreak of BSV-Cav has occurred, as proper management of an infected plantation may still provide an economically acceptable return, for at least one season of the crop.
15. Generally, *Musa* with episomal BSV in their genome, have been present in South African plantations for more that six years without any reports of disease symptoms as a result of BSV infection. Therefore, there does not seem to be a need for the removal of such plantations.
16. Growers and Industry need to be informed and educated on BSD and the risk that B genome cultivars may present.

17. Generally, the majoraty of plantations in South Africa were and still are established with A genome-cultivars, propagated by tissue culture and indexed for known *Musa* viruses. This in itself minimizes the occurrence of BSV in South Africa.
18. Finally, good management practices, rouging of occasionally diseased plants (if necessary) and strict mealybug control (if necessary), would seem sufficient in the prevention and management of BSD in southern Africa.

APPENDIX 2

SUMMARIZED DESCRIPTION OF LOCATIONS, INDICATING SITES SAMPLED AS WELL AS *MUSA* SELECTIONS SAMPLED AT EACH SITE.

LOCATION 1: BURGERSHALL RESEARCH STATION					
Site A. Block A9 East					
Planting date/ Age	**Plant density (m)**	**Layout**	**Selections sampled**	**Sample size***	**Surrounded by selections**
01/03/1996	2.5 x 2	Statistical 23 Selections 3 Replicates	**FHIA 17 (AAAA; Honduras)**	10	Williams (AAA), Grand Nain (AAA), Lancefield (AAA), Gross Michael (AAA), KBC, SH 3641 (AAAB; Honduras), Freelance (AAAB; Honduras), FHIA 02 (AAAB; Honduras), Yangambi (AAA; INIBAP), PV (Pacovan x Calcutta; Brazil), JBW (AAA; local selection, Levubu) Retiella 1 (AAA; local selection, Levubu), CRT (AAA; local selection).
			FHIA 02 (AAAB; Honduras)	10	Grand Nain (AAA), Basiria (AAA, local selection), Gross Michael (AAA), FHIA 02 (AAAB), Yangambi (AAA), KBC (AAA, local selection), Buena Vista (AAA, Canary Islands), JBW (AAA), Retiella 1 (AAA), PA (Prata Ana x Calcutta; Brazil).
Site B. Block A5B West					
22/02/2000	3.0 x 1.75	Statistical 4 Selections 4 Replicates	**Kamaramasenge (AB).**	10	Pisang Mas (AA), Cultivar Rose (AA).
			Pisang Mas (AA)	10	Kamaramasenge (AB), Cultivar Rose (AA).
			Cultivar Rose (AA)	10	Kamaramasenge (AB), Pisang Mas (AA)
Site C. Block A4 West					
02/12/1999	3 x 1.75	Statistical 6 Selections 3 Replicates	**Williams (AAA)**	10	Williams, Grand Nain

Site D. Block A9 WestB					
01/03/1996	2.5 x 2	Not statistical 8 Rows 8 Selections	**Bluggoe (ABB) Row 1**	10	Grand Nain (AAA) and Pelipita (ABB).
			Pelipita (ABB) Row 2	10	Bluggoe (ABB) and Mangaro Torotea (AAB, from INIPAB).
			Ducasse (Pisang awak) (ABB) Row 4	10	Mangaro Torotea (AAB) and Njock Kon (Horn plantain
Site E. Block A9 WestA					
01/03/1996	2.7 x 1.69	Statistical 6 Selections 4 Replicates	**FHIA 01 (AAAB)**	10	Cavendish (AAA) = Grand Nain selections from Novak and Israel, Chinese Cavendish
Site F. Nursery					
Six months old	In pots	Non statistical	**FHIA 23 (AAAB)**	10	
			Lady Finger (AAB)	4	
			Red King (AAA)	5	
			FHIA 25 (AAAB)	10	
Site G. Block A10East					
22/02/1999	2.5 x 2	Statistical 6 Cultivars, 5 Replicates	**High Noon (AAAB)**	10	Local Cavendish selections (AAA)
			FHIA 18 (AAAB)	10	Ten plants sampled in the row
LOCATION 2: DANROC					
Site H. Block 16A					
Planting date/ Age	**Plant density (m)**	**Layout**	**Selections sampled**	**Sample size**	**Surrounded by selections**
23/09/1999	2.64 x 2	Not statistical 2 Rows 9 Plants/row	**Pisang awak (ABB)**	10	Grand Nain, PKZ, PKM and PK4

