BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF POTYVIRUSES INFECTING SWEET POTATO

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Declaration

I hereby declare that this is my original work and has not been submitted for a degree or any other award in any university.

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LIST OF ABBREVIATIONS

A	adenine
aa	amino acid
AP	alkaline phosphatase
AVRDC	Asian Vegetable Research and Development Centre
BBA	Biologische Bundesanstalt für Land- und Forstwirtschaft
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bp	base pair
BSA	Bovine serum albumin
C	cytosine
cDNA	complementary DNA
CIP	International Potato Center
CP	coat protein
DAS	double antibody sandwich
kDa	kilodalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2', 3`deoxyribonucleoside 5'-triphosphate
EDTA	ethylendiamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMBL	European molecular biology laboratory
FAO	Food Agriculture Organisation
G	guanidine
GAR	goat anti rabbit
IC-PCR	Immunocapture polymerase chain reaction
IgG	immunoglobulin G
IgM	immunoglobulin M
IITA	International Institute of Tropical Agriculture
IL-6	Interleukin-6
IPTG	Isopropyl β-D-thiogalactopyanosie
ISEM	immunosorbent electron microscopy
LB	Luria Bertani medium
LL	local lesions

MAb	Monoclonal antibody
MMLV	Murine Maloney-Leukaemia Virus
Mr	relative molecular mass
NBT	nitroblue tetrazolium
NCBI	National Center for Biotechnology Information
NCM	nitro cellulose membrane
Nco I	restriction enzyme isolated from *Nocardia corallina*
nt	nucleotide
NTR	non-translated region
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline supplemented with tween 20
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PVP	polyvinylpyrrolidone
RACE	rapid amplification of cDNA ends
RAM	rabbit anti mouse
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecylsulphate
T	thymine
Taq	*Thermophilus acquaticus*
TAS	triple antibody sandwich
TDT	terminal deoxynucleotydyl transferase
TEMED	N,N,N',N-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
T-TBS	Tris buffered saline supplemented with tween 20
U	unit
UAc	uranyl acetate
X-gal	5-bromo-4-chloro-3-indolyl b-thiogalactopyranoside
XhoI	restriction endonuclease

LIST OF VIRUS NAMES AND ACRONYMS

CMV	Cucumber mosaic cocumovirus
PEMV	pea enation mosaic virus
PVX	Potato virus X
PVY	Potato virus Y
SPCaLV	Sweet potato caulimo-like virus
SPCFV	Sweet potato chlorotic fleck virus
SPCSVS	Sweet potato chlorotic stunt virus
SPFMV	Sweet potato feathery mottle virus
SPLCV	Sweet potato leaf curl virus
SPMMV	Sweet potato mild mottle virus
SPV 2	Sweet potato virus 2
SPVD	sweet potato virus disease
SPVG	Sweet potato virus G
SPVY	Sweet potato Virus Y (SPV 2)
SwPLV	Sweet potato latent potyvirus
ZYMV	Zucchini yellow mosaic virus

Abstract

A survey was conducted to identify the viruses that infect sweet potato in the major growing areas in Kenya. Only *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato mild mottle virus* (SPMMV) and Sweet potato chlorotic fleck virus (SPCFV) were detected. Ninety-two percent and 25% of the symptomatic and asymptomatic plants, respectively, tested positive for at least one of these viruses. SPFMV was the most common and the most widespread, being detected in 74% of the symptomatic plants and in 86% of fields surveyed. Virus incidence was highest (18%) in Kisii district of Nyanza province and lowest (1%) in Kilifi district of Coast province.

A hitherto undescribed isolate from sweet potato in Taiwan, referred to as 'sweet potato virus 2' (SPV2), was characterized. Its filamentous particles were 850 nm in length and induced cytoplasmic cylindrical inclusions consisting of pinwheels and scrolls. SPV2 attained strikingly higher titres and was more uniformly distributed in *Ipomoea setosa* plants dually infected with SPV2 and the crinivirus SPCSV than in plants infected with SPV2 alone. Comparison of the CP and 3'-UTR sequences of SPV2 with those of other potyviruses demonstrated that it is a distinct member of the genus *Potyvirus* (family *Potyviridae*). The name Sweet potato virus Y (SPVY) was proposed for this virus.

To determine the variability within SPVY, the CP-coding region and 3'-UTR sequences of 13 isolates were sequenced. Sequence comparisons of the CP-coding region of these isolates revealed identities ranging from 82 to 99% at the nucleotide level and 86 to 99% at the amino acid level. Bootstrap analysis of sequences distinguished phylogenetic groups, which partially correlated with the geographical origin of the isolates and reflected differences in host range and

symptoms. These results strongly suggest the occurrence of biologically and genetically diverse strains of SPVY.

The CP-coding region and the 3' UTR of four *Sweet potato virus G* (SPVG) isolates from South Africa, China and Portugal was determined. Analysis of CP sequences revealed identities ranging from 81% to 98% and from 90 to 99% at the nucleic and amino acid levels, respectively. Phylogenetic analysis of the CP revealed a cluster comprising closely related isolates from USA, Europe, China and Egypt, as well as two clearly distinct isolates, one from South Africa and the other from China. These data are the first evidence for molecular variability among SPVG isolates and the first report of the occurrence of SPVG in Portugal and South Africa.

The variability of geographically diverse SPFMV isolates in the CP gene and the 3' UTR was determined. The CP genes of the isolates sequenced had amino acid sequence similarities ranging from 82 to 98% and nucleotide sequence identities ranging from 80 to 99%. Phylogenetic analysis of the SPFMV sequences revealed two main distinct clusters, strain groups C and RC (*sensu lato*). The CP sequences of the C group were two amino acids shorter (due to a deletion of 6 nucleotides in the N-terminus) than those of the strain group RC SPFMV. Isolates of strain group O were also shown to occur in East Africa. A high-affinity monoclonal antibody (MAb 1C4) reacting with all SPFMV isolates tested was obtained following immunisation with purified SPFMV virions of a Kenyan isolate (KY115/1S). The determination of the 5'-proximal part of the SPFMV genome indicated that the P3 gene could be the most variable part. Alignment of the P1 sequences of the five African isolates of SPFMV, with that of the previously only known SPFMV P1 sequence from Japan revealed that the latter has a deletion of 75 nucleotides (25 amino acids) and is thus considerably shorter than the P1 of the African isolates. This could indicate a critical source of variation in SPFMV.

1.0 INTRODUCTION

1.1 Importance of sweet potato

Sweet potato (*Ipomoea batatas* L.) is one of mankind's highest yielding crops with higher food value and total production per unit area than other staple crops such as maize, rice, sorghum and millet (FAO, 2002). On a worldwide scale, the importance of sweet potato is exceeded only by cereals (wheat, rice, maize and barley) and potato (*Solanum tuberosum)* (Vietmeyer, 1986; Carey *et al.,* 1997; FAO, 2002). Although the crop is grown in more than 100 countries around the globe, about 90% is produced in Asia with just below 5% in Africa (Horton *et al.,* 1989; Woolfe, 1992). Sweet potato serves as a staple diet in many parts of Uganda, Nigeria, Rwanda, Tanzania, Burundi, Angola, Kenya, and Madagascar (Carey, 1996). With an annual harvest of 100 million tonnes, China is the world's largest producer. Uganda, Nigeria, Indonesia and Vietnam, which follow China in production, each harvests about 2.5 million tons of sweet potato annually (FAO, 2002).

Sweet potato is adapted to a wide range of agro-ecological conditions and performs well in low-input agriculture. It is mostly grown at mid-elevations ranging from 800 to 2000 m above sea level. Africa's production is concentrated in East Africa, particularly around the Great Lakes region (Kapinga *et al.,* 1995; Gibson *et al.,* 1997). Its presence and adaptation to the tropical areas where *per capita* incomes are generally low and its nutritional value make this crop an important component in food production and consumption (Wolfe, 1992; CIP, 1996; CIP, 1998). It is increasingly becoming an important food security and famine relief crop during seasons of crop failure (CIP, 1996; CIP, 1998).

Although the area planted with sweet potato in Africa continues to expand, yields are declining in some areas thereby offsetting what would otherwise constitute higher rates of growth in production. About 2.5 million ha in Africa are under sweet potato with a total production of about 11million tonnes (Appendix 1). Average yields of about 5t/ha for sweet potato in Africa are the lowest and less than a third of the yields in Asia (FAO, 2002, 2003).

Kenya is the seventh largest African sweet potato producer with average yields of 8.2 tons/ha (FAO, 2003) against a potential of 50 tons/ha (Ndolo *et al.,* 1997; FAO, 2002). With diminishing arable land per household, there is need to increase sweet potato yields to meet the current demand for the crop. The main sweet potato producing regions of Kenya are western, areas around Lake Victoria, eastern region, central and coastal areas (MoA, 1999; Matin, 1999) (Appendix 1). In Kenya, sweet potato is grown in a continuous cycle with one planting season overlapping with another. Piecemeal harvesting of tubers commonly extends the cropping season (Bashaasha *et al.,* 1995; Kapinga *et al.,* 1995).

Sweet potato is grown principally for its tuberous storage roots, which are generally steamed or boiled (Carey *et al.,* 1997). The roots provide energy, protein, vitamins A (particularly in the orange fleshed sweet potato varieties), B_1 & B_2, niacin pyridoxine, folic acid and ascorbic acid in diets. Other important nutrients in sweet potato roots include calcium, phosphorus, iron, sodium and potassium (Martin 1984; Woolfe, 1992). Young sweet potato leaves are utilized as vegetables in some countries and are rich sources of vitamin A, B_2, iron and protein (AVRDC, 1976; Woolfe, 1992). The storage roots of sweet potato provide a reliable source of food during times of scarcity and the foliage is an important supplementary fodder in livestock production systems (Ndolo *et al.,* 1997).

Sweet potato is dependable since it can produce despite drought, production can be staggered, while harvesting can be done piecemeal thereby ensuring a continuous source of food for the farm families (CIP, 1998). Sweet potato can be utilized as a salad, a staple, a vegetable, a sweet dessert, a fast food (french fries), an animal feed, or a basic industrial raw material. The crop therefore plays an important role as a food security (famine relief) crop in large parts of Africa, and is expected to become important in food processing and animal feed industries (CIP, 1996; CIP, 1998).

Viruses are the second most important biotic constraint after insects (weevils), which limit sweet potato production both in Africa (Geddes, 1990). Greater than 50% of production losses are attributed to virus infections (Ngeve *et al,* 1991; Melissa *et al,* 1996; Gutiérrez *et al.,* 2003). Viruses reported to infect sweet potato in Africa include Sweet potato feathery mottle virus (SPFMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato mild mottle virus (SPMMV), Sweet potato virus G (SPVG), Cucumber mosaic virus (CMV), Sweet potato chlorotic fleck virus (SPCFV), Sweet potato virus 2 (SPV-2), Sweet potato latent virus (SwPLV) and Sweet potato caulimo-like virus (SPCaLV) (Hollings *et al.*, 1976; Mukiibi, 1977; Hahn, 1979; Geddes, 1990; Wambugu, 1991; Alvarez *et al.*, 1997; Gibson *et al,* 1998).

1.2 Objectives of the study

A good knowledge of the identity of the pathogens and their variability is a prerequisite for the successful formulation and implementation of strategies for the management of plant diseases. Since SPFMV appears to be the most widespread and in co-infection with SPCSV perhaps the most important sweet potato virus, a better understanding of its molecular variation in a representative number of isolates is needed. Moreover, the variabilities and

distributions of SPVG and of SPV2, a hitherto undescribed sweet potato-infecting filamentous virus, are unknown. Therefore, the aims of this study were to:

(i) determine the identity, distribution and incidence of viruses infecting sweet potato in the major growing areas of Kenya

(ii) characterize SPV2 at the morphological, molecular and biological level and to study the interaction between SPV2 and SPCSV

(iii) study the variability amongst geographically diverse isolates of SPV2

(iv) characterize SPVG isolates at the molecular level

(v) determine the variability of SPFMV isolates and to develop diagnostic tools for the detection of SPFMV

(vi) analyze the molecular diversity of the first protein (P1), helper component proteinase (HC-Pro) and third protein (P3) genes regions of African isolates of SPFMV

2.0 LITERATURE REVIEW

2.1 Origin and botany of sweet potato

Sweet potato is thought to have originated from Central or South America, probably in the region between the Yucatan Peninsula of Mexico and the mouth of the Orinoco River in Venezuela. For at least 4000-6000 years, it has been used as human food in this region and spread in pre-Columbus times to many islands of the South Pacific. Its dissemination to Polynesia is possibly associated with voyages of early Peruvian or Polynesian explorers and traders. Sweet potato is thought to have been first brought to Europe by Christopher Columbus on his return journey from South America, and then taken by Portuguese explorers in the 16th Century to Africa, India and eastern Asia (Onueme, 1978; Bassett, 1986; Norman *et al.*, 1989). Today, sweet potato is grown in nearly all parts of the tropical world, and in the warmer areas of the temperate regions (Onueme, 1978; Horton and Knipscheer, 1984; CIP, 1996).

Although usually grown as an annual, sweet potato is a herbaceous perennial with creeping stems (vines) that grow very rapidly and produce a shallow canopy. Genotypes are broadly grouped into bush, intermediate and vining types, which may vary greatly in branching pattern and overall stem length. Leaves may also differ in size, shape (broad and entire or deeply indented) and length of petiole. The plants produce both tuberous and fibrous roots (Onueme, 1978). The flowers of sweet potato are complete with a compound superior pistil, five separate stamens attached to the corolla and with petals united into a trumpet- or bell-shaped corolla. The corolla is usually white at the margin and pink to purple in the throat. Seeds have a hard seed coat and develop within a capsule. The plants usually set few viable seeds; many genotypes do not readily flower, others are sterile and most are self-incompatible (Basset, 1986). Sweet potato is a hexaploid with 90 chromosomes. Since most *Ipomoea*

species have 30 chromosomes, it is suggested that *I. batatas* arose from either a tetraploid (possibly *I. trifida*) or a diploid species (Norman *et al.*, 1989).

2.2 Constraints to sweet potato production

Sweet potato withstands adverse climatic conditions better than most crops in the field, but a wide range of pathogens such as fungi, bacteria, nematodes and viruses infect the crop (Clark and Moyer, 1988; Moyer and Salazar, 1989; Geddes, 1990; Jatala and Bridge, 1990; CIP, 1995; Ames *et al.*, 1997; Carey *et al.*, 1997; Njuguna and Bridge, 1998). Several fungal pathogens such as *Alternaria* leaf petiole and stem blight (*Alternaria* spp), vine and leaf scab (*Elsinoe batatas*), rust (*Coleosporium ipomoeae*), white rust (*Albugo ipomoeae-punduratae*); stem rot (*Fusarium oxysporum*) and leaf spots (*Cercospora* spp.), *Phyllosticta batatas* and *Septoria batatas* cause diseases in sweet potato (Clark, 1987). These foliar diseases reduce yields by reducing photosynthetic area and by interfering with the transport of nutrients and other products to the storage roots. Tuber-rotting fungal pathogens also cause significant losses (Skoglund *et al.*, 1990; Clarence *et al.*, 1990; Skoglund and Smit, 1994; Carey *et al.*, 1996).

Many nematode species have been reported to reduce sweet potato yield and quality including *Meloidogyne* spp., *Rotylenchulus reniformis*, *Pratylenchus* spp. and *Ditylenchus destructor* (Jatala and Bridge, 1990). Njuguna and Bridge (1998) reported the presence of *Meloidogyne* spp., *Rotylenchulus reniformis*, *R. variabilis*, *Scutellonema labiatum* as the main nematode species associated with sweet potato in Kenya. The sweet potato weevil (*Cylas* spp.) is the most important biotic constraint limiting the production of sweet potato worldwide (Ames *et al*, 1997). In Kenya, yield reduction due to weevil infestation is estimated at 20% on a national scale, but can be as high as 80% in individual fields (Matin, 1999). Other biotic

constraints to sweet potato production include vertebrate pests such as monkeys, wild pigs, moles, rats and porcupines (Matin, 1999).

Viruses are a major cause of diseases in sweet potato in Africa (Geddes, 1990). Some of the viruses known to infect sweet potato include *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato mild mottle virus* (SPMMV), Sweet potato latent virus (SPLV), *Cucumber mosaic virus* (CMV), Sweet potato caulimo-like virus (SPCa-LV) and Sweet potato chlorotic fleck virus (SPCFV) (Mukiibi, 1977; Hahn, 1979; Moyer and Salazar, 1989; Geddes, 1990; Gibson *et al.,* 1998). Viral diseases can cause yield reductions of up to 98% thereby greatly constraining production (Ngeve, *et al.,* 1991; Gibson *et al.,* 1997; Gutierrez *et al.,* 2003),

SPVD is the most economically important disease of sweet potato (Geddes, 1990; Melissa *et al.,* 1996; Ngeve *et al.,* 1991; Guiterrez *et al.,* 2003). The disease was probably reported for the first time in 1939 in central Africa near the eastern border of the Democratic Republic of Congo (Sheffield, 1953), and about a decade later in East Africa (Kenya, Uganda and Tanzania) (Sheffield, 1953). It is caused by the dual infection of sweet potato by SPCSV and SPFMV (Schaefers and Terry, 1976; Gibson *et al.,* 1998; Karyeija *et al.,* 2000). Tuber yield losses of greater than 50% due to SPVD have been reported (Mukiibi, 1977; Hahn, 1979; Melissa *et al.,* 1996; Gibson *et al.,* 1998).

2.3.0 Potyviruses: taxonomy and genome organization

The Potyviridae is the largest family of plant-infecting viruses. Virtually every crop of agronomic or horticultural use is infected by one or more species, and numerous additional, non-economic hosts are also susceptible (Berger, 2001). Because of its sheer size, the family *Potyviridae* is considered the most important taxon of plant viruses from an economic

standpoint (Berger, 2001). The vast majority of species in Potyviridae are in the genus *Potyvirus*. All potyviruses have flexuous particles 680-900nm long and about 11-13nm in diameter and have the capacity to form diagnostic cytoplasmic, cylindrical inclusions 'pinwheels' induced by viral proteins in the cytoplasm of infected cells (Edwardson, 1974, 1992). Potyviruses are transmitted by aphids in a non-persistent manner. The potyvirus genome is a single stranded, positive sense RNA encoding a large polyprotein which is proteolytically processed co- and post-translationally by three virus-encoded proteases into nine or ten mature and functional proteins. The potyviral genes encoded include (from 5' to 3' end) the P1 protein (a serine proteinase), the helper component proteinase (HC-Pro), the third protein (P3), 6K1, cylindrical inclusion protein (CI), 6K2, the small nuclear inclusion protein (NIa), the large nuclear inclusion protein (NIb) and the coat protein (CP). The genome of potyviruses also has non-coding regions at the 5' and 3 'termini (Fig 1).

The P1 gene of the *Tobacco vein-mottling virus* (TVMV) has been reported to possess a proteinase activity for C-terminal auto-processing (Verchot *et al.*, 1991), and a single stranded RNA binding activity (Brantley and Hunt, 1993). The P1 is also associated with genome amplification, cell-to-cell movement, and generally virus-host interaction (Shukla *et al.*, 1994; Soumounou and Laliberte, 1994; Verchot, and Carrington, 1995). The P1 protein has also been linked to symptom development and host range (Tordo *et al.*, 1995). Since the P1 is the most variable gene product of the potyvirus genome (Shukla *et al.*, 1994), it is proposed that its sequence be analysed in order to gain a better resolution into strain diversity (Igor and Valkonen, 2001). Phylogenetic analysis of the P1 gene sequences of *Potato virus V* (PVV) and *Potato virus Y* (PVY) grouped isolates according to their geographic origin (Igor *et al.*, 2001; Lin *et al.*, 2001; Tordo *et al.*, 1995).

The helper component proteinase (HC-Pro) is important for virus transmission by aphids (Pirone, 1991; Shukla *et al.,* 1994). The HC-Pro has also been associated with post-transcriptional gene silencing (PTGS) as a counter defensive strategy in potyviruses (Kasschau and Carrington, 1998). The third protein (P3) is associated with inclusion bodies suggesting that it is involved in replication (Rodriguez-Cerezo *et al.,* 1993). The 6K1 and 6K2 are believed to be involved in virus replication (Restrepo-Hartwig and Carrington, 1994). The CI protein with a conserved RNA helicase domain is also involved in genome replication (Shukla *et al.,* 1994). The NIa is composed of two parts, one is the VPg and the other is the proteinase (Murphy *et al.,* 1990; Dougherty *et al.,* 1989). The VPg has been implicated in host genotype-specific long distance movement (Schaad *et al.,* 1997) whereas the NIb is thought to be the RNA replicase (RNA dependent RNA polymerase) responsible for virus multiplication. The coat protein (CP) is the single structural protein in potyviruses and apart from encapsidating the viral genome, is also involved in virus transmission by aphids, cell-to-cell movement and virus spread (Atreya *et al.,* 1990, 1992, 1995; Rojas *et al.,* 1997).

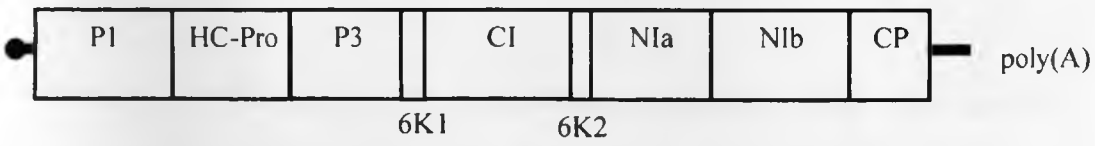


Fig. 1: Genome organization of potyviruses. The genome contains one large open reading frame. The vertical lines represent cleavage sites of the polyprotein. The mature potyviral proteins are P1 proteinase, helper component proteinase (HC-Pro), the third protein (P3), 6 kDa protein 1 (6K1), cylindrical inclusion protein (CI) 6 kDa protein 2 (6K2), nuclear inclusion protein a (NIa), nuclear inclusion protein b (NIb), and the coat protein (CP). The 5' and 3' are at the extreme right and left end of the genome, respectively (Shukla *et al.*, 1994).

The CP represents 95% of the virion by weight and has an extremely variable N-terminal sequence linked to a conserved core domain (Shukla *et al.,* 1988; Shukla *et al.*, 1994). Thus, potyvirus species and strains can be classified on the basis of coat protein size resulting primarily from the differences in the lengths of the N-termini (Shukla *et al.,* 1988: Shukla and Ward, 1989a). Between distinct potyviruses, CP sizes range from 263-355 amino acids, while within a particular species all isolates are believed to share a similar CP length (Shukla and Ward, 1989a). The CP enables the distinction between species and strains (Shukla *et al.,* 1994) since CP amino acid composition is characteristic of the virus group (Fauquet *et al.,* 1986). Furthermore, the CP is the only virus gene product, which exhibits little sequence identity with corresponding protein of other virus groups (Domier *et al.,* 1987).

Distinct potyviruses generally possess a CP sequence similarity of less than about 80% (Van Regenmortel *et al.*, 2000). However, according to Van Regenmortel (1992), it is acceptable for potyviruses showing CP sequence identity of 85% and more to be considered related strains or distinct species depending on the relative significance of other properties such as cross-protection, vector specificity, host range and symptomatology.

While serology is a useful criterion for distinguishing plant viruses, it has often proved unsatisfactory when applied to the Potyviridae (Shukla *et al.,* 1992, 1994). The serological relationships among potyviruses are complex due to the inherent problems associated with the structure of potyvirus particles and their CPs. Variable cross reactions to polyclonal antibodies, inconsistent paired relationships between distinct potyviruses, and lack of cross-reactions between some virus strains cause major inconsistencies in potyvirus serology. Thus, serology on its own is an insufficient criterion for classification of potyviruses (Shukla *et al.,* 1994).

2.3.1 Potyvirus transmission and spread

Aphid transmission has for a long time been considered an essential criterion for potyvirus group membership (Hollings and Brunt, 1981; Matthews, 1982). Potyviruses are transmitted by aphids in a non-persistent, non-circulative, stylet-borne manner. Aphids acquire virus particles after brief feeding periods on an infected host and usually retain the virus for less than an hour, though a potyviruses can survive up to 40 hours. Because of the brief retention of the virus, aphids normally only carry viruses for relatively short distances although occasionally strong winds can blow them over great distances.

More than 200 aphid species are known to transmit one or more potyviruses (Edwardson and Christie, 1991). The superfamily Aphididae that contains most of these species, including those of the genera *Aphis, Myzus* and *Macrosiphum*, are mostly polyphagous (Eastop, 1977, 1983; Murant *et al.*, 1988). It has long been known that aphid species can differ in their efficiency as vectors of potyviruses (Halbert *et al.*, 1981) whereas different populations (biotypes) of the same vector may differ in their ability and/or efficiency (Castle *et al.*, 1992).

The transmission of potyviruses is dependent upon the composition of the particle CP and the helper component , which is encoded by the HC-Pro gene. The non-circulative plant infecting viruses have two molecular strategies regulating their interaction with vectors. The capsid strategy in which the virus CP interacts directly with the vector and the helper strategy in which at least one non-structural viral gene product, the helper component (HC-Pro), is required for successful transmission (reviewed by Froissart *et al.*, 2002 and references therein). A model called the bridge hypothesis (Pirone and Blanc, 1996) is commonly used to explain the mode of action of HC-Pro, and describes the non-structural proteins as molecules capable of interacting with the CP and with a receptor in the vector's mouth parts forming a molecular bridge.

The CP has also been associated with aphid transmission (O'Connell *et al.*, 1986; Atreya *et al.*, 1990; Gal-On *et al.*, 1992). In the capsid strategy, a motif of the CP directly binds to the vector's receptor (Froissart *et al.*, 2002). The interaction between the HC and the CP of potyviruses and with structures in the mouthparts of the vectors has been demonstrated (Blanc *et al.*, 1997). Existence of non-transmissible isolates with functionally active HCs suggested the involvement of CP in the process as another determinant of transmission (Pirone and Thornbury, 1983). A highly conserved motif of three amino acids Asp-Ala-Gly (DAG), located in the N-terminus of the potyvirus CP has been demonstrated to be associated with transmission (Atreya *et al.*, 1990, 1995). However, not all potyvirus isolates with this motif are aphid transmissible, whereas certain modifications of the DAG motif are also tolerated (Johansen *et al.*, 1996; Flasinski and Cassidy, 1998).

2.3.2 Potyvirus infection cycle

Potyviruses naturally enter their hosts through the stylets of their aphid vectors. The aphids regurgitate some saliva onto the plant while feeding and in the process inoculate the plants with potyvirus particles (Martin *et al.*, 1997). As the virus enters the cell, it starts to disassemble while it is simultaneously recognized as messenger RNA (mRNA) and is translated in a process called 'co-translational disassembly' (Shaw *et al.*, 1986). By the time the virus particle is fully disassembled, the first viral proteins, particularly RNA dependent RNA polymerase (RdRP), have already been produced and are ready to start replicating the viral RNA. At first, virus replication is restricted to a single cell (Aranda *et al.*, 1996). However, soon after, the virus moves to neighbouring cells in a process referred to as cell-to-cell movement (short distance movement), and later into the vascular tissue, where it spreads systemically throughout the plant (systemic or long distance movement).

2.3.3 Potyviruses infecting sweet potato

***Sweet potato feathery mottle virus* (SPFMV, genus *Potyvirus*)**

Sweet potato feathery mottle, caused by *Sweet potato feathery mottle virus* (SPFMV; genus *Potyvirus,* family *Potyviridae*;), is the most widely distributed virus infecting sweet potato (Moyer and Salazar, 1989; Karyeija *et al.,* 1998b). Other synonyms include sweet potato chlorotic leaf spot virus, sweet potato internal cork virus, sweet potato russet crack virus and sweet potato virus A (Sheffield, 1957; Hollings *et al.,* 1976). The virus was first described in Beltsville, Maryland, United States of America by Dolittle and Harter (1945). In East Africa (Kenya, Uganda and Tanzania), it was reported for the first time in 1957 (Shefield, 1957). It remained unreported in West Africa until 1976 when it was given the name sweet potato vein clearing virus (Schaefers and Terry, 1976).

As a typical member of the genus *Potyvirus*, the SPFMV virion consists of a monopartite, linear single-stranded positive-sense RNA molecule 11.6 kb and numerous copies of the CP with a molecular weight of M_r 38000 (Moyer and Cali, 1985). The viral RNA encodes a large polyprotein proteolytically processed by viral proteases into mature gene products (Sakai *et al.,* 1997). SPFMV induces pinwheel inclusions in infected cells (Lawson *et al.,* 1971; Edwardson, 1974; Nome *et al.,* 1974).

Symptoms on sweet potato leaves as a result of SPFMV infection are mostly mild and transient but may include vein clearing, chlorotic spots especially on older leaves, which may or may not be characterized by purple pigmented borders (Moyer and Salazar, 1989). Depending on cultivar, virus isolate or strain, infected roots may show external necrosis (Campbell *et al.,* 1974; Moyer and Kennedy, 1978; Cali and Moyer, 1981; Moyer and Cali 1985). When infecting alone, SPFMV in some cultivars seems mostly insignificant in its effects on sweet potato since its movement and/or replication is restricted (Schaefers and

Terry, 1976; Gibson *et al.*, 1998; Karyeija *et al.*, 2000), whearas in others, considerable yield reductions have been reported (Gibson *et al.*, 1997; Cheramgoi, 2003; Njeru *et al.*, 2004). Trials conducted in the United States indicated that plants infected with the russet crack (RC) strain of SPFMV had similar yield as the symptomless ones (Kantack and Martin, 1958). The greater economic impact is realised when SPFMV infects sweet potato in the presence of SPCSV leading to sweet potato virus disease (SPVD), the most important disease of sweet potato (Gibson *et al.*, 1998; Karyeija *et al.*, 2000; Gibson and Aritua, 2002).

Several aphid species including *Aphis gossypii, A. craccivora, Lipaphis erysimi* and *Myzus persicae* are known to transmit SPFMV in a non-persistent manner (Sheffield, 1957; Stubbs and McLean, 1958; Kennedy and Moyer, 1982). The virus is sap and graft transmissible, but is not transmitted by contact between plants or through seed or pollen (Wolters *et al.*, 1990). SPFMV can be perpetuated from one cropping cycle to another through infected sweet potato vines used as planting material. The natural host range of SPFMV is narrow and is limited to the family Convolvulaceae, and especially the genus *Ipomoea.* Some isolates infect *Chenopodium amaranticolor, C. quinoa* or *Nicotiana benthamiana* when inoculated experimentally but others seem to be restricted to *Ipomoea* (Moyer and Kennedy, 1978; Nakashima *et al.*, 1993).

Several strains of SPFMV have been reported such as the Common (C), Russet Crack (RC), Ordinary (O), Severe (S), and the East African (EA) strains (Moyer and Salazar, 1989; Usugi *et al.*, 1991; Abad *et al.*, 1992; Ryu *et al.*, 1997; Kreuze *et al.*, 2000). However, isolates of the strain groups C and RC are the most studied and are distinguished on the basis of variation in CP amino acid sequences, biological and serological characteristics (Abad *et al.*, 1992). The strain groups are distinguished by the symptoms they incite on test plants with only the RC

strain causing local lesions on sap inoculated *Chenopodium quinoa* and *C. amaranticolor* (Cali and Moyer, 1981). The C and RC strain groups share an overall CP amino acid sequence similarity of 82 % (Abad *et al.*, 1992). Since the molecular diversity of SPFMV isolates from Africa has been determined only to a very limited extent (Kreuze *et al.*, 2000; Mukasa *et al.*, 2003b), more isolates from geographically diverse locations need to be characterised to gain a better understanding of this virus.

Sweet potato virus 2 (SPV- 2, genus *Potyvirus*)

In the late 70s, Rossel and Thottappilly (1987) succeeded in transmitting a potyvirus from sweet potato to *N. benthamiana*, in which it induced a mild vein yellowing followed by a chlorotic mottle. Since this was at this time unusual for an isolate of SPFMV from which it also appeared to differ serologically, they referred to this isolate as sweet potato virus 2. SPV-2 was isolated from sweet potato plants showing mild virus-like symptoms such as mottle, vein yellowing and/or ringspots in Taiwan, but did not cause any symptoms in sweet potato clones inoculated under experimental conditions. The SPV-2 isolate failed to induce symptoms when inoculated into plants pre-infected with SPCSV (Rossel and Thottappilly, 1987). The virus has since remained incompletely described since the 70s.

Sweet potato virus G (SPVG, genus *Potyvirus*)

SPVG is a tentative member of the genus *Potyvirus*, family *Potyviridae* (Colinet *et al.*, 1994). The virus was first detected in sweet potato plants originating from China in the early 1990s. Although not much is known about its biological characteristics, the CP amino acid sequence was determined, which allowed its classification into the genus *Potyvirus*. From its molecular characteristics, SPVG appeared to be closely related to, but clearly distinct from SPFMV (Colinet *et al.*, 1994). Based on CP gene sequences, two Chinese strains of SPVG are distinguished (Colinet *et al.*, 1994, 1998). However, there is no information regarding its

distribution.

***Sweet potato latent virus* (SwPLV, genus *Potyvirus*)**

SwPLV has flexuous filamentous particles 700-750 nm long and contains a single-stranded, plus sense RNA genome. The virus induces the formation of cylindrical inclusions in the cytoplasm of infected cells. SwPLV is readily transmitted by sap onto 36 species of Convolvulaceae, Solanaceae and Chenopodiaceae; of these, *N. clevelandii* and *N. benthamiana* are good propagation hosts while *C. quinoa* is a sensitive local lesion assay host (Brown *et al.*, 1988). The CP and 3' non-coding region sequence data of an isolate from China led to the unequivocal assignment of SwPLV as a distinct member of the genus *Potyvirus* (Colinet *et al.*, 1997). The virus, like other sweet potato infecting viruses, is disseminated in infected tubers and other vegetative propagules. SwPLV has only been reported to occur in Africa, Peru and Asia (Taiwan and China) (Wambugu, 1991; Colinet *et al.*, 1997; Gutiérrez *et al.*, 2003). However, the occurrence of this virus in Africa and Kenya in particular has not been confirmed.

***Sweet potato mild speckling virus* (SPMSV, genus *Potyvirus*)**

SPMSV was reported in Argentina infecting sweet potato plants affected by "sweet potato chlorotic dwarf disease". To date, available reports indicate that SPMSV is geographically confined to South America (Alvarez, *et al.*, 1997). The virus is likely to be transmitted by aphids because it has a DAG motif in its CP. The CP gene of SPMSV is 855 nucleotides (285 amino acids) followed by a 3' non-coding region of 248 nucleotides and a poly (A) tail (Alvarez *et al.*, 1997). It is, however, distantly related to other described sweet potato infecting potyviruses based on its CP sequence. SPMSV shares a CP nucleotide sequence identity of less than 55 % with other sweet potato infecting potyviruses (Alvarez *et al.*, 1997).

***Sweet potato mild mottle virus* (SPMMV, genus *Ipomovirus*, family Potyviridae)**

SPMMV was first reported in East Africa from sweet potato plants showing mottling and veinal chlorosis on infected leaves together with dwarfing and generalized poor plant growth (Hollings *et al.*, 1976). The virus is the type member of the genus *Ipomovirus* (Van Regenmortel *et al.*, 2000). SPMMV has flexuous particles and induces the formation of cytoplasmic inclusion bodies in the cytoplasm of infected cells (Hollings *et al.*, 1976). Members of the genus *Ipomovirus* are transmitted by whiteflies, *Bemisia tabaci* (Berger *et al.*, 2000).

Compared to other viruses infecting sweet potato, SPMMV has a wide host range, infecting 14 plant families (Hollings *et al.*, 1976). It has been reported to be prevalent as single or in mixed infections with other sweet potato infecting viruses leading to severe disease symptoms (Mukasa *et al.*, 2003c). Phylogenetic analysis of the CP of SPMMV indicated high genetic variability among isolates from East Africa (Mukasa *et al.*, 2003c).

2.3.4 Other viruses infecting sweet potato

***Sweet potato chlorotic stunt virus* (SPCSV)**

SPCSV (genus *Crinivirus* family *Closteroviridae*) is probably the most damaging virus of sweet potato (Gibson and Aritua, 2002). The crinivirus is transmitted by the whitefly, *Bemisia tabaci* and *Trialeurodes abutilonea* in a semi-persistent manner (Schaefers and Terry, 1976; Cohen *et al.*, 1992). Virions are 850-950 nm long, filamentous, and highly flexuous containing a bipartite, positive–stranded RNA genome (Kreuze *et al.*, 2002). Closteroviruses are generally phloem-limited (Duffus, 1995).

