CROP PROTECTION PROGRAMME

Management strategies for Banana Streak Virus: Variation of BSV in Uganda as an adjunct to diagnosis and epidemiology

R7478 (ZA0342)

FINAL TECHNICAL REPORT

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Executive Summary

Banana streak virus (BSV) is an important limiting factor to the subsistence production of banana in Uganda. The aim of this project was to assess the variation of Ugandan isolates of BSV in order to provide both information and diagnostics for the associated project (R7529) on the epidemiology of the virus. Fifty nine samples of leaves and suckers were taken from 30 farms at 14 sites across the banana producing region of Uganda. Other samples of infector and recipient plants from the epidemiology experiments were provided by the associated project. A new technique was developed for BSV purification and virus preparations were made from the 47 field samples for which there was sufficient material. These virus preparations were analysed by two serological techniques, enzyme-linked immunosorbent assay and immunesorbent electron microscopy. Most of the samples reacted poorly to a broad spectrum BSV antiserum even though other evidence suggested that there was a significant amount of virus present; some samples reacted well with the antiserum. This suggests significant serological variation between the isolates. DNA was extracted from the virus preparations and subjected to cross hybridization which gave complex results. The DNA from 48 field samples was amplified by the polymerase chain reaction (PCR) using degenerate primers covering a conserved part of the BSV genome and several hundred clones were obtained. Most of the isolates could be amplified using the degenerate primers. However, some isolates including those from the epidemiology experiment infectors were not amplified using these primers but were with other primers. Analysis of sequence data on 138 of these PCR clones revealed great variation with 11 virus species being recognized. These species fell into 3 supergroups with relationships to other BSV strains and other badnaviruses from different parts of the world. There was little relationship between cultivar, symptom or region with the BSV species. A diagnostic protocol was developed involving PCR of the DNA from virus particles that had been immune or direct captured from concentrated sap. The selection of PCR primers for this protocol would depend upon the BSV species being analysed.

The significant deductions from this study are: a) The epidemic of BSV in Uganda is not recent though some cultivars may have been infected recently; b) Distinct BSV species may have spread into banana from other crops such as sugarcane and pineapple; c) Distinct species of BSV may have different vectors which could affect epidemiological studies; d) Understanding the variation of the virus is important not only in epidemiological studies but also in the production and tissue culture of disease-free bananas.

Background

Banana streak disease is caused by the badnavirus, banana streak virus (BSV) (Lockhart, 1986) and is becoming increasingly widespread in many countries, especially Uganda (Kariba et al., 1997). BSV is a pararetrovirus with a circular double-stranded DNA genome of about 7.5 kbp. As with most other badnaviruses it is assumed to be mealybug transmitted but there have been few published records of this form of transmission (Lockhart and Autrey, 1988; Su, 1998; Kuriba et al., 2001a). Very recently, the disease spread patterns of BSV in the field have been taken as being indicative of slow spread by a vector (Kuriba et al., 2001b). There is very little known about the specific vectors or the virus-vector relationships of this mealybug transmission. Also very recently, Kuriba et al. (2001a) reported that a Uganda isolate of BSV is transmitted by *Planococcus citri* and *Saccharococcus sacchari*. BSV also resembles several other badnaviruses in being very variable (Lockhart and Olszewski, 1993) but there are few detailed molecular

studies on this variation. The DNA genome of a Nigerian isolate of BSV has been sequenced (Harper and Hull, 1998) showing that this isolate has a very different sequence to that published for a serologically related badnavirus, sugarcane bacilliform virus (ScBV) (Bouhida et al., 1993). Sequences of part of the genomes of BSV from banana cultivars Red Dacca, Williams (Cavendish), Mysore, Goldfinger, IM and Agbagba in Australia differ significantly (Thomas et al., 1998; Geering et al., 1999; Geering et al., 2000). An isolate of BSV has been transmitted to sugarcane giving no symptoms (Lockhart and Autrey, 1988) and an isolate of ScBV has been agroinoculated to banana (Bouhida et al., 1993) giving BSV-like symptoms.

BSV differs from other plant viruses in that sequences of viral DNA have been found integrated in most, if not all, *Musa* cultivars so far examined (LaFleur et al., 1996; Ndowora et al., 1997). In certain cultivars, the integrated viral sequence is activated by stresses such as tissue culture to give the symptom-producing episomal infection (Harper et al., 1999; Ndowora et al., 1999). In other cultivars, integrated BSV-like sequences are found that are incomplete or perturbed and which would be unlikely to produce episomal virus infection (Ndowora et al., 1999; Geering et al., 1999); these have been termed "dead" integrants. Activatable integrated sequences appear to be found only in banana cultivars that contain the B genome and "dead" integrated sequences in cultivars that contain the A genome and probably in some B genomes.

In episomal infections, the virus is in low concentration and symptoms can fluctuate according to season. The low concentration makes diagnosis by the enzyme-linked immunosorbent assay (ELISA) unreliable (Thottappilly et al., 1998; Dahal et al., 1998) and the presence of integrated sequences limits the use of the polymerase chain reaction (PCR) for diagnosis of episomal infections using nucleic extracts from infected plants. An immunecapture - PCR (IC-PCR) technique has been developed for the detection of the Nigerian isolate of the virus (Harper et al., 1999). However, the reliability of this test depends on the antiserum capturing the virus and the primers for PCR being compatible with the viral DNA.

Very little is known about the strains of BSV present in Uganda and of the role of strains of ScBV in causing banana streak disease. A preliminary study indicated that an isolate from Rakai had a different restriction enzyme cutting pattern to that of the Nigerian isolate (Harper and Hull, unpublished observation). For epidemiological studies, it is necessary to a) have a reliable diagnostic system for the strain(s) of virus and b) to have an understanding of the variation so that the possibility of different strains (viruses) having different vectors can be assessed.

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Project Purpose

This project addresses Programme Output for High Potential Production System HP1.11: Virus diseases of banana, cassava, yam, sweet potato, Solanum potato and food legumes characterised and improved methods for diagnosis and management of virus diseases developed and promoted.

The research objective is to assess the strain variation of BSV in Uganda, to provide molecular information which will be of use for epidemiological and loss-assessment studies and to develop diagnostics for the variants.

The outputs of this research will be used for the epidemiological studies proposed in the sister project (R7529). It is considered important to have information on, and diagnostics for, variants of BSV in developing an understanding of the epidemiology of the virus. In the longer term, the diagnostics will be of use to Ugandan and other East African NARS for virus identification and for initiatives on banana improvement by tissue culture in these countries.

Research Activities

1. Sampling

In visits made to Uganda from 3-10 November 1999 and 22-27 July 2000, 30 farms were visited at 14 sites across the main banana growing area of that country (see map in Appendix A). At each farm, samples were collected from plants showing virus or virus-like symptoms. The samples comprised leaves and in about half the cases, suckers. For each farm records were taken of the farm location (GPS) and elevation and the farmer's name together with descriptions of the sample (variety, symptoms (together with photograph) and any other relevant information (see record sheets in Appendix B). The samples were brought back to the JIC for analysis and the suckers were grown on to provide a permanent source of the virus; leaf samples were frozen.

Eighty nine samples from the two epidemiology experimental sites were supplied by Dr. Kenyon May 2001. These were from both the infector and "healthy" plants and showed few if any

symptoms. A further three samples from the infectors in these two experiments were sent by Dr. S. Gowan in July 2001; these were highly symptomatic.

2. Virus preparation

BSV is usually at very low concentration even in strongly symptomatic leaves and so virus was purified before being analyzed. Factors that affect virus yield were determined for a Nigerian BSV isolate (TMP4698), which is in relatively high concentration in glasshouse-grown plants. Most of the previously published purification procedures did not yield much virus. A modification of the purification protocol for rice tungro bacilliform virus (Jones *et al*., 1991. J. Gen. Virol. 72: 757-761) (Appendix C) which uses celluclast to release the virus from vascular tissue proved to give the most reliable yields.

Preparations were made from 47 of the 59 farm samples and from three of the epidemiology experimental site samples; the other samples were either too small or were symptomless to give a workable virus yield.

3. Serology

a. ELISA

Purified virus preparations were analyzed by a standard enzyme-linked immunosorbent assay (ELISA) (Appendix D) using an antiserum against 24 isolates of BSV and other badnaviruses obtained from Dr. Lockhart.

b. ISEM

Purified virus was also analyzed by immune-selection electron microscopy (ISEM) using the protocol outlined in Appendix D. Counts were made on numbers of particles per field for up to 50 fields at 10,000 magnification.

c. Serology results

The results of these two serological tests are summarized in Appendix E. Most of the samples gave very low ELISA readings and ISEM counts but samples 59-65 gave high readings. Some of samples (e.g. 46, 51 and 52) that gave low serological reactions yielded detectable amounts of DNA similar to the amounts from samples 59-65 (Appendix E). This indicates that at least some of the low serological readings were not due to very low amounts of virus but were due to lack of serological reaction with the broad spectrum antiserum.

4. DNA extraction

DNA was extracted from the purified virus and was analyzed by gel electrophoresis using the protocols outlined in Appendix F. The gels were scored as to whether viral DNA bands could be seen on ethidium bromide staining (Appendix E).

5. Cross hybridization

In attempts to group the BSV isolates, gel-electrophoresed DNA was Southern blotted from the gels onto nylon membranes which were probed with ^{32}P-labeled virion DNA of the Nigerian isolate and cloned DNAs from samples 24 and 25. The results from these experiments were complex with cross-hybridization at different intensities in most samples, indicating differing abundances of similar sequences in most samples. In the light of the evidence for mixed

infections (see below) this is not a surprising result but showed that this approach could not be used for grouping of BSV strains in field samples.