Site I. Block 16B					
28/01/2000	2.64 x 2	Statistical 4 Selections 4 Replicates	**PKZ (AAAB)**	10	Grand Nain, PKZ, PKM , PK4 and Pisang awak
Site J. Block 16C					
10/02/2003	1.5 x 1.5 x 2	Not statistical 3 Selections 2 Rows each	**FHIA 25 (AAAB) Row 1 & 2**	7	See Block 16 A and B
10/02/2003			**FHIA 17 (AAAA) Row 3 & 4**	7	See Block 16 A and B
One year old			**FHIA 18 (AAAB) Row 5 & 6**	8	See Block 16 A and B
Site K. Block 2					
19/04/2001	2 x 2 x 6	Not statistical Plants in rows	**High Noon (AAAB) Row 1 & 2**	10	Cavendish (Williams)
1980			**Williams (AAA) Rows 3 & 4**	10	Cavendish and High Noon.
Site L. Block 14					
Eight years old	3.1 x 2	Not Statistical	**FHIA 01 (AAAB)**	10	Cavendish
LOCATION 3: Jan Prinsloo					
Site M					
Planting date/ Age	**Plant density (m)**	**Layout**	**Selections sampled**	**Sample size**	**Surrounded by selections**
23/01/1002	2 x 2 x 4	Statistical 5 Selections 5 Replicates	**High Noon (AAAB)**	10	High Noon (AAAB), Grand Nain (AAA), Du Roi (AAA), PKZ (AAAB) and FHIA 18 (AAAB),
			Williams (AAA)	10	High Noon (AAAB), Grand Nain (AAA), Du Roi (AAA), PKZ (AAAB) and FHIA 18 (AAAB),
LOCATION 4: Hannes van der Walt					

Site N.					
Plantation age	**Plant density (m)**	**Layout**	**Selections sampled**	**Sample size**	**Surrounded by plantations**
Four years	3.1 x 2	Not statistical One hectare	**High Noon (AAAB)**	15	Cavendish
Site O.					
Eight years	3.1 x 2	Not statistical One hectare	**FHIA 01 (AAAB)**	15	Cavendish
LOCATION 5: ADOT RUNNING WATER					
Site P.					
Plantation age	**Plant density (m)**	**Layout**	**Selections sampled**	**Sample size**	**Surrounded by plantations**
Four years	3.1 x 2	Not statistical One hectare	**High Noon (AAAB)**	15	Cavendish
Site Q.					
Eight years	3.1 x 2	Not statistical One hectare	**FHIA 01 (AAAB)**	15	Cavendish
LOCATION 6: Piet Knipe					
Site R.					
Plantation age	**Plant density (m)**	**Layout**	**Selections sampled**	**Sample size**	**Surrounded by selections**
Three years	3 x 1.75	Statistical 4 Replicates	**PKZ (AAAB)**	10	Cavendish varieties
LOCATION 7: Flip Basson					
Site S.					
Plantating date	**Plant density (m)**	**Layout**	**Selections sampled**	**Sample size***	**Surrounded by selections**
2002	2.6 x 1.8	Statistical	**PKZ (AAAB)**	10	Du Roi selection (AAA), BIO-02 (AAA), PKB (AAAB) and

		6 Selections 4 Replicates			Grand Nain (AAA).
			Williams (AAA)	10	PKB (AAAB), PKZ (AAAB) and Grand Nain (AAA).

*For samples taken at statistical sites, representatives from each replicate were sampled. At non-statistical sites, samples representative of a row were sampled. Samples taken at Location 4 and 5 was in an X formation (by walking from one corner of the field to another and sampling every third or fourth row).

ANNEXURE A

STANDARD BUFFERS, CHEMICALS AND MEDIA

Calculation of total grams (g) to be used to obtain a specific molar (M) in solution:

g = Mw x M x Vol (Liter)

1. ELISA coating buffer, (pH 9.6)

Na_2CO_3	1.59g (15mM)
$NaHCO_3$	2.93g (28mM)
NaN_3	0.20 g (0.02 %)

Make up to 1 liter with distilled water

2. Phosphate saline buffer (PBS), (pH 7.4)

NaCl	8.00 g (137 mM)
$Na_2HPO_4.2H_2O$	1.44 g (8 mM)
KH_2PO_4	0.20 g (1.4 mM)
KCL	0.20 g (2.7 mM)
NaN_3	0.20 g (0.02 %)

Make up to 1 liter with distilled water

3. STE Buffer

Tris-Cl, pH 7.5 (Mw 157.6)	0.15 g (10 mM)
NaCl	0.058 g (10 mM)
EDTA (Mw 290.0)	0.290 g (1 mM)

4. BSV Extraction buffer

PBS	1 liter
Polyvinylpyrrolidone (PVP), (Mw 40 000)	20.0 g (2 %)
Na_2SO_3, add just before use	10.0 g (1 %)

5. PBS-Tween (PBST)

PBS	1 liter
Tween-20	0.5ml

6. 2 % MPBS

PBS	1 liter
ELITE low fat milk powder	2.00 g

7. 4 % MPBS

PBS	1 liter
ELITE low fat milk powder	4.00 g

8. General ELISA extraction buffer

PBST	1 liter
Polyvinylpyrrolidone (PVP), (Mw 40 000)	20.0 g (2 %)
Ovalbumin	2.00 g (0.2 %)

9. 50x TAE Buffer (Tris-Acetate-EDTA), (pH 8.5)

Tris base	242.00 g
Acetic acid	57.1 ml
0.5M EDTA	100 ml.