Only two serologically and geographically separated strains of SPCSV are known. The first was isolated from West Africa (Schaefers and Terry, 1976) and has therefore been designated $SPCSV_{WA}$ while the other was first isolated from Kenya in East Africa and is known as $SPCSV_{EA}$ (Hoyer *et al.*, 1996; Vetten *et al.*, 1996). $SPCSV_{WA}$ has also been identified in Egypt (Carey *et al.*, 1999; Israel (Cohen *et al.*, 1992), U.S.A (Pio-Ribeiro *et al.*, 1996), Brazil (Pio-Ribeiro *et al.*, 1994), Argentina and Asia (Winter *et al.*, 1997). The $SPCSV_{EA}$ serotype has been detected in Kenya, Uganda, Tanzania, Zambia, Madagascar (Gibson *et al*, 1998) and Peru (Carey *et al.*, 1999; Gutièrrez *et al.*, 2003). When infecting alone, SPCSV causes mild symptoms such as slight stunting and purpling of lower leaves and mild chlorotic mottle in the middle leaves which may vary with cultivar (Winter *et al.*, 1992; Gibson *et al.*, 1998).

2.4 **Sweet potato virus disease (SPVD)**

SPVD is the most harmful disease of sweet potato in Africa and elsewhere (Geddes, 1990; Gibson *et al.*, 1998; Carey *et al.*, 1999; Gibson and Aritua, 2002). The disease was first reported in 1939 in Central Africa near the eastern border of the Democratic Republic of Congo and in East Africa 14 years later (Sheffield, 1953). However, the first published description of SPVD was from Uganda (Hansford, 1944). SPVD symptoms result from infection caused by two viruses namely SPCSV and SPFMV (Schaefers and Terry, 1976; Gibson *et al.*, 1998).

Most African sweet potato cultivars infected with SPFMV show no symptoms (Gibson, *et al.*, 1997), but co-infection with SPCSV leads to SPVD. Although symptoms in sweet potato plants affected by SPVD differ with genotype, infected plants generally appear very stunted and have small, distorted leaves, which are often narrow (strapped) and crinkled with a chlorotic mosaic especially on mature leaves often with vein clearing and vein feathering (Gibson *et al.*, 1998; Karyeija *et al.*, 2000). In other *Ipomoea* species, including the indicator

species *I. setosa* and *I. nil*, symptoms of SPVD are more pronounced and include necrosis, vein-clearing, mosaic, leaf stunting and distortion. More recent studies indicate that the SPCSV is limited to the phloem and that it unusually mediates an increase in SPFMV titres perhaps by suppressing the plant's resistance to SPFMV (Karyeija *et al.*, 2000).

There have been many appraisals on SPVD-associated production losses, but only a few reliable estimates of such loss are available. Yield reductions exceeding 50% resulting from infection of sweet potato by SPVD in Africa and elsewhere have been documented (Hahn, 1979; Mukiibi, 1977; Melissa *et al.*, 1996; Gibson *et al.*, 1998; Gutiérrez *et al.*, 2003). SPVD causes yield losses through reductions in growth of aboveground parts, and the number and weight of tuberous roots (Hahn, 1979). These data indicate that SPVD diminishes tuberous root yields in sweet potato and therefore constitutes a serious constraint to sweet potato production. In Kenya, loss in production of three commonly grown cultivars of as high as 92% has been reported (Njeru *et al.*, 2004).

2.5 Management of virus diseases

Unlike other pathogens, viruses are obligate parasites utilizing metabolic precursors and the host cell biosynthetic machinery to support their multiplication in plant tissues. Consequently virus control is fundamentally based on the principles that virus diseases are not curable and that infection can only be prevented. Thus, virus control is directed at interrupting infection cycles.

Phytosanitation involves the eradication of sources of infection and inoculum. Sweet potato farmers usually select cuttings relatively unaffected by SPVD as parents of the next crop (Bashaasha *et al.*, 1995; Kapinga *et al.*, 1995; Aritua *et al.*, 1998) and regard this as a main control method for SPVD (Gibson and Aritua, 2002). Most cuttings from symptomless plants

have been found to be virus-free (Gibson *et al.*, 1997) and have been reported overall to yield similar to those from virus-free plants (Carey *et al.*, 1999). Some farmers in East Africa remove diseased plants in young sweet potato crops (Gibson *et al.*, 2000). Roguing, involves the removal and destruction of plants as soon as they become infected and therefore reduces the development of virus sources (Dent, 1995). However, some sweet potato farmers do not rogue for fear that this would cause a proportional loss in yield perhaps due to a lack of knowledge on the cause and spread of viruses. The destruction of surviving crops in old and sometimes abandoned fields can conceivably reduce sources of infection and virus incidence.

Chemical insecticides are seldom used to control vectors of sweet potato viruses especially in Africa. They may be only effective when used to control the spread by vectors of circulatively transmitted viruses where long feeding periods are required for the acquisition and inoculation phases (Antignus, 1987) and especially for the colonizing vectors. The use of chemicals is unsuitable in a non-persistent non-circulative transmission system where the transmission cycle is very short and therefore cannot be affected by the application of insecticides since the vectors would have transmitted the virus before they die (Walkey, 1991; Thottappilly, 1992). Furthermore, vector movement may increase following spraying with insecticides leading to increased levels of virus spread (Berger, 2001). The negative effects resulting from the use of chemicals such as toxicity in the environment and the disruption of the natural balance combined with pesticide resistance development have led to a support for alternative, more environmentally friendly means of control. Moreover, cost and safety are immediate considerations for pesticide usage by smallholder sweet potato growers in developing countries.

One of the most effective and least expensive methods of controlling virus diseases in sweet potato is the use of host plant resistance (Karyeija *et al.*, 1998a; Berger 2001). Since viruses

cause the most important diseases of the sweet potato crop in sub-Saharan Africa (Geddes, 1990), breeding for resistance against viruses should be a priority. In West Africa, SPVD-resistant cultivars have been selected by field exposure and graft-inoculation of storage roots (Hahn *et al.*, 1981). Also in Uganda, cultivars resistant to SPFMV have been identified (Aritua *et al.*, 1998). In Kenya, a single variety showing tolerance to single virus infections by SPFMV, SPMMV, and SPCSV has been identified (Njeru *et al.*, 2004). However, it is known that virus resistance only slows down but does not prevent virus spread in vegetatively propagated crops (Fargette and Vie, 1995). Furthermore, it is difficult to use conventional breeding on polyploid species such as sweet potato which has a low seed set and is self–incompatible (Thompson *et al.*, 1997).

2.6 Pathogen derived resistance (PDR)

Since suitable and useful sources of resistance are either not available or can hardly be introgressed into agronomic cultivars, other methods are now in use. Use has been made of bioengeneering (genetic manipulation) methods to introduce genes for virus resistance obtained from novel sources (Berger, 2001). A plant can be protected from a severe strain of a virus by inoculating it first with a mild strain of the same virus. Abel *et al.* (1986) mimicked cross-protection by transforming plants with a virus derived CP gene. Since then, many virus-derived genes have been used to transform plants so as to obtain resistance (Baulcombe, 1996; Beachy 1997; Powel-Abel *et al.*, 1986; Nishiguchi *et al.*, 1997).

In sweet potato, Nishiguchi *et al.* (1997) introduced the CP gene of a severe strain of SPFMV (SPFMV-S) by electroporation. The plants were highly resistant to SPFMV-S as virus accumulation was significantly reduced in the transgenic plants. Similarly in Africa, the Kenya Agricultural Research Institute (KARI) has in a collaborative venture with Monsanto Inc., incorporated the CP gene of SPFMV to confer resistance to sweet potato. Results from

Kenyan cultivars transformed with an American construct showed similar disease levels in transformed as well as non-transformed checks (Gichuki *et al.*, 2003). It is therefore necessary to determine the strain occurrence in Kenya in order to come up with gene constructs that are likely to be effective in managing SPFMV infection.

In TMV, the large amounts of viral CP expressed in transgenic plants interfered with the uncoating of the virus at infection and with long distance movement (Bendahmane and Beachy, 1999). It has been reported that the capacity of the transgene to confer resistance against the challenge virus directly depends on the sequence similarity between them (Bateson *et al.*, 1994; Tennant *et al.*, 1994). For instance, transgenic tobacco plants expressing the CP of cucumber mosaic virus (sub-group I) were resistant to infection with CMV-C but susceptible to CMV-WL (sub-group II) (Hull and Davies, 1992). It was concluded that the level of resistance conferred by the CP is dependent on the sequence similarity between the transgene and RNA of the virus to be controlled.

2.7 Plant viral synergism

The success of a virus in a given host plant may be influenced considerably by the presence of another virus in mixed infections. Such mixed infections often lead to synergy; a situation characterized by intensified severity of symptoms and/or increased virus accumulation (Karyeija *et al.*, 2000; Hull, 2002). With sophistication in diagnostic techniques, infections of plants by multiple viruses are increasingly better understood.

Synergy is often characterized by effects on the replication of one virus by a second co-infecting virus. In some diseases, enhanced severity may be due to enhanced systemic spread of a restricted virus or spread by vectors. In some isolated cases, two co-infecting viruses may benefit (Scheets, 1998; Fondong *et al.*, 2000), while there is a report of both viruses being at a

disadvantage in a mixed infection (Poolpol and Inuoye, 1996).

Plant viral synergisms may be divided into two main divisions namely, the potyvirus-associated synergisms, in which one of the synergistic pair is a potyvirus; and the other in which neither is a potyvirus. Several such potyvirus-associated synergistic diseases have been characterized, and in each, an appreciable increase in host symptoms is observed in doubly infected plants. The increase in symptoms is often correlated with an increase in the accumulation of the non-potyvirus component of the synergistic pair, without a corresponding increase in the level of the potyvirus (Rochow and Ross, 1955; Calvert and Ghabrial, 1983; Goldberg and Brakke, 1987; Vance, 1991). Examples of mixed virus infections leading to synergy include the co-infection by *Potato virus Y* (PVY) and the potexvirus *Potato virus X* (PVX) in tobacco (Damirdagh and Ross, 1967; Vance 1991; Vance *et al.,* 1995), *Turnip mosaic virus* with *Cauliflower mosaic virus* (CaMV) in radish (Sano and Kojima, 1989) and PVY with the polerovirus *Potato leaf roll virus* in *N. clevelandii* (Barker, 1987). In the case of the PVX and PVY interaction, tobacco plants doubly infected by this pair showed accumulation of PVX with no increase in PVY (Rochow and Ross, 1955; Vance, 1991).

In most of the reported cases of synergistic interaction, the accumulation of the potyvirus did not increase while that of the other virus increased. However, in a recently characterized synergistic interaction in sweet potato, SPFMV, a potyvirus, accumulated 600-fold in plants co-infected with SPCSV (Karyeija *et al.,* 2000). The sweet potato plants infected with both SPCSV and SPFMV contain higher titres of SPFMV, exhibit more severe symptoms and are a better source for aphid acquisition than plants infected with SPFMV alone. In plants infected with SPFMV alone, very small amounts of SPFMV RNA were detected as opposed to large amounts of SPFMV RNA in plants infected with both SPFMV and SPCSV (Schaefers and Terry, 1976; Rossel and Thottappilly, 1987; Karyeija *et al.,* 2000). The titers of SPCSV do

not increase in the dually infected plants and since the plants infected with SPCSV alone are mostly symptomless or show symptoms different from symptoms characteristic of SPVD, the symptoms of SPVD were presumably caused mainly by SPFMV (Karyeija *et al.*, 2000). It is not known whether SPCSV synergizes other viruses or if indeed there are any interactions between other viruses associated with sweet potato.

2.8 Genetic variability of plant RNA viruses

Viruses remain substantially similar to the parents during replication but occasionally give rise to new types (commonly referred to as strains) in order to adapt to new and changing conditions. Adaptation may arise by re-assortment of segmented virus genomes, gene duplication, deletions, frame shift mutations, point mutations or development of gene products from existing, unused reading frames (Hull, 2002). Variation may also results from errors occurring during the replication of viral genomes (Garcìa-Arenal *et al.*, 2001).

Most of the plant infecting viruses possess RNA genomes. The replication of RNA genomes is highly error-prone as a result of the RNA dependent RNA polymerase's (RdRp) lack of proof-reading properties (Domingo and Holland, 1997). This results in a heterogeneous population of any virus within a given host (Eigen *et al.*, 1996; Smith *et al.*, 1997). In time, the genetic structure of a replicating entity changes providing potential for rapid evolution. Recombination is another source of variation, which may take place when two independent segments of genetic information are switched between the nucleotide strands of different genetic variants during replication (Simon and Bujarski, 1994). This way, viruses have the opportunity to exchange or acquire new genes or gene segments into their genomes. Genetic exchange may also result from the re-assortment of genomic segments in viruses with segmented genomes. However, the variants generated undergo a process of selection pressure and only the fittest variants increase their frequency in the population (positive selection),

whereas less fit variants decrease their frequency (negative selection). The latter may lead to certain RNA virus proteins or genes being highly conserved and therefore used for the taxonomic assignment of virus isolates to families, genera and species and to low population diversity. Selection may also increase diversity if similar variants undergo different selection pressures (García-Arenal *et al.*, 2001). Within the Potyviridae for instance, the CP gene sequence is frequently used to determine the taxonomic position of isolates (Shukla *et al.*, 1994).

The property of a virus strain that distinguishes it from other known strain(s) can be manifold. It is often biological, for example, a difference in symptoms in a given host (Hull, 2002). From a practical standpoint, the existence of such strains that cause different kinds of diseases in the field is considerably important. Effective and reliable control strategies against viruses and virus strains will therefore rely upon an accurate detection methodology. So far, conventional approaches based on physical, biological and serological properties have failed to distinguish distinct potyvirus species consistently from strains (Francki *et al.*, 1985; Milne, 1988). Apart from particle morphology, cytopathology and transmission mechanism, molecular genetic characteristics such as genome structure and CP sequence appear to be the ultimate criterion for assigning viruses into groups and designating their strains (Hull, 2002).

Studying the variability and changes in the genetic structure of a plant virus population are important aspects and highly relevant to the development of strategies for control of diseases (Garcìa-Arenal *et al.*, 2001). Nucleotide sequences give valuable information about the extent of relationships between viruses and their strains. Sequences have particularly helped to resolve conflicts brought about by inconsistent biological properties. However, it is not advisable to use only sequences to establish degrees of relatedness without other information since the genetic code is degenerate and many base substitutions may cause no change in the

coded amino acid. Moreover, a base substitution may give rise to an amino acid of very similar properties, leading to little or no change to the protein; although small changes in nucleotide sequence could have profound effects on phenotypic effects (Hull, 2002). Several isolates of a number of plant species have been studied and have shown strain differences that correlate with geographic origins and strain specialization (Ohshima *et al.*, 2002; Aleman-Verdaguer *et al.*, 1997). For potyviruses, the CP gene sequence data presents the most useful criterion for distinguishing viruses from strains and for establishing evolutionary relationships between sets of distinct potyviruses (Shukla *et al.*, 1994).

Although much effort has been directed at the characterization of viruses infecting sweet potato (Schaeffers and Terry, 1976; Hahn, 1979; Cadena-Hinojosa and Campbell, 1981; Rossel and Thottappilly, 1988; Winter *et al.*, 1992; Kreuze *et al.*, 2000), their aetiology is not completely understood. This is particularly so because some of the viruses are not only transmitted by the same vectors (Winter *et al.*, 1992), but also because some viruses occur in more than one serotype or strain in mixed infections.

3.0 CHAPTER THREE

IDENTIFICATION AND DISTRIBUTION OF VIRUSES INFECTING SWEET POTATO IN KENYA

3.1 Introduction

Sweet potato (*Ipomoea batatas* L.) is an important food crop in Kenya. The crop is a staple diet in some parts of the country grown largely by smallholder farmers (Horton, 1988; Carey, 1996). The main sweet potato production areas in Kenya include the western, central and coastal regions where about 75% of total production is concentrated (Ndolo *et al,* 1997). Although the area under sweet potato in Kenya has increased over the years (Matin, 1999), yields fall far below the crop's production potential mainly due to pests and diseases (Ndolo *et al,* 1997; Matin, 1999). Sweet potato yields have shown a steady decline over the last five years (FAO, 2002).

Viruses are the second most important biotic constraint after insects (weevils), which limit sweet potato production both in Africa (Geddes, 1990) and worldwide (Jannson and Raman, 1991). More than 50% of production losses have been attributed to virus infections (Ngeve *et al,* 1991; Melissa *et al,* 1996; Gutiérrez *et al.,* 2003). Viruses reported to infect sweet potato in Africa include Sweet potato feathery mottle virus (SPFMV), Sweet potato chlorotic stunt virus (SPCSV), Sweet potato mild mottle virus (SPMMV), Sweet potato virus G (SPVG), Cucumber mosaic virus (CMV), Sweet potato chlorotic fleck virus (SPCFV), Sweet potato latent virus (SwPLV) and Sweet potato caulimo-like virus (SPCaLV) (Hollings *et al.,* 1976; Mukiibi, 1977; Hahn, 1979; Geddes, 1990; Wambugu, 1991; Alvarez *et al.,* 1997; Gibson *et al,* 1998).

Often, infection of sweet potato by two or more viruses leads to greater damage than the sum of the damage by each virus individually. Sweet potato virus disease (SPVD) is the most damaging disease of sweet potato in the East African region (Geddes, 1990). The disease is caused by dual infection of sweet potato with SPCSV and SPFMV (Schaefers & Terry, 1976; Gibson *et al,* 1998). Most sweet potato cultivars infected with SPFMV or SPCSV alone are respectively, either symptomless or show moderate stunting, purpling or chlorosis of middle and bottom leaves. Symptoms in plants affected by SPVD are, however, much more severe than those in plants infected with either virus alone (Gibson, *et al,* 1997; Gibson *et al,* 1998), with SPCSV synergising the multiplication of SPFMV (Karyeija *et al,* 2000). Depending largely on the cultivar and virus strain, production losses of more than 90% have been attributed to SPVD (Gibson *et al,* 1998; Karyeija *et al,* 1998; Njeru *et al.*, 2004).

There have been only two previously reported surveys on the identity, incidence and distribution of viruses infecting sweet potato in Kenya. Whereas the second survey (Carey *et al.,* 1996) analysed relatively few samples, the first one (Wambugu, 1991) was conducted about a decade ago. Since then, changes in farming practices and population dynamics of virus vectors may have changed virus incidence (Wisler *et al.,* 1998). In addition, several antisera to previously unrecognised sweet potato viruses have become available in recent years. More emphasis also needs to be directed at the co-occurrence of viruses in sweet potato. Consequently, the objective of this study was to determine the identity of viruses infecting sweet potato and the occurrence and distribution of multiple virus combinations in all the major growing regions of Kenya.

3.2 MATERIALS AND METHODS

3.2.1 Survey of viruses infecting sweet potato in Kenya

A survey was conducted in five major sweet potato-growing areas in Kenya to determine the distribution and incidence of viral diseases. At least twenty sweet potato fields were examined in each province (Nyanza, Western, Central, Coast and Eastern). Sweet potato fields with a 3- to 5-month-old crop were sampled along rural roads or paths at approximately 5-km intervals. The number of plants with virus disease symptoms amongst 50 plants examined along a X-shaped transect stretching from the four corners of each field was recorded (Nutter, 1997). Along the transect, about 10 and 5 symptomatic and asymptomatic samples, respectively, were collected in each field. A total of 125 sweet potato fields were assessed for viral diseases. Socio-economic data relevant to sweet potato viral diseases were obtained with the aid of a questionnaire (Appendix 2). To establish farmers' knowledge and perceptions on SPVD in Kenya, information such as source of planting material, age of the crop, name(s) of cultivar(s) grown in the fields, whether the farmer recognizes the diseases, if the farmer considers virus diseases a problem and whether resistant cultivars are available was collected. The exact geographical position and altitude of each farm visited was recorded using the geographical positioning system (GPS). A total of 448 symptomatic and 638 symptomless plants were collected and transferred to the laboratory for serological analysis.

3.2.2 Serological analysis

The diseased and symptomatic samples collected during the survey were tested for SPFMV, SPMMV, SPCFV, SPCaLV, C-6, SwPLV and SPMSV by nitrocellulose membrane enzyme linked immunosorbent assay (NCM-ELISA) employing standard kits and antisera obtained from the International Potato Center (CIP, 2001). The kit contained polyclonal antibodies to SPFMV, SPMMV, SPCFV, SwPLV, SPCaLV, SPMSV, SPCSV, and C-6 as well as *alkaline*

phosphatase-labelled goat anti-rabbit (GAR-AP) IgG and the substrate (NBT/BCIP) nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt. SPCSV and CMV were assayed by triple antibody sandwich (TAS) ELISA.

Nitrocellulose membrane (NCM) ELISA

Test samples collected during the survey were assayed by NCM-ELISA for SPFMV, SPMMV, SwPLV, SPCFV, SPCaLV, C-6 and SPMSV. Two leaf discs (1-cm diameter) from a composite sample of two leaves taken from different points (middle and top) of a sweet potato plant were ground in 1 ml of extraction buffer in plastic bags. The ground sample was allowed to stand for 30-45 min at room temperature for the sap to phase out. Using a clean pipette each time, a drop of clear supernatant of each sample was blotted at the centre of a square made on the nitrocellulose membrane. The membrane was allowed to dry at room temperature for about 15-30 min. Once dry, the membrane was immersed in 30 ml blocking solution in a Petri dish for 1 h. The blocking solution was discarded and the membrane reacted in a Petri dish with the primary antibody diluted (1:1000, v/v) in antibody solution. The membranes were incubated at room temperature, overnight, with a constant agitation on an orbital shaker (50 rpm). The primary antibody solution was discarded and unbound antibodies removed from the membranes by washing with constant agitation in T-TBS four times for three minutes each time at 100 rpm. The membranes were then immersed in 30 ml of GAR-AP solution (1:1000 v/v) in a Petri dish for 1 h. The substrate solution (NBT/BCIP, Appendix 3) was added and the reaction allowed to proceed for 5 to 30 min. Positive and negative reactions were determined by visual assessments with different grades of purple colour indicating positive reactions. The substrate solution was discarded after 30 min incubation and the membranes washed twice with distilled water to stop the reaction.

Triple antibody sandwich (TAS) ELISA

TAS-ELISA was used for the detection of SPCSV and CMV as outlined by Gibson *et al.* (1998) utilizing monoclonal and polyclonal antibodies kindly provided by H.J. Vetten (BBA, Braunschweig, Germany). For SPCSV, microtitre plates (Greiner Bio-One Frickenhausen, Germany) were coated with 100 µl of polyclonal antibody to SPCSV (KY-CP) diluted (1:1000) in coating buffer and the plates incubated for 2-3 h at 37^0C. The plates were thereafter washed 3 times in phosphate buffered saline with 0.05 % Tween 20 (PBS-T), each time leaving the plates filled with PBS-T for 3 min. Samples were ground in sample buffer (1:20 w/v) and tested in duplicate by filling two wells with 100 µl each of leaf extract. The plates were incubated overnight at 4^oC and washed using PBS-T as described above. A mixture (mix 1) containing monoclonal antibodies (MAbs) specific to SPCSV isolates from East Africa ($SPCSV_{EA}$) and another mixture (mix 2) containing MAbs reacting solely to SPCSV isolates from West Africa, Israel and the Americas ($SPCSV_{WA}$), (Vetten *et al.,* 1996; Gibson *et al.,* 1998) were diluted (1:100 w/v) in conjugate buffer and added to each well of the microtitre plate. The plates were incubated at 37^0C for 2 h and then washed before 100 µl of alkaline phosphatase-labelled rabbit anti-mouse IgG (RAM-AP) (DIANOVA, Hamburg, Germany) diluted (1:2500 w/v) in conjugate buffer was added to each well and plates incubated at 37^0C for 2 h. The plates were washed in PBS-T before the addition of 200 µl *p*-nitrophenyl phosphatase substrate (Roche Corporation) in substrate buffer (Appendix 3) to each well. The plates were incubated for colour development at room temperature. Absorbancies (A_{405}) were recorded after 1-h incubation using a microplate reader (Humareader Model 2106, Germany). The intensity of the colour developed was taken to be directly proportional to the virus concentration (Clark and Adams, 1976).

3.2.3 Establishment of sweet potato samples in the screenhouse

Sweet potato cuttings (20-30 cm long) from symptomatic and asymptomatic plants collected during the survey were planted in an insect-proof screenhouse at the University of Nairobi's Kabete Campus field station. The cuttings were planted in 15-cm diameter pots in a sterile soil mixture enriched with di-ammonium phosphate fertilizer.

3.2.4 Grafting of sweet potato onto *Ipomoea setosa*

Since sweet potato infecting viruses often multiply faster in *I. setosa* and attain higher concentrations easily detectable by ELISA (Moyer *et al.*, 1989), cuttings of symptomatic and asymptomatic sweet potato that did not react with any of the available antisera were grafted onto *I. setosa* in the screenhouse. To graft, a scion (with leaves removed) having 2 or more nodes were cut with a sharp blade to make a wedge-shaped cut end that could be inserted easily into an incision made longitudinally in the stem of a 2-3 week old *I. setosa.* The incision and the lower part of the scion were wrapped with parafilm and the whole plant covered with a plastic bag for at least 6 days to minimize moisture loss. The grafted indicator plants with successful graft unions were kept in the screenhouse and observed for symptom development. The *I. setosa* plants were assayed serologically by ELISA for SPFMV, SPMMV, SWPLV, SPCFV, CMV, SPCaLV, SPMSV, C-6, and SPCSV, four weeks after grafting.

3.2.5 Isolation and maintenance of virus isolates

Sweet potato cuttings infected with various viruses and virus combinations were maintained in an insect-proof screenhouse for subsequent experiments. Selected plants infected with SPFMV, SPMMV, SPCFV and SPCSV or with various mixed infections from different locations in Kenya were transferred to the Federal Biological Research Centre for Agriculture and Forestry (BBA), Braunschweig, Germany for virus isolation and further characterisation.

At BBA, pure isolates (except for SPCSV) were established by sap transmission and local lesion transfer.

The main technique of isolating the potyviruses infecting sweet potato was by sap inoculation of various indicator plants, such as *Chenopodium quinoa, C. murale, C. amaranticolor, C. foliosum, Gomphrena globosa, Nicotiana benthamiana, N. debneyi, N. clevelandii, N. occidentalis* P1, *N. occidentalis* ssp. *obliqua, N. hesperis, and N. tabacum.* A small piece of infected sweet potato leaf was ground in a small amount of 0.05 M sodium phosphate buffer, pH 7.4 (inoculation buffer). The resulting suspension was rubbed gently onto the entire surface of the carborundum-dusted leaves using a Q-tip. The leaves were rinsed with tap water to ease the visual assessment of leaf symptoms. All inoculated plants were inspected for symptom development at regular intervals and individually tested at 4-6 weeks after inoculation by DAS-ELISA using virus-specific antibodies.

To ascertain the isolation of (biologically) pure isolates, individual chlorotic or necrotic lesions were excised from the leaves of a local-lesion host and ground singly in 1 ml of inoculation buffer. The resultant extract was mechanically inoculated onto 1-2 leaves of a healthy individual of the same local-lesion host. This type of local-lesion transfer was repeated 3-4 times until the type of local lesions developing on the inoculated leaves appeared uniform.

Double antibody sandwich (DAS) ELISA

For DAS-ELISA, micro-titre plates were coated with 200 µl of virus-specific IgG diluted at 1:1000 in coating buffer and the plates incubated for 2-3 h at 37^0C. The plates were washed 3 times in PBS-T. Sap was extracted by grinding the samples in sample buffer (1:20 w/v) and 100 µl of each test sap pipetted into a pair of wells. The plates were incubated overnight at 4^0C

and washed in PBS-T as above. Aliquots of 200 µl of alkaline phosphatase-labelled antibody diluted at 1:1000 (v/v) was added to each well of the microtitre plate. The plates were washed in PBS-T before addition of 200 µl of *p*-nitrophenyl phosphate in substrate buffer to each well. The plates were incubated for colour development at room temperature. Absorbancies (A_{405}) were recorded after 1-h incubation using a microplate reader (Humareader Model 2106, Germany).

3.3 RESULTS

3.3.1 Farmers' knowledge on sweet potato virus disease

SPVD was present in all the five provinces visited. The highest prevalence (61%) of the disease was observed in Nyanza province whereas the lowest prevalence (4%) was recorded in Eastern province (Fig. 1). Most farmers (66%) recognized plants infected with SPVD as aberrant but they attributed it to various causes. Twenty-five and thirteen percent of the farmers respectively attributed the cause of SPVD to insects and nutritional deficiency. Other factors attributed to the disease by the farmers were use of infected planting material and drought. However, 34% of the interviewed farmers had no idea as to what causes the disease (Fig. 2).

Most farmers (96%) obtained planting material from their stocks or from fellow farmers. Other sources included researchers (KARI), and local markets especially during periods of prolonged dry weather. An estimated 82% of the farmers practiced piecemeal harvesting while the rest, particularly the commercial producers, harvested the entire crop in a single operation.

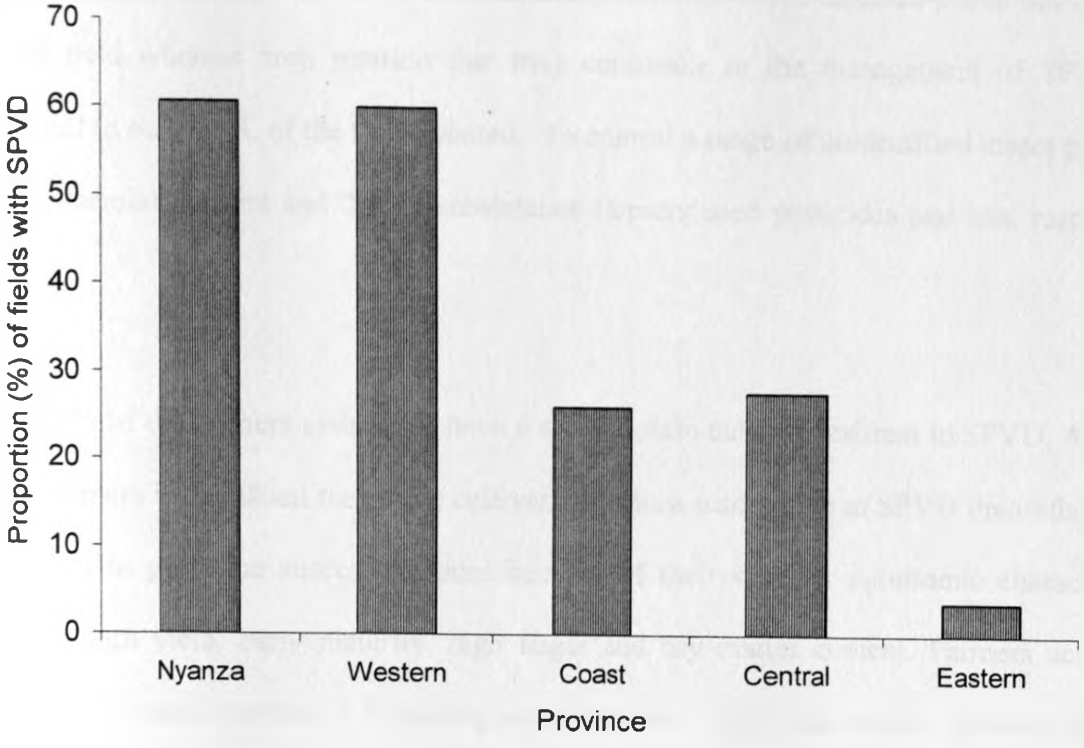


Fig 1: Prevalence of sweet potato virus disease in five provinces of Kenya

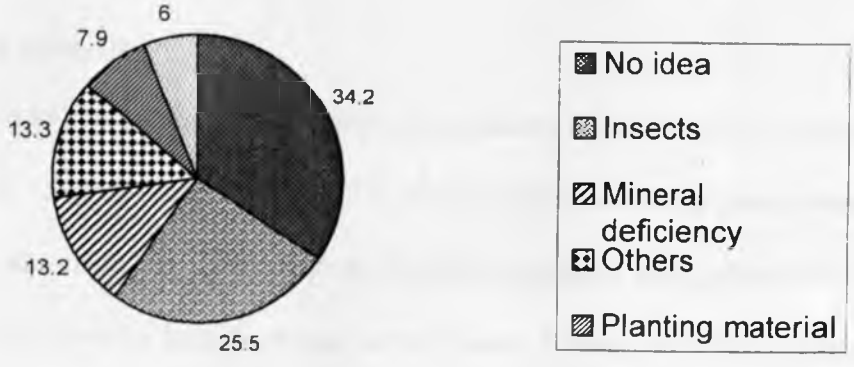


Fig 2: Kenyan sweet potato farmers' perceptions of the causes of sweet potato virus disease

Due to lack of knowledge, no SPVD–specific management practices were consciously used. However, 74% of the farmers selected planting vines from healthy looking and vigorously growing plants. About 16% of the farmers interviewed uprooted diseased plants but left them in the field whereas crop rotation that may contribute to the management of SPVD was practiced in only 12%, of the fields visited. To control a range of unidentified insect pests, 3% of commercial farmers and 2% of subsistence farmers used pesticides and ash, respectively (Fig 3).

Only 20% of the farmers claimed to have a sweet potato cultivar resistant to SPVD. Although some farmers had realised that some cultivars are more susceptible to SPVD than others, they continued to grow the susceptible ones because of their superior agronomic characteristics such as high yield, early maturity, high sugar and dry matter content. Farmers across the country expressed interest in obtaining new cultivars. According to the farmers, the most preferred traits in descending order were high yield, early maturity, high sugar and dry matter content. Only 16% of the farmers interviewed indicated that research should be geared towards the development of SPVD-resistant cultivars.

3.3.2 Serological analysis

A total of 448 and 638 symptomatic and asymptomatic plants, respectively, were serologically analyzed (Table 1). Eighty-three percent (372) of the symptom bearing plants reacted with antisera to at least one virus. Sixty-five of those that tested negative were grafted onto *I. setosa* and of these, 38 were positive to at least one of the viruses. Overall, 410 (92%) symptomatic plants tested positive to at least one virus. Of the 638 asymptomatic plants collected and tested in ELISA, only 28 (4%) reacted with antisera to at least one virus. After grafting those that were negative, a further 133 plants tested positive for at least one of the viruses but mainly

SPFMV. Thus, a total of 161 (25%) of the symptomless plants tested were infected.

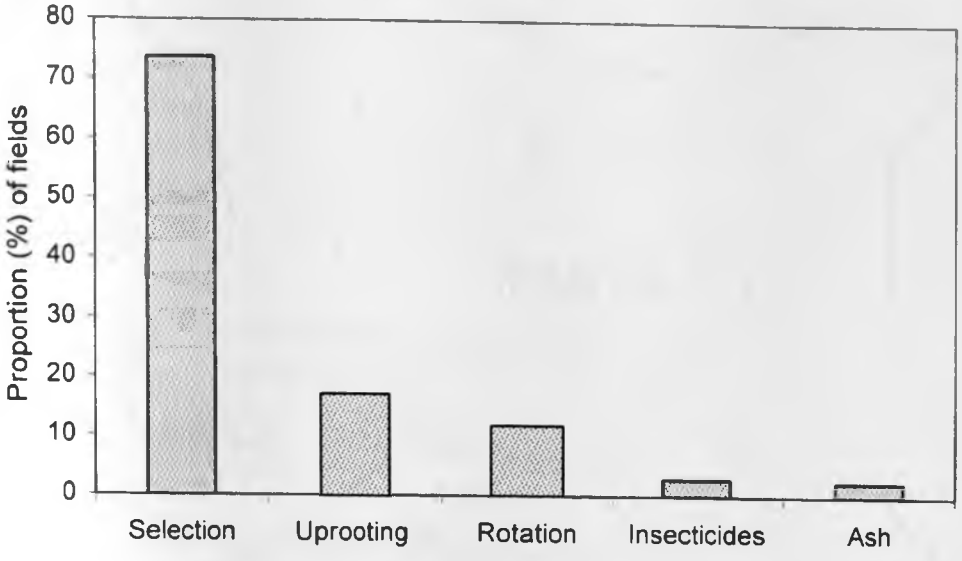


Fig 3: Practices adopted by farmers in the management of sweet potato virus disease

Fig 4: Map of Kenya showing the major sweet potato production areas and the location of the surveyed districts. The Kenyan provinces are 1. Rift Valley, 2. Eastern, 3. North Eastern, 4. Coast, 5. Central, 6. Western, 7. Nyanza, 8. Nairobi

Province	District	Number of fields sampled	Tested	Tested positive for at least one virus	Tested following grafting*	Tested positive after grafting†	Tested	Tested positive for at least one virus	Tested following grafting *	Tested positive after grafting†
Western	Kakamega	10	50	41	4	4	46	2	44	13
	Bungoma	10	61	54	6	1	42	0	42	11
	Teso	5	12	9	3	3	20	2	17	6
	Busia	5	36	36	1	0	22	1	20	5
	Total	**30**	**159**	**140**	**14**	**8**	**130**	**5**	**123**	**35**
Central	Nyeri	8	11	5	5	4	48	2	46	19
	Kirinyaga	10	29	12	14	12	46	2	45	10
	Total	**18**	**40**	**17**	**19**	**16**	**94**	**4**	**91**	**29**
Eastern	Machakos	15	16	9	7	4	97	4	93	19
	Embu	11	6	0	4	4	57	0	57	15
	Total	**26**	**22**	**9**	**11**	**8**	**154**	**4**	**150**	**34**
Nyanza	Rachuonyo	7	35	34	1	1	34	6	28	3
	Kisii	7	55	53	2	0	34	6	26	5
	Homabay	7	43	40	4	0	36	2	34	8
	Kisumu	7	35	31	3	2	31	1	30	3
	Total	**28**	**168**	**158**	**10**	**2**	**135**	**15**	**118**	**19**
Coast	Kilifi	4	5	0	5	0	18	0	18	2
	Malindi	6	2	0	2	2	29	0	29	2
	Kwale	7	39	35	3	1	44	0	44	4
	T Taveta	6	13	13	1	1	34	0	34	8
	Total	**23**	**59**	**48**	**11**	**4**	**125**	**0**	**125**	**16**
Total		**125**	**448**	**372**	**65**	**38**	**638**	**28**	**607**	**133**

- surviving diseased and asymptomatic plants in which no virus was detected by serology were grafted to *I. setosa* for re-indexing.
- † Inducing symptoms in grafted *I. setosa* ; testing serologically positive for at least one virus.