6. PCR

a. Primers and Protocol

A degenerate primer set based on the consensus sequences around the polymerase genes of BSV and other badnaviruses was used:

Primer set 1A-4' (kindly advised by Drs. Lockhart and Olszewski)
Primer 1A (forward) (*nt 5537) 5'CTN TAY GAR TGG YTN GTN ATG CCN TTY GG3'
Primer 4' (reverse) (*nt 6134) 5'TCC AYT TRC ANA YNS CNC CCC ANC C3'
* indicates position in sequence of Harper and Hull (1998)

This primer set, which covers the highly conserved reverse transcriptase and RNaseH regions of the BSV genome, gives a product of about 600 bp.. Initially, PCR was performed on extracted virion DNA but most of the subsequent reactions were on DNA in virus particles trapped on Eppendorf tube walls by either immunecapture (IC-PCR) or direct binding (DB-PCR) using the protocols outlined in Appendix F. The PCR products were analyzed by gel electrophoresis.

Other primer sets that were used were:

Primer set RDfor-RDrev (kindly advised by Dr. Geering)
Primer RDfor (forward) (**nt 402) 5'ATC TGA AGG TGT GTT GAT CAA TGC3'
Primer RDrev (reverse) (**nt 522) 5'GCT CAC TCC GCA TCT TAT CAG TC3'
** indicates position in sequence of Geering (Genbank accession AF215816). These positions are 6499 and 7021 on the sequence of Harper and Hull (1998). The sequence of BSV from banana Red Dacca amplified by these primers is 99% similar to that of the OL strain sequenced by Harper and Hull (1998).

Primer set Ghfor-Ghrev
Primer GHfor (forward) (*nt 5546) 5'TGG YTN GTN ATG CCN TTY GG3'
Primer GHrev (reverse) (*nt 5954) 5'ATG GGW CCW AGK RTT YTT CCC3'
* indicates position in sequence of Harper and Hull (1998)

b. Results

PCR was performed on the all the farm samples except number 4 (which showed no symptoms) using primer set 1A-4'. The results of the reactions are summarized in Appendix E. Most of the samples gave significant to moderate PCR product bands from virus preparations, with a few giving minor bands. Some samples only gave PCR products from captured virus particles from sap extracts but not from virus preparations. Ten samples gave no product either from virus preparation or from sap extracts.

PCR on DNA from the epidemiology experiment plants supplied by Dr. Kenyon gave weak bands in some cases and no bands in others; however, no virus was detected in these samples. The three symptomatic samples from the epidemiology experiment infector plants supplied by Dr. Gowan gave no products using primer set 1A-4'. As virus was observed in these samples

other primer sets were used and those based on variety Red Dhaka (RDfor-RDrev) and a degenerate badnavirus primer (GHfor-GHrev) gave bands.

7. Cloning PCR products

The PCR products were purified and cloned (Appendix F) into T cloning vectors (e.g. Promega pGem Teasy or Invitrogen TA vector) and transformed into *E. coli* TOP10. The resulting plasmids were purified and the size of the insert assessed by cutting with EcoR1 and electrophoresing the products on gels. If there was a number of EcoR1 sites in the insert the insert was amplified by PCR using vector M13for/rev primers to give a product expected to be about 750 bp. Plasmids with inserts of about 600 bp were selected for sequencing

Several hundred clones (20 or more from each successful PCR reaction) were obtained from 48 of the farm samples (Appendix E) with the number of clones per sample ranging from one to nine. Forty one clones were obtained from the epidemiology experiment infector samples

8. Sequencing of clones

The inserts of 172 of the clones from the farm samples were sequenced on an ABI3700 automatic sequencer. Forty nine of the inserts either started with the primer sequence but then had non-badnavirus sequences or were ~50bp shorter than expected; these are listed as “wrong” or “weird” respectively in Appendix E.

The 138 “good” farm sample sequences were aligned using Clustal V computer program and compared using Phylip boot-strapped distance methods. Taking sequence differences of more than 20% as differentiating virus “species”, 11 “species” of BSV were recognized (species A-K) (Appendix G1). A significant observation is that the sequence of members of “species” G from the Mbale region has a deletion of 6 nucleotides (2 amino acids) when compared to the other sequences. The 11 species fell into three “supergroups” (I, II and III) that differed by more than 35% sequence (Appendix G2). Supergroup I contains species that are related to integrated BSV sequences that could be activated to give episomal virus in certain cultivars with the B genome, supergroup II contains integrated BSV sequences that were so perturbed that they were considered not to be activatable (termed “dead”) (mainly in the A genome) and members of supergroup III are closely related to BSV strains having episomal virus from different parts of the world. Thus, the sequenced region of farm samples of Ugandan BSV had representatives in each of the three major groups of BSV variants.

It was observed that some farmer samples, e.g. sample 13, gave only weak PCR bands. Cloning and sequencing of these products showed that they were related to “dead” sequences. Such sequences may well be present in the A genome (A. Geering, pers. comm.) but hybridization experiments on these samples indicated their presence in virion DNA. PCR using epidemiology experiment samples supplied by Dr. Kenyon also gave weak PCR bands, if any band at all. However, in these cases, it appeared that the amplified sequences could be chromosomal.

Attempts were made to obtain comparable sequence from the inserts of the clones from the epidemiology experiment infector samples but technical difficulties were experienced. The DNA was not amplified using the 1A-4’ primers and region of the genome that could be amplified

using the RD primers differed from that amplified by the 1A-4' primers. Further sequence will be obtained from these samples as soon as possible and the results will be passed to Dr. Kenyon.

9. Discussion on variation

The amount of variation of BSV found in the Uganda farm samples was totally unexpected and is unprecedented in plant virus epidemiology. This has many implications including:

1. From Appendix G2 it can be seen that supergroup I is related to another badnavirus, sugarcane bacilliform virus, and supergroup III related to cocoa swollen shoot badnavirus. As noted above, at least one isolate of BSV can infect sugarcane. This suggests that there might have been several introductions of BSV from other plant species. It is not known if this is a "one-off" occurrence or whether it is significant in the epidemiology of the virus.

2. The amount of variation within each supergroup suggests that the initial introduction(s) may be old events. However, if there are infections from other hosts it is not known if there is similar variation within that host. The variation is more likely to occur in perennial hosts that are vegetatively propagated because if it depended on vector transmission to healthy plants there would be likely to be reduction of variability due to Muller's ratchet. This highlights the need in epidemiology studies to consider BSV-like viruses in other hosts.

3. The wide variation of BSV could indicate that there might be different natural vector (mealybug species) for at least members of the three supergroup. This has epidemiological implications.

4. The most frequently found virus species were C (12 samples), F (11 samples) and D (10 samples); virus species A, I, J, and K were the least frequent each being found on one sample.

5. Appendix H shows an analysis of virus species in relation to geographic region of the farm, cultivar of banana, or symptoms produced. In each of 5 localities only one virus species was found but these were only in one sample each from Bukinda and Kambesi. In the other three, species C was found in 3 varieties in Luwero, species D in 4 varieties in Ibanda and species G in 4 varieties in Mbale. The cluster of closely related isolates in Mbale was that which gave the good serological reaction (Appendix E) and also that which has a 6 nucleotide deletion when compared to the other species. The clusters of closely related isolates from the same region in several cultivars suggests relatively recent introduction and spread of the virus. The cultivars were identified by phenotype but before any firm conclusions can be drawn, they should be categorized using molecular techniques.

6. In all 26 varieties of banana and one sample of ensete were examined. The banana cultivars represented 4 genotypes with the AAA East African Highland genotype being divided into 5 clone sets (Karamura, D.A. 1998. PhD Thesis, University of Reading). No relationship could be distinguished between genotype or clone set and virus species (Appendix H).

7. No relationship could be recognized between symptom features and virus species (Appendix H). This is not altogether surprising as BSV symptoms vary with time and cultural conditions of the plant and the observations were only made when the sample was taken.

8. The variation was analyzed by sequencing a region of the BSV genome, the polymerase gene, which is considered to be highly conserved. We have no information on the rest of the genome of any of the species from Uganda. There is a reasonable possibility that there has been recombination between the genomes of the BSV species and between episomal BSV sequences and sequences in the host chromosomes. For instance, sequences previously allocated to supergroup III were regarded as being integrated and "dead". The Ugandan BSV isolates that fell into this supergroup had episomal virus which suggests either that they were the progenitor of the "dead" sequences or that they had acquired the "dead" sequence region from the host genome by recombination.

9. In spite of the apparent close relationship of some Ugandan BSV isolates (supergroup I) to BSV strains that are activatable from integrated sequences, no evidence was found for activatable BSV sequences in East African Highland bananas. This is in accord with the finding that the activatable integrant is limited to the B genome, the East African Highland bananas being AAA genome and suggests that the virus has moved horizontally between bananas containing A and B genomes.

10. As noted above, it has not yet been possible to obtain comparable sequence data from the epidemiology experiment infector samples due to technical difficulties. The sequence data that could be obtained using the RD primers was from a different part of the genome to that obtained from other samples using the 1A-4' primers. The sequence from the products using the RD primers was very similar to that of the OL strain (virus species F). However, the lack of amplification using the 1A-4' primers would suggest that these isolates differed from members of virus species F and that the virus in these infector samples may be a recombinant between the OL strain and an undefined strain. These infectors came from experimental plots at Namulonge and Mbarara and they may represent yet further variation of the virus in Uganda.

10. Development of diagnostic protocol

As explained in Section 3c, serological techniques using the widest spectrum antiserum available were not reliable enough for diagnosing infection with Ugandan BSV isolates. As noted in the Background, Harper et al. (1999) have developed an immune capture-PCR technique and the work in this project has shown that diagnostics based on PCR would seem to be the most reliable. Appendix E shows that products could be obtained with the farm samples that were tested using PCR with primer set 1A-4'. However, this primer set did not function with the samples from the epidemiology experiment infectors but other primer sets did.