Add Distilled water to a final volume of 1 liter

10. 2x TY medium (per litre), (pH 7.4)

Bacto-tryptone	16.00 g
Bacto-yeast extract	10.00 g
NaCl	5.00 g

11. TYE agar plates (per litre)

Agar	15.00 g
NaCl	140 mM

Bacto-Tryptone (Haarlem, England. Cat. # T1332)	10.00 g
Bacto-Yeast extract (Oxoid, England. Cat. # X589B)	5.00 g

12. Glucose, 2M

Glucose	180.16 g
Distilled water	500 ml (final volume)

Filter-sterilize through a 0.2 µm filter unit and store in aliquots at –20°C.

13. IPTG (Promega, V3955) stock solution, 0.1 M

(Isopropyl-beta-D-thiogalactopyranoside) IPTG	1.2 g
Distilled water	50 ml final volume

Filter-sterilize through a 0.2 µm filter unit and store at 4°C.

14. X-Gal (2ml)

5-bromo-4-chloro-3-indolyl-beta-D-galactoside	100 mg
Dimethylfomamide (Promega, V3941).	2 ml

15. LB medium with ampicillin (per litre), (pH 7.5)

Bacto-Tryptone	10.00 g
Bacto-Yeast Extract	5.00 g
NaCl	5.00 g

Autoclave and allow medium to cool to 55°C before adding ampicillin (final concentration 100 µg/ml). For LB plates, include 15 g agar prior to autoclaving.

16. LB/amp/IPTG/X-Gal

Make the LB plates with ampicillin as above; then supplement with 0.5 mM IPTG and 80 µg/ml X-Gal and pour the plates. Alternatively, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal may be spread over the surface of an LB-ampicillin plate and allowed to absorb for 30 min at 37°C prior to use.

17. Mg^{2+} Stock solution, 2 M

$MgSO_4.7H_2O$	123.30 g
$MgCl_2.6H_2O$	101.50 g

Make up to 500 ml with distilled water, filter-sterilize through a 0.2 µm filter unit.

18. SOC medium, (pH 7.0).

Bacto-Tryptone	2.00 g
Bacto -Yeast extract	0.50 g
1M NaCl	1 ml
1M KCl	0.25 ml
Mg^{2+} Stock	1 ml
2 M Glucose	1 ml

Add distilled water to a final volume of 100 ml.

19. Ethidium bromide

Add 5mg to 1ml water.

20. Trisoduim citrate buffer (per litre)

Trisoduim citrate (Mw 210.14)	147.05 g (0,5 M)
Triton-X 100	100 µl (0.1 %)
Thioglycollic acid	100 µl (0.1 %)

Adjust pH 0.5 M citric acid to pH 6.5, add distilled water to a final volume of 1 litre.

21. TPS 1 buffer, (pH 7.4)

Tris-HCL (Mw 157.6)	1.576 g (100 mM)

KCL	7.456 g (1 M)
EDTA (Mw 372.2)	0.372 g (10 mM)
Add distilled water to 100 ml	

22. CTAB buffer, (pH 8.0), (500 ml)

Tris-HCL (Mw 157.6)	6.055 g (200 mM)
EDTA (Mw 372.2)	3.722 g (50 mM)
NaCl (Mw 58.44)	40.91 g (2 M)
CTAB	20.00 g (4 % (w/v))
Distilled Water	447.31 ml
10 µl of Mercaptoethanol added fresh	

23. Potyvirus tissue extraction buffer

SDS	1 g (2 %)
Tris, pH8 (Mw 157.6)	0.788 g (0.1 M)
EDTA (Mw 186.1)	0.292 g (2 mM)
Sterilile distilled water to a volume of 50 ml	

24. Loading dye

Sucrose	4 g
Bromophenol blue	0.025 g
Make up to 10 ml with distilled water	

Table A1: Composition of modified Murashige and Skoog medium for *in vitro* propagation of *Musa* plants

Nutrient components		**(mg/L)**
Macronutrients	Ammonium nitrate	1650.0
	Potassium nitrate	1900.0
	Calcium chloride.$2H_2O$	440.0
	Magnesium sulfate.$7H_2O$	370.0
	Potassium phosphate	400.0
Micronutients	Boric acid	6.2
	Manganese sulfate • H_2O	16.9
	Zinc sulfate • $7H_2O$	8.6
	Potassium iodide	0.83
	Molybdic acid (sodium salt) • $2H_2O$	0.24
	Cobalt chloride • $6H_2O$	0.024
	Cupric sulfate • $5H_2O$	0.025
Iron	Na_2-EDTA	37.26
	Ferrous sulfate • $7H_2O$	27.8
Organic components		
Vitamins	Glycine (free base)	2.0
	Nictotinic acid (free acid)	0.1
	Pyridoxine • HCl	0.5
	Thiamine • HCl	0.5
Growth regulators	Indole-3-acetic acid	0.175
	N^6-benzylaminopurine	2.25^a or 0.225^b
Carbon source	Sucrose	30 000
Gelling Agent	Gelrite	2000
Anti-Fungal	Activated charcoal	2000^b
Antioxidant	Ascorbic acid	10.0

a = for multiplication, b = for rooting

ANNEXURE B

NUCLEIC ACID EXRACTIONS AND PCR PROTOCOLS

B.1.1 IMMUNOCAPTURE (IC) STEP IN PCR.