Virus disease incidence

Viral diseases were common in most of the areas surveyed (Fig. 4). The highest mean disease incidence (18%) was observed in Kisii district and confirmed following serological detection (Table 2). The lowest incidence (1%) was recorded in Kilifi and Malindi districts of Coast province. Considerable differences were observed in virus incidence between the districts surveyed and even among fields within a district. In terms of provinces, incidence was highest (14%) in Nyanza province closely followed by Western province with a mean of 13%. Central and Coast provinces had an incidence of 11% and 6%, respectively, while Eastern province had the lowest (4%) incidence of virus infection. The highest (48%) incidence of viral diseases in an individual field was observed in Kirinyaga district of Central province in which sweet potato had been cultivated for several consecutive seasons. The visually assessed incidence levels were consistently lower than those based on serological assays (Table 2) indicating a fairly constant proportion of symptomlessly infected plants. The relationship between incidences as assessed visually and serologically is presented in figure 5. Overall, Kisii district had the highest disease incidence irrespective of whether the incidence was estimated visually or serologically. Correlations between virus disease incidence and altitude, mean rainfall or mean temperature were not significant ($P \leq 0.05$).

Table 2: Comparison of the incidence (%) of virus diseases in sweet potato crops as assessed visually and serologically in 16 Kenyan districts

Province/ District	Incidence (%) of virus-infected plants based on	
	visual assessment	serological detection
Eastern		
Embu	3 (0-8) *	4 (0-8)
Machakos	4 (0-20)	4 (0-12)
Mean	**4**	**4**
Central		
Nyeri	2 (0-8)	6 (2-12)
Kirinyaga	14 (0-48)	16 (0-48)
Mean	**8**	**11**
Western		
Busia	11 (0-18)	15 (6-22)
Kakamega	9 (0-32)	13 (0-38)
Teso	4 (0-12)	8 (0-20)
Bungoma	16 (0-40)	17 (4-42)
Mean	**10**	**13**
Nyanza		
Rachuonyo	12 (0-38)	14 (0-42)
Kisii	18 (6-24)	18 (6-22)
Homabay	13 (4-22)	15 (2-26)
Kisumu	10 (2-20)	10 (4-18)
Mean	**13**	**14**
Coast		
Kilifi	2 (0-4)	1 (0-2)
Malindi	1 (0-4)	1 (0-4)
Kwale	15 (4 –30)	13 (2-30)
Taita Taveta	4 (0-16)	4 (0-16)
Mean	**6**	**6**

* Figures in parentheses give the incidence range (in %)

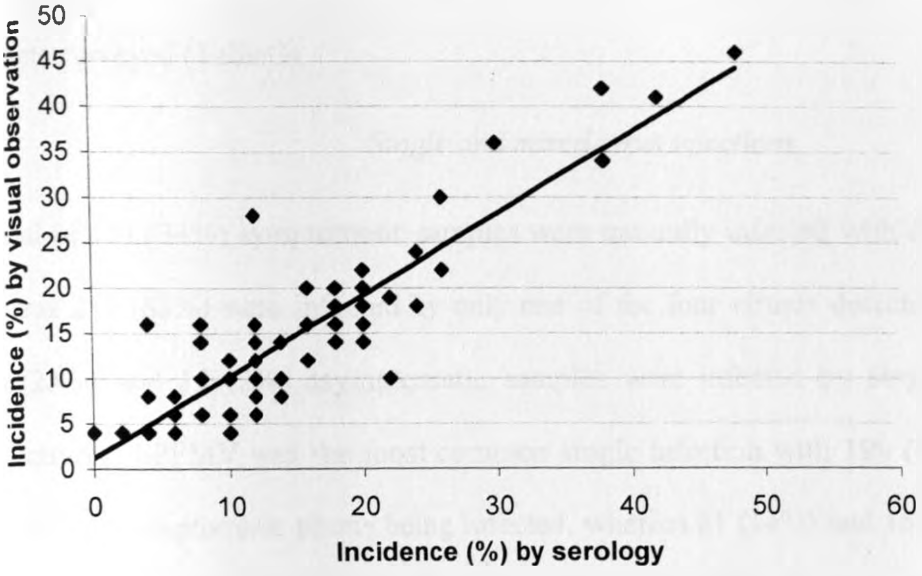


Fig. 5: Relationship between incidence (%) estimated serologically and by visual observation

Four viruses, namely SPFMV, SPCSV, SPMMV, and SPCFV, were detected in samples from 448 symptom-bearing and asymptomatic sweet potato plants collected from the five provinces surveyed (Table 3). As already shown in Table 1, the vast majority (92%) of the symptomatic samples reacted with antisera to at least one of the viruses.

Since a total of 334 (75%) and 123 (19%) symptomatic and asymptomatic samples, respectively, reacted with the SPFMV antibodies, SPFMV was the most frequently detected virus. SPCSV was detected in 173 (39%) of the 448 symptomatic plants and was the second most frequently detected virus. Of the 638 asymptomatic samples, only 16 (3%) were infected by $SPCSV_{EA}$, consistent with it being the most severe sweet potato-infecting virus, with most infected plants exhibiting distinct virus symptoms. SPCSV was not detected in samples collected from Embu, Kilifi and Malindi districts. SPMMV was detected in 47 (10%) symptomatic and 32 (5%) asymptomatic plants but was not detected in plant samples collected from Malindi and Kilifi

districts. SPCFV had the most restricted distribution being found only in seven out of the 16 districts surveyed (Table 3).

Single and mixed virus infections

A total of 151 (34%) symptomatic samples were naturally infected with different virus mixtures whereas 259 (58%) were infected by only one of the four viruses detected (Fig. 6). In contrast, 146 (23%) and 15 (2%) asymptomatic samples were infected by single and mixed viruses, respectively. SPFMV was the most common single infection with 190 (42%) symptomatic and 109 (17%) asymptomatic plants being infected, whereas 61 (14%) and 15 (2%) symptomatic and asymptomatic plants were singly infected with SPCSV (Fig. 6). As expected, the numbers of single and mixed infections were consistently higher in symptomatic samples. However, single infections by SPMMV were higher in asymptomatic samples (Fig. 6).

Seven different types of mixed virus infections were detected in the symptomatic plants tested. SPFMV and SPCSV (= SPVD) was the most common virus combination which occurred in 98 (22%) of the symptomatic samples collected (Fig. 6). SPVD was most frequent in samples collected from Western and Nyanza provinces though also present in Coast, Central and Eastern provinces. Infection by SPFMV and SPMMV was the second most prevalent dual infection in symptomatic and asymptomatic plants and was detected in 29 (7%) and 10 (3%) plants, respectively. SPCSV and SPMMV were observed to dually infect sweet potato in only 6 (1%) of the symptomatic samples collected.

A mixed infection involving four viruses (SPFMV, SPCSV, SPMMV and SPCFV) was detected in one symptomatic plant collected from Homa Bay district of Nyanza province. Unlike

symptomatic plants, mixed infections were extremely uncommon in the symptomless plants tested. Only four different virus complexes were detected in the symptomless plants (Fig. 6).

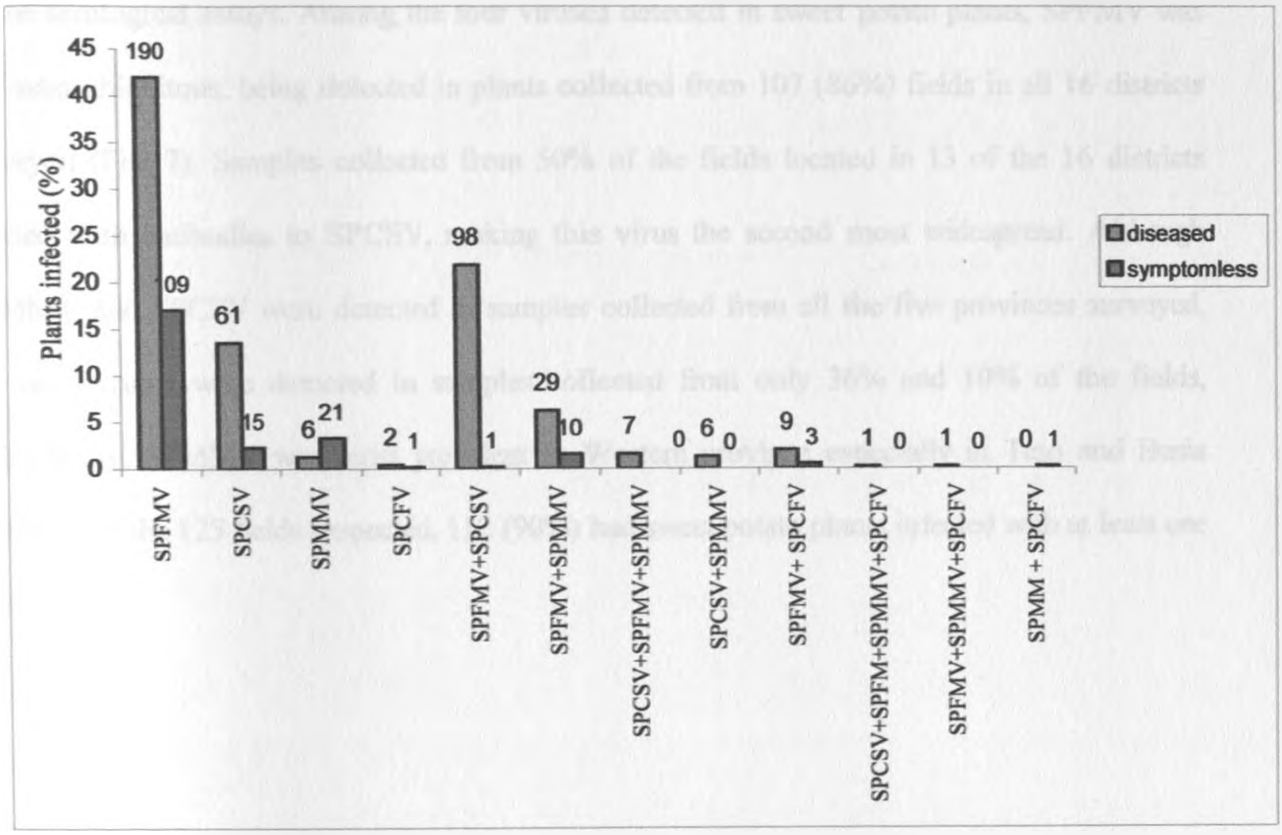


Fig. 6: Proportion (%) of single and multiple infections in symptomatic and asymptomatic sweet potato plants collected from 16 districts of Kenya. The numbers above each bar indicate the number of plants infected with single or multiple viruses.

The correlation between the number of plants infected with SPCSV and SPFMV was highly significant ($P \leq 0.01$; $r = 0.8$). Similarly, the correlation between the number of plants infected with SPCSV and SPMMV was also significant ($P \leq 0.01$; $r = 0.6$). Virtually all plants infected with SPCSV and SPFMV showed disease symptoms, whereas a high proportion of plants infected with single viruses or with the combination SPFMV and SPMMV had no obvious symptom when inspected in the field. This was particularly evident for SPMMV (Fig. 6).

Distribution and relative importance of individual viruses

The distribution of sweet potato infecting viruses in Kenya was determined based on the results of the serological assays. Among the four viruses detected in sweet potato plants, SPFMV was the most ubiquitous, being detected in plants collected from 107 (86%) fields in all 16 districts surveyed (Fig. 7). Samples collected from 50% of the fields located in 13 of the 16 districts reacted with antibodies to SPCSV, making this virus the second most widespread. Although SPMMV and SPCFV were detected in samples collected from all the five provinces surveyed, the two viruses were detected in samples collected from only 36% and 10% of the fields, respectively. SPMMV was most prevalent in Western province especially in Teso and Busia districts. Of the 125 fields inspected, 112 (90%) had sweet potato plants infected with at least one virus.

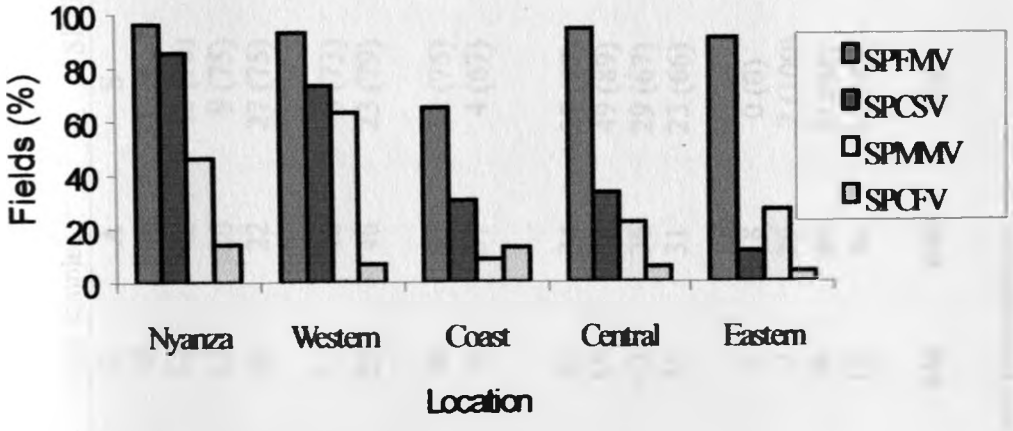


Fig. 7: Proportion (%) of fields with plants infected with SPFMV, SPCSV, SPMMV and SPCFV in five provinces of Kenya

Table 3: Number and types of virus infections of the 448 symptomatic (S) and 638 asymptomatic (A) plants collected from five provinces of Kenya

		No of samples		SPFMV		SPCSV		SPMMV		SPCFV	
		S	**A**	**S**	**A**	**S**	**A**	**S**	**A**	**S**	**A**
Western	Kakagema	50	46	33 (66) *	13(28)	20 (40)	1(2)	5 (10)	3(7)	1 (2)	0(0)
	Bungoma	61	42	43 (70)	9(21)	29 (48)	1(2)	9 (15)	2(5)	0(0)	0(0)
	Teso	12	20	9 (75)	3(25)	4 (33)	0(0)	3 (25)	5(25)	0 (0)	1(5)
	Busia	36	22	27 (75)	3(14)	27 (75)	0(0)	6 (17)	4(18)	0 (0)	0(0)
Central	Nyeri	11	48	8 (73)	19(40)	3 (27)	0(0)	0 (0)	2(4)	2 (18)	1(4)
	Kirinyaga	29	46	23 (79)	12(26)	6 (21)	1(2)	0 (0)	1(2)	0 (0)	0(0)
Eastern	Machakos	16	97	12 (75)	15(15)	2 (13)	6(6)	1 (6)	3(3)	1 (6)	0(0)
	Embu	6	57	4 (67)	13(23)	0 (0)	0(0)	0 (0)	4(7)	0 (0)	0(0)
Nyanza	Rachuonyo	35	34	27 (77)	5(15)	9 (26)	4(12)	7 (20)	0(0)	0 (0)	0(0)
	Kisii	55	34	49 (89)	8(24)	18 (33)	2(6)	0 (0)	1(3)	0 (0)	0(0)
	Homabay	43	36	29 (67)	7(19)	16 (37)	0(0)	6 (14)	3(8)	4 (9)	0(0)
	Kisumu	35	31	23 (66)	1(3)	15 (43)	1(3)	8 (23)	3(10)	2 (6)	0(0)
Coast	Kilifi	5	18	0 (0)	2(11)	0 (0)	0(0)	0 (0)	0(0)	0 (0)	0(0)
	Malindi	2	29	2 (100)	2(7)	0 (0)	0(0)	0 (0)	0(0)	0 (0)	0(0)
	Kwale	39	44	33 (85)	4(10)	20 (51)	0(0)	1 (3)	0(0)	0 (0)	0(0)
	T.Taveta	13	34	12 (92)	7(21)	4 (31)	0(0)	1 (8)	1(3)	4 (31)	2(6)
Total		**448**	**638**	**334**	**123**	**173**	**16**	**47**	**32**	**14**	**4**

* Figures in parentheses are percentages

3.4 DISCUSSION

The data on farmer perceptions and management of SPVD in Kenya indicated that the majority of the farmers do not know the cause of the disease. Consequently, no deliberate or conscious management practices are practiced. A high proportion of farmers select planting material from symptomless plants and this is an important practice towards reducing the incidence and spread of SPVD. Indeed, symptomless plants have not only been reported to be relatively virus-free (Gibson *et al.,* 1997), but have also been observed to yield similar to cuttings taken from known virus-free plants (Carey *et al.,* 1999). Few farmers uproot infected plants although roguing has been recommended (Aldrich, 1963). In Uganda, most farmers do not rogue either because of fear that this may cause a proportional loss in yield or due to the fact that they attribute the disease to abiotic causes. In Kenya, the latter is perhaps the case. Although a few farmers acknowledged that some cultivars are more susceptible to SPVD than others, they continued to grow them because of their superior agronomic characteristics such as high yield, early maturity, and high sugar and dry matter contents. The observation that farmers grow a mixture of landraces, which seemed to vary in resistance, indicates that resistant cultivars with acceptable agronomic traits could be identified.

Four viruses, SPFMV, SPCSV, SPMMV and SPCFV were detected in sweet potato samples from farmers' fields in the major growing areas in Kenya. The occurrence of SPFMV in all the areas surveyed indicates that this virus is widely distributed in Kenya. This observation is consistent with previous reports, suggesting that SPFMV occurs wherever sweet potato is grown (Moyer and Cali, 1985; Moyer and Salazar, 1989; Sakai *et al.,* 1997; Colinet *et al.,* 1998).

SPCSV was the second most prevalent virus infecting sweet potato in Kenya. All the SPCSV-positive samples reacted only with monoclonal antibodies specific to the east African serotype but not with those specific to the west African serotype, suggesting that, similar to Uganda (Gibson *et al.*, 1998), only the east but not the west African serotype occurs in Kenya. Based on the survey results, SPMMV was the third most common virus infecting the crop in Kenya. Although an earlier survey (Carey *et al.*, 1996) failed to detect SPCFV in Kenya, these new data indicate a low incidence of this virus. This survey was the first in Kenya to include SPMSV antibodies but no sample reacted with antiserum to this virus and so far, SPMSV seems to be confined to Argentina or the Americas (Alvarez *et al.*, 1997). CMV and SwPLV were not detected in the samples tested although an earlier survey (Wambugu, 1991) indicated that these viruses were common in East Africa. In Uganda, the same range of viruses, namely SPFMV, SPCSV, SPMMV and SPCFV, was detected in a recent sweet potato virus survey (Mukasa *et al.*, 2003a). SPCaLV, which occurred in one plant in Uganda (Aritua *et al.*, Unpublished), was not detected in Kenya. In Kenya and Uganda, SPFMV and SPCSV, the two most common viruses, often occurred together to produce SPVD, suggesting that it is the most important disease of sweet potato in both countries.

Incidences of all viruses were highest in Nyanza and Western provinces, in accord with Sheffield (1953). In Western and Nyanza provinces, farmers grow sweet potato continuously, thus ensuring a continuous availability of virus inoculum. Moreover, these areas are fairly warm throughout the year, and thus favour vector survival and the build-up of high vector populations. Disease incidence was low in Eastern and Coast provinces, probably because of frequent spells of drought, making the crop not to be continuously grown and hence less likely for viruses to be spread between crops, and perhaps also for the vectors. Furthermore, the long and intense dry season in the Eastern and Coast provinces particularly in Kilifi and Malindi

districts also completely destroys the sweet potato foliage, so eliminating the food supply of the virus vectors. The varieties grown in these areas for instance cvs. Ex-Shimba Hills, Kemb 10, Ex-Diani, Muibai and Mtwapa 8 were found to be infected in other locations, suggesting that the low infection rates in these provinces was due to the low numbers of the insect vectors and virus source plants but not to the growing of especially virus-resistant sweet potato varieties. Data of Sheffield (1953) suggested that virus incidence is negatively correlated with altitude but the data obtained in the current study found no significant correlation between virus incidence and altitude or temperature although there seemed to be a trend towards less SPVD at higher, cooler locations such as Nyeri, Embu and Kirinyaga districts of central Kenya.

It has been reported that SPFMV on its own causes mild or no symptoms in east African sweet potato cultivars (Gibson *et al.,* 1997). This was also observed in this study, with a substantial proportion of samples from asymptomatic plants reacting with antiserum to SPFMV. In such samples, SPFMV generally occurred at concentrations too low to be detected in ELISA. This was consistent with the observation that most single SPFMV infections were serologically detected only following graft inoculation from sweet potato onto *I. setosa* (Aritua *et al.,* 1998; Karyeija *et al.,* 2000). The higher incidence and wider distribution of SPFMV as compared to the other three viruses could be due to the relative abundance of its aphid vectors over the whitefly vectors of SPCSV (Schaefers and Terry, 1976) and SPMMV (Hollings *et al.*, 1976), and the (unknown) vector(s) of SPCFV, a carlavirus (Aritua *et al.*, unpublished data). An alternative explanation might be that, since sweet potato singly infected with SPFMV exhibit slight or no symptoms, farmers inadvertently use SPFMV-infected cuttings as planting material. In Kenya, the subsistence sweet potato growers select symptomless cuttings, which serve as planting material for the next crop. Other viruses particularly SPMMV and SPCFV were observed to infect some sweet potato cultivars without

inducing any obvious symptoms. It is surprising that incidences of such viruses remain low in sweet potato crops as the faintness or absence of symptoms in singly infected sweet potato plants make it difficult for farmers to select virus-free planting material (Brunt *et al.*, 1996). The observation that the incidence of SPFMV (and these other viruses) remains so low supports the likelihood that some cultivars of sweet potato possess a resistance mechanism capable of limiting its multiplication and perhaps even of eliminating it (Gibson *et al.*, 1997; Karyeija *et al.*, 1998b).

Natural infections of sweet potato by most combinations of viruses almost always resulted in plants showing virus-like symptoms. The observation that SPVD, the disease caused by SPFMV and SPCSV, is widely distributed confirms earlier reports by Geddes (1990) that SPVD is the most serious disease of sweet potato in Africa. It is noteworthy that most of the plants infected with SPCSV were also co-infected with another virus(s) usually SPFMV. This is consistent with earlier evidence that SPCSV synergistically interacts with SPFMV, leading to SPVD (Schaefers and Terry, 1976; Gibson *et al.*, 1998; Karyeija *et al.*, 2000; Gibson and Aritua, 2002). SPMMV occurred more frequently in mixed infection with SPFMV than alone, although they are vectored by different types of insects. Since they are both members of the *Potyviridae*, although belonging to different genera, it is conceivable that they can interact synergistically in some manner.

A small number of symptomless plants reacted with more than one virus antiserum. This could be attributable to either a late infection with respect to the plant age, or an inherent ability of the plant to tolerate the effects of virus infection (Hollings *et al.*, 1976; Karyeija *et al.*, 2000) or a combination of both. However, SPCSV and SPFMV, the most common

combination of mixed infection was detected in only one symptomless plant, confirming that SPVD symptoms typically are severe (Gibson *et al.*, 1998; Karyeija *et al.*, 2000). No virus was detected in about 8% of the apparently virus-diseased sweet potato plants, even following grafting to *I. setosa*. The significance of these observations is currently unclear as they may indicate either genetic abnormalties in the plants or the presence of unknown virus(es).

The correlation between the proportion of plants infected by SPCSV and those infected by SPFMV was highly significant, consistent with previous reports from neighbouring Uganda (Mukasa *et al.*, 2003a). Since these two viruses are transmitted independently by whiteflies (SPCSV) and aphids (SPFMV), the cause of this association is not immediately obvious, despite the synergism of SPFMV by SPCSV in mixed infections. Three possible explanations are (i) since the titre of SPFMV increases markedly when plants are co-infected with SPCSV (Karyeija *et al.*, 2000), SPFMV becomes more easily detected and, hence, there is an apparent linkage (ii) the inoculation of sweet potato with SPFMV by viruliferous aphids results in a higher transmission rate if plants are pre-infected with SPSCV and (iii) Some sweet potato cultivars have an inherent resistance mechanism, which enables them to eliminate an infection by SPFMV alone (Aritua *et al.*, 1998). This could result in a higher proportion of plants infected with SPFMV and SPCSV than can be attributed to chance. The current data, however, cannot distinguish the most suitable explanation.

Unlike the previous study in Uganda (Mukasa *et al.*, 2003a), there was also a significant association between SPMMV and SPCSV. In this case, the special association may be due to the fact that (i) both viruses are transmitted by the same whitefly species, (ii) SPCSV occurs at a much higher frequency than SPMMV in sweet potato fields, and (iii) SPCSV possibly

also synergizes SPMMV (Mukasa *et al.*, 2003b). Since SPMMV and SPFMV are both members of the *Potyviridae*, a similar set of explanations as for SPFMV may also apply for the association between SPMMV and SPCSV. It is likely that the concentration of SPMMV increases in plants infected with SPCSV and SPMMV, rendering SPMMV more easily detectable in a fashion similar to the SPFMV and SPCSV pathosystem.

The findings on farmer perceptions and management of SPVD, the first reported in Kenya, indicates that farmers lack accurate information on the cause, spread and management of the disease. This information needs to be availed to the farmers to avoid further losses due to degeneration of sweet potato. The identity and relative importance of viruses and virus complexes infecting sweet potato in Kenya were determined in this study. Resistance breeding and other control strategies are urgently needed in the areas with high virus disease incidences. Since SPFMV and SPCSV were the most commonly detected viruses and are known to interact synergistically (Gibson *et al.*, 1998; Karyeija *et al.*, 2000), future control strategies should focus on these two viruses as a priority.

4.0 CHAPTER FOUR

CHARACTERIZATION OF SWEET POTATO VIRUS 2, A HITHERTO UNCHARACTERIZED POTYVIRUS

4.1 INTRODUCTION

Sweet potato is an adaptable and versatile crop that has gained increasing importance as human food worldwide, in particular in developing countries. While in Africa the major areas of sweet potato production are concentrated in the Great Lakes region, sweet potato is also important in several West African countries and in South Africa. Its presence and adaptation to the tropical areas, where the *per capita* income is generally low, and its nutritional value make this crop an important component in food production and consumption (CIP, 1996; CIP, 1998; Woolfe, 1992). However, the crop is faced with numerous production constraints including insect pests and diseases.

Virus diseases are the second most significant biotic constraint after the sweet potato weevil Geddes, 1990; Matin, 1999). Some of the viruses reported to infect sweet potato have been characterized to a large extent and appear to cause severe viral diseases (Carey *et al.*, 1996; Karyeija *et al.*, 1998; Moyer and Salazar, 1989; Woolfe, 1992). However, the significance and taxonomic assignment of other virus isolates remain unclear; for instance, a potyvirus isolated from sweet potato in Zimbabwe (SPV-Zw) (Chavi *et al.*, 1997) shares CP amino acid sequence similarities of < 75 % with established SPFMV isolates and other sweet potato potyviruses, suggesting that it should be regarded as a distinct potyvirus.

In the 80s, Rossel and Thottappilly (Rossel and Thottappilly, 1988) succeeded in transmitting a potyvirus from sweet potato to test plants. In *Nicotiana benthamiana* it induced mild vein yellowing followed by chlorotic mottle. This was unusual for an isolate of SPFMV at this

time. Since the isolate also differed serologically from SPFMV, it was referred to as 'sweet potato virus 2' (SPV2) (Rossel and Thottappilly, 1988). Although SPV2 was isolated from sweet potato plants in Taiwan showing mild virus-like symptoms such as mottle, vein yellowing and /or ringspots, it did not cause any symptoms in some sweet potato clones under experimental conditions (Rossel and Thottappilly, 1988). The fact that this virus remained incompletely described since the 80s, prompted further characterization so as to generate a basis for its classification.

4.2 MATERIALS AND METHODS

4.2.1 Isolates used

A SPV-2 isolate obtained from G. Thottappilly, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria in the late 80s was cultured at BBA Braunschweig, Germany. The isolate was used for production of an antiserum and has since been maintained in *N. benthamiana* by mechanical inoculation. Before starting its characterization, the isolate was tested for purity using the SPV-2 antiserum and antisera to other sweet potato infecting viruses such as SPFMV, SwPLV, SPMMV, SPMSV, SPCFV, SPCaLV and a filamentous virus provisionally named 'C6', all obtained from CIP. The isolate appeared to be free of viruses other than SPV2.

4.2.2 Determination of SPV-2 particle length

To determine the particle length of SPV2 in adsorption preparates, leaf tissues of infected *N. benthamiana* leaves were triturated in 0.1 M sodium phosphate buffer (Appendix 3). Pioloform-coated and carbon stabilized copper grids were floated on leaf extracts for 5 min, washed with about 40 drops of distilled water and stained with 5 drops of aqueous 1% uranyl acetate solution. The length of 150 particles was measured at a magnification of 50,000x

directly in a Zeiss EM906 electron microscope using an online attached image analysing system (Digivision SIS, Münster, Germany) with histogram production and normal length calculation. This procedure was repeated three times and the mean of the three length measurements served as the representative normal particle length of SPV2 (Brandes, 1964). The cytopathological effects of SPV 2 on *N. benthamiana* were determined following the method described by Koenig and Lesemann (1985).

4.2.3 Serological relationship of SPV2 with other viruses

To determine the serological relationship between SPV2 and other potyviruses in ELISA, the SPV 2 isolate was tested using antisera against several sweet potato infecting viruses. SPCSV was detected by triple antibody sandwich (TAS) ELISA, using polyclonal antibodies to recombinant capsid protein of SPCSV (Hoyer *et al.*, 1996) for coating of high-binding microtitre plates (Greiner Bio-One, Frickenhausen, Germany) and a mixture of monoclonal antibodies specific to East African isolates of SPCSV for antigen detection (Hoyer *et al.*, 1996; Vetten *et al.*, 1996), followed by alkaline phosphatase-labelled rabbit anti-mouse IgG (DIANOVA, Hamburg, Germany). Similarly, SPMMV was tested by TAS–ELISA. A polyclonal antiserum was used for coating and a monoclonal antiserum for antigen detection. SPFMV, SPCFV, SPMSV, SPVG, CMV were tested by DAS-ELISA. The serological relatedness of SPV2 to other sweet potato infecting potyviruses was further determined in decoration titre experiments according to the method described by Milne (1988) using two-fold dilution series (from 1:25 to 1:12,800) of antisera to SPV2, SPFMV, and SwPLV. Antisera to a SwPLV isolate from Taiwan and the SPFMV isolate KY46b from Kenya were from the stock of BBA, while antisera to SwPLV and the SPFMV strains RC and C were kindly provided by the International Potato Centre (CIP), Lima, Peru.

A small leaf disc of infected *N. benthamiana* macerated on 1-2 drops of extraction buffer. A grid was placed on the plant extract with the coated surface touching the extract for a period of 5 min. The grid was washed with 20 drops of phosphate buffer. The grids were drained with dry filter paper (without drying the grid completely). The grids were placed on drops of antisera all serially diluted from 1:25 to 1:12,800 for 15 minutes up to 1h. The grids were then washed with 40 drops of double distilled water and stained with 5 drops of Uranyl acetate. The grids were drained using dry filter paper and let to dry. The intensity of decoration was visualized under the electron microscope (EM 906). The highest antiserum dilution leading to a recognizable decoration effect is defined as the decoration titer.

4.2.4 Host range studies

The experimental host range of SPV2 was determined by mechanical inoculation of test plants. Leaves of infected *N. benthamiana* were homogenized in 0.1 M phosphate buffer, pH 7.8, and the homogenate was rubbed onto the carborundum-dusted leaves of test plants (Table 1). For each species tested, three plants were inoculated when the first true leaf was fully expanded (generally 10-20 day post emergence). The inoculated leaves were rinsed with tap water and observed for symptom development over a period of 4 weeks in an insect-free glasshouse. Non-inoculated plants of each species were included as controls. All plants with or without symptoms were analysed in DAS-ELISA using antiserum to SPV2.

4.2.5 Aphid transmission

Two biotypes of *Myzus persicae* (green and red biotypes) reared on healthy oil-seed rape plants were used. The aphids were placed on moist filter paper in a Petri-dish and starved for 1 h. Symptomatic leaves of SPV2-infected *N. occidentalis* P1 and *I. setosa* plants mechanically inoculated 2-3 weeks previously were detached and used as a virus source. Following a 5-min acquisition access feeding period on infected leaves, groups of five aphids

were transferred onto healthy 10-day-old *I. setosa* seedlings with a camel hair brush and given an inoculation access feeding period of 10 min. Aphids given an acquisition feeding on healthy *I. setosa* plants served as a control. The plants were sprayed with imidacloprid and observed for symptom development in an insect-proof glasshouse. The number of seedlings that developed SPV2 symptoms was recorded and, at about three weeks after inoculation, all symptomatic and asymptomatic plants were tested in DAS-ELISA using the SPV2 antiserum.

4.2.6 Mixed infection with SPCSV

The effect of SPCSV on SPV2 infection in *I. setosa* was investigated. The treatments comprised plants infected with SPV2 alone, plants infected with SPCSV only, plants dually infected with SPV2 and SPCSV, and non-inoculated control plants. Sixteen *I. setosa* plants were mechanically inoculated with SPV2. Seven days later, SPCSV-Ky38 was graft inoculated onto eight SPV2- infected and eight non-inoculated *I. setosa* plants which were about two weeks old. Eight noninoculated *I. setosa* plants were included as a control. The treatments were arranged in a completely randomised design (CRD) with eight replications. Symptom development was monitored and, at 21 days post inoculation, a composite sample of three symptomatic leaves was taken from each plant and analysed by DAS- and TAS-ELISA for SPV2 and SPCSV, respectively. Results indicated that the plants were infected by the respective viruses used for inoculation. Since symptoms due to SPV2 were not uniformly distributed in SPV2-infected plants, three different leaf stages (bottom, intermediate, and top) were sampled from three randomly selected plants of each treatment and individually analyzed for SPV2 and SPCSV. Growth parameters such as leaf number, fresh shoot weight was determined for all the treatments and subjected to analysis of variance (ANOVA).

4.2.7 RNA extraction, RT- PCR and sequencing of 3' genome of SPV 2

Total RNA was extracted from SPV2-infected leaves of *N. benthamiana* plants using the Nucleospin® Plant kit (Macherey-Nagel, Düren, Germany) and following the manufacturer's instructions. Synthesis of complementary DNA (cDNA) from the 3' region of the viral genome was done using an oligo(dT) primer and M-MLV reverse transcriptase (GIBCO) according to the manufacturer's instructions. The 3′ end of the SPV2 genome comprising part of the NIb gene, the coat protein gene and the 3′-nontranslated region (NTR) was amplified in a MJ Research (Massachusetts, USA) thermocycler using the potyvirus-specific, degenerate primers POT1 and POT2 of Colinet *et al.* (1994) as well as an oligo(dT) primer and the viral anti-sense primer 5'- TGAGGATCCTGGTGYATHGARAAYGG-3' (CPUP) of Langeveld *et al.* (Langeveld *et al.*, 1991). For determining the correct sequence of the 'WCIENG' region (binding site of the POT1 and CPUP primers), the SPV2 specific primers 5'-CGAACTTGCTCGAGTAGGCAG-3' (viral sense) and 5'-TCCGTCCATCATCACCCA-3' (viral anti-sense) were used.