Sample preparation is an important aspect of diagnostics. The best method should be simple and reliable. Although much of the characterization was performed on purified virus preparations because of the low titer of the virus in infected plants, for routine diagnosis it was not necessary to go through this procedure. The diagnostic protocol that was developed is listed in Appendix I. Basically, this involves extraction of sap from the sample, concentration of the virus (and other proteins) by polyethylene glycol precipitation, binding of virus to an Eppendorf tube either by immunecapture or direct binding, PCR using primer set 1A-4' and gel electrophoresis of the PCR products.

This basic protocol can be varied according to the requirements of the diagnostician. In the case of the epidemiology experiments in the associated project R7529 the primers will have to be either RDfor-RDrev or GHfor-GHrev listed above. If one was studying aspects of the virus variation either the PCR product could be characterized by specific restriction enzymes identified from the sequences or by designing primers based on the sequences of the various "species". For diagnostics related to tissue culture the protocol in Appendix I should be used, but if there is any suspicion that the plant was infected but not giving a product, further sets of primers should be tested. It must be recognized that the reliability of any diagnostic test depends on the current knowledge of the pathogen and that the full range of variation of BSV may not yet have been determined.

Outputs

On two visits to Uganda 59 samples were collected from 30 farms at 14 sites covering the major banana growing regions of that country. In addition, 92 samples were sent from the two epidemiology sites used by project R7529. A modified purification procedure was developed for BSV and virus preparations were made from 47 of the 59 farm samples and from three of the epidemiology site samples; the other samples were too small or symptomless. Serological analysis by ELISA (enzyme-linked immunosorbent assay) and ISEM (immune-specific electron microscopy) showed that few of the samples reacted strongly with a broad spectrum antiserum that detects BSV isolates from other parts of the world. DNA extraction and PCR using degenerate primers (covering a region that is considered to be highly conserved among this group of viruses) (1A-4') indicated that, at least in some cases, the poor serological reaction was not due to low virus titre. The inserts in 187 clones were made from the PCR products of 48 of the samples (ranging from 1 to 9 per sample) were sequenced. The sequences were compared with each other and with published and other BSV sequences. The results were:

1) If sequence differences of more than 20% differentiate between virus species, 11 BSV species could be recognised in the Ugandan samples.
2) These species fell into three supergroups that differ by more than 35% sequence. One supergroup contains BSV strains that are integrated and can be activated to give episomal infection, one supergroup contains BSV sequences that have been found integrated in a form that is not activatable ("dead") and the third supergroup contains strains that have no integrated analogue.
3) The samples from the infectors in the epidemiology experiment would not amplify using the degenerate primers that gave products for the other samples but did so with other primer sets (Rdfor-Rdrev and Ghfor-Ghrev). However, PCR of asymptomatic samples from epidemiology sites gave minor products that had "dead" sequences; it is possible that PCR was amplifying such sequences from the plant chromosome and that there was no episomal virus.
4. There was no correlation between the virus "species" or supergroup and banana cultivar or symptoms. One site, in the East of Uganda, had one virus species but all the other sites had more than one species. Eleven of the samples were infected by more than one virus "species".

In developing a robust diagnostic technique, it was shown that serological techniques such as ELISA were not reliable but that PCR using primer set 1A-4' could detect BSV in most cases; however, three cases were found in which primer set 1A-4' did not amplify a product from samples with obvious symptoms but other primer sets gave products. To distinguish between episomal virus and integrated viral sequences and to remove potential inhibitors of PCR, techniques involving

capture of virus particles from sap extracts either on antiserum-coated tubes (immune-capture, IC) or directly onto the plastic tube surface (direct binding, DB) were developed. In samples with low virus titre, IC/PCR or DB/PCR was improved by concentrating the virus by polyethylene glycol (PEG) precipitation.

Contribution of Outputs to developmental impact

This project has exceeded the research goals. It has shown that there is much more variation of BSV in Uganda than was originally expected. This has implications in four areas. Specific to projects such as that on the epidemiology of BSV (R7529) it has demonstrated that the BSV epidemic in Uganda is not due to one virus strain or one introduction but indicates a large number of variants and introductions. The range of variation suggests long-standing infections with BSV. Thus, for project R7529, it is important to characterize the virus(es) in the infector plants. In such experiments, it should be possible in many cases to distinguish between spread from the infector plants and spread from outside the experimental plot. Secondly, it highlights the importance of good diagnostics for obtaining healthy material from tissue culture systems. Not only has one to be aware of the virus variability already shown but also it should be recognized that there might be virus variants not detected by the current primer sets. Thirdly, this is the first example of a detailed analysis of BSV variation in an individual country and it is likely that there would be similar variation in other countries or regions where the virus has been established for a long time. Fourthly, there is the basic scientific question as to how this variation arises. There has not been enough time in the project to sequence full genomes of several of the virus species found in Uganda but it is likely that at least some of the variation is due to recombination. It would be interesting to know whether any recombination is between episomal variants or whether there is recombination between an episomal variant and integrated BSV sequences.

The basics of a diagnostic technique have been developed. For general diagnosis this involves extraction of sap from the test plant, concentration of the virus in that sap by PEG precipitation, binding of the virus to a plastic tube (IC or DB), PCR using primer set 1A-4' and analysis of the PCR products by gel electrophoresis. If a symptomatic sample does not give a PCR product, other primer sets should be used. For specific diagnosis of virus "species", the PCR product could be analysed by restriction enzyme cutting or by sequencing or specific PCR primers can be designed from the sequence information gained in this project.

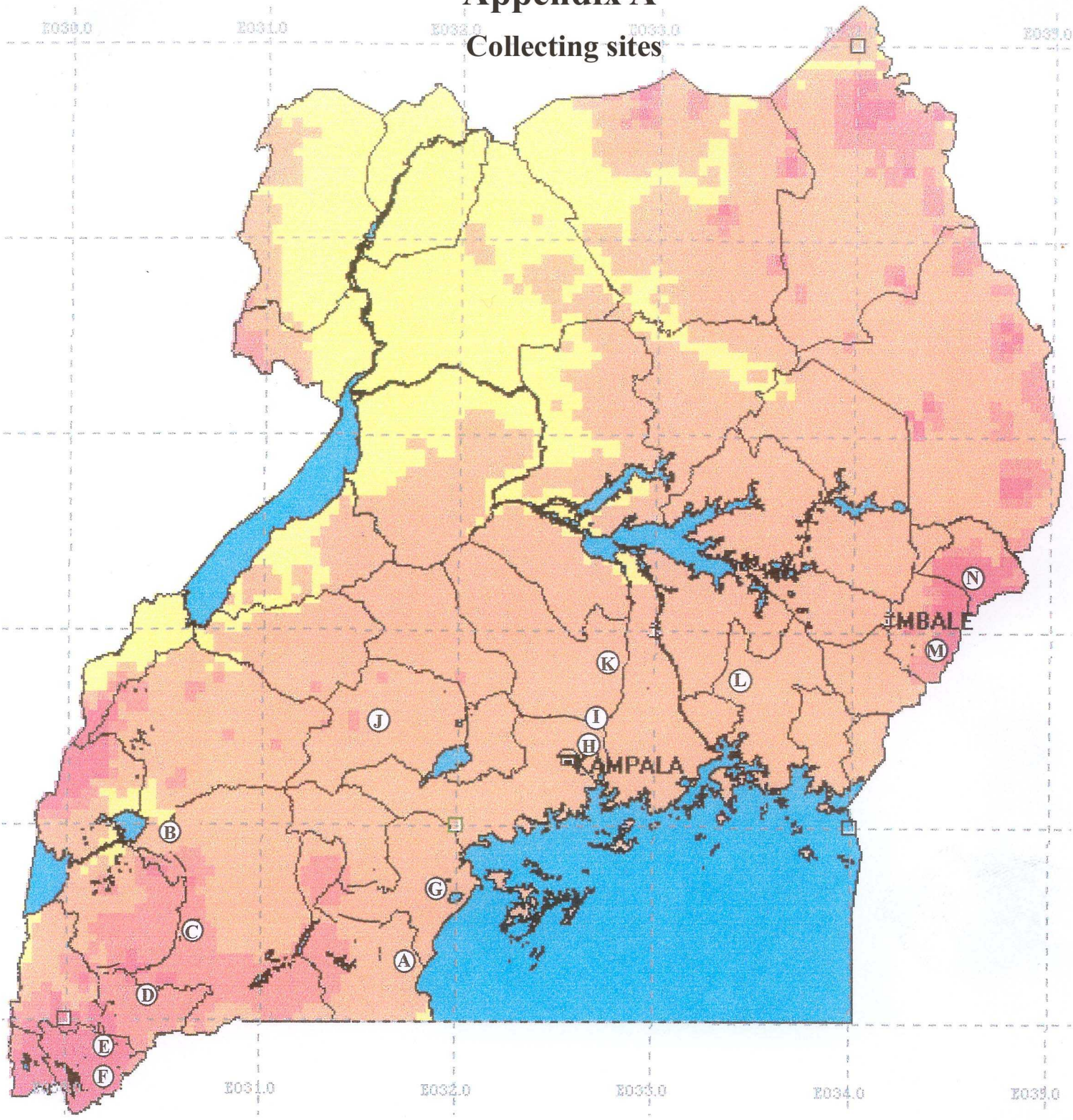
Appendix A

Collecting localities in Uganda

A. Rakai
B. Ibanda
C. Mbarara
D. Ntungamo
E. Bukinda
F. Kambesi
G. Msaka
H. Kawanda
I. Namulonge/Basukuma
J. Mubende
K. Luwero
L. Iganga
M. Mbale
N. Kapkwirwo