The IC-PCR detection method was followed in studies where it was necessary to detect only episomal BSV. In this procedure antiserum, specific to BSV was used to trap BSV. After sample addition, standard PCR was performed. Antiserum from Prof, Lockhart, diluted at 1:500 (v/v) or antiserum of Goat 2F Bleed 1, diluted 1:4000 or 1:8000 (v/v) in ELISA coating buffer was used. The antiserum type used is specified in the chapter texts (Chapters 3, 4, 6 and 7). Procedure for IC was as follow: 0.6ml thin wall PCR tubes were incubated with 50 µl seroreagent in each tube. Incubation was for 4 hours at 30°C or overnight (approximately 16 hours) at 4°C. Tubes were washed two times with 200 µl PBST [Annexure A] and 50 µl sample added. After incubation for 4 hours at 30°C or overnight (approximately 16 hours) at 4°C, tubes were washed 3 times with PBST.

B.1.2 PCR PROTOCOL USED FOR THE DETECTION OF BSV-OL (ONNE') (GEERING *ET AL.*, 2000).

Primers:

BSV 5317 5' AGT CAT TGG GTC AAC CTC TGT CCC 3' (24bp)

BSV 4673 5' GGA ATG AAA GAG CAG GCC 3' (18bp)

A PCR master mixture was prepared containing the BSV-OL specific primer pairs. The PCR master mixture for each reaction contained 50µM of each NTP, 0.4 µM of each primer, 1.5 mM $MgCl_2$, 1 x ammonium buffer, 1.25 U Biotaq™ DNA Polymerase (BIOLINE, UK), in a total volume of 25 µl. Samples were incubated in a PCR-Express thermocycler (Hybaid). Thermocyler conditions for BSV Onnè was denaturation at 94°C for 30 seconds, 35 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 1 min, followed by a final elongation step of 72°C for 5 min.

B.1.3 PCR PROTOCOL USED FOR THE DETECTION OF BSV-GF, BSV-MYS, BSV-RD AND BSV-IM (GEERING *ET AL.*, 2000).

Primers:

IRFA 914 specific	BSV-IM F1	TGC CAA CGA ATA CTA CAT CAA C
IRFA 914 specific	BSV-IM R1	CAC CCA GAC TTT TCT TTC TAG C
Goldfinger specific	BSV GF F1	ACG AAC TAT CAC GAC TTG TTC AAG C
Goldfinger specific	BSV GF R1	TCG GTG GAA TAG TCC TGA GTC TTC
Mysore specific	BSV Mys F1	TAA AAG CAC AGC TCA GAA CAA ACC
Mysore specific	BSV Mys R1	CTC CGT GAT TTC TTC GTG GTC
Red Dacca	BSV RD F1	ATC TGA AGG TGT GTT GAT CAA TGC
Red Dacca	BSV RD R1	GCT CAC TCC GCA TCT TAT CAG TC

Separate PCR master mixtures were prepared containing the desired strain-specific primer pairs. The PCR master mixture for each reaction contained 100 µM of each dNTP, 0.1 µM of each primer, 2.0 mM $MgCl_2$, 1 x ammonium buffer, 1.25 U Biotaq^{TM} DNA Polymerase (BIOLINE, UK), in a total volume of 25 µl. The thermocycler (PCR-Express thermoclycler (Hybaid)) conditions for the detection of these strains of BSV was similar and was as follow: denaturation at 94^{o}C for 30 seconds and 35 cycles of denaturation at 94^{o}C for 10 seconds, annealing at 64^{o}C for 30 seconds and elongation at 72^{o}C for 1 min, with a final elongation of 72°C for 5 min.

B.1.4 PCR PROTOCOL USED FOR THE DETECTION OF BSV-CAV (GEERING *ET AL.*, 2000).

Primers:

BSV Cav F1 AGG ATT GGA TGT TAA GTT TGA GC

BSV Cav R1 ACC AAT AAT GCA AGG GAC GC

The PCR master mixture for each reaction contained 50µM of each NTP, 0.4 µM of each primer, 1.5 mM $MgCl_2$, 1 x ammonium buffer, 1.25 U Biotaq^{TM} DNA Polymerase (BIOLINE, UK), in a total volume of 25 µl. Using a PCR-Express thermoclycler (Hybaid) PCR was performed as follows: denaturation at 94^{o}C for 30 seconds followed by 35 cycles of denaturation at 94^{o}C for 10 seconds, annealing at 64^{o}C for 30 seconds and elongation at 72^{o}C

for 1 min, with a final elongation of 72°C for 5 min. Ten microlitres of the amplified products were mixed with loading dye [Annexure A] and loaded on a 1 % agarose gel incorporated with 8µl of ethidium bromide (5 mg/ml solution) and viewed with a UV transilluminator.