Agarose gel electrophoresis

Standard agarose 1% (w/v) was dissolved by heating in TAE electrophoresis buffer (appendix 3). The gel was allowed to cool for a few minutes before adding ethidium bromide to a final concentration of 0.001% (w/v). The gel was then poured into a horizontal gel tray fitted with an appropriate comb. After about 40 minutes of gel polymerization, the comb was gently removed and the tray immersed in an electrophoresis tank containing electrophoresis buffer.

To 50µl of PCR reaction, 6µl of sample loading buffer was added and mixed by pipetting before loading the resulting mixture in the pre-formed sample slots on the gel. A digested λ psti phage DNA (Farmentas) was used as a molecular weight marker. The gel was run at a

constant voltage of 100 V until the bromophenol blue migrated to about 1 cm from the bottom end of the gel before viewing it under UV light. The amplified fragments were excised from the gel using a clean blade and purified using the Nucleospin extract kits, (Macherey-Nagel, Düren Germany) essentially following the manufacturer's protocol.

Cloning of PCR fragments in pGM®-T

The purified PCR products were ligated onto the pGM®-T vector (Promega Corporation) according to the manufacturers instructions. A ligation mix containing 1 µl (50ng/ml) of the cloning vector and 1µl (3 Weiss U/µl) of the T4 DNA ligase was made in 5µl of 5x rapid ligation buffer (Promega). Three µl of the PCR product was added into the ligation mix and mixed by pipetting. The ligation mixture was incubated at 4°C overnight.

Preparation of competent cells and transformation of amplified DNA onto Escherichia coli: A single colony of DH5α cells was grown in 10ml LB overnight at 37° with shaking. Two ml of this culture was added to fresh 50ml LB and grown for 2.5 h or until an OD at A_{600} was reached. The cells were pelleted by centrifugation at 4000xg for 10min at 8°C, resuspended gently in 50ml cold 0.1M $MgCl_2$, and then pelleted by centrifugation. Cells were resuspended in 25ml cold 0.1M $CaCl_2$ and incubated on ice for 25min. Cells were pelleted by centrifugation at 4000xg for 10 min at 8°C and finally resuspended in 1.6ml 0.1M$CaCl_2$ containing 15% glycerol. The cells were stored in 150µl aliquots at –70°C till use.

To transform, 5µl of the ligation reaction was added into a sterile 1.5ml tube on ice. A tube containing *E. coli* strain DH5∝ competent cells were transferred from -70°C into an ice bath until just thawed (ca. 5 min). Competent cells (50µl) were carefully transferred into the tube containing ligation reactions and gently flicked to mix the contents. The tubes were incubated on ice for 20 min. The cells were heat-shocked for 45-50 seconds in a water bath at exactly

42°C and the tubes immediately placed on ice for 2 min. LB medium (950µl) was added to the tubes containing transformed cells. The tubes were incubated for 1.5 h at 37°C with shaking (150 rpm).

The transformation culture was centrifuged at 4000 rpm to concentrate the transformants and the pellet resuspended in 200µl LB medium before plating in duplicate LB plates supplemented with ampicillin/IPTG/X-GAL (Appendix 4). The plates were incubated overnight at 37°C. Colonies with insert were identified by the blue/white screening. Single white bacterial colonies were picked and suspended in 25µl of sterile deionized water and screened for insert in PCR using the SP6 and T7 universal primers (MWG D-85560 Ebersberg, Germany). Colonies that contained the correct size of insert were grown in 5ml LB medium containing ampicillin overnight at 37°C with shaking at 150 rpm.

Isolation of plasmid DNA from E. coli

Three ml of saturated culture of *E. coli* was pelleted in a standard microcentrifuge for 30s at full speed and the supernatant discarded. Plasmid extraction was done using the Nucleospin plasmid kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. The size and yield of the extracted plasmid DNA preparations, was analyzed in a 1 % agarose gel stained with ethidium bromide. Plasmid minipreps containing the correct size of DNA fragment were selected for sequence analysis.

Sequencing and sequence analysis

Sequencing was done by a commercial company (MWG D-85560 Ebersberg, Germany). The obtained sequence comprising part of the Nib, the entire CP and 3' NTR was compared with corresponding sequences of other sweet potato infecting potyviruses by pairwise alignments and bootstrap phylogenetic analysis. The sequence was also compared with sequences

deposited in the gene bank using the basic local alignment search tool (BLAST) (Altschul *et al.*, 1997).

4.3 RESULTS

Morphology and cytopathology

Particles of SPV2 are filamentous with a normal length of approximately 850 nm. Cylindrical inclusions comprising pinwheels and scrolls were observed in SPV2-infected cells of *N. benthamiana* (Fig. 1a). Virus induced crystalline structures presumably formed by other non-structural proteins were also observed in the cytoplasm of infected cells (Figs. 1b, 1c).

Serological relationships

In DAS-ELISA, antibodies to SPV2 gave a strong homologous reaction but did not react with extracts from SPFMV-, SwPLV-, and SPMSV-infected *Nicotiana* spp.. Conversely, antibodies to SPFMV, SwPLV, and SPMSV gave strong DAS-ELISA reactions with homologous antigen but failed to react with SPV2 antigen and in all other heterologous combinations.

In immunoelectron microscopy, particles of SPV2 were strongly decorated by the homologous antiserum, reaching a decoration titer of 1:12,800. Antisera to the common (C) and russet crack (RC) strains of SPFMV decorated the SPV2 particles only at a dilution of 1:25, whereas the antiserum to SPFMV-KY46b did not decorate the SPV2 particles (Plate 1). No decoration of SPV2 particles was observed with the SwPLV antiserum from CIP, whereas the SwPLV antiserum from BBA gave a heterologous decoration titre of 1:200, which, however, differed by five dilution steps from the homologous titre. The decoration titre experiments indicated that SPV2 is only distantly related to the other sweet potato potyviruses SPFMV and SwPLV (Table 1).

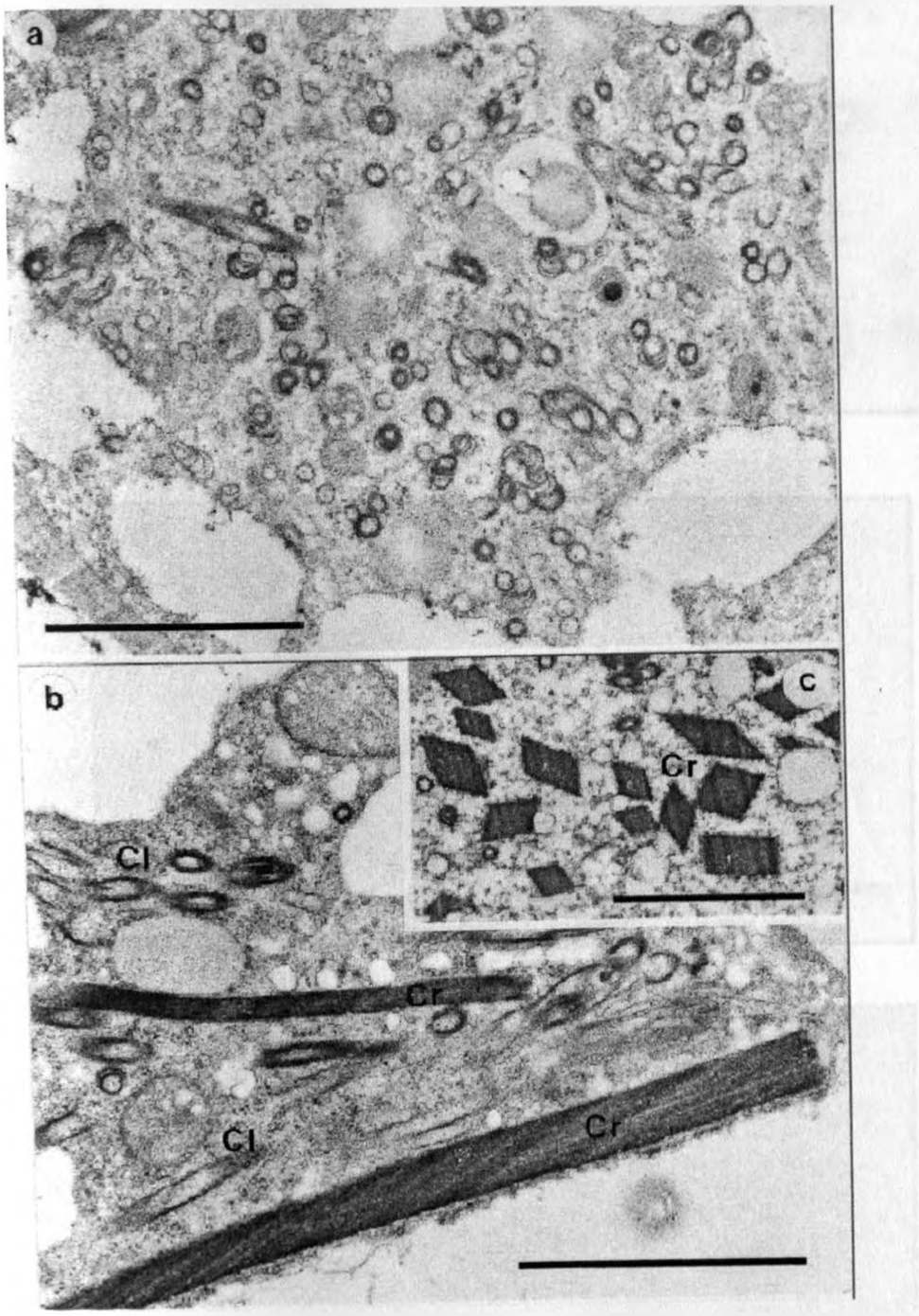
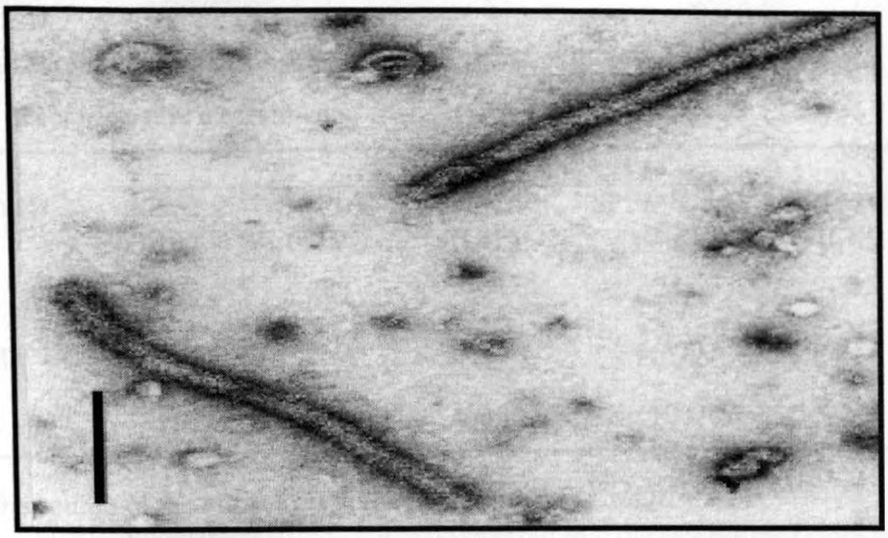
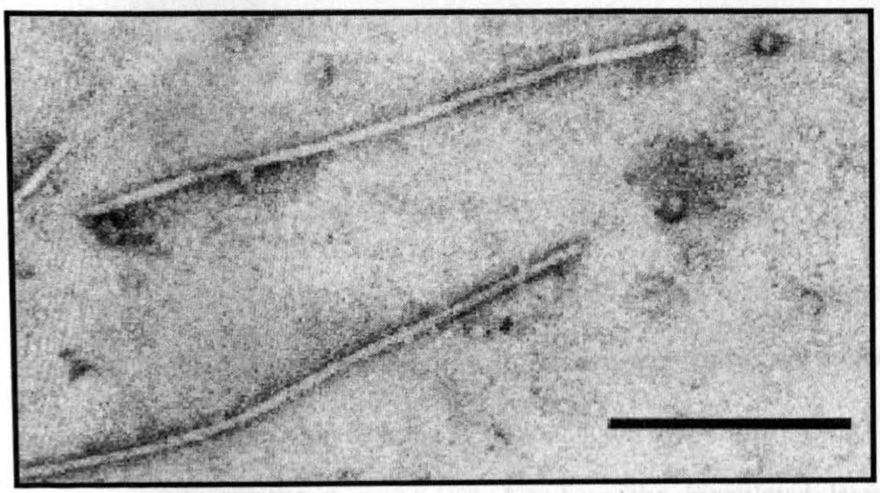


Fig. 1: Cytopathological effects induced in the cytoplasm of 'sweet potato virus 2'-infected mesophyll cells of *N. benthamiana*. (a) Cross sections of cylindrical inclusions consisting of pinwheels and scrolls. (b) Cross to oblique sections of cylindrical inclusions (CI) and oblique to longitudinal sections of crystalline inclusions (Cr) presumably formed by other viral non-structural proteins. (c) Cross sections of the crystalline inclusions (Cr) shown in (b). Each bar equals 2 µm.





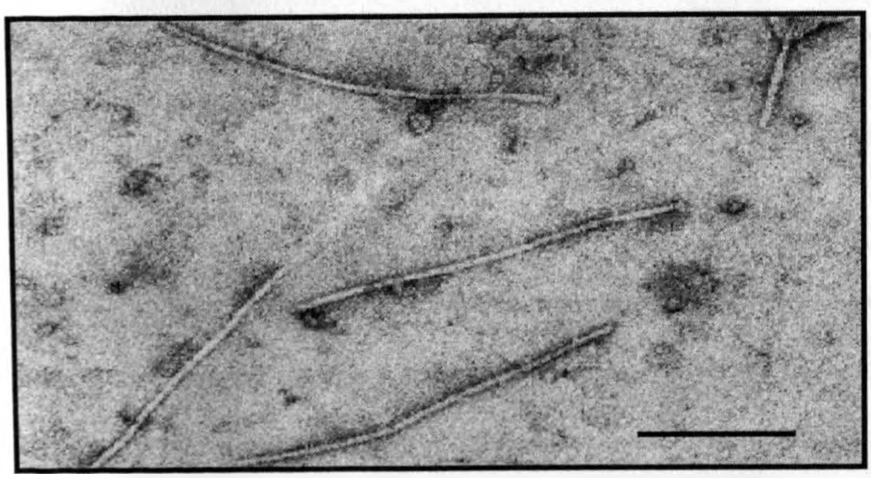


Plate 1: Electron micrographs showing negatively stained preparations from *N. benthamiana* of flexuous filamentous particles of (A) SPV2 incubated with homologous antiserum (B) SPV2 incubated with SPFMV antiserum and (C) SPFMV particles incubated with SPVY antiserum. Each bar is 2 µm.

Table 1. Serological relationships among SPV2, SPFMV, and SwPLV as assessed by decoration titre experiments

Isolates	Antisera to					
	SPV2 (BBA) [1]	SwPLV (BBA)	SwPLV (CIP)	SPFMV-RC (CIP)	SPFMV-C (CIP)	SPFMV-46b (BBA)
SPV2	12,800 [2]	200	- [3]	25	1:25	-
SPFMV-RC	-	-	-	6,400	400	1,600
SwPLV	50	6,400	6,400	100	25	50

[1] The source of the antisera is given in parentheses.

[2] Reciprocals of the highest antiserum dilution leading to a recognizable decoration of virions. Homologous reactions are underlined.

[3] No decoration observed (-).

Host range

SPV2 infected 12 of the 17 plant species tested (Table 2). Five species of the genus *Ipomoea* and six species of the genus *Nicotiana* were infected systemically and showed various types of symptoms, ranging from vein clearing to mosaic and leaf malformations (Table 2; Plate 2, 3 and 4). Chlorotic local lesions were induced on the inoculated leaves of the three *Chenopodium* spp. tested. However, SPV2 failed to infect *Gomphrena globosa, N. glutinosa, N. debneyi* as well as *Datura metel* and *D. stramonium*.

Insect transmission

Both the green and red biotypes of *M. persicae* transmitted SPV2 in a non-persistent manner from both *N. occidentalis* P1 and *I setosa* onto *I setosa*. After 10 days, vein clearing and mosaic symptoms developed in 9 out of 10 *I. setosa* plants inoculated with the red biotype and in 2 out of the 10 plants inoculated with the green biotype. When assayed by ELISA, all the plants showing symptoms tested positive for SPV2 while the symptomless ones were negative.

Table 2. Host range and symptoms incited by SPV2 in different test plants

Host	Symptomatology [1]
Chenopodium amaranticolor	chlorotic local lesions at 12 dpi
C. murale	chorotic local lesions at 21 dpi
C. quinoa	chlorotic local lesions (14 dpi), turning necrotic at 21 dpi
Datura metel	no symptoms (not infected)
D. stramonium	no symptoms (not infected)
Gomphrena globosa	no symptoms (not infected)
Nicotiana benthamiana	SI [2], vein clearing, leaf distortion, mosaic at 10 dpi
N. clevelandii	SI, chlorotic mosaic at 21 dpi
N. debneyi	no symptoms (not infected)
N. glutinosa	no symptoms (not infected)
N. hesperis	SI, chlorotic mosaic, vein clearing at 14 dpi
N. occidentalis P1	SI, chlorosis, necrotic ringspots
N. occidentalis ssp. obliqua	SI, chlorotic leaf spots, leaf deformation and chlorosis
Ipomoea coccinea	SI, chlorotic mosaic, vein clearing at 14 dpi
I. nil	SI, chlorotic mosaic, vein clearing at 14 dpi
I. purpurea	SI, vein clearing, leaf chlorosis and leaf distortion at 14dpi
I. setosa	SI, vein clearing, chlorotic mosaic at 13 dpi

[1] Infections were confirmed by DAS-ELISA 21 dpi using antiserum to SPV2;

[2] Systemic infection (SI)

Plate 2: Necrotic ringspots on *N. occidentalis* P1 caused by SPVY-Tw isolate, 21 days after sap inoculation

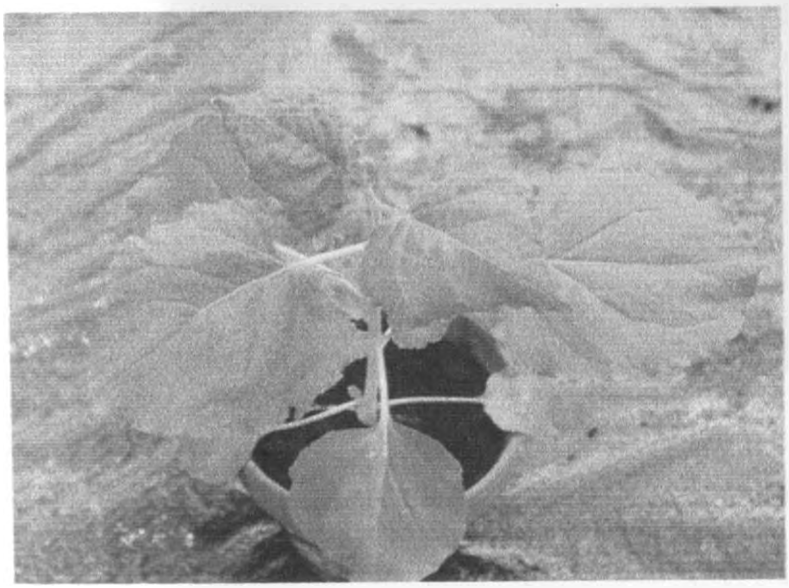
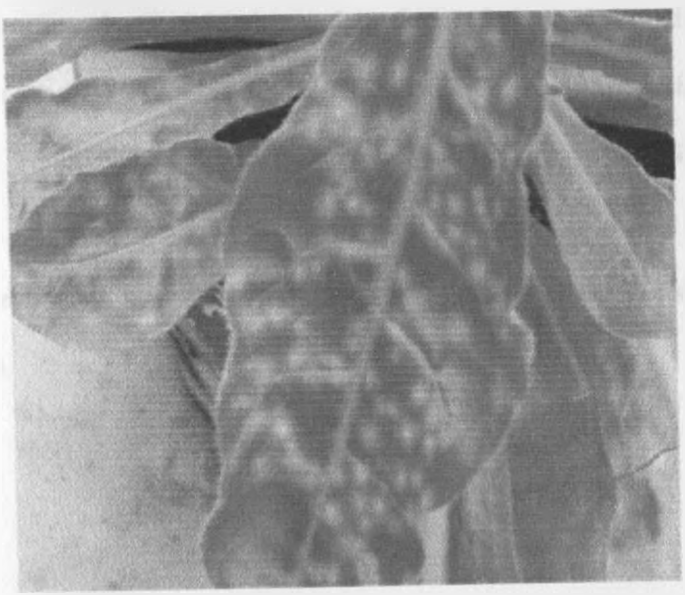


Plate 3: Typical vein clearing, leaf distortion and chlorosis on *N. benthamiana* caused by SPV2 isolate Nigeria 14 days after sap inoculation

(A)



(B)

Plate 4: Chlorotic local lesions caused by SPV2 on (A) *C. amaranticolor* and (B) *N. occidentalis* Obliqua, 21 days after sap inoculation

SPV2 caused more severe symptoms in mixed infections with SPCSV

I. setosa plants infected with SPV2 alone developed vein clearing and mosaic symptoms only on few leaves. These were, in general, the only leaves that had ELISA-detectable concentrations of SPV2, whereas the asymptomatic leaves of SPV2-inoculated plants tested negative in ELISA. The SPCSV alone caused nearly imperceptible symptoms in infected *I. setosa* and was reliably detected by ELISA in the intermediate and top leaves but not in the bottom leaves of infected plants (Table 3). However, plants co-infected with SPV2 and SPCSV showed very conspicuous symptoms on all leaves, such as severe veinal chlorosis, mosaic (Plate 5), leaf distortions, leaf size reductions, and plant stunting.

In addition, the number of leaves and the fresh shoot weights of dually infected *I. setosa* plants were significantly ($P \leq 0.05$) lower than those of singly infected plants, whose growth parameters were largely indistinguishable from each other and from those of non-inoculated plants (Fig. 2). The SPV2 concentrations in plants infected only with SPV2 were often below the detection limit, particularly in the top leaves (Table 3). In dually infected plants, SPV2 attained higher concentrations and appeared to be more uniformly distributed than in singly infected plants.

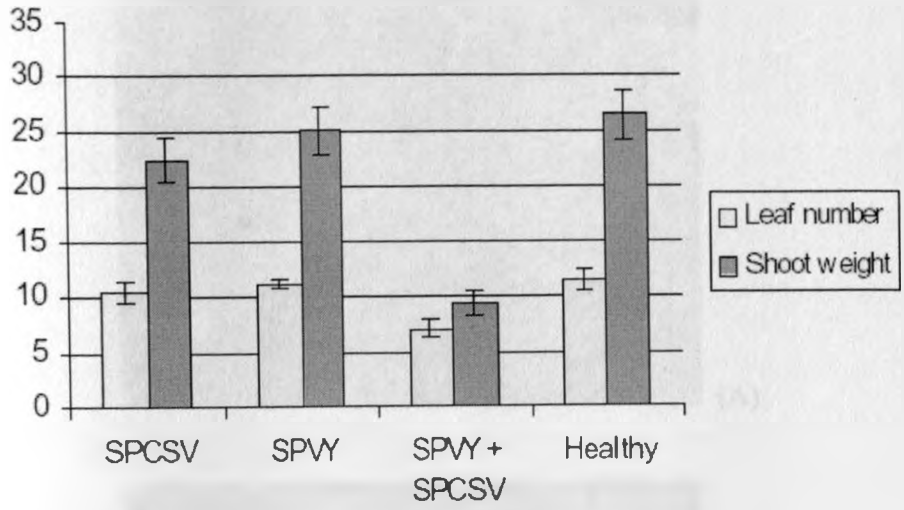
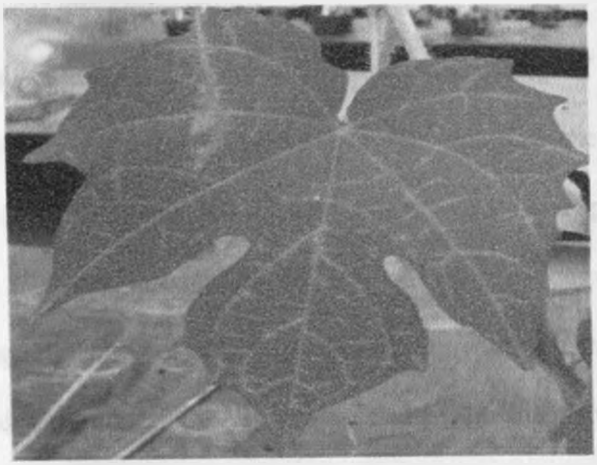
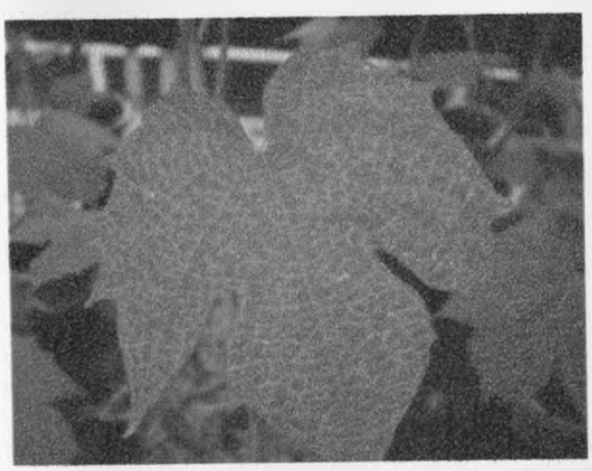


Fig. 2. Leaf number and mean fresh shoot weight (g) of plants inoculated with *Sweet potato chlorotic stunt virus* (SPCSV), 'sweet potato virus 2' (SPV2), both viruses (SPCSV + SPVY), and of non-inoculated plants (healthy). Vertical bars represent standard errors of means of 8 plants for each treatment. Treatments were compared by single-factor ANOVA and means separated at P=0.05 level.



(A)

(B)



(C)

Plate 5: *I. setosa* showing (A) vein clearing caused by SPVY (B) chlorosis caused by SPCSV which is hardly discernible (C) severe vein clearing caused by the mixed infection of SPVY and SPCSV