Appendix A

Collecting sites



A: 1-6	E:17-19	I: 27-31	M: 59-63
B: 7-10	F: 20	J: 40-50	N: 64-66
C: 11-12	G: 21-25	K: 51-55	
D: 13-16	H:26A & B	L: 56-58	

Sample numbers at the collecting sites

Appendix B

Record sheets for samples

SITE # 1

Site: Nabigasa-Rakai GPS 0-34-25S/31-26-53-E Altitude 1250 masl

Farmer: Rubambura Date visited: 6/11/99

Samples:

No. 1

Variety: Kibuzi
Symptoms: Moderate symptoms;
largish chlorotic and necrotic streaks
Slide:
Sample: Leaves (wt. 225 gm), 1sucker
Notes: Adjacent to sugar cane

No 2 Variety: Nakabululu
Symptoms: Severe at leaf edge;
largish chlorotic and smaller necrotic
streaks over all lamina
Slide: 10, 11
Sample: Leaves (wt. 225 gm), 1 sucker

No. 3 Variety: Nakitembe
Symptoms: Milder than 1 or 2; short chlorotic streaks
Slide: 12
Sample: Leaves (wt. 305 gm), 1 sucker

No 4

Variety: Gros Michel
Symptoms: ? no symptoms
Slide:
Sample: 1 sucker

General notes:

a) First noted here about 15 years ago
b) Various EAHs which have different symptoms
c) Gros Michel relatively new introduction
d) General view of site Photo 13:

SITE # 2

Site: Nabigasa-Rakai GPS 0-34-14S/31-26-48E Altitude 1250 masl
Farmer: Sekyondura Vincent Date visited: 6/11/99

Samples:
No: 5

Variety: Ntukura (beer banana)
Symptoms: Severe; chlorotic streaks fusing and becoming necrotic
Slide:
Sample: Leaf (wt. 130 gm)

General notes:

a) Jerome's spread site

SITE # 3

Site: Luabuguma-Rakai GPS 0-34-22S/31-27-21E Altitude 1250 masl
Farmer: Kawarabu Emanuel Date visited: 6/11/99

Samples:
No: 6
Variety: Kibuzi
Symptoms: Moderate chlorotic streaks becoming necrotic and then leaf splitting
Slide: 14,15
Sample: Leaf (wt. 560 gm), 1 sucker.

General notes:
a) TC plants on this site. No BSV symptoms observed
TMPx5295
TMBx1378
Local cvs
b) Pisang Awak nearby – no BSV symptoms

SITE # 4

Site: Kyamoshe cell, Ibanda GPS 0-07-31S/30-31-03E Altitude 1380 masl
Farmer: Byamkole Date visited: 7/11/99

Samples:
No: 7
Variety: Enzwiga (beer banana)
Symptoms: Mild; fine short chlorotic streaks going necrotic
Slide: 16, 17
Sample: Leaf (wt. 150 gm), 1 sucker

No. 8
Variety: Enzuma (cooking)
Symptoms: Severe; broad yellow streaking going necrotic; pseudostem splitting
Slide: 18
Sample: Leaf (wt. 215 gm)

General notes:
a) First seen on this farm in 1980, reported to “Germans” but no action taken
b) Yield from “healthy” up to 50 kg; from infected 15-20 kg
c) 4 acres > 50 years old
d) 29/50 infected
e) Most cvs have leaf symptoms

SITE # 5

Site: Nsasi, Nyabuhikye, Ibanda GPS 0-06-35S/30-31-55E Altitude 1380 masl
Farmer: Mugumba Sebastiano Date visited: 7/11/99

Samples:
No: 9 Variety: Enzuma
Symptoms: Severe; short chlorotic to necrotic streaks
Slide:
Sample: Leaf (wt. 285 gm)

No: 10
Variety: Kabucuragye
Symptoms: Severe; yellow lozenge flecks fusing to give chlorotic areas
Slide: 20, 21
Sample: Leaf (wt. 370 gm)

General notes:
a) 20/25 and 18/25 infected on counts
b) Emburura with same symptoms as 10
c) Gros Michel, 2 plants no obvious symptoms

SITE # 6

Site: NARO farm, Busheni GPS 0-33-48S/30-11-31E Altitude 1620 masl
Farmer: Date visited: 7/11/99

Samples: No samples taken
No:
Variety:
Symptoms:
Photo:
Sample:

General notes:

a) Mixture of EAH well cultivated. Low % showing mild symptoms

SITE # 7

Site: Stock Farm, Mbarara GPS 0-35-56S/30-36-76E Altitude 1460 masl
Farmer: Date visited: 7/11/99

Samples:
No: 11
Variety: Sukali Ndizi
Symptoms: Moderate; short, relatively broad, chlorotic streaks
Slide:
Sample: Leaf (wt. 75 gm)

No: 12
Variety: Nasuma
Symptoms: ? genetic but like BSV except that also on fruit
Photo: 23
Sample: Leaf (wt. 20 gm), 1 sucker (sucker lost)

General notes:
a) Culture collection (see separate list) from Kawanda

SITE # 8

Site: Karegeya - Ntungamo GPS 0-52-57S/30-14-00E Altitude 1440 masl
A.
Farmer: Kassim Date visited: 8/11/99

Note: 10% BSV infection with mild symptoms

B.
Farmer: Ravaheru Daniel

Samples:
No: 13
Variety: Enyeru
Symptoms: Mild interveinal mottle
Slide:
Sample: Leaf (wt. 180 gm)
No 14 Variety: Enyeru
Symptoms: Mild interveinal mottle + yellow blotching at leaf base
Slide:
Sample: Leaf (wt. 170 gm)

C.
Farmer: Katachira

Samples:
No. 15
Variety: Enyeru
Symptoms: Very severe; chlorotic streaks turning necrotic
Slide:
Sample: Leaf (wt. 295 gm), 1 sucker (sucker lost)
No. 16
Variety: Enyeru
Symptoms: Severe; strong speckled yellow to necrotic
Slide: 24, 25, 26
Sample: Leaf (wt. 260 gm)

Note: 36/50 (72%) plants infected

General notes:
a) Farm A – high management; Farm B – high management; Farm C – Low management
b) At farm B a plant of Enyeru

From cigar leaf: leaf 1 no symptoms; leaf 2 v. sl. Symptoms at base; leaf 3 no symptoms; leaf 4 no symptoms; leaf 5 severe infection;leaf 6 moderate symptoms mid-leaf; leaf 7 slight symptoms at base; leaf 8 slight symptoms at base. Leaf emergence at about 14 days

c) New benchmark site

SITE # 9

Site: Buknida GPS 1-13-28S/30-07-12E Altitude 1880 masl
Farmer: Pokomo Date visited: 8/11/99

Samples:
No: 17
- Variety: Ensete
- Symptoms: Necrotic streaking
- Slide: 27, 28, 29
- Sample: Leaf (wt. 185 gm)

No. 18
- Variety: Enyeru
- Symptoms: No BSV symptoms
- Slide:
- Sample Leaf (wt. 65 gm)

No 19
- Variety: Enyeru
- Symptoms: Mild interveinal mottle
- Slide:
- Sample: Leaf (wt. 65 gm)

General notes:
- a) Sample 18 taken for DNA test

SITE # 10

Site: Bwenjaji, Kamwesi GPS 1-10-35S/30-07-12E Altitude 1750masl
Farmer: Date visited: 8/11/99

Samples:
No:
Variety:
Symptoms:
Slide:
Sample:

General notes:
a) No BSV

SITE # 11

Site: Rwenyangye-Kamwesi GPS 1-10-49S/30-10-26E Altitude 1680masl
Farmer: Jonathan Anho Date visited: 8/11/99

Samples:
No: 20 Variety: Enyonga (beer type)
Symptoms: Moderately mild BSV symptoms;
short chlorotic streaks turning necrotic
Slide:
Sample: Leaf (wt. 270 gm)

General notes:
a) People moved here from Rakai/Masaka/Mbarara from 1921 - now

SITE # 12

Site: Busubi-Kissekka, Masaka GPS 0-23-58S/31-32-36E Altitude 1250masl
Farmer: Mrs Kakinda Date visited: 9/11/99

A.
Samples:
No: 21 Variety: Musakala
Symptoms: Moderate streaking; short to very short chlorotic streaks, relatively narrow
Slide:
Sample leaves: (wt. 285 gm + 180 gm midribs)

No 22 Variety: Kibuzi
Symptoms: Very mild; chlorotic speckling
Slide
Sample: leaves (wt. 160 gm)

No 23 Variety: Mbwazarume
Symptoms: Mild; fine chlorotic streaking turning necrotic
Slide: 30, 31
Sample: Leaves (wt. 360 gm)

B.
Samples
No 24
Variety: Kibuzi
Symptoms: Very severe streaking; broad chlorotic streaks turning necrotic
Slide: 32, 33
Large leaf sample (wt. 1110 gm), 1 sucker
No 25
Variety: Nakyinyika
Symptoms: Moderate severe streaking; finer chlorotic streaking than 24, turning necrotic
Slide:
Sample: leaf (wt. 690 gm),1 sucker (sucker lost)

General notes:
a) Plantation A > 20 years old; plantation B 2 years old planted from A
b) Plantation A 13/25 infected; plantation B 15/25 infected