B.1.5 PCR PROTOCOL USED FOR THE DETECTION OF *CUCUMBER MOSAIC CUCUMOVIRUS* (CMV)

IC was performed as described in section B 1.1, using an antiserum specific to CMV (ARC-PPRI, Pietersen, personal communication). The antiserum was diluted 1:100 v/v. The samples were macerated in Trisoduim citrate buffer [Annexure A].

PCR reaction conditions:

CMV-specific primers, which amplify approximately a 500bp fragment of the putative coat protein gene of CMV, were used. The primers detect strains in both CMV subgroups 1 and 2 (Wylie *et al.*, 1993). The upstream primer used was CMV-primer 2 of Wylie *et al* (1993) (5' TATGATAAGAARCTTGTTTCGCG 3') and the downstream primer was a slight modification of their CMV-primer 1: (5' GTAAGCTGGATGGACAACCCGTTC 3'). (The primer was modified as multiple, non-specific amplification products were obtained in healthy and infected material. This was overcome by removing excess G and C bases at the 3' end of the primer).

The PCR reaction mixture contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL (pH 8.8), 0.01 % Tween-20, 1,5 mM $MgCl_2$, 10 mM DTT, 175 µM of each dNTP, 1 µM of each oligonucleotide primer, 0,5 U Biotaq^{TM} DNA Polymerase (BIOLINE, UK), 18 U of HPRI RNAse Inhibitor (Amersham) and 10 U of MMLV Reverse Transcriptase (Amersham) in a total volume of 50 µl . The reverse transcription step was performed at 42^{o}C for 45 min followed by a denaturation step of 94^{o}C for 2 min in a PCR-Express thermoclycler (Hybaid). Following PCR cycle conditions were: denaturation at 94°C for 2 min, 40 cycles of denaturation 94^{o}C at 30 seconds, annealing 50^{o}C at 60 seconds and elongation 72^{o}C for 40 seconds followed by a single cycle of elongation of 72^{o}C for 10 min.

B 1.6 DETECTION OF *BANANA BUNCHY TOP BABUVIRUS* (BBTV)

The primers and extraction protocol of (Thompson and Dietzgen, 1995) were used as described in the article. The PCR reaction mixtures were adapted for our conditions and enzymes. BBTV infected material were obtained from Queensland Department of Primary Industries, Australia as positive controls (freeze dried [Annexure E.1.2] vials # 602 19/08/1999), with the necessary import permits.

Sample preparation and extraction protocol:

Leaf material was macerated in liquid nitrogen to a powder using sterile mortars and pestles. The macerates were transferred to storage vials and immediately placed at –80^{o}C. This approach minimised variation in virus distribution.

Approximately 0.5-1.0 mg of the ground tissue was added to 100µl TPS 1 buffer [Annexure A] and incubated at 95^{o}C for 10 min. Thereafter the samples were placed on ice and a 10-fold dilution of the supernatant of each sample was made in sterile water. The dilution of the sample extract was found to be crucial to dilute inhibitory substances, which influence the PCR reaction. A half a microlitre of this diluted extract was added to the PCR reaction mix of each sample, which was performed in 50µl reaction volumes.

PCR reaction conditions:

BBTV-specific primers BBT-1 and BBT-2, which amplify a 349bp fragment of the replicase gene of BBTV, were used. The reaction mixture contained 16mM $(NH_4)_2SO_4$, 67mM Tris-HCL (pH 8.8), 0.01 % Tween-20, 1,5 mM $MgCl_2$, 175 µM of each dNTP, 1 µM of each oligonucleotide primer, 0,5 U Biotaq^{TM} DNA Polymerase (BIOLINE, UK) in a total volume of 50 µl. PCR reaction conditions were a denaturation step of 94^{o}C for 2 min followed by 40 cycles at 94^{o}C 20s, 60^{o}C 30 seconds and 72^{o}C 30 seconds and single cycle of elongation of 72^{o}C for 10 min. A PCR-Express thermoclycler (Hybaid) was used.

B.1.7 DETECTION OF *BANANA BRACT MOSAIC VIRUS* (BBrMV) (RODONI *ET AL.*, 1997).

Control: BBrMV infected material was obtained from Queensland Department of Primary Industries, Australia as positive controls (freeze dried [Annexure E.1.2] vials # 679 19/08/1999), with the necessary import permits.