Sequence analyses and comparisons

Sequence analysis of the 3'-terminal 2006 nucleotides of the SPV2 RNA (Acc. No. AY232437) showed that this genome fragment contains the coding sequence for the C-terminal half (296 aa) of the NIb protein and the entire coat protein (CP; 332 aa), which is followed by a 3' untranslated region (UTR) of 216 nucleotides excluding the poly(A) tail (Fig 3). The coding sequence not only shared similarity with NIb and CP genes of potyviruses but also contained a characteristic NIb protease cleavage site (VYHQ/S). In close proximity to this cleavage site, the CP amino terminus carries the amino acid triplet DAG that is involved in aphid transmission of potyviruses (Atreya *et al.*, 1995). BLAST search analysis of the SPV2 sequences gave the highest scores with potyvirus isolates from sweet potato. CP size comparisons showed that the SPV2 CP (332 aa) is the third largest CP of all sequenced sweet potato potyviruses. It is 23 aa smaller than that of SPVG (355 aa) and 3 aa smaller than that of a potyvirus (SPV-Zw) isolate (335 aa) reported from Zimbabwe (Chavi *et al.*, 1997).

```
1          TCGGGCACTGAAGAAACAAAAGACGCTGGAACCCCAACACCAGCGAAATCAGGTAAGACA
1           S  G  T  E  E  T  K  D  A  G  T  P  T  P  A  K  S  G  K  T

61         AGAACAGGACAAACCCAACCGCTTAAAGCACCAGAATGGAGCACGGATCCAACAGATCCA
21          R  T  G  Q  T  Q  P  L  K  A  P  E  W  S  T  D  P  T  D  P

121        CCACCTCCAACAGTTGAAGAGATAATTGAAGAAGAAACACCAGCACAAAAAGCATTGAGG
41          P  P  P  T  V  E  E  I  I  E  E  E  T  P  A  Q  K  A  L  R

181        GAAGCCCGTGATAAGCAACCAGCAACACAGCCCTCATACACTTATGGGCGAGACACAGGA
61          E  A  R  D  K  Q  P  A  T  Q  P  S  Y  T  Y  G  R  D  T  G

241        CCACGTAGCCCAAGGCAAGTTACAACAACAAATGGAGTTAGGGATAGAGATGTCAATGCT
81          P  R  S  P  R  Q  V  T  T  T  N  G  V  R  D  R  D  V  N  A

301        GGAACAGTAGGGACGTTTACAGTTCCAAGACTTCAAATTACATCAAGCAAGAAGAGATTG
101         G  T  V  G  T  F  T  V  P  R  L  Q  I  T  S  S  K  K  R  L

361        CCAATAGTTGACGGACGTCCAGTAATCAACCTGGATCACTTGGCAGTTTACGATCCAGAG
121         P  I  V  D  G  R  P  V  I  N  L  D  H  L  A  V  Y  D  P  E

421        CAAACAAATCTTGCAAATACCAGATCAACACAAGAACAGTTTAAGGCATGGTATGAAGGT
141         Q  T  N  L  A  N  T  R  S  T  Q  E  Q  F  K  A  W  Y  E  G

481        GTGAAGGGCGATTATGGGGTATCTGATGCTGAAATGGGCATACTCCTTAATGGCCTCATG
161         V  K  G  D  Y  G  V  S  D  A  E  M  G  I  L  L  N  G  L  M

541        GTTTGGTGTATTGAGAATGGTACATCACCGAATATTAATGGAATGTGGGTGATGATGGAC
181         V  W  C  I  E  N  G  T  S  P  N  I  N  G  M  W  V  M  M  D

601        GGAGAAGAACAAGTAACTTATCCAATAAAACCTCTATTGGATCATGCTGTCCCCACATTT
201         G  E  E  Q  V  T  Y  P  I  K  P  L  L  D  H  A  V  P  T  F

661        AGACAGATAATGACACACTTCAGCGACATAGCTGAAGCGTACATTGAAAAGAGAAACAGG
221         R  Q  I  M  T  H  F  S  D  I  A  E  A  Y  I  E  K  R  N  R

721        ATAAAGGCCTATATGCCAAGGTATGGCCTACAGAGGAATTTGACTGATATGAGTCTTGCG
241         I  K  A  Y  M  P  R  Y  G  L  Q  R  N  L  T  D  M  S  L  A

781        CGGTATGCATTCGATTTCTATGAACTCCACTCAAACACACCAGTGAGAGCAAGGGAGGCA
261         R  Y  A  F  D  F  Y  E  L  H  S  N  T  P  V  R  A  R  E  A

841        CATATGCAAATGAAAGCAGCAGCTTTAAAGAATGCACAGAATCGCCTGTTTGGTTTGGAT
281         H  M  Q  M  K  A  A  A  L  K  N  A  Q  N  R  L  F  G  L  D

901        GGAAACGTCTCCACGCAAGAAGAAGACACGGAGAGGCATACAACAACTGATGTTACAAGA
301         G  N  V  S  T  Q  E  E  D  T  E  R  H  T  T  T  D  V  T  R

961        AATATACATAACCTGTTAGGAATGAGAGGTGTGCAGTAAACAATATATTGCTCGTACTTT
321         N  I  H  N  L  L  G  M  R  G  V  Q  *

1021       TAATTTCAGTTGGTCTTTAATTTAAATTCGTGTCTTTCGGTCCCGAAGAGTGTTGGTTGT

1081       ATGTAGTAACTATGTGTGGTTGTACCACCGTTGCTACATATAAGAAAACCTCTTTCTATT

1141       ACGTATCATAAGGGACTCTTAAAAGTGAGTCTTTGACTCGTAAGAAAAGCCTTTTTGGTT

1201       CGTGATCGAGCC(A)
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Fig 3: Coat protein nucleotide sequence and deduced amino acid sequence of a SPV2 isolate. Motifs highly conserved in potyvirus coat proteins are underlined.

However, the SPV2 CP is considerably larger than that of SPFMV (315 aa), SwPLV (292 aa) and SPMSV (285 aa). Based on the phylogenetic tree of the CP amino acid sequences (Fig. 4A), the closest relatives of SPV2 appear to be SPV-Zw and SPVG, which share CP amino acid sequence similarities with SPV2 of 80 % and 77 %, respectively (Table 4). These three viruses have CP core amino acid sequence identities of 86-90%. The considerably higher N-terminal amino acid sequence identity observed for SPV2 and SPV-Zw (58%) than for SPV2 (or SPv-Zw) and SPVG (47-50%) (Table 5; Fig. 5) may suggest that SPV2 and SPV-Zw are two divergent strains of the same species. Much lower CP amino acid sequence similarities were observed between SPV2 and SPFMV (~75%), SwPLV (69%), and SPMSV (63%) (Table 4). Very recently, unpublished data on partial CP amino acid sequences of two isolates (AY178989 and AY178992) of a potyvirus (LSU2; LSU5) infecting sweet potato in the United States have been released in the database. Sequence comparisons indicate that LSU2/LSU5 has an amino acid sequence identity of about 93 % with SPV2 (Table 5), indicating that they can be confidently considered isolates of the same virus. Phylogenetic analysis (Fig. 4B) and pairwise alignments (Table 4) of 3' UTR sequences of sweet potato potyviruses also supported the notion that SPV2 is more closely related to SPV-Zw (82%) and SPVG (82%) than to SPFMV (78%), SwPLV (56%) and SPMSV (44% only). The 3' UTR size (without poly(A) tail) of SPV2 (216 nt) was very similar to that of SPV-Zw (215 nt), SPVG (221 nt), and SPFMV (222 nt), whereas it differed considerably from that of SPMSV (248 nt) and SwPLV (196 nt).

Table 4. Percent coat protein amino acid sequence (lower diagonal) and 3`UTR nucleotide sequence identities (upper diagonal) following pairwise alignments of SPV2 and other potyviruses infecting sweet potato

Virus	SPV2	SPV-Zw	SPVG	SPFMV isolate					SwPLV	SPMSV
				-6	-C	-RC	-S	-O		
SPV2		82.1	82.2	56.3	79.0	78.6	77.8	77.4	55.5	44.0
SPV-Zw	80.0		79.5	55.7	77.9	77.5	75.8	75.3	45.6	43.5
SPVG	77.3	75.0		47.5	75.3	75.3	75.1	74.7	51.8	38.8
SPFMV-6	74.4	71.3	72.3		71.0	70.1	72.1	73.1	44.1	49.5
SPFMV-C	74.7	72.4	72.4	92.7		97.7	99.1	98.6	50.3	45.6
SPFMV-RC	76.4	73.3	74.4	81.2	84.7		98.2	96.4	50.8	45.6
SPFMV-S	76.3	73.3	74.1	82.3	84.4	99.0		97.8	50.3	46.1
SPFMV-O	76.4	72.3	74.5	80.8	83.1	95.6	95.2		51.3	45.2
SwPLV	62.3	61.6	61.6	63.1	62.2	64.1	64.1	62.3		46.1
SPMSV	57.7	57.4	60.4	59.8	60.9	58.7	59.3	60.2	58.8	

Table 5 . Percent coat protein (CP) core (lower diagonal) and CP N-terminal (upper diagonal) amino acid sequence identities following pairwise alignments of SPV2 and other potyviruses infecting sweet potato

Virus	SPV2	LSU2	SPV-Zw	SPVG	SPFMV isolate					SwPLV	SPMSV
					-6	-C	-RC	-S	-O		
SPV2		92.8	57.9	50.5	54.9	45.5	51.3	50.0	55.8	36.8	29.8
LSU2	- [a]		58.5	48.4	55.6	48.1	52.6	51.3	53.8	36.8	24.0
SPV-Zw	88.9	- [a]		47.3	45.3	49.4	55.3	55.3	55.3	36.4	31.3
SPVG	90.2	- [a]	86.0		47.3	46.2	50.0	48.7	51.4	38.6	35.0
SPFMV-6	83.0	- [a]	79.6	80.4		82.1	62.8	64.5	60.3	29.5	37.5
SPFMV-C	84.7	- [a]	80.0	82.1	96.2		67.1	65.8	62.0	34.0	37.5
SPFMV-RC	84.7	- [a]	79.1	82.6	88.1	90.6		96.2	86.2	42.9	35.4
SPFMV-S	84.7	- [a]	79.1	82.6	88.1	90.6	100.0		85.0	42.9	40.4
SPFMV-O	84.3	- [a]	78.7	82.1	87.7	90.2	98.7	98.7		34.7	43.5
SwPLV	68.9	- [a]	68.9	67.7	69.4	69.8	68.9	68.9	68.5		31.1
SPMSV	62.8	- [a]	62.8	63.5	64.4	66.1	63.9	63.9	63.5	63.7	

[a] The complete CP core sequence of LSU2 is not available

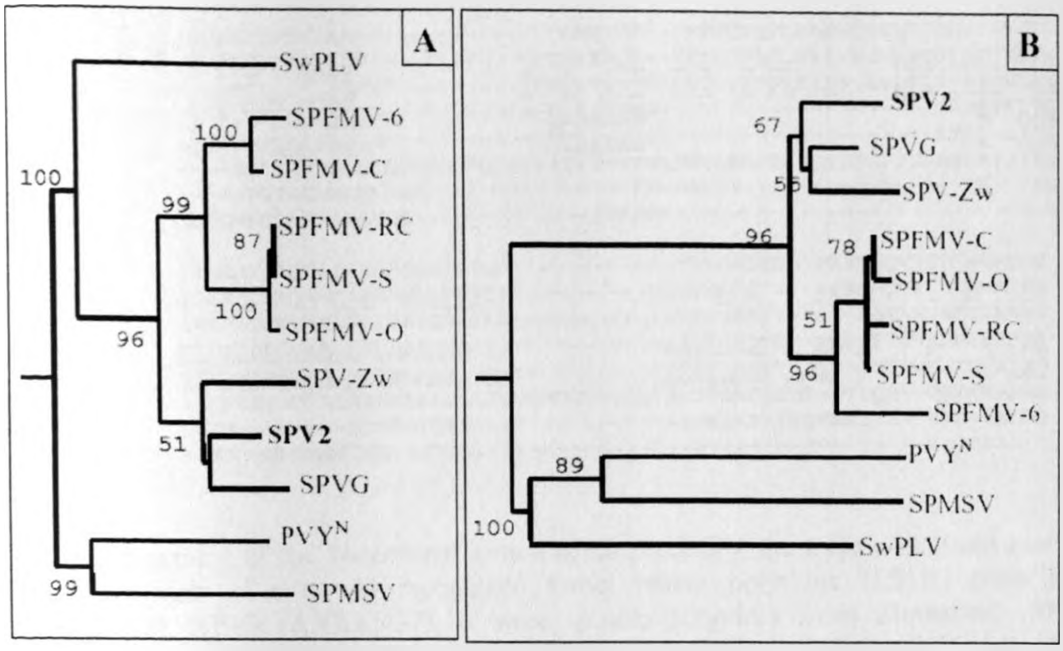

Fig. 4: Neighbour-joining relationship dendrograms of the coat protein (CP) core amino acid (A) and 3' UTR nucleotide sequences (B) of SPV2 and other sweet potato infecting potyviruses, with the CP and 3' UTR sequences of PVY_N used as outgroup sequences. Vertical branch lengths are arbitrary, horizontal branch lengths are proportional to percent sequence differences, and numbers at each node indicate bootstrap scores higher than 50%. Virus (isolate) designations and accession numbers are SPV2 (AY232437), SPVZw (AF016366), SPVG (X76744), SPFMV-RC (S43450), -C (S43451), -S (D86371), -O (D16664), -6 (U96625), SwPLV (X84011), SPMSV (U61228), and PVY_N (AY166867).

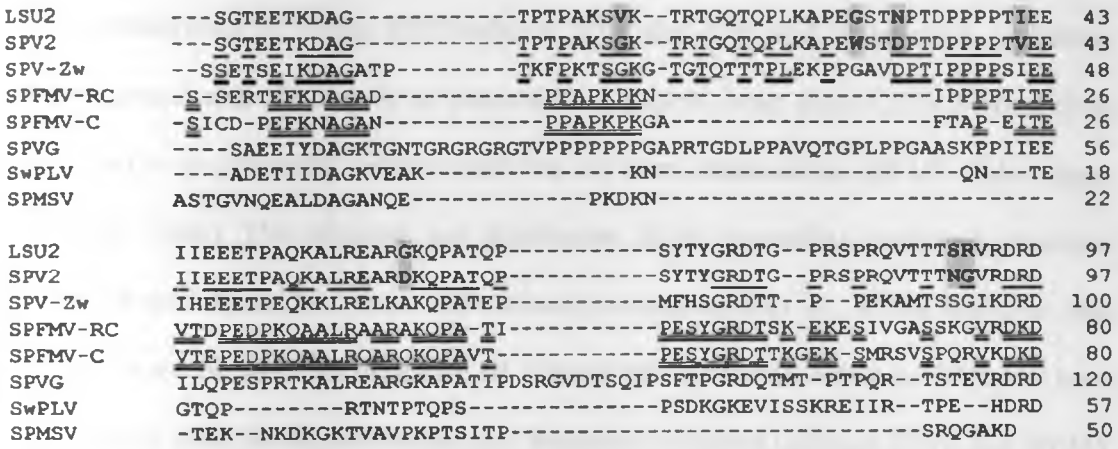

Fig. 5. Alignment of the N-terminal amino acids preceding the trypsin-resistant core of the capsid proteins of a newly recognised sweet potato potyvirus (LSU2) from the US (AY178992), SPV2 (AY232437), a sweet potato potyvirus from Zimbabwe (SPV-Zw) (AF016366), SPFMV-RC (S43450), SPFMV-C (S43451), SPVG (X76944), SwPLV (X84011), and SPMSV (U61228). The seven amino acid exchanges between SPV2 and LSU2 are shaded. Identical amino acids shared by SPV2 and LSU are underlined and those shared by the SPFMV strains RC and C are double underlined.

4.4 DISCUSSION

In order to further characterize SPV2, an incompletely described potyvirus isolate from sweet potato in Taiwan, some of its morphological, biological, serological and molecular properties were determined. Based upon the data obtained, SPV2 can be assigned to the genus *Potyvirus* of the family *Potyviridae* and appears to be a potyvirus species distinct from the other major potyviruses infecting sweet potato worldwide. The SPV2 virions are filamentous and have a normal particle length of 850 nm, which is at the upper end of the 680- to 900-nm range reported for potyviruses (Berger *et al.*, 2000). The observation that SPV2 induced cytoplasmic cylindrical inclusions (CI) comprising pinwheels and scrolls not only identified it as a member of the family *Potyviridae* but also placed it in the CI subdivision 1 (Edwardson, 1974) that is different from that of SPFMV. The latter has been reported to induce pinwheels

plus laminated aggregates (Edwardson, 1974; Porth, 1986) and, hence, belongs to another CI category (subdivision 2). Since SPV2-infected cells also contained cytoplasmic crystalline inclusions presumably formed by a non-structural protein other than CI, it differed from SPFMV also in its cytopathic effect, which has not been observed for SPFMV (Edwardson, 1974; Porth, 1986). The structure and distribution of the crystalline inclusions somehow resembled those induced by bean yellow mosaic virus (Riedel *et al.*, 1998). However, this similarity cannot be taken as indicative of a taxonomic affiliation between SPV2 and bean yellow mosaic virus, as the CP amino acid sequence similarity between SPV2 and BYMV (Acc. no. S77515) is only 56%.

The observation that SPV2 was readily transmitted by two biotypes of *Myzus persicae* in a non-persistent manner was consistent with its classification as a member of the genus *Potyvirus*. In agreement with the aphid transmissibility of SPV2 was the observation that its CP amino terminus contained the amino acid triplet DAG which is involved in aphid transmission of potyviruses (Atreya *et al.*, 1995). The two aphid biotypes used in this study appeared to differ in their SPV2 transmission efficiencies. Although this was not studied here in detail, differences between vector species populations in their efficiency to transmit potyviruses have been reported (Castle *et al.*, 1992).

Although SPV2 infected 12 of the 17 plant species tested, its host range appeared to be smaller than that of SPMMV (Hollings *at al.*, 1976), similar to that of SwPLV (Liao *et al.*, 1979), and much wider than that of SPFMV. The latter infects, apart from *Ipomoea* spp., only some *Chenopodium* spp. and, depending on the isolate, *N. benthamiana* (Brunt *et al.*, 1996). Since no host range data are available for SPVG, SPV-Zw, and SPMSV, comparison of host range data for SPV2, SPVG, and SPV-Zw, which shared highest levels of sequence similarity was not feasible.

In synergy studies using *I. setosa* as a model host, plants co-infected with SPV2 and SPCSV showed very severe and conspicuous symptoms on all leaves and strikingly enhanced levels in SPV2 accumulation and distribution as compared to singly infected plants. Since the symptoms observed on the dually infected plants were typical of potyviruses and the titre and distribution of SPV2 but not those of SPCSV were dramatically enhanced, it is conceivable that the greater damage observed for the dually infected plants was due to SPV2. These observations and conclusions are in agreement with those made for SPFMV, another sweet potato potyvirus that caused more severe symptoms and accumulated several-fold in sweet potato and *I. setosa* plants co-infected with SPCSV (Gibson *et al.*, 1998; Karyeija *et al.*, 2000, Schaefers and Terry, 1976). It was suggested that the crinivirus suppresses the resistance mechanism of sweet potato to the potyvirus leading to its accumulation (Karyeija *et al.*, 2000). In this type of synergistic interaction, SPCSV-encoded factors might be responsible for the suppression of the host's defense mechanism thereby permitting an enhanced multiplication of potyviruses, such as SPV2 and SPFMV.

Whereas SPV2 did not cross-react with other sweet potato potyviruses in DAS-ELISA, decoration titer experiments indicated that SPV2 is distantly related to SwPLV and SPFMV. This suggested that it is a distinct member of the genus *Potyvirus*. Antisera to SPVG and SPV-Zw with which SPV2 shared highest levels of sequence similarity were not available for comparison. The taxonomic assignment of SPV2 to the genus *Potyvirus* was further corroborated by the sequence analysis of the 3'-terminal 2006 nucleotides of the SPV2 RNA. The genomic organization and the sequence motifs of this genome fragment were typical for a potyvirus. Based on the partial CP amino acid sequences released in the database for potyvirus isolates (LSU2; LSU5) from sweet potato in the United States, SPV2 shares CP N-terminal amino acid sequence identities of about 93% with LSU2 and LSU5, indicating that

all these isolates are very closely related to each other and merely isolates or strains of the same virus. Based on one of the major demarcation criteria for potyvirus species ("CP amino acid identity of < about 80%") (Berger *et al.*, 2000), SPV2 is clearly different from SPMSV (58%), SwPLV (62%), and SPFMV (~75%). Furthermore, the CP size of SPV2 (332 aa) is much larger than that of SPFMV (315 aa), SwPLV (292 aa), and SPMSV (285 aa). In view of the notion that distinct potyvirus species have 3'-UTR sequence identities of < 80% (Frenkel *et al.*, 1989, Shukla *et al.*, 1994), it should also be pointed out that the 3'-UTR nucleotide sequence identities between SPV2 and the other potyvirus species SPMSV (38%), SwPLV (49%), and SPFMV (77%) did not exceed 80% (Table 4).

Based on the aforementioned molecular criteria, however, SPV2 appears to be closely related to SPV-Zw and SPVG. These three viruses share CP amino acid sequence similarities of 75-80% and 3'-UTR nucleotide sequence identities of 80-82% (Table 4). On the basis of the phylogenetic trees of the CP core amino acid (Fig. 3A) and 3'-UTR nucleotide sequences (Fig. 3B), the branching relating to these three viruses had very low or insignificant bootstrap scores, indicating that they are closely related to one another. Furthermore, SPV2 and SPV-Zw have very similar CP (332 aa vs. 335 aa) and 3' UTR sizes (216 nt vs. 215 nt; without the poly(A) tail). In these respects, they differ from SPVG which has a slightly longer 3' UTR (221 nt) and a considerably larger CP size (355 aa), which results from a 20 aa larger N terminus (Fig. 5). This is the reason why SPVG should perhaps be considered a potyvirus species distinct from SPV2 and SPV-Zw. This notion is further supported upon close examination of the N-terminal amino acids of sweet potato potyviruses (Fig. 5). From this it is evident that SPV2 and SPV-Zw have a similar number (~55) of identical amino acids as the two very divergent SPFMV strains RC and C. Since the CP N termini of the two SPFMV strains are somewhat smaller (80 aa) than that of SPV2 (97 aa) and SPV-Zw (100 aa), this results in CP N-terminal amino acid sequence identities of 67% for SPFMV-RC and -C and of

58% for SPV2 and SPV-Zw (Table 5). This might suggest that the two latter virus isolates can also be regarded as two divergent strains of the same potyvirus. In addition, both SPV2 and SPV-Zw infect *N. benthamiana* (Chavi *et al.*, 1997). In conclusion, based on the properties determined in this study, SPV2 is a distinct member of the genus *Potyvirus* for which the name Sweet potato virus Y (SPVY) is proposed.

5.0 CHAPTER FIVE

BIOLOGICAL AND MOLECULAR VARIABILITY AMONGST GEOGRAPHICALLY DIVERSE ISOLATES OF SWEET POTATO VIRUS Y

5.1 INTRODUCTION

Viruses of sweet potato have attracted considerable attention in recent years and have become topical internationally because the crop has become recognised as economically important in developing countries and also because its vegetative propagules are distributed worldwide. Some of the more important viruses infecting sweet potato belong to the genus *Potyvirus* of the family *Potyviridae* and include *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato latent virus* (SwPLV), *Sweet potato mild speckling virus* (SPMSV), *Sweet potato virus G* (SPV G) and Sweet potato virus Y (SPVY) (Moyer and Salazar, 1989; Colinet *et al.,* 1994; Alvarez *et al* 1997; Colinet *et al.,* 1998). Generally, potyviruses induce mild or no symptoms on sweet potato (Gibson *et al.*, 1998; Karyeija *et al.*, 2000). However, mixed infections of SPFMV together with the crinivirus *Sweet potato chlorotic stunt virus* (SPCSV) induce severe symptoms (Gibson *et al.*, 1998; Karyeija *et al.,* 2000).

SPVY, initially referred to as Sweet potato virus 2 (SPV-2), was first isolated from sweet potato plants from Taiwan that exhibited mild symptoms consisting of mottle, vein yellowing and/or ring spots (Rossel and Thottappilly, 1987). It caused mild vein yellowing, a chlorotic mottle and leaf distortion in *Nicotiana. benthamiana* but no symptoms were apparent on some sweet potato cultivars following experimental inoculation. In recent studies, SPVY was shown to be aphid transmissible, and like SPFMV, it is synergised by SPCSV. Based on molecular studies, it is a distinct potyvirus to be named SPVY (Chapter 4).

As part of a survey of viruses in sweet potato crops in South Africa in 2001, extracts from some sweet potato plants were inoculated to herbaceous host plants. Test plants infected with different SPVY isolates exhibited differences in symptoms. These observations prompted the assessment

of the molecular and biological variability of SPVY isolates/strains from diverse geographical regions of South Africa and Zambia. Additional sequence information of SPVY isolates from China and Portugal were obtained in the course of this study. Recently, two sequences of SPVY originating from the United States of America became available in the sequence databases, indicating a worldwide distribution of this virus. These sequences were used in comparisons and phylogenetic analyses to determine the biological and molecular diversity of SPVY strains.

5.2 MATERIALS AND METHODS

3.2.1 Isolation and maintenance of SPVY isolates

Cuttings of SPVY-infected sweet potato plants were obtained from farmers' fields in different regions of South Africa. Another cutting was obtained from a sweet potato plant from Zambia infected with SPVY, SPCSV and SPFMV. A sample of sweet potato from China (XN3) infected with SPVY, SPVG and SPFMV was kindly provided by Pierre Detrixhe, Belgium, and cuttings from several SPVY-infected sweet potato plants from Portugal were kindly provided by D. Louro. Further details of the samples are given in Table 1. Plants were grown in a temperature-controlled insect-proof glasshouse. Isolates were obtained from individual local lesions obtained by sap inoculation to *Chenopodium* species. Single lesion isolates were produced in *C. quinoa* to ensure the separation of individual pathotypes from a possible mixed population. These were tested and confirmed to be solely SPVY by DAS- and TAS-ELISA using polyclonal and monoclonal antibodies to common mechanically transmissible sweet potato viruses namely SPVY, SPFMV, SPMMV, SPMSV, SPLV and Sweet potato chlorotic fleck virus (SPCFV). All the isolates were subsequently maintained in *N. benthamiana* by sap inoculation.

5.2.2 Host range studies

The experimental host range of six selected isolates of SPVY namely SPVY-Tw, J5/1, Thomas 1.6A, PD1/2, M5/2/1 and Zambia was determined by sap-inoculating them onto a range of commonly used indicator plant species. Infected N. benthamiana leaves were macerated in a

mortar containing 0.1 M potassium phosphate buffer, pH 7.8, and rubbed on carborundum-dusted leaves of four young plants of each species. Non-inoculated plants of each species were included as checks. The plants were observed for symptom development and tested in DAS-ELISA using SPVY antiserum. The inoculated and top leaves of the indicator plants were tested separately to distinguish between localized and systemic infections.

Table1: Origin of SPVY isolates used for phylogenetic analysis

Isolate	Country	Location	Sequence accession no.
PD1/2	South Africa	KwaZulu Natal	AY459607
J5/1		KwaZulu Natal	AY459603
MD2		Kwazulu Natal	AY459606
Thomas1.6A		Mpumalanga	AY459608
M5/2/1		Kwazulu Natal	AY459605
VTSB Tschilombo		Northern	AY459609
Tschilombo IV 1-1		Northern	AY459612
Zambia	Zambia	Silowezi	AY459610
XN3	China	China	AY459611
260702/1	Portugal	-	AY459614
Louro 1	Portugal	-	AY459604
300902	Portugal	-	AY459613
SPVY Tw	Taiwan	-	AY232437

5.2.3 RNA extraction, RT- PCR, cloning and sequencing

For cloning and sequencing of the 3' end of the SPVY genomic RNA, the total RNA of the 12 isolates (Table 1) was extracted from 100 mg infected *N. benthamiana* leaf tissue using either the Nucleospin® Plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol or the modified plant total nucleic acid extraction with silica.

5.2.3.1 Modified plant total nucleic acid extraction with silica

Infected leaf (100 mg) was ground in liquid nitrogen and grinding buffer (1 ml/100 mg tissue, Appendix 3). The homogenate (500 µl) was transferred to an Eppendorf tube and 100 µl of 10% sarkosyl (Carcorcine) added. The homogenate was incubated at 70°C with intermittent shaking for 10 min. The tube was then incubated on ice for 5 min and centrifuged at 13,000 rpm for 10

min. The supernatant (300 µl) was transferred to a new tube, to which 150 µl absolute alcohol, 300 µl 6M NaI, and 25 µl resuspended silica were added. The mixture was incubated at room temperature for 10 min with intermittent shaking and centrifuged at 6,000 rpm for 1 min. The pellet was washed in 500 µl of wash buffer (Appendix 3) followed by centrifugation at 6,000 rpm for 1 min. The wash step was repeated and the pellet resuspended in 150 µl of autoclaved distilled water. This was incubated at 70°C for 4 min, centrifuged for 3 min at 13,000 rpm and the supernatant stored at -20°C till use.

cDNA synthesis

Synthesis of complementary DNA (cDNA) was done using an Oligo(dT) primer as cDNA primer, and maloney murine leukemia virus reverse transcriptase (MMLV-RT) (Invitrogen/Gibco) as outlined earlier (Section 4.2). The resulting cDNA was amplified by PCR using *Potyviridae* specific degenerate upstream primer TAATACGACTCACTATAGGGAAYAAYAGYGG5CARCC and a downstream primer GATTTAGGTGACACTATAGTTTTTTTTTTTTTTTTV described by Gibbs and Mackenzie (1997). Two µl of cDNA were mixed with 42 µl of H_2O, 5 µl of 10x PCR buffer (Invitrogen/Gibco), 2.5 µl of $MgCl_2$ (50mM), 0.5 µl of each primer (100pmol/ul), 0.5 µl of dNTPs (25 mM each) and Taq polymerase (0.5 U/µl). Incubation was done in a PTC 200 thermocycler (MJ Research Inc., Watertown, MA). PCR conditions were denaturation at 94°C for 3 min followed by 30 cycles of 30 s at 94°C, 1 min at 58°C primer annealing, extension for 3 min at 72°C and a final extension step at 72°C for 8 min. The amplification products were analysed by electrophoresis in a 1% agarose gel, excised and purified using the Nucleospin® Extract kit (Macherey-Nagel, Düren, Germany. The eluted DNA fragments were cloned into pGEM®-T vector (Promega Corp., Madison, WI) and subsequently transformed into *Escherichia coli* strain DH5α competent cells (Promega) according to the manufacturer's instructions. Plasmids were isolated from recombinant *E. coli* using the Macherey-Nagel Kits. Sequencing was done using the M13 reverse and M13uni universal primers. A degenerate primer

SPVYsequp GCATGGRTTYTKGATCAGGC was derived from the obtained sequences and was subsequently used in combination with the oligo(dT) primer to amplify the entire CP and 3' NTR of SPVY from sweet potato plants originating from China and Portugal.

Sequence comparisons and phylogenetic analysis

Pairwise alignments and identity matrices were generated using DNAMAN software (Lynnon BioSoft Version 4.02). Phylogenetic analyses were done for the CP nucleotide as well as the 3' NTR nucleotide sequences using the default settings of the ClustalX software (Jeanmougin *et al.*, 1998). One thousand bootstrapped data sets were generated to estimate the statistical significance of the branching. The consensus tree was visualized using the Treeview programme (Page, 1996). The CP aa sequences of the SPVY isolates were further compared in a phylogenetic analysis with SPFMV strains –C and –RC, SPFMV-Zw and SPVG.

5.3 RESULTS

5.3.1 Host range and symptoms

Nicotiana glutinosa, N. debneyi, Gomphrena globosa and *Datura stramonium* were not infected by any of the six isolates under glasshouse conditions. *N. clevelandii, N. benthamiana* and *C. quinoa* were all infected by the six isolates, developing systemic mosaic, vein clearing plus leaf deformation and chlorotic local lesions, respectively. *N. occidentalis* sub-species P1 was susceptible to all the isolates but PD1/2. J5/1 caused systemic chlorotic lesions on *N. occidentalis* P1, whereas the other isolates induced more severe symptoms with additional systemic necrotic ringspots. PD1/2 and J5/2 differed from the other isolates also by not infecting *N. occidentalis* ssp. obliqua and *N. hesperis*. However, these isolates (PD1/2 and J5/1) differed from each other in that PD1/2 did not infect *C. murale*. Isolate Zambia was the only one that failed to infect *C. amaranticolor* whereas only Thomas 1.6A infected *D. metel*. Two isolates, namely Zambia and Thomas 1.6A, caused more severe symptoms on virtually all the infected test plants and especially in *N. hesperis*, which showed severe stunting, leaf distortion, and

chlorosis (Plate 1). Conversely, mild symptoms were induced by isolates SPVY-Tw and M5/2/1, which caused chlorotic lesions only. The symptoms incited by the six isolates on a number of test plants are presented in Table 2.

Table 2: Reactions of indicator plants 21 days after sap inoculation with 6 selected SPVY isolates

Host*	SPVY isolates					
	SPVY Nig.	**PD1/2**	**Zambia**	**Thomas 1.6A**	**M5/2/1**	**J5/1**
N. clevelandii	m**	m	m	m	m	m
N. occid. Pl	chl, nrs	ni	chl, nrs	chl, nrs	chl, nrs	chl
N. benth.	vc, ld	vc, ld	vc, ld	vc, ld	vc, ld	vc, ld
N. occi. Obli.	chl	ni	chl	chl	chl	ni
N. hesperis	chl	ni	chl, st, ld	chl, st, ld	chl	ni
C. quinoa	cll	cll	cll	cll	cll	cll
C. murale	cll	ni	cll	cll	cll	cll
C. amarant.	cll	cll	ni	cll	cll	cll
D. metel	ni	ni	ni	cll	ni	ni

D. stramonium, G. globosa and *N. glutinosa* did not show any symptoms and tested negative for SPVY in DAS-ELISA.

* Infection of *Nicotiana* species by the various SPVY isolates always led to a systemic infection whereas the *Chenopodium* species and *D. metel* only formed chlorotic local lesions.

** The symptom codes used are: chl-chlorosis, cll -chlorotic local lesions, ld-leaf distortion, m-mosaic, ni-not infected, nrs- necrotic local lesion, st-stunting and vc-vein clearing

5.3.2 Sequence comparisons

To test whether biologically and geographically diverse isolates of SPVY show corresponding genetic variability, the CP gene and the 3' NTR nucleotide (nt) sequences of the six biologically characterised isolates and an additional six from different geographical regions were determined. All isolates possessed a CP coding region of 996 nt encoding a putative protein of 332 amino acid (aa) residues. The 3' NTR was 216 nt except for Zambia, which had a deletion of 2 nt at the 5' end. Alignment of the CP sequences obtained including two SPVY sequences from the USA recently deposited in the database revealed several motifs highly conserved in the potyviral CP; which included the DAG amino acid triplet associated with aphid transmissibility (Atreya *et al.*, 1990, 1992, 1995) that was located eight amino acids downstream of the NIb/CP cleavage site,

Plate 1: Severe symptoms incited by sweet potato virus Y isolate Zambia on *Nicotiana hesperis* 21 day after sap inoculation

the WCIENG and the AFDF motifs (Jagadish *et al.*, 1991; Pappu *et al.*, 1993).

The comparison of the CP encoding nt sequences gave identities ranging from 81-100%, whereas the CP aa similarities ranged from 86 to 100% (Table 3), indicating many silent or synonymous nt changes. The N-termini of the SPVY CP varied considerably. The aa sequence similarity in the CP N-terminal portion ranged from 67 to 99% (Table 4, Fig. 3) whereas the rest of the CP sequences were highly conserved (93 to 100%). The percent identities of the 3' NTR nucleotide sequences were higher than those of the CP nucleotide sequences and ranged from 85 to 100% (Table 4). The phylogenetic relationships among the isolates based on the CP comparison followed a pattern similar to the one based on the 3' NTR comparison as no major differences were observed in the topology of the trees. The most divergent CP was that of Zambia while the most divergent 3' NTR sequence was that of Thomas 1.6A. This difference was mainly due to the considerable divergence of the Zambian CP N-terminus. Thomas 1.6A and Zambia consistently took a position divergent from all the other isolates. Apart from Thomas 1.6A, all isolates from South Africa were closely related (94-100%) to one another and to isolates from China, Portugal and the type isolate from Taiwan. The phylogenetic analyses of the CP and 3' NTR nucleotide sequences (Fig. 1 and 2) reflect these groupings with the Zambian isolate and isolate Thomas 1.6A being phylogenetically distinct from the rest of the SPVY isolates.

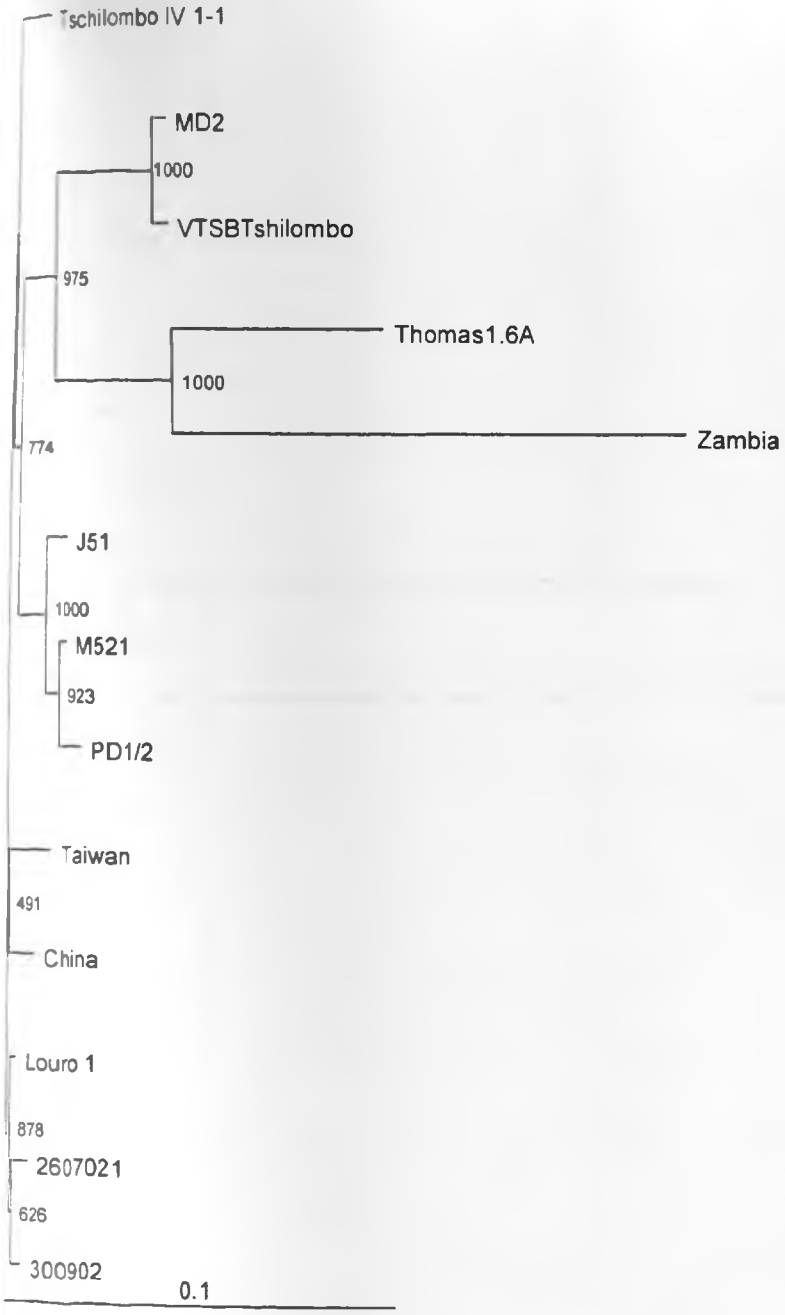

Fig. 1: Phylogenetic analysis illustrating the relationships between SPVY isolates. Coat protein nucleotide sequences were aligned and neighbour-joining trees were constructed with Clustal X v1.83. Bootstrap values were calculated from 1000 replicates. Trees were drawn with Tree View v 1.66. The scale bar represents a genetic distance of 0.1 for the horizontal branch lengths.

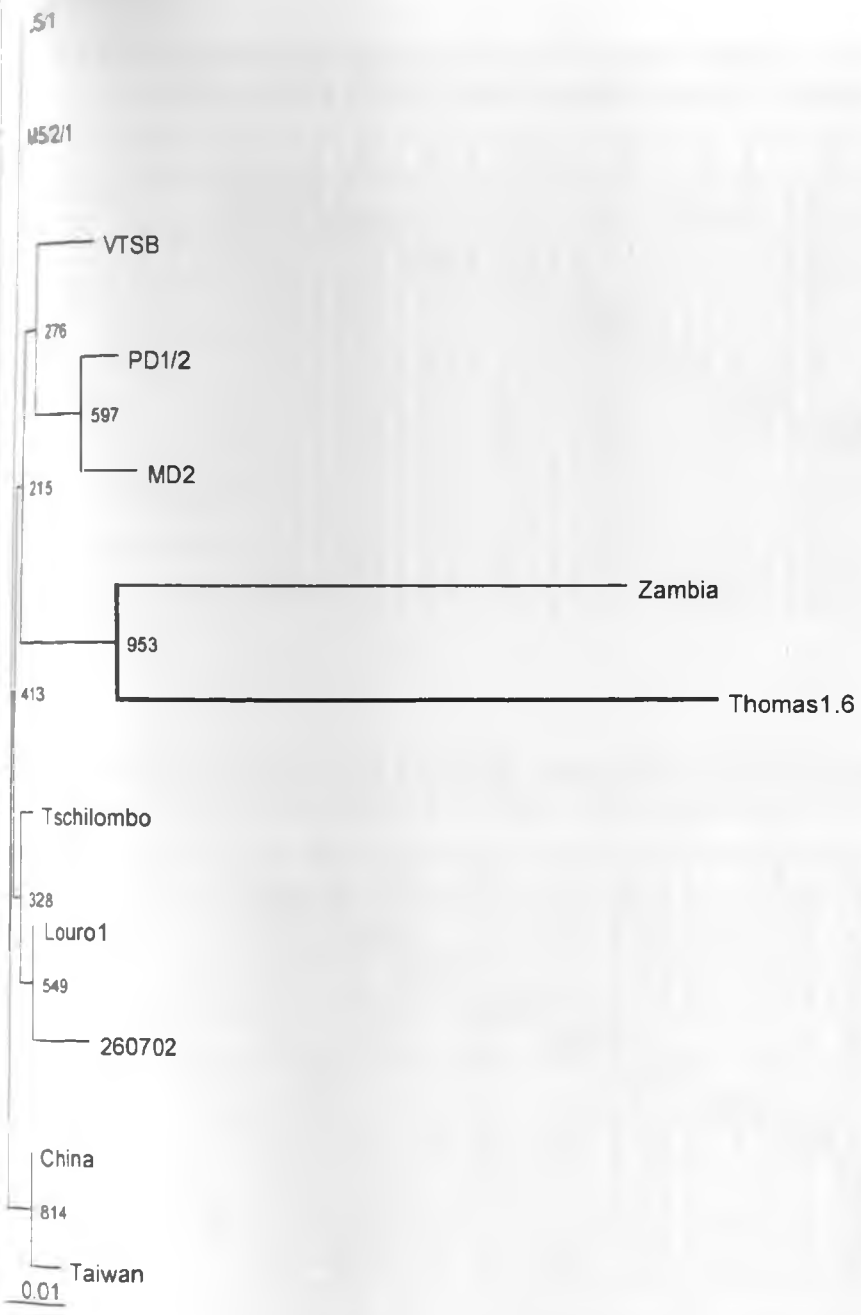

Fig. 2: Phylogenetic analysis illustrating the relationships between SPVY isolates. The 3'NTR nucleotide sequences were aligned and neighbour-joining trees were constructed with Clustal X v1.83. Bootstrap values were calculated from 1000 replicates. Trees were drawn with Tree View v 1.66. The scale bar represents a genetic distance of 0.1 for the horizontal branch lengths.

Table 3: Pairwise percent coat protein amino acid sequence similarity (lower diagonal) and nucleotide sequence identity (upper diagonal) among 13 isolates of SPVY

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13
1. 260702/1		97.7	94.7	97.2	97.5	97.9	82.1	89.1	98.2	94.6	98.4	98.5	99.2
2. M521	96.4		93.6	99.1	98.8	96.8	81.9	89.4	97.1	93.7	97.3	97.2	97.7
3. MD2	95.5	94.0		93.2	93.8	94.8	81.3	88.1	94.8	99.2	94.8	94.6	94.9
4. PD1/2	95.8	98.8	93.7		98.3	96.5	81.9	89.3	96.6	93.2	96.8	96.7	97.2
5. J5/1	95.8	98.2	93.4	97.6		98.8	82.0	89.1	97.1	93.8	97.3	97.2	97.7
6. SPVY Tw	97.9	96.1	95.8	95.5	95.5		81.5	89.1	98.0	94.9	97.9	97.6	98.1
7. Zambia	87.0	86.4	86.4	86.1	86.4	87.0		82.4	81.7	80.9	81.5	81.4	81.9
8. Thomas	94.3	95.8	93.4	95.2	94.9	94.0	86.1		88.9	87.9	89.1	88.7	89.4
9. Tschlombo IV 1-1	98.2	96.4	97.0	95.8	95.8	97.9	87.3	94.3		94.8	98.0	97.9	98.4
10. VTSB Tschilombo	95.2	93.4	98.5	92.8	92.8	95.2	85.8	92.8	96.4		94.9	94.5	94.9
11. XN3	97.6	95.8	95.2	95.2	95.2	97.3	86.1	94.0	97.6	94.6		98.1	98.6
12. Louro 1	98.5	96.1	95.2	95.5	95.2	97.6	86.7	94.0	97.9	94.9	97.3		98.7
13. 300902	98.8	96.4	95.5	95.8	95.8	97.9	87.0	94.3	98.2	95.2	97.6	98.5	

Table 4: Pairwise percent 3' NTR nucleotide sequence identity (below diagonal) and CP N-terminal amino acid sequence similarity (above diagonal) of 14 SPVY isolates

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. 260702/1		89.7	85.6	87.6	88.7	93.8	71.1	88.7	94.8	86.6	96.6	99.0	99.0	94.8
2. M5/2/1	98.6		80.4	97.9	96.9	87.6	69.1	93.8	88.7	80.4	90.7	90.7	90.7	89.7
3. MD2	97.2	97.7		79.4	79.4	85.6	68.0	84.5	89.7	96.9	87.6	86.6	86.6	84.5
4. PD1/2	97.7	98.1	98.6		94.8	85.6	68.0	91.8	86.6	78.4	88.7	88.7	88.7	87.6
5. J5/1	98.6	100.	97.7	98.1		86.6	68.0	91.6	87.6	79.4	89.7	89.7	89.7	88.7
6. SPVY	97.7	99.1	96.8	97.2	99.1		70.1	86.6	92.8	85.6	94.8	94.8	94.8	92.8
7. Zambia	90.2	91.6	89.7	89.7	91.6	90.7		67.0	71.1	68.0	71.1	72.2	72.2	72.2
8. Thomas	88.9	90.3	88.4	89.4	90.3	89.8	85.5		87.6	84.5	90.7	89.7	89.7	88.7
9. Tschilombo IV 1-1	98.6	99.5	97.7	98.1	99.5	98.6	91.5	89.8		89.7	87.6	87.6	95.9	84.5
10. VTSB Tschilombo	97.2	98.6	98.1	96.8	98.6	97.7	91.1	89.8	98.1		87.6	87.6	87.6	84.5
11. XN3	98.1	99.5	97.2	97.7	99.5	99.5	91.1	90.3	99.1	98.1		97.9	97.9	95.9
12. Louro 1	99.1	99.5	97.7	98.1	99.5	98.6	91.1	89.8	99.5	98.1	99.1		100.0	95.9
13. 300902	*													
14. LSU-2	*													

* The 3' NTR sequences of LSU-2 and 300902 are not available

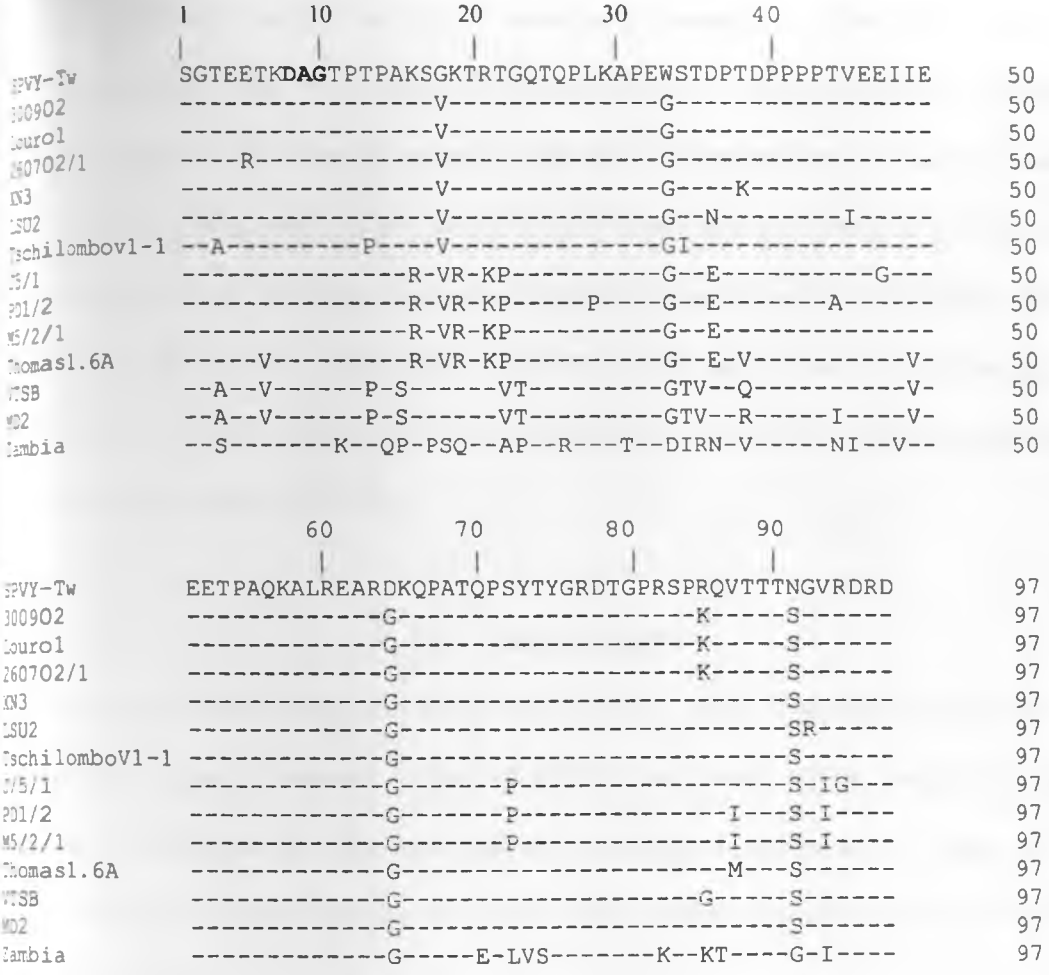

Fig. 3: Multiple alignments of CP N- terminal amino acid sequences of SPVY isolates

A further phylogenetic analysis of the CP amino acid sequences of the SPVY isolates and SPFMV -C and -RC (Abad *et al.*, 1992), SPV-Zw (Chavi *et al.*, 1997) and SPVG (Colinet *et al.*, 1994) was performed. Whereas the majority of the SPVY isolates formed a compact clade, the SPVY isolate Zambia appeared to be phylogenetically intermediate between the SPV-Zw and the SPVY isolates (Fig. 4). In terms of sequence similarity, Zambia was 87% and 76% similar to SPVY-Tw and SPFMV-Zw, respectively. The SPV-Zw CP was 67 and 71% similar to that of SPFMV (both C and RC strains) and SPVG, respectively. All the SPVY isolates tested reacted strongly with the PAbs to SPVY-Tw.

5.4 DISCUSSION

This study shows the host range and nucleotide sequences of the CP-encoding region and the 3' non-translated region of selected isolates of SPVY from South Africa, Zambia, China, and Portugal and constitutes the first biological and molecular comparison of a range of SPVY isolates. The study also provides the first report of the occurrence of this potyvirus in the afore-mentioned countries.

Host range and symptoms have been used for the identification and differentiation of strains and pathotypes of viruses (Hamptom and Provvidenti, 1992; Xiao *et al* 1993; Shukla *et al.*, 1994; van Regenmortel *et al.*, 2000). Sap inoculation of six selected isolates of SPVY onto a range of test plants revealed differences in symptoms and host range. Some isolates showed similar biological properties suggesting that they are closely related, others appeared to be exceptional strains and these observations seem to confirm phylogenetic groupings. Isolates PD1/2 and J5/1 exhibited a similar trend in infection and symptoms and shared 98% CP sequence identity. The Zambian isolate and isolate Thomas 1.6A from South Africa caused more severe symptoms on most test plants particularly in *N. hesperis* and were rather divergent from other isolates based on CP and 3' NTR sequences. Moreover, isolate Zambia was the only isolate that did not infect *C. amaranticolor* despite repeated tests, while only isolate Thomas 1.6.A infected *D. metel*.

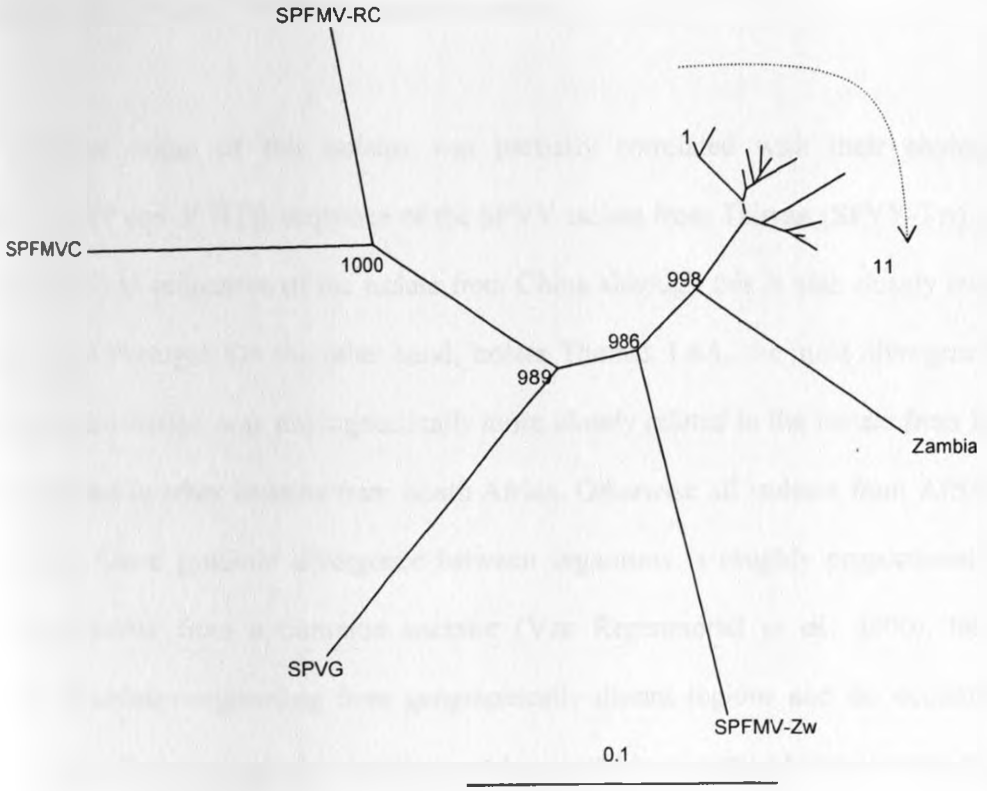

Fig. 4: Unrooted neighbour-joining tree obtained from the alignment of the CP aa sequences using the ClustalX programme. Vertical distances are arbitrary. The SPVY isolates designated 1-11 on the phylogenetic tree in ascending order are; MD2, VTSB, Tschilombo, SPVY-Tw, Louro 1, 260702, XN3, Thomas, J5/1, M5/2/1, PD1/2.

Experimental results and circumstantial evidence indicated that differences in the CP and the 3' NTR of potyvirus isolates were biologically significant (Shukla and Ward, 1991; Shukla *et al.*, 1994). It is likely that some of the observed differences in the amino acid sequences of the CP and in the nt sequences of the 3' NTR might be responsible for the symptom differences observed between the isolates. However, the extent to which biological properties are reflected by variations in the CP and 3' NTR sequences is unclear.

The geographical origin of the isolates was partially correlated with their phylogenetic clustering. The CP and 3' NTR sequence of the SPVY isolate from Taiwan (SPVY-Tw) is very similar (ca. 97 %) to sequences of the isolate from China although this is also closely related to the isolates from Portugal. On the other hand, isolate Thomas 1.6A, the most divergent SPVY isolate from South Africa, was phylogenetically more closely related to the isolate from Zambia than it was related to other isolates from South Africa. Otherwise all isolates from Africa were closely related. Since genomic divergence between organisms is roughly proportional to the evolutionary distance from a common ancestor (Van Regenmortel *et al.*, 2000), the close relationship of isolates originating from geographically distant regions and the occurrence of divergent isolates in one geographic location, predominated by a certain homogeneous group of isolates, may suggest a recent evolutionary event. However, information based on other potyvirus genes, such as the P1 and P3 protein sequences might provide a more sensible separation of biologically and/or geographically distinct isolates (Tordo *et al.*, 1995; Igor *et al.*, 2001; Lin *et al.*, 2001).

The CP, 3' NTR, and CP N-terminus sequences are considered species-specific and have been used extensively for the classification of potyviruses and potyvirus strains (Frenkel *et al* 1989; Shukla and Ward, 1988, 1989a; Shukla *et al.*, 1994). CP aa sequence identities between virus strains range from about 80 to 99% whereas distinct species have CP aa sequence identities of less than about 80% (Shukla *et al.*, 1994; Berger *et al.*, 2000). From the results, the CP aa

sequence similarities among the SPVY isolates studied ranged from 86 to 100%, whereas in the 3' NTR, nt sequence identities ranged from 85 to 100% which is within the suggested thresholds of distinct virus species (Frenkel *et al.*, 1992, 1989; Shukla *et al.*, 1994). Other studies have reported similar wide variations in sequence among potyvirid strains (Shukla *et al.*, 1994; Abad *et al.*, 1992; Kreuze *et al.*, 2000).

This study describes the genetic diversity and host range data of the SPVY isolates and herewith provides the basis for proposing the existence of SPVY strains. The isolates from Zambia and Thomas 1.6A each represent distinct strains of SPVY whereas the other 12 isolates belong to a third strain of closely related isolates. It is unknown how widespread strains similar to Zambia and Thomas 1.6A or other divergent strains are. More work needs to be done to get a better understanding of the range of variability in SPVY, and to determine if certain deviant strains escape serological detection.

6.0 CHAPTER SIX

IDENTIFICATION OF SWEET POTATO VIRUS G STRAINS BASED ON THEIR 3' TERMINAL GENOME SEQUENCES

6.1 INTRODUCTION

Sweet potato virus G (SPVG) is one of the potyviruses infecting sweet potato (Colinet *et al.*, 1994). The virus was first identified in sweet potato plants originating from China by RT-PCR using degenerate primers (Colinet *et al.*, 1994). Based on sequence information of the 3' terminal portion of its genome, SPVG was classified as a distinct member of the genus *Potyvirus* and family *Potyviridae*, closely related to SPFMV (Colinet *et al.*, 1994). However, little information is available with regard to geographical distribution, biological properties and genetic variability. SPVG was initially detected in samples from China but has very recently been reported from Egypt (Is Hak *et al.*, 2003) and the United States of America (De Souto *et al.*, 2003).

The coat protein (CP) gene is a highly variable region of the potyvirus genome that has widely been accepted for classification of isolates in the genus (Ward and Shukla, 1991; Shukla *et al.*, 1994). The 3' NTR sequences have also been used to identify and classify potyviruses (Frenkel *et al.*, 1989; Habera *et al.*, 1994). The CP sequences have been used to determine the phylogenetic relationships among isolates and strains. In some of the studies, isolates of the same species show genetic differences that correlate with geographical origins and host specialization (Ohshima *et al.*, 2002; Sánchez *et al.*, 2003). This chapter reports the serological detection and the 3' terminal nucleotide sequence variability of SPVG isolates from Asia, Africa, Europe, and North America.

6.2 MATERIALS AND METHODS

6.2.1 Sources of infected sweet potato and serological tests

Sweet potato cuttings (XN3) originally collected from China were obtained from Pierre Detrixhe, Belgium, while several SPV-G infected sweet potato cuttings from Portugal were obtained from D.

Louro, Portugal. Infected cuttings were obtained during a survey of sweet potato fields in South Africa carried out in May 2003. These sweet potato accessions were assayed by DAS- and TAS-ELISA as outlined in section 3.2.2 using antibodies against SPVG, *Sweet potato feathery mottle virus* (SPFMV), Sweet potato virus Y (SPVY), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato mild mottle virus* (SPMMV), *Sweet potato chlorotic fleck virus* (SPCFV), *Sweet potato mild speckling virus* (SPMSV), *Sweet potato latent virus* (SwPLV) and *Cucumber mosaic virus* (CMV).

6.2.2 RNA extraction, RT-PCR and cloning of PCR products

Extraction of total nucleic acid from SPVG-infected sweet potato plants was done using the modified total RNA extraction method with silica (Section 5.2.3.1). Synthesis of cDNA was carried out using MMLV-RT and an oligo(dT) cDNA primer as outlined in section 4.2.7. Using an upstream primer SPVGNibup1 5'-ATGATGAACTACTCGCAAGG-3' derived from the published SPVG sequences (Colinet *et al.*, 1994, 1998) and an oligo(dT) primer, the entire CP and 3' NTR of the SPVG isolates from China and Portugal were amplified. Since the primer pair failed to amplify SPVG sequences from South African sweet potato, the general potyvirus primers POT1 and POT2 (Colinet *et al.*, 1994) were used to amplify a DNA fragment comprising the 3' terminal half of the NIb gene and the 5' terminal half of the CP gene of SPVG. Subsequently, a degenerate upstream primer (at the 'wcieng' region) 5'-TGAGGATCCTGGTGYATHGAARAAYGG-3' was used in combination with an oligo(dT) primer (Langeveld *et al.*, 1991) to amplify the 3' terminal half of the CP gene and the entire 3' NTR. A sequence specific primer was consequently derived from the CP N-terminus and used to obtain an overlap. A mix of 2 µl cDNA, 42 µl of H_2O, 5 µl of 10x PCR buffer (Invitrogen/Gibco), 2.5 µl of $MgCl_2$ (50 mM), 0.5 µl of each primer (100 pmol/µl), 0.5 µl of dNTPs (25 mM each) and 0.5 µl Taq polymerase (0.5 U/µl) was incubated in a PTC 200 thermocycler (MJ Research Inc., Watertown, MA) as follows: first template denaturation at 94°C for 4 min followed by 30 cycles of 30 s at 94°C, 1 min at 58°C primer annealing, extension at 72°C for 1.5 min and a final extension step at 72°C for 8 min. The amplification products of approximately 1300 bp were separated by agarose gel electrophoresis, excised from the gel and purified using the Nucleospin

Extract kits (Macharey-Nagel, Düren Germany). DNA fragments were ligated into the pGEMT cloning vector (Promega) and subsequently transformed into *Escherichia coli* following standard procedures (Sambrook *et al.*, 1989). Recombinant plasmid DNA was isolated from overnight bacterial cultures using the Nucleospin Plasmid kit (Macharey-Nagel, Düren Germany) following the manufacturer's protocol. Sequencing was done by a commercial company (MWG D-85560 Ebersberg, Germany). Multiple sequence alignments were done using ClustalX (version 1.83) software and bootstrap neighbour-joining (NJ) trees were drawn using 1000 replicates. Phylogenetic trees were viewed using the TreeView programme.

Table 1: Isolates of sweet potato virus G used for sequence comparisons and phylogenetic analysis

Isolate	Accession no	Origin	Reference
CH1	X76944	China	Colinet *et al.*, 1994
CH2	Z83314	China	Colinet *et al.*, 1998
XN3	-	China	This study
EG	AJ515380	Egypt	Is Hak *et al.*, 2003
Po-2	-	Portugal	This study
Po-1	-	Portugal	This study
SA	-	S. Africa	This study
LSU 3	AY178990	N. America	De Souto *et al.*, 2003
LSU 1	AY178991	N. America	De Souto *et al.*, 2003

6.3 RESULTS AND DISCUSSION

SPVG was detected by double antibody sandwich ELISA in sweet potato plants originating from South Africa, Portugal, and China (Table 2). Most of the plants were co-infected with SPFMV and SPVY (Table 2) and seldom with SPCSV. Mixed virus infections in sweet potato is a common phenomenon (Schaefers and Terry, 1976; Moyer and Salazar, 1989; Gibson *et al.*, 1998; Karyeija *et al.*, 2000). The co-occurrence of these three potyviruses is not surprising since they are most likely transmitted by the same vector (aphids). SPFMV and SPVY are known to be transmitted by aphids in a non-persistent manner (Moyer and Kennedy, 1978; Chapter 4). Whereas SPCSV synergizes the infection of SPFMV (Schaefers and Terry, 1976; Gibson *et al.*, 1998; Karyeija *et al.*, 2000) and

SPVY (Chapter 4), the effect of the mixed infections involving three potyviruses in the presence of SPCSV is unknown. It is hypothesized that such mixed infections may lead to more severe symptoms, and that SPCSV may similarly synergize the infection of SPVG. More severe symptoms were observed in mixed infected sweet potato plants also infected with SPCSV (Table 2). The symptoms of SPVG in sweet potato have not been described. However, the sweet potato cultivar Bosbock commonly grown in S. Africa showed chlorotic spots when infected only with SPVG (Plate 1).

Sequence analysis: The CP-encoding regions of the SPVG sequences obtained in this and previous studies span 1065 nucleotides (nt), except for the isolate from South Africa, which had a CP-coding region of 1062 nt. The DAG motif associated with aphid transmission (Atreya *et al.*, 1990, 1992, 1995) was conserved in all the analysed sequences thereby indicating the likelihood of SPVG transmission by aphids. Multiple alignment of the nt and deduced CP amino acid (aa) sequences revealed sequence similarities ranging from 81 to 98% and from 90 to 99%, respectively (Table 3 and 4), indicating a high level of synonymous (silent) nt substitutions. The isolate SA had an insert of one aa at CP position 15 and a deletion of 2 aa at position 37 and 38 (Fig. 3). The 3' NTR, sequence identities among the isolates ranged from 84 to 99% (Table 4).

Table 2: **Detection of SPVG in sweet potato accessions by ELISA**

Accession	Origin	Symptoms in sweet potato	Antibodies and source			
			SPV-2* BBA	SPVG BBA	SPFMV CIP	$SPCSV_{WA}$ BBA
15070/18	Portugal	ld, p, st,	++	+	++	0
PO-1	Portugal	ld, p, st,	+++	+++	+++	0
PO-2	Portugal	ld, p, st,	+++	+++	+++	0
1310	Portugal	- (not explained)	+++	+++	+++	0
300902/33	Portugal	ld, chl, vc, m, st	0	+++	+++	++
300902/37	Portugal	ld, chl, vc, m, st	+++	+++	+++	+++
XN3	China	prs	+++	+++	+++	0
Frank 5.2mx	S. Africa	cs	++	++	+++	0
GW2/2/1	S. Africa	ns	0	+++	+++	0
GW2/5/3	S. Africa	ns	0	+++	+++	0
437	S. Africa	cs	0	+++	0	0
445	S. Africa	cs, m	0	+++	0	0
SA	S. Africa	cs	0	+++	0	0

* Strength of DAS- and TAS-ELISA reaction, measured at $A_{405\ nm}$ after a substrate incubation period of 1h, was classed as +++ (>0.7), ++ (0.2-0.7), + (>three times the mean value for uninfected control to 0.2) or 0 (<three times mean of uninfected controls). Symptom codes: ld (leaf distortion), p (purpling), st (stunting), chl (chlorosis), vc (vein clearing), prs (purple ring spots), cs (chlorotic spots), m (mosaic), ns (no symptoms). $SPCSV_{WA}$ refers to the West African strain of SPCSV.

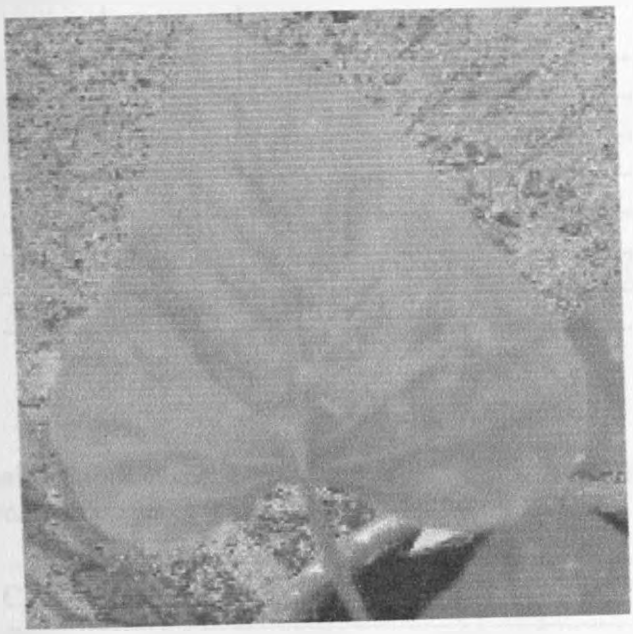

Plate 1: Chlorotic spots on sweet potato cv. Bosbok caused by isolate 437 of SPVG

Table 3: Percent coat protein nucleotide sequence identity (below diagonal) and amino acid sequence similarity (above diagonal) among seven SPVG isolates

	CH2	CH1	XN3	PO-2	PO-1	EG	SA
CH2		93.5	93.8	94.6	95.8	94.4	91.5
CH1	85.4		97.2	97.5	97.2	98.0	89.8
XN3	85.0	98.0		97.5	97.2	98.0	89.8
PO-2	85.7	97.8	97.8		98.6	90.4	90.4
PO-1	85.6	97.9	98.0	98.5		97.5	91.2
EG	85.3	98.3	98.0	97.9	97.9		90.4
SA	81.2	81.7	81.9	81.9	82.3	81.9	

Table 4: Percent CP N-terminal amino acid sequence identity (below diagonal) and 3' NTR sequence identity (above diagonal) among SPVG isolates

	CH2	CH1	XN3	PO-2	PO-1	EG *	SA
CH2		92.3	92.3	91.4	91.9		88.6
CH1	88.3		99.1	97.3	97.7		86.3
XN3	90.0	97.5		98.2	98.6		85.8
PO-2	89.2	96.7	97.5		98.6		84.0
PO-1	91.7	95.0	95.8	96.7			84.9
EG	90.0	98.3	99.2	96.7	95.0		
SA	78.3	75.4	76.3	74.6	76.3	76.3	
LSU-3*	88.3	76.7	97.5	95.0	93.3	98.3	76.4

* The complete 3' NTR sequence of EG is not available and that of LSU-3 is missing

Bootstrap phylogenetic analysis of the entire CP nt sequences revealed that the two isolates from Portugal (PO-1 and PO-2) were closely (98%) related to each other (Fig. 1). The two SPVG CP encoding sequences from the Chinese isolates (CH1 and XN3) were closely related (98% nt identity) whereas each was only 85% identical to a third isolate (CH2) from China. Both XN3 and CH1 were genetically closely related to the isolate EG from Egypt. The CP nt sequence of the isolate SA was the most divergent (Fig. 1) with identities of about 81% with all other SPVG sequences. Phylogenetic analysis of the 3' NTR sequences revealed similar groupings to those based on the CP nt sequence analysis (Fig. 2). Again, the isolate, SA and CH2 formed divergent branches whereas the rest of the isolates formed a large compact clade.

Since only partial CP coding sequences of two SPVG isolates from Louisiana, USA (LSU 1 and 3) are available in the databases, their CP N-terminal aa sequences were included in a phylogenetic analysis (Table 4). The CP N-terminal aa sequences of the isolates LSU-1 and LSU-3 were genetically most closely related (98%) to the Egyptian isolate and was also closely related to the isolates from China (XN3 and CH1) (ca. 97%) and Portugal (93%). The major differences in the CP aa sequences emanate from the N-terminal portion of the CP (Fig. 3; Table 4), whereas the CP aa sequences were highly conserved in the core region. The CP N-terminal aa sequence of the S. African isolate was 74-78% similar to the sequences of all the other isolates included in this comparison, and had a unique 15 aa stretch strikingly different from position 92 to 108 of the CP (Fig. 3).

The CP N-terminal aa sequence of the isolate CH2 on the other hand was 78-90% similar to the rest of the isolates further reinforcing the observation that these two isolates are genetically distinct from the rest and from each other.

No relationship was observed between the geographic origin of the isolates and their phylogenetic relatedness. From the analysis, a low level of variation amongst the isolates PO-1, PO-2, CH1, XN3, LSU-1. LSU-3 and EG was observed and these were considered to belong to the common (C) strain. This low level of diversity could be attributable to the rapid and perhaps recent exchange of vegetative propagation sweet potato material resulting in the introduction of this strain in those areas. The fact that the CH2 strain and S. Africa (SA) are strikingly deviant from the rest in CP sequences suggests that they may have undergone different selection pressures and therefore represent two different lineages in the evolution of SPVG. The other possibility is that these isolates with low genetic variability may have emerged later in the evolution of SPVG and have had less time to differentiate from each other following recent introduction in those areas.

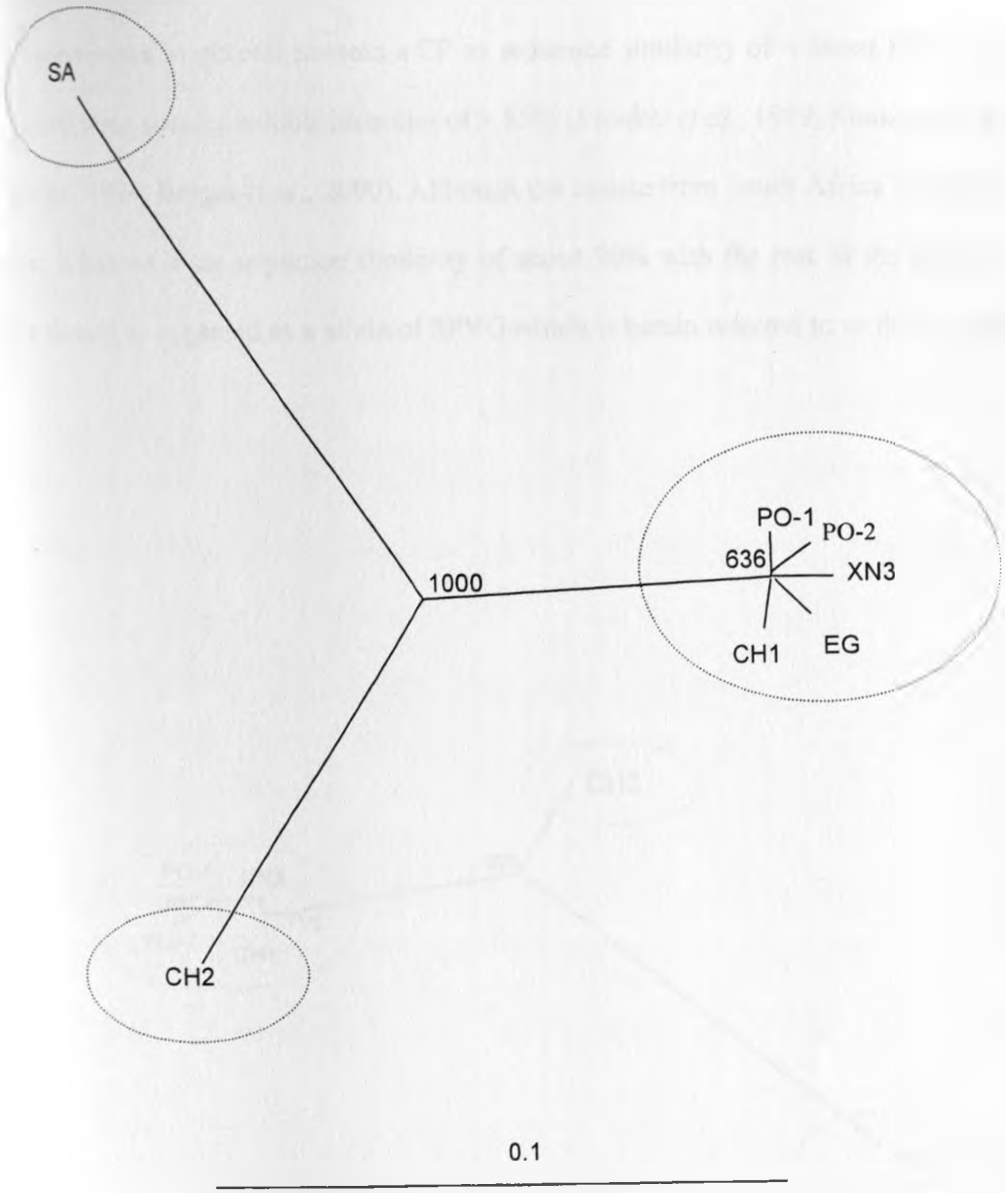

Fig. 1: Unrooted neighbour-joining tree of the CP nucleotide sequences of SPVG constructed with ClustalX v1.83. Bootstrap analysis was performed using 1000 bootstrap samples. Trees were viewed with TreeView v 1.65.

The analysis of sequence information demonstrated a wide variability of SPVG. Three distinct genetic groups of isolates emerged from the phylogenetic analyses of the CP and 3' NTR sequences. Distinct potyviruses in general possess a CP aa sequence similarity of < about 80% whereas strains of individual virus species exhibit identities of > 85% (Frenkel *et al.*, 1989; Shukla and Ward, 1989b; Shukla *et al.*, 1994; Berger *et al.*, 2000). Although the isolate from South Africa is clearly genetically divergent, it has a CP aa sequence similarity of about 90% with the rest of the SPVG isolates and therefore should be regarded as a strain of SPVG which is herein referred to as the SA strain.

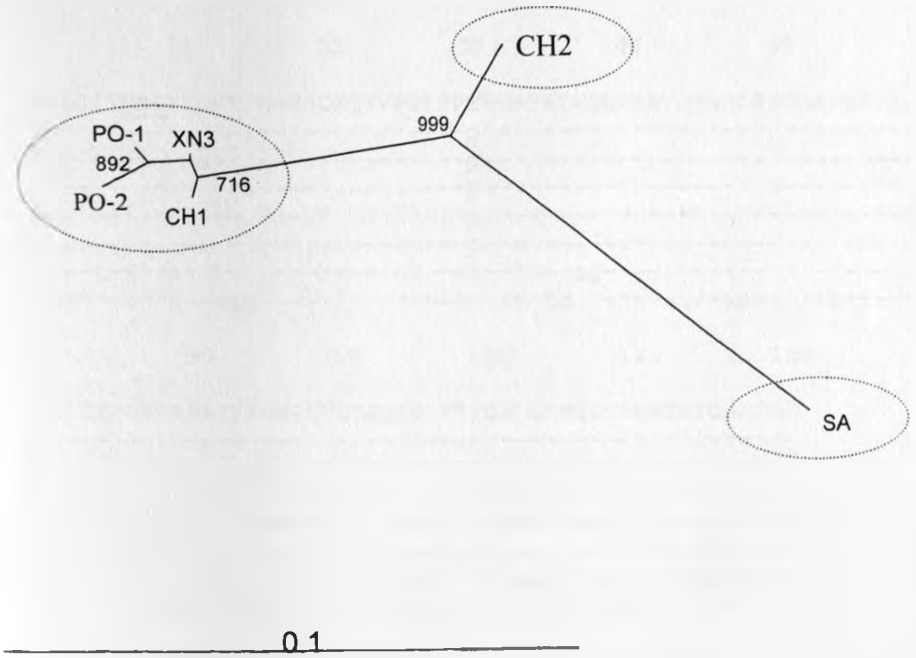

Fig. 2: Unrooted neighbour-joining tree of the 3' NTR nucleotide sequences of SPVG constructed with ClustalX v1.83. Bootstrap analysis was performed using 1000 bootstrap samples. Trees were visualized with TreeView v 1.65.

Furthermore, the 3' NTR sequence identity among SPVG isolates (84-99%) studied is within the range (83-99%) given for strains of a virus (Frenkel *et al.*, 1989; Shukla *et al.*, 1994).

The sequences of SA and CH2 from China were consistently demonstrated to be divergent in the CP, 3' NTR and CP N-terminal aa sequences, and they should therefore be regarded as distinct strains while the rest of the isolates comprise a genetically distinct but highly homogeneous group (strain). The observation that several ELISA-positive samples from S. Africa did not yield a PCR product with the SPVG primers derived from CH1, CH2 sequences and those of other isolates suggests that this aberrant type (strain) of SPVG is widespread in S. Africa. It is also likely that there are other intermediate or more deviant strains (with regard to sequence variability) that may have escaped detection in serological tests and/or PCR. Further work on the biological variability among these SPVG strains will expand the knowledge of this potyvirus.

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                  10         20         30         40         50         60         70
LSU-1     SAEEIYDAGKTGNT.GRGRGRGTVPPPPPTPGAPRTGDLPPAVQTGPLPPGAASKPPIIEEIPQPESPRT
PO-1      -t--t----n----.--------------p---------------------------------l------a
PO-2      ----t------e--.--------------p---------------------------------l------a
EG        --------------.--------------p-----------------------------------------
XN3       --------------.--------------p-----------------------------------------a
CH1       --------------.--------------p---------------------------------l-------
CH2       -t--t----n----.--------------p--t---tg-------------------------------s
SA        -t-------n--agg--------------p--t-ts..--------vp-----a--------k------s

          80         90         100        110        120        130

LSU-1     KALREARGKAPATIPDSRGVDTSQIPSFTPGRDQTMTPTPQRTSTGARDRD
PO-1      ----------------------------------------------i-v----
PO-2      ----------------------------------------------v----
EG        ----------------------------------------------v----
XN3       ----------------------------------------------v----
CH1       ----------------------------------------------ev----
CH2       -------e-----v-----------------e-----a-----a-v----
SA        -------------v-e-----a-dr-q-re-atp-gaivn------v----
```