SITE # 13

Site: NARO, Kawanda GPS Altitude
Farmer: Date visited: 5/11/99

Samples:
No: 26A
Variety: ? Siira
Symptoms: Moderate; fine chlorotic streaking
Slide:
Sample: Leaf (wt. 55 gm)
No 26B
Variety: Mbwazarume
Symptoms: Moderate; chlorotic streaking
Slide:
Sample: leaf (wt. 145 gm)

General notes:
a) Samples taken first day and kept in fridge

SITE # 14

Site: Namulonge GPS 0-13-48N/32-36-46E Altitude 1180 masl
Farmer: IITA/ESARC Date visited: 10/11/99

Samples:
No: 27
Variety: Musa ornata
Symptoms: No obvious symptom
Slide:
Sample: leaf (wt. 25 gm), 1 sucker (sucker lost)
No. 28
Variety: Mbwazarume
Symptoms: moderate streaking; relatively broad chlorotic streaks
Slide:
Sample: leaf (110 gm), 1 sucker

General notes:
a) Sample 27 for DNA PCR etc
b) Sample 28 from EET block and for strain id for Jerome's transmission work

SITE # 15

Site: Kabumba-Basukuma GPS 0-31-43N/32-35-13E Altitude 1180 masl
Farmer: Date visited: 10/11/99

Samples:
No: 29
Variety: Ndizi (apple banana)
Symptoms: Moderate; interveinal mottle
Slide:
Sample: Leaf (wt. 50 gm)

General notes:

SITE # 16

Site: Kasazi-Busukuma GPS Altitude
Farmer: Date visited: 10/11/99

Samples:
No: 30
Variety: ? Musakola
Symptoms: Relatively mild; chlorotic streaking
Slide: 34,35 of plant for cv id
Sample: leaf (wt. 190 gm)
No 31
Variety: ?
Symptoms: Moderate; chlorotic streaks
Slide: 35
Sample: leaf (wt. 310 gm)

General notes:

SITE # 17

Site: Bulera-Mubende GPS 00.50508N/32.01306E Altitude 1519 masl
Farmer: Mr. Mabike Jacks Date visited: 23/07/00

Samples:
No: 40
Variety: Nabweru
Symptoms: Chl. Streaks, some going necrotic on old leaves; severe infection of cigar leaf of one plant.
Photo: dig. 40, 43, 44 (two of cigar leaf and one of chl i/v patch cf adjacent streaked patch
Sample: Leaves (190 g)

No: 41
Variety: Nabweru
Symptoms: Chl. Streaks going necrotic. (? Mix with sigatoga)
Photo:dig, 46, 47
Sample: Leaves (378 g) + sucker

General notes:

SITE # 18

Site: Kibale-Mubende GPS: 00.53876N/32.03531E Altitude: 1334 masl
Farmer: Lowben Senyondo Date visited: 23/07/00

Samples:
No: 42

Variety: Nakyetengu

Symptoms: Mild patches of chlorotic streaks. Only a few plants with these symptoms, many with mild mottle

Photo: Nil

Sample: Leaves (172 g)

No: 43

Variety: Nakytengu

Symptoms:One plant with overall more general chlorotic streaking, including cigar leaf – symptoms severe

Photo: dig. 50, 51

Sample: leaves (396 g)

General notes
Plants with side bunches – dig. Photos 48, 49

SITE # 19

Site: Kibogo-Mubende GPS: 00.52718N/32.01234E Altitude 1363 masl
Farmer: Mary Nantume Date visited: 23/07/00

Samples:
No: 44
Variety: Nakabululu
Symptoms: General chlorotic streaking going necrotic with age
Photo: None
Sample: leaves (87 g)

General notes:
About 10% infection

SITE # 20

Site: Kirumbi-Kitenga-Mibende GPS: 00.44237N/31.61585E Altitude: 1308 masl
Farmer: Nsereko Musa Date visited: 23/07/00

Samples:
No: 45

Variety: Gonja (AAB)

Symptoms: Very strong chlorotic streaks, especially in cigar leaf and next young leaf. Other leaves, severe near tip and less sev ere towards base. Older leaves have more spaced chlorotic streaks.

Photo: dig. 56, 57

Sample: Leaves (148 g) + sucker

General notes:
Use this sample for 25 nt work

Sample
No. 46

Variety: Nfuuka

Symptoms: Extensive but milder streaking than # 45

Photo: dig 58, 59

Sample: leaves (678g) + sucker.

Sample
No: 47.

Variety: Nasaba

Symptoms: Chlorotic to necrotic streaking

Photo: nil

Sample: leaves (230 g)

Sample
No: 48

Variety: Nasaba

Symptoms: From bottom leaf: 1. Blotchy streaks; 2. General and severe fine streaking; 3. Patchy and fine streaking with some necrosis

Photo: dig 62, 63 (leaf # 3)

Sample: leaves (136 g)

General notes

Compare the isolates in these four samples. They are from plants 3-4 m apart

SITE # 21

Site: 300 m from site 20 GPS Altitude
Farmer: Musoke Wood Date visited: 23/07/00

Samples:
No: 49
Variety: Kisansa
Symptoms: Broad chlorotic streaks turning necrotic (red)
Photo: dig. 64
Sample: leaves (423 g)

No. 50
Variety: Kisansa
Symptoms: Extensive mild streaking
Photo: No photo
Sample: leaves (75 g)

General notes:
Plantation 8 years old

SITE # 22

Site: Kakora - Luwero GPS: Altitude
Farmer: NARO Date visited: 24/07/00

Samples:
No: 51

Variety: Dwarf Cavendish
Symptoms: Patches of fine chlorotic streaks. Cigar leaf heavily infected.
Photo: dig. 65, 66
Sample: Leaves (541 g) + sucker

General notes:
1. This is a NARO demonstration site
2. D.C tissue culture plants from South Africa or KUL
3. Infected patch in corner of block. No symptoms seen in adjacent FHIA23 or local varieties

?? Was this infection through tissue culture or spread from adjacent plants.
** Consider as a site for monitoring spread.

SITE # 23

Site: Bamunanika-Luwero GPS: 00.73465N/32.61292E Altitude: 1284
Farmer: Kisamba Mugwera Date visited: 24/07/00

Samples:
No: 52
Variety: Ntika
Symptoms: Sucker leaf showing broad chlorotic streaking on one half of leaf near tip and fine chlorotic streaking on other half.
Photo: dig. 69, 70
Sample: Leaf (108 g)

No. 53
Variety: Kisasa
Symptoms: similar to sample 52
Photo: dig 71 (broad streaking), 72 (fine streaking)
Sample: leaves (140 g) + sucker

General notes:

SITE # 24

Site: Kayunga - Makona GPS: 00.67934N/32.88103E Altitude: 1284 masl
Farmer: Mrs. Damulira Date visited: 24/07/00

Samples:
No:
Variety: Many but main one Ndibwabalangira
Symptoms:
Photo:
Sample:

General notes:
No BSV symptoms. Pineapple and sugar cane in plantation samples. Some sugar cane had MSV-like symptoms.

SITE # 24B

Site: GPS Altitude
Farmer: Date visited: 24/07/00

Samples:
No:
Variety:
Symptoms:
Photo:
Sample:

General notes:
About 1 km from site 24 but not able to take GPS because of cloud. Previous visits showed BSV (4/25). None found this time. Similar range of cultivars to # 24

SITE # 25

Site: Ntoke-Koyunga GPS: 00-68540N/32.89824E Altitude 1284
Farmer: Kibugo Lameck Date visited: 24/07/00

Samples:
No: 54
Variety: Ndibwubalangira
Symptoms: Fine chlorotic streaking – quite mild.
Photo: No photo
Sample: Leaves (131 g)

No. 55
Variety: Gros Michel
Symptoms: Interveinal chlorosis giving green vein banding.
Photo: dig. 73, 74
Sample: leaves (88 g)

General notes:
1. This is one of the sites for nematode clearing with a two year break of cassava. The one Ndibwubalangira plant showing symptoms was from TC plants from Kawanda 2.5 years ago.
2. Gros Michel not on site but adjacent. Symptoms viral but not characteristic BSV ? another virus

SITE # 26

Site: Bulongo - Iganga GPS: 00.7569N/33.3397E Altitude: 1100 masl
Farmer: Katumba Sali Date visited: 25/07/00

Samples:
No: 56
Variety: Mafuka
Symptoms: Well dispersed short chlorotic streaks with two interveinal areas with streaks over whole half leaf.
Photo: dig. 79
Sample: leaves (49 g)

No. 57
Variety: Mbwazirume
Symptoms: severe chlorotic streaking
Photo: dig. 80
Sample: leaf 206 g) + sucker

No. 58
Variety: Mafuka
Symptoms: finer streaking than # 57, going necrotic
Photo: none
Sample leaves (345 g)

General notes:
This farm has interplanted banana and pineapple – well looked after.

SITE # 27

Site: Butiru - Mbale GPS: 00.8763N/34.2888E Altitude: 1230 masl
Farmer: Omulaku Stephen Date visited: 25/07/00

Samples:
No: 59
Variety: Mbwazarume
Symptoms: Severe chlorotic to necrotic symptoms
Photo: dig. 81, 82, 83, 84, 85
Sample: leaves, large sample of leaves of type in photo 85 (365 g)

Sample: 60
Variety: Mbwazarume
Symptoms: similar to # 59
Photo: none
Sample: leaf (172 g) + sucker.

General notes:
1. Plantation 9 – 10 years old, material obtained locally
2. Counts on infected plants 3/10, 2/ 10, 7/10 (overall 12/30). This shows that there is a BSV patch. Count in 1997 of 20%
3. Farmer said yield loss about 30% per year.