Sample preparation: Crude Nucleic acid extractions and G-25/STE column fraction

Crude nucleic acid extractions were prepared using a modified protocol of Pappu *et al.,* (1993). For each sample, approximately 3 cm^2 of leaf tissue was macerated 1 ml of potyvirus tissue extraction buffer [Annexure A] using a mortar and pestle. 300 µl of the macerated sample mixture and 300 µl phenol-chloroform (80 % phenol in STE buffer [Annexure A], add an equal volume of chloroform) were then added together in an eppendorf tube and vortexed for a few seconds. The tubes with the mixture were then incubated at 70°C for 5 min and thereafter centrifuged (10 000rpm) for 5 min at room temperature. The supernatant was added to a G25/STE saturated column for nucleic acid purification. Column preparations were done as follows. The bottom of a 0.6 ml thin wall PCR tube were perforated with a sterile needle (+/- 2-4 holes) and placed in a 2 ml eppendorf tube which served as a collection basket. One gram of Sephadex G-25 powder, per 4-8ml of STE buffer, was weighed into an Erlenmeyer flask and heated in a microwave on low power for 1 min. The G-25/STE preparation was cooled and stored at 4°C. 500 µl of the G-25/STE preparation was added to the perforated eppendorf tube on top of the collection tube (2ml eppendorf tube) and centrifuged at 5000 rpm for 3 min. Buffer in the bottom of the collection tube was discarded and the sample carefully loaded to the G-25 eppendorf tube column. After centrifugation (5000 rpm) for 3 min the fractioned sample was collected in the collection tube and kept on ice for immediate PCR or frozen at -80°C for further use.

BBrMV-specific primers, which amplify a 600bp fragment of the coat protein gene and the 3' untranslated region of the BBrMV genome, are used. The primers are modifications of the primers designed by Rodoni *et al.,* (1997).

The following primers were used:

5' CACTCGATCAATACCTCACAG 3' and 5' TGGTGYATMGAGAATGGRACATCAC 3'.

The reaction mixture contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL (pH 8.8), 0.01 % Tween-20, 1,5 mM $MgCl_2$,10 mM DTT, 175 µM of each dNTP, 1 µM of each oligonucleotide primer, 0,5 U Biotaq^{TM} DNA Polymerase (BIOLINE, UK), 18 U of HPRI RNAse Inhibitor (Amersham) and 10 U of MMLV Reverse Transcriptase (Amersham). The reverse transcription step was performed at 42^{o}C for 45 min followed by a denaturation step

of 94°C for 2 min. PCR cycle conditions were: 40 cycles at 94°C 30 seconds, 46°C 60 seconds and 72°C 40 seconds followed by a single cycle of elongation of 72°C for 10 min.

B.2.1 CTAB PROCEDURE TO EXTRACT TOTAL DNA FROM *MUSA* LEAVES (GAWEL AND JARRET, 1991)

Ten milliliters of CTAB buffer [Annexure A] were heated at 65°C in a water-bath. Leaf material (200 mg per sample) was grounded in liquid nitrogen within a 1.5 ml microcentrifuge tube using a micropestle. Preheated CTAB (700µl) buffer were immediately added to the sample and the mixture was incubated at 65°C for 1 hour. A volume of 600µl of Chloroform:Isoamylalcohol (24:1) were added to each tube and mixed by inversion for 5 min at room temperature. After centrifugation at 13000 rpm for 5 min the supernatant was moved to a new tube together with 500µl of ice-cold isopropanol. The sample was incubated at -20°C for 1 h or overnight and centrifuged at 13000 rpm for 5 min. Resulting pellet was washed with 300 µl of 70 % ethanol and left at 4°C overnight. After centrifugation of 13000 rpm for 5 min, tubes were left to dry and 100 µl of DEPC water added. Finally the tubes was incubated at 37°C for 1 h and stored at -20°C.

ANNEXURE C

TAS-ELISA PROTOCOL, DEVELOPED IN CHAPTER 4 OF THIS THESIS, FOR THE DETECTION OF *BANANA STREAK BADNAVIRUS*

C1.1 PROTOCOL FOR THE DETECTION OF *BANANA STREAK BADNAVIRUS*

1. Prepare goat antiserum **(Goat 1F Bleed 2)** at a dilution of 1:8000 in ELISA coating buffer. Mix well and add 100µl to each well of a 96 well maxisorb ELISA immuno-plate (Nunc, Amersham, cat nr. 442404). *Incubate at 30°C for 4 hours.
2. Discard the contents from the plate and rinse 3 times with 3 min intervals, with PBST [Annexure A].
3. Macerate samples in BSV extraction buffer [Annexure A] to give a ratio of 1:10 (sample weight: buffer volume). Prepare the sample as follows: add one gram of leaf lamina and one ml of BSV extraction buffer to a mortar, along with a sprinkling of coarse carborundum powder, macerate to a fine paste with the pestle before adding the rest of the buffer (9ml), mix well).
4. Add 100µl of each sample separately to each well according to a planned experimental layout. Incubate the plate overnight at 4°C.
5. Wash 5 times with 3 min intervals with PBST.
6. Prepare rabbit antisera **(R210 Bleed 1)** at a dilution of 1:8000 in general ELISA extraction buffer [Annexure A]. Add 100µl to each well and incubate at 30°C for 4 hours.
7. Wash plate 3 times with 3 min intervals with PBST.
8. Prepare a commercial conjugate (goat-anti-rabbit with alkaline phosphatase enzyme, GAR-AP, Sigma A3687) to the desired dilution in general ELISA extraction buffer. Add 100µl to each well. Incubate plate at 4°C overnight.
9. Wash 5 times with 3 min intervals followed by the addition of ρ-nitrophenyl phosphate (PNP) substrate at 1mg PNP per milliliter of substrate buffer.
10. Read absorbance values at OD 405nm after incubation at room temperature for 30 min and one hour with an ELISA plate reader.
11. The reaction can be stopped with 25µl of 3M NaOH.