Fig. 3: Alignment of the N-terminal amino acid (aa) residues preceeding the trypsin-resistant core of the capsid protein of SPVG. Amino acid positions identical to those of LSU-1 are indicated by dashes. The aa deletions of isolate SA are indicated by dots whereas the DAG motif is underlined and in bold. The origins and accession numbers of the isolates are all presented in Table 1.

7.0 CHAPTER SEVEN

BIOLOGICAL, SEROLOGICAL AND MOLECULAR VARIABILITY OF GEOGRAPHICALLY DISTINCT ISOLATES OF *SWEET POTATO FEATHERY MOTTLE POTYVIRUS*

7.1 INTRODUCTION

Sweet potato feathery mottle virus (SPFMV; Genus *Potyvirus;* Family *Potyviridae*) is the most widely distributed virus of sweet potato worldwide (reviewed by Karyeija *et al.,* 1998). The virus was first described in USA by Doolittle and Harter (1945). It was first reported in 1957 in East Africa (Kenya, Uganda and Tanzania), and named sweet potato virus A (Shefield, 1957) and in West Africa in 1976 under the name Sweet potato vein clearing virus (Schaefers and Terry, 1976). Several aphid species including *Aphis gossypii, A. craccivora, Lipaphis erysimi,* and *Myzus persicae* transmit SPFMV in a non-persistent manner (Moyer and Kennedy, 1978; Stubbs and McLean, 1958).