SITE # 28

Site: Bugavero - Mbale GPS 00.888N/34.2760E Altitude 1230 masl
Farmer: Khaukha David Date visited: 25/07/00

Samples:
No: 61
Variety: Nakitembe (Nasirembe)
Symptoms: Mod. severe chlorotic to necrotic streaking
Photo: None
Sample: leaves (357 g) + sucker

No. 62
Variety: Namanamunga (Gisu language)
Symptoms: Broader chlorotic streaks than # 61, going necrotic
Photo: dig 89, 90
Sample: leaves (252 g)

No. 63
Variety: Kibusi
Symptoms: very mild
Photo: none
Sample: leaves (144 g)

General notes:
Symptoms vary between these three cultivars in the same plantation – are they all the same strain?

SITE # 29

Site: Sipi, Kapchorwa, Kapkwirwok GPS:01.3311N/34.3712E Altitude:1837 masl
Farmer: Lazarus Muki Date visited: 26/07/00

Samples:
No: 64

Variety: Njeryandet (Nyeru)
Symptoms: Broad chlorotic interveinal streaking. One leaf streaking at edge
Photo: dig. 95
Sample: leaf (312 g) + sucker

No. 65

Variety: Mudware
Symptoms: “mosaic” of broad chlorotic streaking
Photo: dig 97
Sample: leaf (150 g)

No. 66

Variety: Gros Michel
Symptoms: very severe chlorotic streaking to necrosis
Photo: dig 98 and 99
Sample: leaves (722 g) + sucker

General notes:
1. Sample 66 from same farm but some distance (GPS 01.3330N/34.3744E; 1815 masl)
2. Plantation about 4 years old planted from local material.
3. Photo dig 100 across valley towards this site

Appendix C

Virus purification

Method

1. Grind 20g of leaf tissue in liquid nitrogen with a small amount of sand added.

2. Thaw to –20°C for 30min.

3. Add 100ml of 50mM PO_4 extraction buffer pH6.1 with 0.5% 2-ME.

4. Blend for 1min.

5. Add 2ml Celluclast and incubate at 30°C for 3hr.

6. Squeeze through double layer of muslin.

7. Dispense into 250ml bottles, and centrifuge in Superspeed centrifuge using GSA rotor with rubber cushions at 8,000 rpm for 20min at 4°C.

8. Pour off supernatant.

9. Add 20% Triton to 2%. Stir for 30min at room temperature.

10. Keep at 4°C overnight.

11. Dispense into 250ml bottles, and centrifuge in Superspeed centrifuge using GSA rotor with rubber cushions at 8,000 rpm for 10min at 4°C.

12. Pour off supernatant.

13. Dispense into 30ml Nalgene Oak Ridge centrifuge tubes. Underlay with 5ml 20% sucrose cushions, made from sucrose dissolved in extraction buffer without 2-ME.

14. Centrifuge in Ultracentrifuge using type 30 rotor at 27,000 rpm for 90min at 4°C.

15. Resuspend pellets with 500µl of 0.01M PO_4 resuspension buffer pH7.0 per sample. Loosen pellet with glass rod before adding buffer.

Extraction buffer

500mM PO_4 pH6.1

diluted to 50mM
1M urea
4% PVP
with 0.5% 2-Mercaptoethanol added prior to using

0.01M Phosphate resuspension buffer

0.5M Na_2HPO_4
0.5M NaH_2PO_4

pH 7.0
diluted to 10mM

Appendix D

Serological methods

ELISA

Method

1. Coat wells with 100µl of antisera diluted 1:1000 in carbonate coating buffer. Leave first column blank.

2. Incubate at 37°C for 2hrs.

3. Plate washed 4x with PBS-T. Tapped on blue paper after each wash.

4. Add to each well 100µl of virus prep diluted 1:10 or 1:100 with PBS.

5. Incubate at 4°C overnight.

6. Plate washed 4x with PBS-T. Virus wells washed initially with pipette, and then emptied with suction line. Tapped on blue paper after each wash.

7. Add to each well 300µl of 2.5% dried milk in PBS.

8. Incubate at 37°C for 30min.

9. Plate washed 4x with PBS-T. Tapped on blue paper after each wash.

10. Add to each well 100µl of conjugated anti BSV diluted 1:200 with ECl buffer.

11. Incubate at RT for 2hrs.

12. Substrate made by dissolving one 5mg PNP tablet in 5ml of PNP buffer. Buffer should be at RT. Prepare before washing plate to allow tablets to dissolve fully.

13. Plate washed 4x with PBS-T. Tapped on blue paper after each wash.

14. Add to each well 100µl of substrate.

15. Cover plate to exclude light and read at 405nm after 30min (10min if colour develops quickly), and again after 1hr and 2hr when colour has developed further. If necessary leave at 4°C overnight and read again the following morning.

Carbonate coating buffer pH9.6

Na_2CO_3	1.59g
$NaHCO_3$	2.93g

make up to 1000ml

PBS x1 or x10 stock

	x1	x10
Sodium chloride NaCl	8.0g	80g
di-Sodium hydrogen orthophosphate (anhydrous) Na_2HPO_4	1.15g	11.5g
Potassium dihydrogen orthophosphate (anhydrous) KH_2PO_4	0.2g	2g
Potassium chloride KCl	0.2g	2g

dissolve in distilled water to 1000ml
adjust pH to 7.4

PBS-T

PBS + 0.1% tween

ECl buffer

Bovine serum albumin (BSA)	2.0g
Polyvinylpyrrolidone (PVP) MW 24-40,000	20.0g

add to 1000ml 1x PBS-T
adjust pH to 7.4
store at 4°C

PNP buffer

Magnesium chloride	0.1g
Sodium azide	0.2g
Diethanolamine	97.0ml

dissolve in 800ml distilled water
adjust pH to 9.8 with hydrochloric acid
adjust final volume to 1000ml with distilled water
store at 4°C.

Immunosorbent Electron Microscopy - ISEM

Method

1. Place 20µl drop of antisera, diluted 1:1000 in 0.06M phosphate buffer pH6.5*, on coating plate in a Petri dish with damp filter paper.

2. Float grid carbon side down on drop.

3. Leave at room temperature for 2-4 hrs, or 37°C for 1 hr.

4. Wash off excess antisera by floating grid on 20µl drops of 0.06M phosphate buffer pH6.5*. Two changes of 10min each. Or run 20 drops of PBS over the grid.

5. Place 20µl drop of sample (spin sample for 1min) on grid plate, or dilution of 10µl sample plus 10µl 0.06M phosphate buffer pH6.5*.

6. Float grid carbon side down on drop.

7. Leave at 4°C overnight.

8. Hold grid in forceps and wash five times using a disposable Pasteur pipette of distilled water.

9. Without drying, stain with 20 drops of uranyl acetate dropped onto face of grid.

10. Blot, but do not dry completely.

* If 0.06M phosphate buffer pH6.5 is not available, PBS can be used.
If also preparing antisera for ELIZA then coating buffer can be used.

Phosphate buffer 0.6M (stock solution) pH6.5

$Na_2HPO_4.12H_2O$	21.48g
KH_2PO_4	8.16g
distilled H_2O	200mls

dilute 1:10 with sterile distilled water to make 0.06M solution

Uranyl acetate

2% solution in distilled water

Appendix E

Characterization of BSV samples

Virus prep.

+ indicates virus prepared from sample

Serology

ISEM: numbers of particles per field at 10, 000 x magnification

ELISA: Number of + indicates strength of reaction

PCR

Number of + indicates strength of product band on gel electrophoresis

DB = PCR from direct binding from sap; IC = PCR from immunocapture from sap; direct virus DNA = PCR from DNA from virus preparation

Sequences

Good: clone numbers that gave relevant sequences

Wrong and weird: clone numbers that gave suspect sequences

Sample No.	Locality	Virus prep	Serology ISEM No./F	Serology ELISA	degenerate PCR DB-virus prep	degenerate PCR IC-virus prep	degenerate PCR direct virus DNA	degenerate PCR DB- and IC on sap	Sequences Good	Sequences wrong	Sequences weird
1	Rakai	+	<0.1	+			+++		-2, -3, -4, -5, -6	-1	
2	"	+	<0.1	+ [1]	+++		+++		-1,-4 -5,-7,-10,-11,-12	-3,-8, -9	
3	"	+	<0.1	-	+++		+++		-1, -2,-6,-7	-3,-4,-5,-8	
4	"										
5	"	+	<1	-	-		+++		-1,-2,-3, -5, -6	-4,-7,-8,-9	
6	"	+	<0.01	-	++		+		-1, -3	-2,-4	
7	Ibanda	+	<0.01	++			+++		-8, -10, -11	-4,-6	
8	"	+	<1	+			+++		-3, -4, -6		
9	"	+	2	-			+++		-1,-2, -3, -4		
10	"	+	<0.01	-			+++		-2, -3,-4	-1	
11	Mbarara							+	-1,-2, -4		
12	"							-			
13	Ntungamo	+	<0.01	-	++		+		-2,-3, -4,-5,-6	-7	-8,-9
14	"	+	0	-	+	+	-	+	-2		
15	"	+	2	+ [1]			+++		-1,-2, -5	-4	
16	"	+	<1	-			+++		-3,-5,-6	-1,-7	-8
17	Bukinda	+	<0.01	-	-	-	+		-3, -4		
18	"							-			
19	"							-			
20	Kambesi	+	<1	-			+++		-2, -4, -5		
21	Msaka	+		+			+++		-1, -2, -3		
22	"	+	<1	-	++		-		-1, -2, -4	-3,-5,-6	
23	"	+	5	++	++		-		-1, -3,-4	-5,-6,-7	-8
24	"	+	30	+++ [2]			+		-3, -6	-1,-2	
25	"	+	2	+ [2]			+		-1, -3, -4, -5, -6	-2	
26A	Kawanda										
26B	"	+	<0.1	-			-	+	-1, -2, -5		
27	Namulonge							-			
28	"	+	<0.01	-	-	-	-	-			
29	Basu Kuma							+	-1, -2		
30	"	+	5	+	++		-	+	-4		
31	"	+	<1	-	++		-	-			
40	Mubende	+	<1	-	+++		-	+	-3		
41	"	+	4	-	++		-		-1		
42	"	+	3	+	+++		-		-3, -4, -5		
43	"	+	1	-	++		-		-1,-3,-6	-4,-5	
44	"							+			
45	"	+	12	-			+++		-1, -2, -3	-4,-5,-6	
46	"	+	37	+ [1]	+/-	+++	-	+	-1		
47	"	+	9	+	-	++	-	+	-1, -2	-3	
48	"	+	2	+			+++		-1, -2, -3,		
49	"	+	12	+			+++		-1, -2, -4, -6		
50	"							+			
51	Luwero	+	<1	-			++		-2, -3, -4, -5	-1	
52	"	+	<1	-			+++		-1,-2,-3,-4	-5,-6,-7	
53	"	+	<1	-			+++		-1,-2, -3	-4	
54	Koyunga	+	<0.01	-			+++		-1		
55	"							+	-2		
56	Iganga							-			
57	"	+	<0.01	-			+++		-1, -2, -3		
58	"	+	<0.01	+	++		-		-2,-6		
59	Mbale	+	89	+++	+++		-		-3,-4,-5	-2,-6	
60	"	+	79	+++	+++		-		-2, -3, -6		
61	"	+	74	+++	++		-		-6		
62	"	+	68	+++			+	+	-2, -3		
63	"	+	41	+	++		-		-1, -2,-5	-4	
64	Kapkwirwok	+	44	+	+	+++	-		-2,-4,-6		-3
65	"	+	58	+++	+++		-		-1, -3, -5		
66	"	+	<1	-	+++		-		-1, -5, -6		
=59		=47							=138	= 49	