**Specified incubation times were used for detection and no other incubation-time variations were tested. However, the 4h or overnight periods could be reduced to 2h incubation periods. Likewise 2h or 4h incubation periods could be replaced by overnight periods at 4°C , if desired. With each incubation step, plates should be put into a moist chamber (plastic bag -, or plastic box with damp paper towel)*

ANNEXURE D

PANNING WITH SYNTHETIC LIBRARIES

D.1.1 PANNING, ROUND ONE

This panning method involved a solid surface method, where the antigen was "trapped" directly unto a maxisorb-microplate well (Nunc). A maxisorb plate well was filled with 300 µl of the antigen (the concentration used was 2µg/ml BSV in PBS [Annexure A]) and incubated at 4°C overnight. Simultaneously a single colony of TG1 *E. coli* was inoculated into 5 ml of 2xTY [Annexure A] and incubated at 37°C overnight with shaking. The plate with antigen was washed two times with PBS by simply pouring PBS into the well and then flipping the plate downwards in order to discard the liquid immediately. The plate well was then filled to the brim with 2 % ELITE milk powder in PBS w/v (2 % MPBS), covered and incubated at room temperature for 2 hours. Milk powder (MP) blocks any open spaces on the surface of the well in order to prevent non-specific binding of phage particles to the surface of the ELISA well walls. After incubation at 37°C for 2 hours the plate was washed 2 times with PBS. M13KO7 (Pharmacia Biotech) helper phage in 1 ml of 2 % MPBS was then added at 10^{12} to 10^{13} transducing units (TU) and the plate was incubated at room temperature for 30 min with occasional mixing by pipetting. After another 90 min at room temperature the plates was washed 20 times with PBST (0.1 % Tween-20) and again 20 times with PBS to remove all traces of detergent. Each washing step was performed by pouring buffer in and immediately out.

Three hundred microlitres of freshly prepared triethylamine (l00 mM) was added to the well and incubated for 10 min at room temperature in order to elute the bounded phage. During the incubation, 0.5 ml Tris (1 M), pH 7.4, was added to an eppendorf tube to which eluted phages were added immediately after the incubation. This step allows neutralization of the pH and eluted phages can be stored at 4°C or used to infect the prepared TG1 *E. coli*.

Panning with eluated phages: After dilution of the overnight TG1 bacterial culture, 0.5ml of TG1 was added to 50 ml of fresh 2x TY. Cells were incubated at 37°C with shaking until an absorbance of 0.5 - 0.6 was reached at an optical density of 600nm. This exponentially growing culture of TG1 (0.5ml) was then added 1.0 ml of the eluted phage. During the following incubation step of 37°C for 30 min (without shaking) phage infection of the TG1 bacteria occurs. After centrifugation of the infected TG1 cells at 3,000 g for 10 min at room

temperature, the cells were resuspended in 1 ml of 2x TY and plated on three large Petri dishes (25 cm diameter) with TYE agar [Annexure A] containing 100 µg/ml ampicillin (AMP) and 2 % glucose (GLU, Annexure A). Plates, onto which the cells were evenly spread, were incubated overnight at 30°C.

The following morning, the density of cells on the plate was evaluated. Ideally a dense layer of cell colonies should have grown on the agar plate surface. All the colonies were resuspended into 5 ml of 2xTY, which was added to a plate. Cells on the agar plate were loosened with a glass spreader and transferred to the next plate, the process was repeated in order to recover all the colonies. A 100µl aliquot of the scraped bacteria was added to a cryovial with an equal amount of 15 % glycerol for storage at -70°C. The remaining scraped cells were used as input cells (with pages) for the next round of selection.

Further Rounds of Selection: Scraped bacteria were grown at 30°C overnight in liquid culture medium (100 ml of 2xTY containing 100 µg/ml ampicillin and 2 % glucose to which 100-200 µl of cells were added). The culture was diluted into the same growth medium to obtain cells in log phase, by incubating the culture at 37°C with shaking until the optical density at 600 nm was 0.4 -0.5. This culture was again infected with M13KO7 helper phage by adding helper phage in the ratio of 1:20 (number of bacterial cells:helper phage particles, taking into account that 1ml bacteria at 600nm equals around $8x10^8$ bacteria/ml. After incubation without shaking in a 37°C water bath for 30 min, the infected cells were centrifuged at 3,300 g for 10 min. The pellet was resuspended in 50 ml of 2xTY containing 100 µg/ml AMP and 25 g/ml kanamycin (KANA) and incubated at 30°C overnight with shaking.