Leaf symptoms caused by SPFMV are mostly mild but may include vein clearing, irregular chlorotic patterns (feathering) along the leaf mid-rib and chlorotic spots which may have purple pigmented borders especially on older leaves. Depending on the sweet potato cultivar and virus strain, the storage roots of infected plants may show external necrosis (Cali and Moyer, 1981; Campbell *et al.,* 1974; Moyer and Cali, 1985; Moyer and Kennedy, 1978; Clark and Moyer, 1988). When infecting alone, effects of SPFMV infections are not conspicuous since its movement and/or replication in most sweet potato cultivars is restricted (Gibson *et al.,* 1998; Karyeija *et al.,* 2000). Although SPFMV has been associated with insignificant or no yield reduction in most sweet potato cultivars, there are reports that virus-free sweet potato plants yield more than those infected by SPFMV (Over de Linden and Elliott, 1971; Joubert *et al.,*

1979; Pozzer *et al.*, 1994; Gibson *et al.*, 1997; Cheramgoi, 2003). The main economic impact is, however, realised when SPFMV infects sweet potato in the presence of SPCSV leading to sweet potato virus disease (SPVD). SPVD causes yield losses of up to 90% (Melissa *et al*, 1996, Gibson *et al.*, 1998; Karyeija *et al.*, 2000; Gibson and Aritua, 2002; Gutiérrez *et al.*, 2003).

The complete nucleotide sequence and genome organization of a severe isolate of SPFMV (SPFMV-S) from Japan has been determined (Sakai *et al.*, 1997), indicating that the SPFMV genome is about 11 kb which is somewhat larger than that of a typical potyvirus which is10kb, due to its considerably larger P1 gene. Isolates of SPFMV have been assigned to the Common (C), Russet Crack (RC), Ordinary (O) and East African (EA) strain groups on the basis of their genetic characteristics (Moyer and Salazar, 1989; Abad and Moyer, 1992; Querci *et al.*, 1992; Ryu *et al.*, 1997; Kreuze *et al.*, 2000).

Differences/similarities in sequences have been a useful criterion for differentiating closely related virus species or virus strains. The distinction of virus strains is particularly relevant when employing the pathogen-derived resistance (PDR) concept, such as use of virus segments for plant transformation. A number of CP gene sequences of African SPFMV isolates are available, the majority of which are from Uganda (Kreuze *et al.*, 2000; Mukasa *et al.*, 2003b). However, the molecular diversity of SPFMV isolates from Kenya has not been determined and more information of isolates from geographically diverse locations could lead to a better understanding of the variability of SPFMV and the prospects of using PDR against SPFMV. It is hypothesised that there may be more variability among East African SPFMV similar to that reported from USA.

In this study, the biological, serological and molecular properties of geographically diverse isolates of SPFMV are presented and compared to assess its variability. To date, there are no means to differentiate the strains of SPFMV in diagnostic tests and therefore attempts were made to raise differentiating antibodies using purified virus preparations and/or bacterially expressed CPs of SPFMV for immunisation of mice. Possible variations in host range among selected isolates of SPFMV were also studied.

7.2 MATERIALS AND METHODS

7.2.1 *Virus isolates*

Virus-infected sweet potato (*Ipomoea batatas* L.) cuttings were obtained from Kenya, Uganda and South Africa during surveys of sweet potato crops in 2000-2001. SPFMV-infected sweet potato cuttings from Zambia, Tanzania, and Madagascar were obtained from BBA's isolate collection. D. Louro and R.W. Gibson provided infected sweet potato plants from Portugal and Spain, respectively (Table 1). Detrixhe P. provided an isolate from China. The plants were confirmed to be infected with SPFMV by DAS-ELISA. Isolation of SPFMV was achieved by sap transmission on to a range of indicator plants followed by serial local lesion transfer onto local lesion hosts namely *Chenopodium quinoa, C. murale* or *C. amaranticolor* and graft inoculation onto *I. setosa.*

7.2.2 RNA extraction, RT-PCR and cloning of PCR products

Total RNA was isolated from 100 mg of SPFMV-infected leaves using the Nucleospin® Plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesised as follows; a mixture containing 0.3 µl oligo(dT) primer (100 µ*M*), 3 µl of total nucleic acid (TNA) and 9.5 µl RNAse-free water was

incubated at 70 °C for 10 min and on ice for 2 min. The reverse transcription mixture (1.9 µl H_2O, 4 µl of 5x RT buffer, 0.3 µl of 25 m*M* dNTPs, 0.5 µl ribonuclease inhibitor (Promega) and 0.5 µl of maloney murine leukaemia virus reverse transcriptase (MMLV-RT) (Farmentas) in a total volume of 7.2 µl was added, followed by incubation at 42°C for 1 h, and 70°C for 10 min.

Table1: *Sweet potato feathery mottle virus* isolates used in this study

Isolate	Country	Location	Reference	Acc. No
MD1/1	South Africa		This study	AY459601
Canar 3	Canary Islands		This study	AY459600
TZ 4	Tanzania	Mwanza	This study	AY459598
Rak 6e	Uganda	Rakai	This study	
Nam12	Uganda	Namulonge	This study	AY459596
Ruk 4	Uganda	Rukungiri	This study	
Arua 10	Uganda	Arua	This study	AY459595
KY 97/5S	Kenya	Kisumu	This study	AY459594
KY 25/4A	Kenya	Nyeri	This study	
KY 85/7S	Kenya	Kisii	This study	AY459593
KY 115/1S	Kenya	Kwale	This study	
KY 51/9S	Kenya	Kakamega	This study	AY459591
KY 46b	Kenya	Busia	This study	
KY 54/9S	Kenya	Kakamega	This study	AY459592
KY 45/3S	Kenya	Machakos	This study	
Put	Madagascar		This study	AY459597
Por	Portugal		This study	AY459599
Spain1-C	Spain		This study	
Spain 1-EA	Spain		This study	
XN3	China		This study	AY459602
Zambia	Zambia		This study	
SPFMV-C	USA		Abad *et al.*, 1992	S43451
SPFMV-RC	USA		Abad *et al.*, 1992	S43450
MAD	Madagascar		Kreuze *et al.*, 2000	AJ010700
SPFMV-O	Japan		Mori *et al.*, 1994	D16664?
SPFMV-S	Japan		Sakai *et al.*, 1997	D38543
Sor	Uganda	Soroti	Mukasa *et al.*, 2003	
MBL	Uganda	Mbale	Kreuze *et al.*, 2000	AJ010701
Nam 1	Uganda	Namulonge	Kreuze *et al.*, 2000	AJ010704
SPFMV-6	Argentina		Alvarez *et al.*, unpubl.	U96624
BAU	Nigeria	Bauchi	Kreuze *et al.*, 2000	AJ010699
TZ2	Tanzania		Mukasa *et al.*, 2003	
JIII	South Africa		This study	

Table 2: ***Sweet potato feathery mottle virus*** antisera used in this study

Antiserum	Source
SPFMV-46b	BBA
SPFMV-C	CIP
SPFMV-C	J. Moyer
SPFMVRC	J. Moyer
SPFMV-1C4-B3	BBA
SPFMV-4F5-G6	BBA
SPFMV-1A10-D2	BBA
SPFMV-7H8G2	CIP

Two µl of the cDNA were used for PCR in a MJ Research thermocycler (Massachusetts, USA). Potyviridae-specific degenerate primers (Gibbs and Mackenzie 1997) were used for the amplification of an approximately 1800 nt long fragment from the 3' end of the genome. The amplified DNA fragments were gel purified (1.0% agarose gel) using the Nucleospin® Extract kit (Macherery-Nagel, Düren, Germany) and ligated into pGEM®-T cloning vector (Promega Corp.) and transformed into *Escherichia coli* (strain DH5α) following standard procedures (Sambrook *et al.*, 1989). The recombinant plasmids were isolated from overnight bacterial cultures using the Nucleospin® Plasmid kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. DNA sequencing was done using the universal SP6/T7 primers by a commercial company (MWG Biotech, Ebersberg Germany). Nucleotide and predicted amino acid sequences were aligned using the DNAMAN (Lynnon BioSoft, Version 4.02) programme. Phylogenetic analyses were done for the CP and 3' NTR sequences. Phylogenetic trees were constructed using the neighbour joining method of Clustal X version 1.83 (Jeanmougin *et al.*, 1998). One thousand bootstrapped samples were generated to estimate the statistical significance of the branching. Trees were visualized using the TreeView program (Page, 1996).

7.2.3 Experimental host range of selected SPFMV isolates

Based on their geographical origin and CP sequences, 6 isolates were selected to assess the differences in their biological properties. The isolates KY46b, KY115/1S, KY24/4A MD 1/1, Rak6e and Zambia were sap-inoculated onto 15 species of indicator plants belonging to 5 genera (Table 1). Mechanical inoculation was carried out as outlined in chapter 4. Two non-inoculated plants of each test plant species were included as checks. The plants were observed for symptom development and tested individually for SPFMV infection in DAS-ELISA 21 days post inoculation (dpi).

7.2.4 Monoclonal antibody (MAb) production against bacterially expressed CP and purified virions

For the production of MAbs, two isolates KY115/1S and KY25/4A representing the O and C strain groups, respectively, were selected for propagation in indicator hosts. Isolate KY115/1S was propagated in *C. quinoa* but isolate KY25/4A did not infect any of the test plants used and, attempts to purify it from *I. setosa* leaves yielded very small amounts of virus. Attempts were therefore made to express the CP gene of both isolates in *E. coli* and to use the bacterially expressed CP as immunogens for the immunization of mice.

Expression of *Sweet potato feathery mottle virus* CP in *E. coli*

A 945 and 939-bp DNA fragment of isolate KY115/1S and KY25/4A, respectively, comprising the entire CP gene were amplified using two isolate-specific primer pairs. The primer pair for KY25/4A was upstream 25/4A *Nco*I 5' AC**C/CATGG**CATCTGGCAA 3' and downstream 25/4A *Xho*I 5' CA**C/CTCGAG**CTGCACACC 3'; the primer pair for KY115/1S was upstream 115/1S *Nco*I 5' AC**C/CATGG**CATCTAGTGAGAA 3' and downstream 115/1S *Xho*I 5' CAC/**CTCGAG**TTGCACAC 3' with *Nco*I and *Xho*I restriction sites (in bold). The 50 µl

reaction volume contained 5 µl of 10x Pfu DNA polymerase buffer, 0.4 µl dNTPs (25mM each), 1.4 µl of each primer (100 p*M*/µl), 0.5 µl *Pfu* DNA polymerase (3 U/µl) (Farmentas), 43 µl of sterile deionized water, and 2 µl of cDNA. PCR amplification was done at 94°C for 4 min, followed by 30 cycles at 94°C for 30 sec, 55°C primer annealing 1 min, extension at 72°C for 3 min and a final extension step at 72°C for 8 min. The amplified products were excised from the gel and purified using the Nucleospin extract kit.

Digestion of DNA

The expression vector pET 28(a) (Novagen) carrying a C-terminal tag of six histidine residues was digested by *Xho*I and *Nco*I restriction enzymes (Fermentas). In a final volume of 10 µl, 2 µl of 10x Y^+ Tango buffer (Fermentas) was mixed with 1 µl each (10 U/µl) of *Nco*I and *Xho*I. Two µl of the plasmid DNA was made up to 10 µl using sterile deionized water. The reaction was incubated at 37°C for 1.5 h. The digested DNA was gel-purified following the manufacturer's protocol. The cut vector was eluted in 25 µl of elution buffer and stored at –20°C till use. PCR products (30 µl) were digested in 8 µl of 10x Tango buffer, 1 µl of sterile deionized water and 0.5 µl (10 U/µl) each of *Xho*I and *Nco*I. The reaction was similarly incubated at 37°C for 1.5 h. and gel-purified using the Nucleospin Extract kit.

Cloning of CP fragments into pET (28a) and transformation into **E. coli**

The recombinant DNA was ligated into the pre-digested pET-28a expression vector. To 5 µl of 2x rapid ligation buffer (Promega), 1 µl of T4 DNA ligase (Promega) and 2 µl of the digested pET-28a vector were added to a 0.5 ml tube. Digested PCR product (3 µl) was added to the ligation reaction mixture. The ligation reactions were incubated at 4°C overnight and transformed into *E. coli*. Competent cells of *E. coli* strain BL21 DE (50 µl) were added just after thawing into a 1.5 ml tube containing 5 µl of the ligation reaction. The tube was gently flicked to

mix the contents and incubated on ice for 20 min. The cells were placed in a water bath at 42°C for 45-50 s and immediately returned to an ice bath for 2 min. About 950 µl of LB medium was added to the tubes and incubated for 1.5 h at 37°C with constant shaking (150 rpm). After incubation, bacterial cells were pelleted at 4,000 rpm for 5 min and resuspended in 200 µl of LB medium before plating in duplicate LB plates supplemented with kanamycin (30 µg/ml). The plates were incubated at 37°C overnight. Colony screening for inserted target DNA was done by PCR using the T7 promoter and the T7 terminator standard primers. Colonies that contained the correct size of insert were inoculated in 5 ml of LB medium containing kanamycin and incubated overnight at 37°C with constant shaking at 150 rpm. Plasmids were isolated from overnight bacterial cultures using the Macherey-Nagel kit following the manufacturer's protocol. The recombinant clones were sequenced to confirm that the inserts were ligated in-frame and contained no stops.

Expression of target protein

A colony containing the desired CP gene was selected and streaked on fresh LB agar supplemented with kanamycin at 30µg/ml and allowed to grow at 37°C overnight. A single colony was used to inoculate 50 ml of LB medium containing kanamycin in a sterile 250 ml Erlenmeyer flask. The cultures were allowed to grow with shaking at 37°C until an OD_{600} of 0.70 was reached (after *ca.* 3 h). One ml of non-induced culture fluid was taken before addition of IPTG (induction) to a final concentration of 1 mM and growth continued for additional 4 h. Before harvesting the cells, one ml of the induced culture was collected by centrifugation at 8000 rpm for 20 min at 4°C. The pellet was stored at -20°C till needed.

Protein purification

The frozen cell pellets were thawed on ice for 15 min and resuspended in 5 ml of buffer B (lysis buffer, Appendix 3). Lyzozyme (1 mg/ml) (Boehringer Mannheim, Germany) was added to the cell suspension and incubated on ice for 30 min. The cell suspension was sonicated (200-300W) on ice by 6- to 10-sec bursts with pauses of 10 sec in between. Using a sterile syringe, the lysate was drawn several times through a narrow gauge needle to reduce its viscosity. The lysate was centrifuged at 8000 rpm for 25 min at 4°C. The supernatant was saved as the soluble protein fraction while the pellet was resuspended in buffer B (lysis buffer, Appendix 3) as the insoluble protein fraction.

The purification of the histidine-tagged proteins was done using 50% a Ni-NTA slurry (Qiagen Corp) following the manufacturer's instructions. Ni-NTA slurry (1 ml) was added to 4 ml of clarified lysate and mixed gently by shaking at 200 rpm on a rotary shaker for 45 min. The lysate-NTA mixture was filled into a column with the bottom outlet capped. The column was washed twice with 4 ml of wash buffer C (Appendix 3) and eluted as eluate E_1. The proteins were first eluted (E_2) in 2 ml of elution buffer D and then 2 ml of elution buffer E (Appendix 3) to collect eluate E_3.

Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) and electro-blot immunoassay (EBIA)

SDS-PAGE was performed in a vertical gel electrophoresis apparatus (Mighty small II, Hoefer Scientific Instruments, San Franscisco) using the buffer systems of Laemmli *et al.* (1970). For SDS-PAGE analysis, 10 µl each of (i) uninduced, (ii) induced, (iii) soluble, and (iv) insoluble fractions were mixed with 2.5 µl of 5x sample buffer and boiled for 5 min in a water bath. Samples were loaded into sample slots and electrophoresed alongside low range protein

standards (Biorad Laboratories, CA) as a molecular size marker at 80V until the bromophenol blue reached the resolving gel and continued at 120V till it reached the bottom of the gel. Protein bands were visualized after staining the gel with Coomassie™ brilliant blue for at least 3 h followed by destaining overnight in destaining solution (Appendix 3).

Preparation of samples for electro-blot immuno assay (EBIA)

About 100 mg of infected and healthy *C. quinoa* leaf material was ground in liquid nitrogen and homogenized in 4 ml of 1x sample buffer (Laemmli *et al.*, 1970) and boiled for 5 min. The boiled homogenates were clarified by centrifugation at 13,000 rpm in a centrifuge for 5 min. Aliquots of 10 µl of expressed protein and purified virus preparations were similarly boiled for 5 min.

EBIA was conducted essentially according to the procedure described by Towbin *et al.* (1979). SDS-PAGE was conducted as outlined above; however, the size marker used were pre-stained marker proteins (Biorad, Munich). Following SDS-PAGE, the gel was carefully removed from the glass plates and immediately used for transfer of the protein bands onto the nitrocellulose membrane (electro-blotting). The assembly parts for electroblotting were pre-wetted in transfer buffer [25 mM Tris base, 192 mM glycine, pH 8.3, containing 20% (v/v) methanol] and mounted in the following order; Cathode, sponge pads, filter paper (Whatman 3 mm), SDS-PAGE gel, nitrocellulose membrane, filter paper, sponge pads, anode. Care was taken to avoid entrapping air bubbles between the membrane and the gel before submerging in transfer buffer. The proteins were electro-blotted from the polyacrylamide gel onto the nitrocellulose membrane overnight at 220 mA and at 4°C.

After transfer, the membrane was placed in blocking solution containing 3% gelatin (Biorad) in TBS (Appendix 3) and agitated on a shaker for 60 min at room temperature to block all free protein binding sites on the membrane. The lane containing the pre-stained marker proteins was cut off and washed three times in T-TBS. The membrane was then washed three times in T-TBS with 5 min between washes before incubation for 1 h with alkaline phosphatase labelled antibodies to SPFMV-RC (Moyer) diluted 1:1000 (v/v) in buffer with shaking at room temperature. The membrane was washed three times for a minimum of 5 min between washes in T-TBS and rinsed with water before immersion in substrate (NBT/BCIP) solution (Appendix 3). The reaction was stopped after 20 min by rinsing the membranes in double distilled water and drying them on paper.

7.2.4.2 Propagation and purification of SPFMV KY 115/1S

The SPFMV isolate (KY115/1S) obtained from a sweet potato plant containing a mixed infection with SPCSV in Kwale district, Kenya. The isolate was obtained by serial transfer of a single local lesion onto *C. quinoa*. The isolate was confirmed to be pure as assessed by DAS-ELISA using antisera against sap-transmissible sweet potato viruses such as SPFMV, SPMMV, SwPLV, SPCFV, SPV2, CMV and SPVG. The isolate was sap-inoculated onto 200 seedlings of *C. quinoa*. Three to four weeks later, leaves bearing chlorotic local lesions were harvested and stored at -80°C until used for purification.

SPFMV was purified following the procedure described by Lisa *et al.* (1981) with slight modifications. Using a warring blender at full speed, 200 g of frozen *C. quinoa* leaves infected with SPFMV were homogenized in 400 ml 0.5 M K_2HPO_4 (pH 8.5), containing 0.02 M Na_2SO_3, 0.01 M DIECA, and 5 mM EDTA. Following the addition of 60 ml of chloroform, the mixture was again homogenized briefly. The homogenate was clarified by low speed centrifugation (8,000 rpm) for 15 min at 4°C in a Sorvall GSA rotor. The aqueous phase was filtered through a

large laboratory tissue and then subjected to high-speed centrifugation (30,000 rpm, Beckman 45Ti rotor, 4°C; 2 h). The resulting pellet was resuspended in 0.05 M Na-citrate buffer containing 5 mM EDTA. For immunization of mice, the virus was purified by two successive isopycnic gradient centrifugations (40% w/v caesium chloride; 35,000 rpm, Beckman SW55Ti rotor; 10°C; 17 h). The opalescent bands observed under incident light illumination were withdrawn using a peristaltic pump. Virus particles were diluted in 0.05 M Na-citrate buffer and sedimented by centrifugation (35,000 rpm; Beckman 45Ti rotor; 10°C; 4 h). At all purification steps, the purified virion preparation was checked by electron microscopy for yield and purity. The yield of the virus preparation was analyzed by UV spectrophotometry using a Hitachi U-3200 spectrophotometer. The yield was calculated using the formula:

$$\text{Virus yield [mg/ml]} = \frac{A_{260} \times \text{dilution factor}}{2.8\ [\text{Extinction coefficient of potyviruses (Noordam, 1973)}]}$$

Virus pellets were resuspended in 0.5 ml Na-citrate buffer and stored at –20°C until use. Purified virus was used for the immunization of mice, serological tests and SDS-PAGE analysis.

7.2.4.3 Production of monoclonal antibodies

A 100-µl aliquot of purified virus preparation and 150 µl of dialysed CP fusion protein were emulsified in an equal volume of Freund's incomplete adjuvant and administered sub-cutaneously to each of 3 mice. A further subcutaneous injection was administered after approximately 4 weeks. Three days prior to the fusion experiment, a booster injection containing 200 µl of purified virus preparation and fusion protein (without adjuvant) was intraperitoneally administered to induce the proliferation of antigen specific B-lymphocytes.

Seven days prior to the fusion, myeloma (FOX-NY) cells were taken from a stock in liquid nitrogen and thawed in a water bath (37°C). The cells were first washed in DMEM and then grown at a 6% CO_2 atmosphere in 10 ml of Dulbecco's modified eagle medium (DMEM, Appendix 5) containing 20 % foetal calf serum (FCS) in a Petri dish at 37°C. After about 3 days (when the cells had multiplied enough to almost cover the bottom of the Petri dish), the cells were transferred to three 250 ml culture bottles filled with 20 ml of DMEM supplemented with 20 % FCS. When about 70% of the bottom surface of the bottle was covered, the FOX-NY cells were considered ready for fusion.

Production of hybridomas

Thymus glands of two 3- to 4-week-old mice were aseptically dissected and macerated in 10 ml DMEM in a sterile Petri dish. The resultant cell suspension was centrifuged at 900 rpm for 10 min. The pelleted thymocytes were resuspended in 10 ml of HAT medium [DMEM containing 20 % fetal calf serum, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine], and incubated at 37°C till use. Using a sterile pasteur pipette, the myeloma cells were gently rinsed off from the walls of the culture bottle and centrifuged at 900 rpm for 10 min. An immunized mouse was sacrificed and its spleen cells were suspended in 10 ml DMEM as outlined above for the thymus cells. The cells were centrifuged at 900 rpm for 10 min. The resulting pellet was resuspended in 10 ml DMEM containing the myeloma cells (above) and centrifuged again for 10 min at 900 rpm in a sterile falcon tube. The supernatant was decanted and the spleen cells were fused with the mouse myeloma cell line FOX-NY using standard procedures (Galfrè and Milstein, 1981). The tube was half-immersed in a water bath at 37°C. Using a 5 ml sterile pipette and with gentle stirring, one ml of 50 % (w/v) polyethylene glycol 4000 (Serva) solution was slowly added over a period of 1 min. Then the cell suspension was further diluted by slowly

adding 1 ml, 2ml and 6 ml of DMEM over periods of 1 min, 1 min and 3-5 min, respectively. The 10-ml cell suspension resulting from the fusion was centrifuged and the sedimented cells mixed with the 10-ml thymocyte suspension and diluted in 60 ml of HAT medium. The cells were seeded in six 96-well culture plates (100 µl/well). The culture plates were incubated at 37°C and 6% carbon dioxide. Four days later, 100 µl of HAT culture medium was added to each well of the culture plate. The culture medium was removed from the plates by aspiration one week after the fusion and fresh medium (200 µl) was added to each well.

Screening for antibody producing hybridomas

Supernatant fluids from the culture wells were screened for the production of SPFMV-specific antibodies by testing the supernatant of each well against SPFMV infected and healthy *C. quinoa* extracts in a TAS-ELISA format (Galfrè and Milstein, 1981; Adam *et al.*, 1991; Franz *et al.*, 1996). Plates were coated with 100 µl of IgG to SPFMV (1:1000 v/v) in coating buffer and incubated at 37°C for 3 h. Extracts from SPFMV-infected (homologous antigen) and healthy *C. quinoa* leaves in sample buffer (1:20 w/v) were loaded in alternate rows of wells of the coated plates and incubated at 4°C overnight. The plates were washed and two 100-µl aliquots of culture supernatant from each well of the culture plates were added to two adjacent wells and treated with SPFMV-infected sap and the other with the non-infected sap. Plates were incubated for 2 h at 37°C, washed and 100 µl of alkaline phosphatase-conjugated rabbit anti-mouse (RAM) IgG (Jackson Immuno Research Laboratories Inc.) diluted (1:2500 v/v) in conjugate buffer added to each well. The plates were again incubated (1.5 h, 37°C) washed and 200 µl of *p*-nitrophenyl phosphate substrate (Roche Diagnostics Corp.) in substrate buffer (1 mg/ml) added to each well. Positive reactions were assessed visually and measured at A_{405} using an ELISA plate reader. Cell cultures secreting antibodies that reacted specifically to SPFMV were transferred to a 24-well plate containing HT medium (HAT without aminopterin).

Cloning of cells producing virus specific antibodies

Cell cultures that were confirmed to secrete SPFMV specific antibodies on the 24-well plates were cloned by the limited dilution method. In position A1 of a 96-well culture plate, 200 µl of DMEM was mixed with 100 µl of cell culture. One hundred µl of the resulting dilution was serially diluted (1:1) from wells A1 to H1. The cells were allowed to settle at the well bottom (ca. 10 min) and then counted under the phase contrast microscope. A well containing about 100 cells was selected and the resuspended cells diluted in a further 18 ml DMEM containing 20% FCS and 20 U/ml interleukin 6 (IL-6). Each well of the culture plate was filled with 200 µl of cell suspension and incubated at 37°C and 6% CO_2. After 5 days, all the wells of the culture plate that contained single-cell colonies were identified under the microscope. The culture supernatants from single-cell colonies were first tested against infected and non-infected leaf extracts as described above. After transfer of selected sub-clones from 24-well plates, the resulting culture media were tested similarly against several SPFMV isolates in a TAS-ELISA format as described earlier. Cell lines that reacted specifically to SPFMV were re-cloned and tested in TAS-ELISA. Isotyping of the three MAbs raised against purified virions of SPFMV was done using the mouse antibody isotyping kit (HyCult Biotechnology, Netherlands) following the manufacturer's instructions.

7.3 RESULTS

Sequence comparisons and phylogenetic analysis

The sequences of the 3' region of the SPFMV RNA comprising the CP gene and 3' non-translated region were determined for 21 field isolates. Fifteen of the isolates had CP coding

regions that were 945 nucleotides (nt) long and encoded 315 amino acids (aa), whereas 7 of the isolates had CP coding regions spanning 939 nt encoding 313 aa. This difference in length results from a deletion of 6 nt (2 aa) from position 190 to 195 of the CP gene (Fig. 1).

Alignment of the deduced CP aa sequences of the above mentioned isolates along with previously published CP sequences of SPFMV revealed conserved domains characteristic of potyviral CPs. Except for the C strain isolate reported from the USA (Abad *et al.*, 1992), the CP aa sequences of all the isolates contained at + 9-11 position of the CP the aspartic acid-alanine-glycine (DAG) amino acid triplet (Fig. 1). The majority of non-synonymous nt changes and aa variability occurred at the CP N-terminus (Fig. 1), a region known to be highly variable (Shukla and Ward, 1989b), whereas the CP core and carboxyl terminus was highly conserved (data not shown). The cleavage site between the NIb and CP genes of all the isolates was VHYQ/S, whereas at the CP carboxyl end, the sequence was variable with the majority of the isolates' CP ending with MRGVQ/- and only isolates KY97/5S, KY51/9S, MD1/1, and Spain1-C terminating with MRGVH/-.

Comparison of the CP genes revealed nt sequence identities ranging from 76.0 to 97.8% and aa sequence similarities ranging from 78.9% to 98.4% between the isolates (Table 2). Surprisingly, a few isolates showed a higher level of aa than nt sequence variability; for instance Zambia and KY46b differed from each other by 7.9% and 5.9% at the aa and nt levels, respectively (Table 2).

Phylogenetic analysis of the CP nt sequences revealed two distinct clusters. One large cluster was sub-divided into three groups, namely the O, RC and EA strain groups (Fig. 2). The other very distinct cluster comprised all SPFMV isolates of strain group C. Isolates of strain group C had CP sequence similarities ranging from 95.2 to 98.4 % and from 94.4 to 96.8 % at the aa and

at sequence levels, respectively. The SPFMV-C isolates sequenced originated from South Africa (MD1/1), Kenya (KY25/4A, KY97/5S, KY51/9S, KY45/3S), Uganda (Nam 12) and Spain (Spain1-C) indicating its widespread nature. The overall CP aa sequence similarity between the strain group C and the other 3 phylogenetic groups was 82%. The two sequences Spain 1-EA and Spain1-C were obtained from one sweet potato cutting (Spain 1) indicating that strain mixtures occur.

```
SPFMV-RC        SSERTEFKDA GADPPAPKPK NIPPPPTITE VTDPEDPKQA ALRAARAKQP 50
SPFMV-O         SGEKTEFKDA GANPPDPKSK INPPPPTITE IVDPEDPKQA ALKAARAKQP
KY115/1S        SSEKTEFKDA GVNPPAPKSN INPPPPTITE VVDPEDPKQA ALKAARAKQP
Zambia          SDEKTEFKDA GADPPAPRPK NIPPPPTTTE ITDPEDPKQA ALKAARAKQP
KY46b           SNERTEFKDA GADPPAPKPK SDPPPPTITE ITDPEDPMQA TLKAARAKQP
SPFMV-C         SICDPEFKNA GANPPAPKPK GAFTAPEITE VTEPEDPKQA ALRQARQKQP
KY51/9S         SNNPTEFKDA GANPPAPKPK GPYAAPEITE VTEPEDPKQA ALREARQKQP
KY45/3S         SGNPPEFKDA GANPPAPKPK GPFTAPEITE VTEPEDPKQA ALREARQKQP
KY25/4A         SGNPPEFKDA GANPPAPKPK GPYTAPEITE VTEPEDPKQA ALREARQKQP
Nam 12          SGNPPEFKDA GANPPAPKPK GPYTAPEITE VTEPEDPKQA ALREARQKQP
Spain1          SGNPLEFKDA GANPPAPKPK GPFVAPEITE VTEPEDPKQA ALREARQKQP
KY97/5S         SGNPPEFKDA GANPPAPKPK GPFTAPEITE VTEPEDPKQA ALREARQKQP
MD1/1           SGNPPEFKDA GANPPAPKPK GPYTAPEITE ITEPEDPKQA ALREARQKQP
consensus       *    *** * *  ** *          *  **    **** **  *   * ***

SPFMV-RC        ATIPESYGRD TSKEKESIVG ASSKGVRDKD  80
SPFMV-O         ATIPESYGRD TSKEKESIVG TSSKGVRDKD
KY115/1S        ATIPESYGRD TSKEKESIVG ASSKGVRDKD
Zambia          AVIPESYGRD TSKERESIVG TSSKVVRDKD
KY46b           AVIPESYGRY TSKERESIVG TSSKGVRDKD
SPFMV-C         AVTPESYGRD TTKGEKSMRS VSPQRVKDKD
KY51/9S         AVTPEPYGRD T..GEKSMRS VSPRRVKDKD
KY45/3S         AVTPESYGRD T..GEKSMRS VSPQRMKDKD
KY25/4A         AVTPESYGRD T..GEKSMRS VSPQRVKDKD
Nam 12          AVIPESYGRD T..GEKSMRS VSPQRVKDKD
Spain1          AVTPESYGRD T..GEKSMRS VSPQRVKDKD
KY97/5S         AVIPESYGRD T..SEKPMRS VSPQRVKDKD
MD1/1           AVTPESYGRD T..GERPMRS VSPQRVKDKD
Consensus       *  ******* *           *     ***
```

Fig. 1. Alignment of N-terminal CP sequences of selected SPFMV isolates. Dots (.) represent deletions of amino acids whereas * depicts identical amino acids in the consensus sequence. The NAG motif in the SPFMV-C (Abad *et al.*, 1992) is underlined. The accession numbers of the sequences used in this alignment are shown in Table 1.

Phylogenetic analysis grouped the isolate XN3 from China together with the RC isolate from North America (Abad *et al.*, 1992) and the S isolate from Japan (Sakai *et al.*, 1997). These isolates can therefore clearly be assigned to strain group RC (Fig. 1). The three isolates KY115/1S, Arua 10 and TZ4 from Kenya, Uganda and Tanzania, respectively, clustered with the isolate O from Japan (Mori *et al.*, 1994) and the isolate BAU from Nigeria (Kreuze *et al.*, 2000), thereby providing the first evidence for the occurrence of the strain group O isolates in East Africa. As expected, isolates KY85/7S, KY54/9S and KY46b (all from Kenya), Ruk55-2 and Rak6e (both from Uganda) and Put from Madagascar were assigned to the 'East African' strain group (Kreuze *et al.*, 2000). Although the isolate from Zambia was slightly divergent, it also clearly belonged to strain group EA. Surprisingly, isolates from the Canary Islands, Portugal and Spain also clustered together with the 'East African' strain group. No correlation was thus established between any of the strain groups and the geographical origin of the isolates.

Caption to Fig 3 next page ➡

Fig. 3. Neighbour-joining tree showing the relationships of SPFMV coat protein nucleotide sequences. The sequences of isolates in bold were obtained from the sequence databases and their accession numbers are show in Table 1.