[1] = positive on earlier ELISA test

[2] = results from earlier ELISA test

Appendix F

Protocols for BSV DNA extraction and PCR

Virus DNA Isolation

Spin virus prep for 15minutes @ 13K in bench microfuge. Remove supernatant to fresh tube.
Spin 1hr 30 minutes @ 100,000 g on bench t-100 ultracentrifuge.
Resuspend pellet in 200ul 1x Dnase Buffer (40mm Tris (pH 7.9) 10mm NaCl, 6mm $MgCl_2$, 10mm $CaCl_2$)
Add 5ul DNase (1U/ul). Incubate @ 37°C for 15 minutes. Add stop buffer as manufacturers instructions (or EDTA to 20mm).
Add 5ul RNase A (10mgs/ml). Incubate at 37°C for 15 minutes.
Add 6.5ul SDS (10%) and 10ul Prot K. (140U/ml) Incubate at 65°C for 2hrs.
Add equal volume phenol, vortex and then spin @13K for 5 mins and remove aqueous layer to fresh tube.
Add equal volume phenol/Chloroform/Isoamyl Alcohol (24:24:1). Vortex then spin @13K for 5 mins and remove aqueous layer to fresh tube.
Add equal volume Chloroform/Isoamyl Alcohol (24:1). Vortex then spin @13K for 5 minutes. Remove aqueous layer to fresh tube.
Ethanol precipitate (usually o/n @-20)(2.5vols 100% EtOH, 10% 3M NaOac pH 5.2 + 1ul Blue Dextran @10mgs/ml)
Spin 15mins @ 13K.
Remove supernatant, Resuspend pellet in 20ul TE.

Usually have to run out at least 25% of total extract in order to see a band on a gel.

ICPCR

Coat thin wall tubes with 100µl antisera diluted 1:1000 in carbonate coating buffer
Incubate at 37°C for 2hrs, or room temperature for 4hrs
Wash tubes 3x in PBS-T

Add samples to tube
50 or 100µl of concentrated or normal sap
or 10µl of concentrated or normal sap made up to 100µl with PBS/2-ME
or 0.01, 0.1, 1 or 10µl of purified virus prep made up to 100µl with PBS/2-ME

Prepare control tube of 100µl PBS/2-ME

Incubate overnight at 4°C

Wash tubes twice with PBS-T
Add PCR reaction mix

Direct binding (DB) PCR

Add samples to tube
50 or 100µl of concentrated or normal sap
or 0.1, 1 or 10µl concentrated or normal sap made up to 100µl with PBS/2-ME
or 0.1, 1 or 10µl of purified virus prep made up to 100µl with PBS/2-ME

Prepare control tube of 100µl PBS/2-ME

Incubate overnight at 4°C

Do not wash tubes - remove sample by pipette
Add PCR reaction mix

Direct PCR

Add samples to tube
0.1, 1 or 10µl of purified virus prep or sap

Add PCR reaction mix

PCR reaction mix per tube

10x buffer		10µl
$MgCl_2$		3µl
dNTP		2µl
Primer badna 1a (10pmole/µl)		10µl
Primer badna 4' (10pmole/µl)	10µl	
Taq		0.5µl
(template		2µl)
Sterile dH_2O to		100µl

Prepare tubes for +ve (2µl bright BSV; positive control) and -ve (2µl sterile distilled H_2O) controls

Add 98µl to each tube

Run PCR

Pre-heat lid to 80°C

94°C for 10mins

94°C for 30secs
37°C for 30secs
72°C for 1min 30secs
x 5 cycles

94°C for 30secs
50°C for 30secs
72°C for 1min
x 30 cycles

72°C for 10mins

Run out on gel

2µl hyperladder1
5µl loading buffer
10µl PCR sample

PBS x1 or x10 stock

	x1	x10
Sodium chloride NaCl	8.0g	80g
di-Sodium hydrogen orthophosphate (anhydrous) Na_2HPO_4	1.15g	11.5g
Potassium dihydrogen orthophosphate (anhydrous) KH_2PO_4	0.2g	2g
Potassium chloride KCl	0.2g	2g

dissolve in distilled water to 1000ml
adjust pH to 7.4

PBS-T

PBS + 0.1% tween

PBS/2-ME

PBS + 0.1% 2-ME

Plasmid purification QIAprep spin miniprep kit protocol

1ml L-broth per tube with 1:1000 ampicillin (100mg/ml) added
Pick colonies with 200µl tip - white colonies, not blue or opaque

Drop tip into tube
Incubate at 37°C in orbital incubator overnight, tubes wedged in at an angle

Pour tube contents into microfuge tube
Spin 1min
Siphon off - no need to change tip

Add 250µl P1 buffer
Resuspend on vortex

Add 250µl P2 buffer
Invert 4-6 times to mix

Add 350µl N3 buffer - use fresh tips each time
Invert immediately 4-6 times
Spin for 10min

Prepare columns
Pour supernatant into blue filter

Spin for 1min
Tip out supernatant from bottom tube
Wash by adding 0.75ml buffer PE

Centrifuge for 1min
Discard flow through

Centrifuge 1 min

Place blue filters in fresh microfuge tube
Add 50µl buffer EB
Stand for 1min
Centrifuge 1min
Discard blue filters

Reaction mix

1µl REACT3 buffer
1µ EcoR1 enzyme
4µl sterile water (to 10µl)

Add 6µl reaction mix to empty microfuge tube
Add 4µl plasmid, and pipette up and down

Flick tube
Centrifuge 10secs

Incubate at 37°C for 1hr in water bath

Run out on gel

5µl hyperladder1
Add 5µl loading buffer to tube and centrifuge for 10secs

Appendix G

Analyses of BSV sequences

G1 Groupings of BSV isolates in relation to each other and to other badnavirus sequences. This shows 11 BSV groups that differ in sequence from each other by more than 20%. These are considered to be distinct virus species and are designated A – K
BSV Mys = Banana streak virus – Mysore strain; CoMV = Commelina yellow mosaic virus; CSSV = cacoa swollen shoot virus; DaBV = Dioscorea alata bacilliform virus; RTBV = Rice tungro bacilliform virus; SCBV = Sugarcane bacilliform virus.

G2. Groupings of Ugandan BSV isolates in relation to other BSV isolates and other badnaviruses. This shows the three supergroups I – III. Supergroup I contains BSV-IM, BSV-Mys and BSV-OL, which have been shown to be integrated into the banana B genome and which are activatable to give episomal infections; Supergroup II contains BSV sequences that are integrated into the A and possibly into the B genome and which are not potentially activatable (AG are sequences kindly supplied by Dr A. Geering); Supergroup III contains sequences which give episomal infections and for which there is no evidence for integration. For clarity, only some representatives of each BSV group are shown. Note that the arrangement of species is different to that in G1 with supergroup I comprising species G, E, F, H and BSVmys, supergroup II comprising species I, J, and K and supergroup III comprising species A, B, C and D

Appendix H

Lack of correlation between locality, cultivar, symptoms and virus species

Locality.

As identified in Appendix A

Site number

Given in Appendix B

Symptoms

Mod = moderate; sev. = severe; c = chlorotic; n = necrotic; broad, fine, large, lozenge, mod, short refer to the streaks; interv. mot. = interveinal mottle; speckling refers to interveinal chlorotic speckling; gn. vb = green vein-banding; mos = mosaic.