Overnight culture were spinned at 10,800 g for 10 min or 3,300 g for 30 min and a 1/5 volume PEG/NaCl (20 % Polyethylene glycol 6000, 2.5 M NaCl) was added to the supernatant. This was incubated for 1 hr or more at 4°C, spinned at 3,300 g for 30 min and supernatant aspirated. The pellet was resuspended in 2 ml PBS and centrifuged at 11, 600 g for 10 min in a micro centrifuge in order to remove most of the remaining bacterial debris. A portion of these recovered phages was stored at 4°C and a one milliliter aliquot used for the next round of selection. Phage selection was repeated for another 2 rounds.

ANNEXURE E

STANDARD METHODS

E.1.1 ACCESSING OF VIRUS SAMPLES

Musa samples were accessed according to a system developed at ARC-PPRI. A numerical system was followed by which the first two digits indicate the year in which the samples were received and accessed. The last three digits is a code that follows in numerical order. Therefore a sample such as 02/0030 would indicate that the sample was received in 2003 while it is the thirtieth sample accessed that year. Sample 99/2722 is a positive control for BSV and contains BSV-GF, BSV-RD and BSV-OL. This sample together with sample 94/1609 (a virus free Grand Nain plant) was frequently used as controls in studies reported in this thesis.

E.1.2 FREEZE-DRYING

Freeze-drying is a method by which plant samples, infected with viruses can be stored for a long period of time. It is also a safe way for storing foreign viruses. The material was dried and stored using a method described by Hollings and Leliott (1960). Infected plant material was macerated in a solution of 7 % glucose and 7 % peptone. Samples were pre-frozen in glass-vials at -70°C and dried overnight under vacuum with a Virtis freeze drying apparatus. Using a flame, vials were sealed off while still under vacuum and stored at 4°C.

E.1.3 AGAROSE GEL ELECTROPHORESIS

In order to view the amplicons obtained after PCR or IC-PCR agarose gel electrophoresis was performed. Samples mixed with 2 % loading dye [Annexure A] were loaded on a 1.0 % agarose gel (w/v) in 1 x TAE buffer [Annexure A], with 10 ul of ethidium bromide [Annexure A] added. Electrophoresis was at 100 V for 30 min. The gel was viewed with an UV transilluminator and processed using the software programme; Laboratory imaging and analysis systems (UVP).

E.1.4 TRANSFORMATION OF COMPETENT CELLS

In this method, plasmids were transferred to *Eserichia coli* (Promega, High efficiency JM 109) cells by using heat shock. Five microlitres of product, obtained after ligation of PCR products into the pGEM-T easy vector system (Promega, A1360), were added to a sterile Falcon tube and kept on ice. Forty microlitres of thawed competent cells were added to each tube. After mixing the contents and incubating the tubes on ice for 20 min, cells were heat-shocked for 45-50 seconds in a water-bath at 42°C. Tubes with samples were incubated on ice for 2 min and 950µl of SOC medium added, followed by an incubation step of 1.5hours at 37°C, while shaking. For each tube, two LB/Ampicillin/IPTG.X-Gal plates [Annexure A] were prepared. On each plate 20 µl of 50 mg/ml X-Gal and 100 µl of 100 mM IPTG were plated and incubated for 30 min at 37°C. Transformed culture (100 µl of each culture) was spread onto separate plates and incubated overnight at 37°C.

E.1.5 QUICK SCREENING OF TRANSFORMANTS

The indication system used to distinguish transformants for non-transformed cells, involves a colour reaction. Colonies, in which the β-galactosidase gene, of the pGEM-T easy vector was interrupted by a PCR product insert, will usually display a white colour. White colonies were selectively 'picked-up' with sterile toothpicks and individually inoculated into 5ml of LB medium [Annexure A] containing ampicilin under aseptic conditions. One blue colony was isolated for each PCR clone-group, serving as a control. Cultures were grown overnight at 37°C, with agitation. The following day, 1ml of bacteria was transferred to a sterile eppendorf tube and centrifuged at 5000rpm for 3 min. Resulting pellets were extracted with 40µl of chloroform/phenol (1:1) and centrifuged for 1 min at 12 000rpm. Cleared supernatant were transferred to a new sterile eppendorf tube, to which 1µl of RNAse A (10 mg/ml) was added. For screening, 9µl of product were mixed with 2µl loading dye [Annexure A] and loaded to a one percent agarose gel. After electrophoresis [see Annexure E, section E.1.3] gels were viewed under a UV transilluminator. Transformants were selected on the basis that the sizes of plasmids with inserts were higher than those of the control (blue colonies with no inserts).

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