Species

This refers to the virus species identified in Appendix G1

Sample No	Locality	Site No.	Cultivar	Symptoms Type	Necr/Chl	Size	No. of Clones	Species
1	Rakai	1	Kibusi	mod	c/n	large	5	B
2	"	"	Nakabululu	sev	c/n	large	9	B
3	"	"	Nakitembe	mild	c/n	short	7	B, D
4	"	"	Gros Michel	nil			x	
5	"	2	Ntukura	sev	c/n	large	6	B, F
6	"	3	Kibusi	mod	c/n	mod	5	B
7	Ibanda	4	Enzwiga	mild	c/n	fine	5	D
8	"	"	Enzuma	sev	c/n	broad	3	D
9	"	5	Enzuma	sev	c/n	short	4	D
10	"	"	Kabucuragye	sev	c	lozenge	4	D
11	Mbarara	7	Sukali Ndizi	mod	c	short/broad	3	C, H
12	"	"	Nasuma	?genetic			0	
13	Ntungamo	8	Enyeru	mild		interv. mot.	2	I, J
14	"	"	Enyeru	mild		interv. mot.	1	C
15	"	"	Enyeru	v. sev.	c/n	fine	3	C
16	"	"	Enyeru	sev	c/n	speckle	4	C
17	Bukinda	9	Ensete	mod	c/n		2	F
18	"	"	Enyeru	nil			0	
19	"	"	Enyeru	mild	c	interv. mot.	0	
20	Kambesi	11	Enyonga	mild	c/n	fine	4	C
21	Msaka	12	Musa kala	mod	c	short/very short	6	B, F
22	"	"	Kibusi	v mild	c	speckling	6	F
23	"	"	Mbwazirume	mild	c/n	fine	6	B
24	"	"	Kibusi	v. sev.	c/n	broad	6	H
25	"	"	Nakyinyika	sev	c/n	fine	6	C. H
26A	Kawanda	13	?Sera	mod	c	fine	0	
26B	"	"	Mbwazirume	mod	c	fine	3	D, K
27	Namulonge	14	Musa ornata	nil			0	
28	"	"	Mbwazirume	mod	c	broad	0	
29	Basu Kuma	15	Ndizi	mod		interv. mot.	2	F,H
30	"	16	? Musa kala	mild	c	fine	1	F
31	"	"	?	mod	c	fine	0	
40	Mubende	17	Nabweru	sev	c/n		1	C
41	"	"	Nabweru	mod	c/n		2	D
42	"	18	Nakytengu	mild	c/n		4	D, F
43	"	"	Nakytengu	sev	c/n		3	A
44	"	19	Nakytengu	mod	c/n		0	
45	"	20	Gonja	sev	c/n		6	C
46	"	"	Nfuuka	mod	c		1	F
47	"	"	Nasaba	mod	c/n		2	C. F
48	"	"	Nasaba	mod	c/n	fine	6	D
49	"	21	Kisansa	mild	c/n	broad	4	D, F
50	"	"	Kisansa	mild	c		0	
51	Luwero	22	Dwarf Cavendish	mod	c		6	C
52	"	23	Ntika	mod	c	fine/broad	6	C
53	"	"	Kinsasa	mod	c	fine/broad	5	C
54	Koyunga	25	Ndibwubalangira	milde	c	fine	1	H
55	"	"	Gros Michel	mild	c	gn vb	1	F
56	Iganga	26	Mafuka	mod	c	short	0	
57	"	"	Mbwazirume	sev	c	broad	5	B
58	"	"	Mafuka	mod	c/n	fine	2	B
59	Mbale	27	Mbwazirume	sev	c/n		4	G
60	"	"	Mbwazirume	sev	c/n		3	G
61	"	28	Nakitembe	mod-sev	c/n		2	G
62	"	"	Namanamunga	mod	c/n	broad	2	G
63	"	"	Kibusi	v mild	c		3	G
64	Kapkwirwo	29	Njeryandet	mod	c	broad	3	H
65	"	"	Mudware	mod	c	broad/mosaic	4	E
66	"	"	Gros Michel	v. sev.	c/n		4	C

Analysis of Virus species, locality, cultivar and symptoms

LOCALITY (see Appendix A)

Rakai	B,D,F
Ibanda	D
Mbarara	C,F,H
Ntungano	C,F,I,J
Bukinda	F(1)
Kambesi	C(1)
Masaka	B,C,F,H
Kawanda	D,K
Namulonge	F,H
Mubende	A,C.D,F,G
Luwero	C
Iganga	B,F
Mbale	G
Kapkwiro	E,H

CULTIVAR GENOME

AB

Ndizi	F,H
Sukali Ndizi	C,F,H

AAB

Gonga	C

AAA Desert

Dwarf Cavendish	C
Gros Michel	E,E

AAA East African Highland

Beer clone set

Enyonga	C
Enzwiga	D
Kabuchuragye	D
Nabweru (probably Bwara)	C,D

Musakala clone set

Kisansa	C,D
Mudwale	E
Musakala	B,F
Namanamunga (Namunga)	G

Nakabululu clone set	
Kibuzi	B,B,F,G,H
Nakabululu	B
Nakitembe clone set	
Nakitembe	B,D,G
Nakytengu	A,D,F
Nfuuka clone set	
Enyeru	I,J,C
Enzuma	D
Enzwiga (?Enzinga)	D
Nakinyika	C,H
Nassaba	C,D,F
Nasuma	-
Ndyabalangira	-
Nfuuka (Mafuka)	G
Ntika	C
Ntukura	B,F
Ensete	F

SYMPTOMS

Severity

V sev: C,E
Sev: B,B/F,D,D,D,C,H,C,A,C,B,G,G
Mod: B,B,C/F/H,F,B/F,C/H,F/H,D,G,C/F,D,C,C,C,B,G,G,H,E
Mild: B/D,D,C,C,C,B,D/K,F,F,D,F
V mild:F,G

Chlorosis/necrosis

c/n: B,B,B/D,B/F,B,D,D,D,C,C,F,C,B,H,C/H,C,D,F,A,C,C/F,D,D,B,G,G,G,G,E
n: D,C/F/H,B/F,F,D/K,F,G,C,C,C,F,B,G,H,E

Morphology (size of streak or other)

Large:	B,B,B,H,B
Mod:	B
Short:	B/D,D,C/F/H,B/F
Fine:	D,C,C,B,C/H,D/K,F,D,C,C,B
Broad:	D,H,D,G,H.E
Lozenge:	D
IV mot:	I/J,C,F/H
Speckle:	C,F
Gn vb:	F

VIRUS SPECIES IN SAMPLES

A: 43
B: 1, 2, 3, 5, 6, 21, 23, 57, 58
C:11, 14, 15, 16, 20, 25, 40, 45, 47, 51, 52, 66
D: 3, 7, 8, 9, 10, 26b, 41, 42, 48, 49
E: 65
F: 5, 17, 21, 22, 29, 30, 42, 46, 47, 49, 55
G: 59, 60, 61, 62, 63
H: 11, 24, 25, 29, 54, 64
I: 13
J: 13
K: 26b

Appendix I

Diagnostic protocol

A. Preparation and concentration of sap samples

1. 1g of frozen leaf tissue ground for 1min in Agdia sap extraction pouch or pestel and mortar with 4ml PBS + 0.1% 2-ME (or 1.5g in 5mls).
2. Sap spun in microfuge for 10min.
3. Supernatant taken off.
4. Some sap kept back as 'normal' sample.
5. To remainder added PEG to 4% and NaCl to 0.1M.
6. Stirred for 2hr at RT.
7. Spun in microfuge for 20min.
8. Pellet resuspended in PBS + 0.1% 2-ME at $1/10^{th}$ original volume.

B. The virus particles are absorbed onto the surface of an Eppendorf tube either by immune-capture (IC) or by direct binding (DB and the DNA in the particles amplificed by PCR.

B1 ICPCR

1. Coat thin wall tubes with 100µl antisera diluted 1:1000 in carbonate coating buffer Incubate at 37°C for 2hrs, or room temperature for 4hrs. Wash tubes 3x in PBS-T
2. Add samples to tube
   50 or 100µl of concentrated or normal sap
   or 10µl of concentrated or normal sap made up to 100µl with PBS/2-ME
   or 0.01, 0.1, 1 or 10µl of purified virus prep made up to 100µl with PBS/2-ME
3. Prepare control tube of 100µl PBS/2-ME
4. Incubate overnight at 4°C
5. Wash tubes twice with PBS-T
6. Add PCR reaction mix

B2 Direct binding (DB) PCR

1. Add samples to tube
   50 or 100µl of concentrated or normal sap
   or 0.1, 1 or 10µl concentrated or normal sap made up to 100µl with PBS/2-ME
   or 0.1, 1 or 10µl of purified virus prep made up to 100µl with PBS/2-ME

2. Prepare control tube of 100µl PBS/2-ME

3. Incubate overnight at 4°C

4. Do not wash tubes - remove sample by pipette

5. Add PCR reaction mix

PCR reaction mix per tube

10x buffer	10µl
$MgCl_2$	3µl
dNTP	2µl
Primer badna 1a (10pmole/µl)	10µl
Primer badna 4' (10pmole/µl)	10µl
Taq	0.5µl
(template	2µl)
Sterile dH_2O to	100µl

Prepare tubes for +ve (2µl bright BSV) and -ve (2µl sterile distilled H_2O) controls

Add 98µl to each tube

PCR primers

Primer set 1A-4' (kindly advised by Drs. Lockhart and Olszewski)
Primer 1A (forward) 5'CTN TAY GAR TGG YTN GTN ATG CCN TTY GG3'
Primer 4' (reverse) 5'TCC AYT TRC ANA YNS CNC CCC ANC C3'

Run PCR

1. 94°C for 10mins

2. 94°C for 30secs
   37°C for 30secs
   72°C for 1min 30secs

x 5 cycles

3. 94°C for 30secs
   50°C for 30secs
   72°C for 1min
   x 30 cycles

4. 72°C for 10mins

Run out on gel
1.5% agarose gel in TBE
2µl hyperladder1
5µl loading buffer
10µl PCR sample