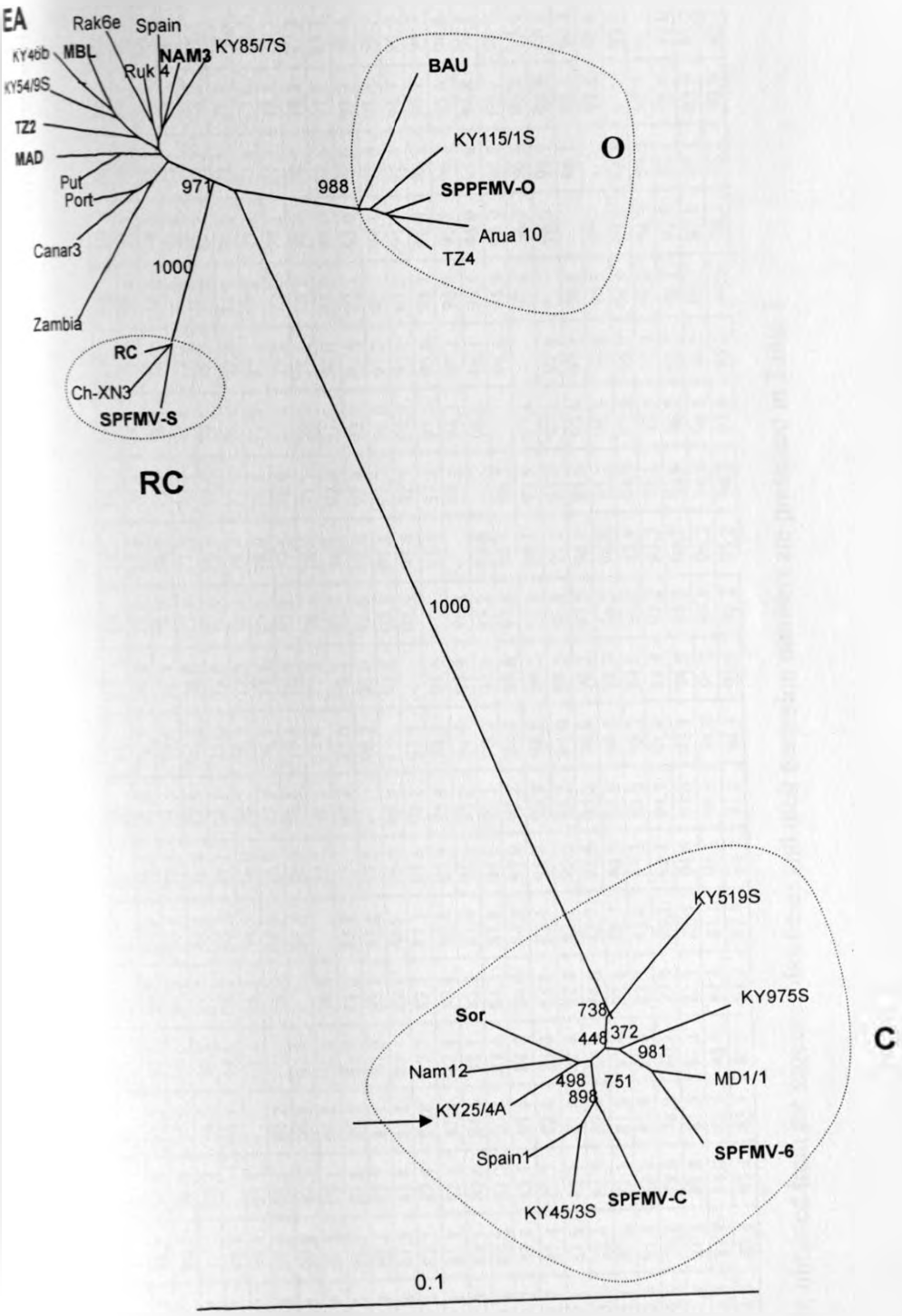
EA
Rak6e
Spain
KY46b
MBL
NAM3
KY85/7S
KY54/9S
Ruk 4
TZ2
MAD
Put
Port
971
988
BAU
KY115/1S
O
SPPFMV-O
Arua 10
TZ4
Canar3
1000
Zambia
RC
Ch-XN3
SPFMV-S
RC
1000
KY519S
738
KY975S
Sor
448
372
981
Nam12
498
751
MD1/1
898
KY25/4A
Spain1
SPFMV-6
KY45/3S
SPFMV-C
C
0.1

Table 2: Pairwise percent coat protein amino acid sequence identity (below diagonal) and nucleotide sequence identity (above diagonal), among 25 isolates of SPFMV

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 SPFMV-C*	-	95.2	96.3	94.4	93.3	94.7	96.8	93.9	76.6	77.6	76.5	76.9	77.4	77.8	76.6	78.1	77.5	77.4	77.2	77.2	77.1	76.2	77.4	77.4	77.1
2 KY25/4A	96.2	-	96.8	96.4	95.0	96.6	96.6	96.1	77.3	78.1	77.0	77.4	78.0	78.7	77.2	78.5	78.1	78.0	78.1	78.5	78.3	77.4	78.0	77.7	77.5
3 KY 45/3S	96.2	96.4	-	95.7	94.5	96.2	97.7	95.4	76.4	77.5	76.0	76.5	77.1	77.3	76.1	77.4	77.0	77.2	76.9	77.1	76.9	76.3	76.8	77.0	77.0
4 KY 97/5S	94.6	96.2	98.1	-	94.9	95.5	96.2	96.3	77.1	78.1	77.0	77.6	77.6	78.5	77.0	78.5	78.0	77.8	77.7	78.2	78.2	77.1	77.8	77.4	77.4
5 KY 51/9S	93.3	95.2	95.2	95.5	-	94.4	95.1	94.6	76.9	78.2	76.7	77.2	78.0	78.4	77.2	78.5	77.8	78.2	77.8	78.5	78.3	77.0	77.1	77.8	77.8
6 Nam 12	95.8	96.5	97.8	98.4	95.2	-	96.0	94.9	76.9	77.5	76.7	77.1	77.2	78.0	76.3	77.5	77.3	77.4	77.2	77.8	77.6	76.7	77.4	77.2	77.4
7 Spain 1-C	96.5	96.5	97.8	97.8	95.8	97.4	-	95.5	76.9	77.4	76.4	76.8	77.3	77.5	76.4	77.8	77.2	77.2	77.1	77.3	77.1	76.5	77.2	77.2	77.0
8 MD1/1	94.6	97.8	96.8	97.1	98.8	96.8	96.8	-	77.3	78.1	77.6	77.8	78.4	78.6	77.3	78.7	78.3	78.2	78.0	78.4	78.3	77.3	78.1	77.5	77.3
9 SPFMV-o*	82.2	81.5	81.5	81.8	80.5	81.7	81.8	81.8	-	97.2	97.6	98.1	92.9	94.1	92.6	92.5	94.0	93.0	94.0	92.6	91.7	91.3	93.7	91.9	91.1
10 KY 115/1S	82.5	82.7	82.4	82.7	82.1	83.0	83.1	81.8	97.8	-	96.2	97.0	92.6	93.0	92.4	92.0	93.5	92.3	93.0	92.0	91.9	90.4	92.7	91.5	91.0
11 Arua 10a	81.6	81.8	81.5	81.8	81.2	82.1	82.1	815	96.2	95.9	-	97.6	92.3	93.5	92.2	91.6	93.3	92.3	94.1	92.0	91.4	90.3	92.9	91.3	90.6
12 TZ4	82.2	82.4	82.2	82.4	81.8	82.7	82.7	82.1	97.8	98.1	97.1	-	92.6	93.4	92.7	92.4	94.1	93.2	93.5	92.3	91.7	91.0	93.0	91.7	91.0
13 MBL*	83.2	82.1	82.1	82.4	81.5	82.4	82.4	82.4	95.9	95.9	94.3	95.6	-	95.4	97.5	97.5	96.6	95.7	96.5	96.2	95.7	94.7	95.7	93.3	92.6
14 KY 85/7S	83.2	82.7	82.1	82.4	84.4	83.1	83.1	83.4	95.9	95.2	94.6	95.9	96.8	-	95.2	94.2	97.2	96.0	96.8	96.1	95.6	94.6	96.8	93.2	92.9
15 KY 46b	81.6	81.2	80.8	81.2	80.8	81.4	81.5	81.8	94.0	93.3	93.0	94.3	95.2	95.6	-	96.8	96.1	95.4	95.9	95.6	94.8	94.1	95.2	92.5	91.7
16 KY 54/9S	84.1	83.7	83.4	83.7	83.4	84.0	84.0	84.3	95.9	95.2	94.3	95.6	97.5	97.5	96.2	-	96.5	95.6	95.9	95.8	95.2	99.3	95.3	92.8	92.1
17 Ruk55-2	83.2	82.7	82.4	82.7	82.4	83.0	83.1	83.4	95.9	95.9	94.6	96.5	97.1	98.1	95.9	97.8	-	97.4	96.7	96.8	96.3	95.1	97.4	93.9	93.1
18 Rakai 6e	82.5	82.1	81.8	82.1	81.8	82.4	82.4	82.7	95.6	95.2	94.0	95.2	96.5	97.5	95.2	97.1	97.5	-	96.0	95.7	94.9	94.4	95.9	93.8	93.0
19 Put	84.1	83.1	83.4	83.7	82.4	84.0	84.0	82.7	95.6	95.2	94.9	96.2	97.5	96.8	95.6	96.8	97.1	96.8	-	96.1	95.3	94.4	97.0	93.4	92.9
20 Portugal	82.5	82.1	81.8	82.1	81.9	82.4	82.4	82.7	94.9	94.6	94.3	94.9	96.5	96.8	85.2	96.5	96.8	96.5	97.1	-	97.8	95.8	96.5	94.7	94.0
21 Canar 3	82.2	82.7	81.8	82.1	82.4	82.4	82.7	83.4	93.3	93.3	93.3	94.0	95.2	95.9	94.0	95.9	95.9	94.9	95.6	96.5	-	95.7	95.8	93.8	93.0
22 Zambia	80.0	79.6	79.2	79.6	78.9	80.1	79.9	80.2	92.4	91.7	91.4	92.4	93.2	93.7	92.1	93.3	93.7	93.0	94.3	94.3	92.7	-	94.2	92.8	92.1
23 Spain 1-EA	82.9	81.8	81.5	81.8	81.5	82.1	82.1	82.4	94.6	94.6	93.7	94.9	96.2	96.2	94.9	96.2	96.5	96.2	97.5	97.1	95.6	93.0	-	93.8	93.2
24 SPFMV-RC*	83.8	84.0	83.7	84.0	83.4	83.4	84.3	83.1	95.6	96.5	94.9	96.2	97.1	96.2	94.9	96.2	96.2	95.6	97.1	97.1	95.9	93.7	96.2	--	98.4
25 XN3	83.2	84.0	83.1	83.4	83.1	83.7	83.7	82.4	94.9	95.9	94.3	95.6	96.2	95.2	94.3	95.9	95.2	94.6	96.2	96.2	94.9	92.7	92.2	99.0	-

* Sequences were obtained from the sequence databases and their accession numbers are presented in Table 1.

Comparison of the 3' NTR sequences of the 21 SPFMV isolates gave identities ranging from 79.9% to 99.6% (Table 3). Phylogenetic analysis of the 3' NTR sequences revealed two distinct clusters (Fig. 3) slightly different from those obtained from the CP sequence analysis. The isolates of strain group C were clearly and consistently distinguished from the rest of the isolates (Fig. 3) with the exception of the C isolate from North America, which not only had a nt sequence identity of 98% with the isolate RC (Abad *et al.*, 1992), but also clustered with strain groups EA and O. The 3' NTR sequence identities between the non-C isolates ranged from 94.2 to 97.3% whereas the overall 3' NTR sequence identity between the two main groups was 79% which is comparable to the CP sequence identity of 82% between the C and RC strains (Abad *et al.*, 1992). The analysis of the 3' NTR sequences did not subdivide the non-C isolates into the strain groups EA, O and RC similar to that observed for the CP nt sequences; for instance, the 3' NTR sequence of the isolate from the Canary Islands was very similar to that of a Chinese isolate (XN3) (99.6 %), Zambia (99.1%), Put (97.3%) and Ruk55-2 (98.2 %). Similarly, the 3' NTR of the isolate from Portugal was more closely related to that of the isolates from Zambia and Canary Islands although they originated from geographically distinct areas. Moreover, the low bootstrap support among this group of isolates may suggest high identities among the isolates in their 3' NTR sequences. Preliminary analysis of sequences for recombination among the isolates gave evidence of possible recombination events between the C strain isolate from North America (Abad *et al.*, 1992) and other strains and between the isolate Rak6e and Canar 3.

Table 3: Pairwise percent 3' NTR sequence identities among 24 selected SPFMV isolates

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. SPFMV-C*	-																							
2. KY 25/4A	85.1	-																						
3. KY 45/3S	82.8	94.6	-																					
4. KY 97/5S	81.4	96.4	94.2	-																				
5. KY 51/9S	83.7	96.9	94.6	95.1	-																			
6. Nam 12	82.0	96.4	94.2	95.5	95.1	-																		
7. Spain 1-C	83.7	97.3	95.6	96.9	96.4	96.9	-																	
8. MD1/1	83.3	97.3	96.0	95.5	96.0	95.6	96.1	-																
9. SPFMV-O*	98.6	83.4	81.2	82.4	82.1	81.2	82.1	82.5	-															
10. KY 115/1S	97.3	82.5	80.3	79.7	81.2	81.2	81.2	81.6	96.4	-														
11. Arua 10a	98.6	83.3	81.2	80.6	82.0	81.1	82.1	82.4	8.2	96.4	-													
12. TZ4	98.2	84.3	82.1	81.5	83.0	82.1	83.0	83.4	97.8	96.4	97.8	-												
13. KY 85/7S	97.3	83.0	80.3	80.2	81.6	81.6	81.6	81.6	96.9	95.0	96.9	96.4	-											
14. KY 46b	99.5	83.9	81.6	81.1	82.5	82.5	82.5	83.0	98.7	96.9	98.7	98.2	97.3	-										
15. KY 54/9S	99.1	83.9	81.6	81.1	82.5	82.5	82.5	83.0	98.7	96.9	98.7	98.2	97.3	99.0	-									
16. Ruk55-2	98.2	83.8	81.6	81.1	82.4	81.5	82.5	82.9	97.8	96.0	97.8	97.8	96.4	98.2	98.2	-								
17. Rakai 6e	99.1	81.6	81.6	81.1	82.5	81.6	82.5	83.0	98.7	96.9	98.7	98.2	97.3	99.1	99.1	98.2	-							
18. Put.	98.2	81.6	78.6	78.5	79.8	78.9	79.9	80.7	97.8	96.0	99.6	97.3	96.4	98.2	98.2	97.3	98.2	-						
19. Portugal	98.2	85.1	83.0	82.4	83.8	82.9	83.0	84.2	96.9	95.5	96.9	96.4	95.5	97.3	97.3	96.4	99.3	96.4	-					
20. Canar 3	99.0	85.1	83.0	82.4	83.8	82.9	83.9	84.2	97.8	96.4	97.8	97.3	96.4	98.2	98.2	97.3	98.2	97.3	99.1	-				
21. Zambia	98.2	8x.3	82.1	81.5	83.0	82.1	83.0	83.4	96.9	95.5	96.9	96.4	96.4	97.3	97.3	96.4	97.3	96.4	98.2	99.1	-			
22. Spain -EA	97.7	83.9	81.0	82.0	82.5	96.9	82.5	82.6	98.2	95.5	97.3	96.9	96.0	97.8	97.8	96.9	97.8	96.9	96.9	97.8	96.9	-		
23. SPFMV-RC*	97.7	82.0	81.9	80.5	80.6	81.1	82.8	82.4	96.4	95.0	96.4	95.9	95.0	97.3	96.8	95.9	96.8	5.9	97.3	98.2	97.3	95.9	-	
24. XN3	98.6	84.7	96.0	82.0	83.3	82.4	83.4	83.8	97.3	96.0	97.3	96.9	96.0	97.8	97.8	96.1	97.8	96.9	98.7	99.6	98.7	97.3	97.7	-

* Sequences of isolates were obtained from the sequence databases and their accession numbers are shown in Table 1.

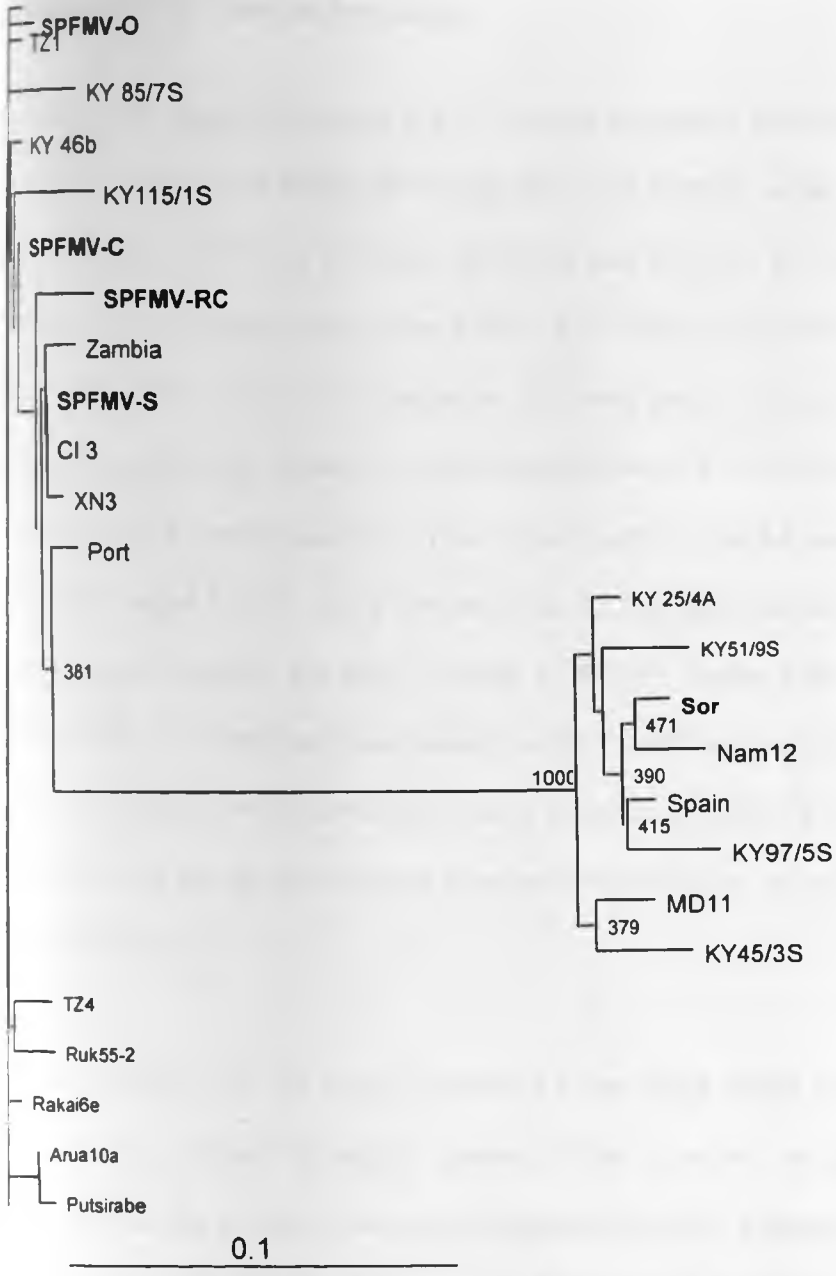

Fig. 3. Neighbour-joining tree showing the relationships of SPFMV 3' NTR nucleotide sequences. The sequences of isolates in bold were obtained from the sequence databases and their accession numbers are show in Table 1.

Comparisons of SPFMV isolates from Kenya

The similarity in CP sequences between SPFMV isolates originating from different regions of Kenya (Table 2) ranged from 80.0 to 98.1% and from 76.4 to 96.8% at the aa and nt levels, respectively. Isolates KY25/4A, KY45/3S, KY97/5S and KY51/9S all clustered with the strain group C isolates, whereas the isolates KY85/7S, KY46b and KY54/9S belonged to the EA group. Only isolate KY115/1S belonged to the strain group O (Fig. 1). Within strain group C, sequence similarity among the isolates ranged from 95.2 to 98.1% and from 94.5 to 96.8% at the aa and nt levels respectively. The similarity between the EA and O groups of the Kenyan isolates ranged from 93.3 to 97.5% and from 92.0 to 96.8% at the aa and nt levels, respectively, clearly showing the wide variation of SPFMV strains and their geographic distribution (Table 1). Overall sequence identity in the 3' NTR between isolates of SPFMV from Kenya ranged from 79.7% to 99.1% (Table 3). The isolates KY85/7S, KY46b, KY54/9S and KY115/1S were clearly distinguished from the remaining four, which belonged to the strain group C (Fig. 2).

Host range differences: Of the isolates selected for host range studies (Table 4), isolates KY46b and Zambia infected the highest numbers of the inoculated test plants, with only isolate Zambia infecting *D. metel.* None of the isolates infected *C. foliosum, D. stramonium* and *S. demissum* (Table 4). The host range of Rak6e and KY115/1S was restricted to *Chenopodium* and *Ipomoea* species. Isolates KY25/4A and MD1/1 both belonging to strain group C did not infect any of the test plants except *I. setosa* which was included as a positive check in this test. On *I. setosa,* however, no discernible differences in symptomatology were observed between these six isolates. The symptoms in *I. setosa* included vein clearing and feathering which are typical of SPFMV.

On *Chenopodiaceae*, chlorotic local lesions were clearly recognizable 14 days post inoculation (dpi) on the leaves of plants inoculated with isolates Zambia, KY46b, Rak6e and KY115/1S. Neither were symptoms observed nor was SPFMV detected by serological assays in *Chenopodium* plants inoculated with isolates KY25/4A and MD1/1. Systemic infection of *N. benthamiana* inoculated with KY46b and Zambia caused leaf distortion, stunting and vein clearing, whereas on *N. occidentalis* ssp. *obliqua* and *N. occidentalis* P1, these two isolates caused chlorotic lesions on the inoculated plants followed by systemic chlorosis. Both isolates Zambia and KY46b caused severe stunting, leaf distortion, vein clearing and leaf necrosis in *N. hesperis*. Although no symptoms were visible on *N. clevelandii* and *N. debneyi* inoculated with KY46b and Zambia, these plants were clearly positive for SPFMV in DAS-ELISA after testing both top and inoculated leaves (Table 4).

Table 4: Experimental host range of selected SPFMV isolates

	Isolates and their strain assignment to groups					
Test plant	KY46b EA	Zambia EA	Rak 6e EA	KY115/1S O	KY25/4A C	MD1/1 C
Nicotiana benthamiana	+*	+	-	-	-	-
N. clevelandii	+	+	-	-	-	-
N. debneyii	+	+	-	-	-	-
N. hesperis	+	+	-	-	-	-
N. occidentalis P1	+	+	-	-	-	-
N. occidentalis ssp. obliqua	+	+	-	-	-	-
Chenopodium amaranticolor	LL+	LL+	LL+	LL+	-	-
C. murale	LL+	LL+	LL+	LL+	-	-
C. quinoa	LL+	LL+	LL+	LL+	-	-
Datura metel	-	+	-	-	-	-
Ipomoea setosa	+	+	+	+	+	+

* Systemic (+), local lesion (LL+), and no (-) infections were confirmed by DAS-ELISA. *C. foliosum, D. stramonium and S. demissum* were not infected by any of the 6 isolates. *I. setosa* was included as a positive control

Production and characterization of MAbs

Recombinant SPFMV CPs expressed in *E. coli* were visualized by SDS-PAGE. The fusion proteins of KY115/1S and KY25/4A differed somewhat in size but were similar in size to the purified virions and/or infected leaf extracts. The protein fractions eluted from the Ni-NTA columns were examined by EBIA (Western blot) for protein yields using IgG to SPFMV as shown in Fig. 4. Satisfactory yields of recombinant protein were obtained. However, three fusion experiments using mice immunised with recombinant CP derived from isolate KY25/4A did not result in hybridoma lines secreting virus specific MAbs. Therefore no further attempts were made to raise MAbs against bacterially expressed CP of isolate KY115/1S.

Following immunization of mice with purified virions of isolate KY115/1S, three stable cell lines were obtained which secreted antibodies against SPFMV. Table 5 summarizes the specificities of the MAbs and PAbs in TAS- and DAS-ELISA, respectively. MAb 1C4-B3 was broad-reacting, giving strong reactions with all SPFMV isolates tested. MAb 4F5-G6 and 1A10-D2 reacted selectively with the homologous antigen (Isolate KY115/1S) and sweet potato samples from the same field or from nearby fields in Kwale area of coastal Kenya suggesting that the SPFMV isolate in these samples may share the same epitopes. The MAbs did not react with other sweet potato potyviruses.

Apart from reacting with all SPFMV isolates tested, MAb 1C4-B3 gave strong TAS-ELISA reactions, suggesting sensitive detection of SPFMV isolates. The RC, C (Moyer) and 46b (BBA) PAbs reacted with all other SPFMV isolates irrespective of their strain group assignment although they slightly differed in reaction strength.

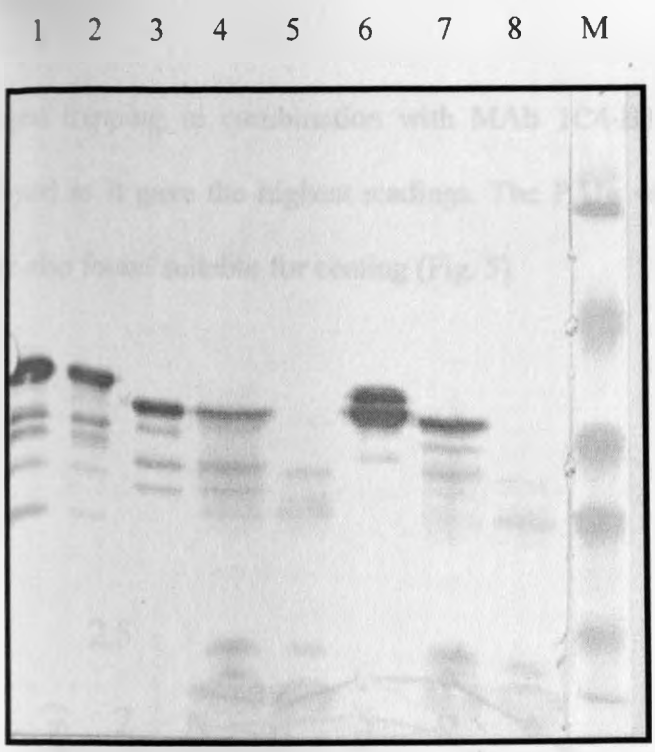

Fig. 4. Electroblot immunoassay analysis of the bacterially expressed capsid proteins of KY115/1S (lane 1 and 2 [but dialysed]) and KY25/4A (lane 6). For comparison, extracts from KY115/1S–infected (lane 3), KY25/4A-infected (lane 7), and non-infected *C. quinoa* leaves (lanes 5 and 8) as well as purified virus preparation of isolate KY-115/1S (lane 4) were also analysed.

The SPFMV-C antiserum (CIP) generally gave weaker reactions than other PAbs but did detect all isolates. The MAb 7H8G2 (CIP) reacted with only 7 out of 27 accessions indicating specificity to a limited number of isolates. Similarly, MAb 4F5-G6 and MAb 1A10-D2 reacted only with 8 and 4 isolates, respectively. The MAb and PAb reactions did not correlate with the grouping of isolates based on CP sequence comparisons. Isotyping of MAbs 1C4-B3 and 1A10-D2 revealed that they are of the IgM and IgG3 types, respectively (Table 5).

Since MAb 1C4-B3 appeared to be a high-affinity antibody that reacted with all SPFMV isolates (strains), it was tested in combination with several trapping antibodies for the most sensitive detection of SPFMV in TAS-ELISA. The most appropriate PAb to be used for

antigen trapping in combination with MAb 1C4-B3 was the SPFMV-RC antiserum (Moyer) as it gave the highest readings. The PAbs 46b (BBA), C (Moyer) or C (CIP) were also found suitable for coating (Fig. 5).

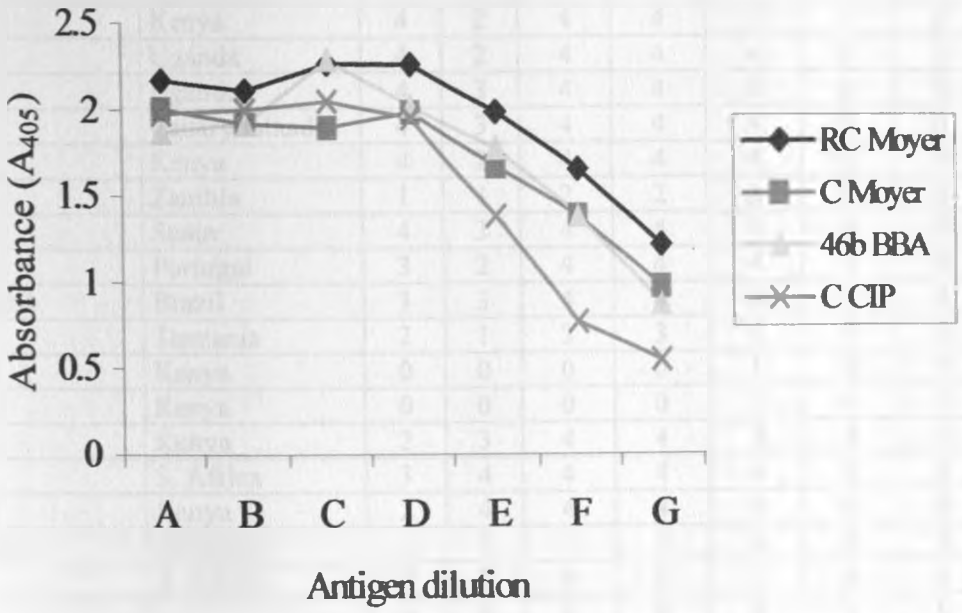

Fig. 5. Sensitivity of TAS-ELISA using 4 different antisera for coating in combination with MAb 1C4-B3. The homologous antigen KY115/1S was tested at dilutions of 1: 20 (A), 1: 50 (B), 1:100 (C), 1:200 (D), 1:1000 (E), 1:2000 (F), and 1:4000 (G).

Table 5: Reactions of geographically diverse field samples with polyclonal and monoclonal antibodies to SPFMV isolates in DAS- and TAS-ELISA

Sample or isolate	Origin	DAS-ELISA reaction * with SPFMV PAbs				TAS-ELISA reaction * with SPFMV MAbs			
		46b BBA	C CIP	C Moyer	RC Moyer	1C4-B3 §	4F5-G6 §	1A10-D2 §	7H8G2 CIP
XN3	China	4	2	4	4	4	4	0	4
KY115/1S	Kenya	4	2	4	4	4	4	4	0
KY118/1S	Kenya	3	4	4	4	4	4	4	1
KY116/4A	Kenya	1	1	2	2	4	2	0	2
KY115/6S	Kenya	4	4	4	4	4	4	4	2
KY58/1S	Kenya	4	2	4	4	4	4	0	2
SPFMV Togo	Togo	2	3	4	4	4	2	2	1
KY85/7S	Kenya	4	2	4	4	4	1	0	1
Ruk 55-2	Uganda	4	2	4	4	4	0	0	0
Rak 6e	Uganda	4	3	4	4	4	0	0	0
Canar 3	Canary Islands	4	3	4	4	4	0	0	0
KY46b	Kenya	4	2	4	4	4	0	0	4
Zambia	Zambia	1	1	2	2	3	0	0	1
Spain 1	Spain	4	3	4	4	4	0	0	0
300902/35	Portugal	3	2	4	4	4	0	0	0
SPVD Brazil	Brazil	3	3	4	4	4	0	0	4
TZ4	Tanzania	2	1	3	3	4	0	0	1
KY25/4A	Kenya	0	0	0	0	1	0	0	1
KY25/4A	Kenya	0	0	0	0	1	0	0	1
KY97/5S	Kenya	2	3	4	4	4	0	0	2
MD1/1	S. Africa	3	4	4	4	4	0	0	0
KY45/3S	Kenya	2	4	4	4	4	0	0	2
SPVG -Eth	Ethiopia	2	3	4	3	4	0	0	1
JIII	S. Africa	0	0	0	0	3	0	0	0
Healthy	-	0	0	0	0	0	0	0	0

* The strength of DAS- and TAS-ELISA reactions, measured as A_{405} after substrate incubation period of 1h, was classed as 4 (> 1.5-), 3 (0.7-1.5), 2 (0.2-0.7), 1(> three times the mean value for uninfected control up to 0.2) or 0 (< three times mean of uninfected controls). § MAbs were obtained following immunization of mice with SPFMV isolate KY115/1S.

7.4 DISCUSSION

The molecular, biological and serological properties of geographically diverse isolates were analysed to assess the variability within SPFMV. The variability of SPFMV has

never been assessed on such a broad basis before and the characterization of the diversity of SPFMV is of considerable importance for the development of diagnostic tools and disease management strategies against the main viral disease, SPVD. The 3' terminal part of the genome (CP coding region and NTR) of 21 SPFMV isolates was analysed. Comparisons and the phylogenetic analysis of the CP sequences revealed two main clusters: the fairly homogeneous C cluster and the second heterogeneous cluster comprising the strains groups RC, O and EA. Because of its sub-division into RC, O and EA strains, the second main cluster is further on referred to as the non-C cluster, despite the historic first molecular differentiation of C- and RC strains (Abad *et al.*, 1992). The two main clusters share a CP sequence similarity of only 82%, which is just above the threshold value (< about 80%) for the discrimination of potyvirus species (Aleman-Verdaguer *et al.*, 1997; van Regenmortel *et al.*, 2000). The CP sequences were most variable in the N-terminus. However, there was still considerable conservation within the regions adjacent to the DAG motif, which has been associated with aphid transmission (Atreya *et al.*, 1990, 1992, 1995). Quite exceptionally, the C strain from North America (Abad *et al.*, 1992) shows a NAG at the corresponding position.

The range of variability between the C strain isolates and the EA, O and RC strain groups for the 3' NTR nucleotide sequences was similar to those of the CP nt sequences. No major differences were observed in the topology of these trees except that the 3' NTR sequence of the C strain from North America clustered with those of the non-C strain group. This discrepancy, supported by high bootstrap values, might indicate that the C strain reported in North America (Abad *et al.*, 1992) is a recombinant isolate that has a C-

like CP gene and an RC-like 3' NTR. The 3' NTR sequences of the other isolates of strain group C clearly grouped together and were separated from the rest. Recombination involving the CP and 3' NTR of distinct strains has been reported for potyviruses such as *Potato virus Y, Bean common mosaic virus* and *Zucchini yellow mosaic virus* (Revers *et al.*, 1996). Mixtures of strains do occur in sweet potato plants as the accession Spain1 was the source of the C- and EA-like sequences (see Spain1-C and Spain1-EA in Fig. 1) and this might facilitate the occurrence of recombination events. Since the RC, O and EA strain groups were not clearly distinguishable based on the 3' NTR sequences, it is suspected that recombination among these strains has been taking place in naturally occurring mixed infections and preliminary analysis for the possibility of recombination seems to support this. Therefore, results presented here demonstrated the usefulness of the 3' NTR sequences for the differentiation of strain group C isolates from other SPFMV isolates.

In previous phylogenetic studies based on the comparison of SPFMV CP sequences, East African isolates (6 Ugandan and 1 Madagascan) formed a distinct cluster separate from other SPFMV isolates (Kreuze *et al.*, 2000; Abubakar *et al.*, 2003). This study, with a broader spectrum of isolates, could only partly confirm this geographical separation. Most of the isolates from East Africa belonged to strain group EA (Kreuze *et al.*, 2000). However, several isolates originating from outside East Africa also belonged to this phylogenetic group, whereas some East African isolates grouped together with those of strain groups C or O (Fig. 2). Based on the CP phylogenetic analysis, only RC strain has not been detected in East Africa, whereas all other described biological and/or molecular

strains were found. The absence of distinct geographical clades clearly shows that phylogenetic groups do not exactly reflect geographical origin of SPFMV isolates and that the diversity of SPFMV amongst East African isolates is perhaps as high as is amongst SPFMV isolates found elsewhere in the world. Possibly the EA strain isolates of Europe and the Canary Islands were introduced to these places (Europe and East Africa) together with sweet potato cuttings from the Americas, the center of origin of sweet potato. It is speculated that the EA type strain is as widespread in Europe's sweet potato growing regions as they are in East Africa. The only sequence of a South African isolate of SPFMV was that of a C strain, which is not sap transmissible to non-*Ipomoea* spp. contrasting to RC strains. All these results may suggest that C strains are predominant in South Africa. However, this hypothesis has to be verified either by more sequence data or preferably by a diagnostic test that permits the discrimination of C- and non-C strains of SPFMV in a large-scale screening.

Lyerly *et al.* (2003) has suggested that SPFMV-C may be a distinct virus species on the basis of CP gene sequences. However, despite the high CP aa sequence difference of ca. 20% between C and non-C strain isolates, both groups should be regarded as SPFMV because of their close serological relationship and their CP variability being less than what has been reported for other potyviruses. For example, *Yam mosaic vi*rus with CP nucleotide sequence differences as great as 28% (Bousalem *et al.,* 2000). Also, the shorter SPFMV-C coat protein sequence of two aa compared to other SPFMV strains does not seem to warrant the differentiation of strain C as a distinct species, as also the

Papaya ringspot virus CP-encoding sequences vary in size from 840 to 870 nucleotides (Bateson *et al.*, 2002).

The host range experiment with six selected isolates revealed three main biotypes with the Kenyan isolate KY46b and Zambia, both of the EA cluster, systemically infecting *N. benthamiana* and exhibiting the widest host range. These two isolates seemed to be exceptional since all other SPFMV isolates failed to infect *N. benthamiana* (data not shown). There are only two other reports of SPFMV isolates infecting *N. benthamiana* from Peru (Nakashima *et al.*, 1993) and Guatemala (Moyer, 1986). Possibly, because of the scarcity of this biotype, Karyeija *et al.* (1999) reported that isolates from East Africa do not infect *N. benthamiana.* The second group, isolates KY115/1S and Rak6e, were indistinguishable in host range but based on CP sequences belonged to the strain groups O and EA, respectively. However, at least some isolates from East Africa incited local lesions on *Chenopodium* species and most isolates belonging to the EA cluster infected and were maintained in *C. quinoa*, which contrasts with findings by Karyeija *et al.* (1999) that East African SPFMV do not infect *Chenopodium* species. The C strain isolates KY25/4A and MD/1/1 infected *I. setosa* only. This is in line with findings of Cali and Moyer (1981) who reported failure to transmit the C strain onto non-*Ipomoea* spp. by sap inoculation. Finally, the host range differences observed in this study did not reflect the groups as based on the phylogenetic analysis of the CP and 3' NTR sequences. The phylogenetic differentiation of the C and non-C strain isolates and among the O, RC and EA strain groups suggested that there is a correlation between the molecular differentiation and biological properties. However, since there was little correlation with

the geographical origin of SPFMV isolates, the naming of an East African group seems inappropriate.

Efforts to produce differentiating monoclonal antibodies against SPFMV strains resulted in three MAbs with different specificities. MAb 1C4-B3 reacted strongly and specifically with all SPFMV strains. In a screening of over 500 geographically diverse samples (data not shown), the reactions of MAb 1C4-B3 consistently confirmed the reactions of the polyclonal antibodies, and the MAb gave more sensitive reactions than the SPFMV-C antiserum from CIP and the antiserum 46b raised against the SPFMV isolate KY46b. Although MAb 1C4-B3 performed best when antiserum SPFMV-RC from Moyer was used as the coating antibody, sensitive detection of SPFMV was achieved with all tested antisera. These properties rendered MAb 1C4-B3 a powerful tool for the universal detection of SPFMV, especially in singly infected sweet potato plants, notorious for their low virus concentrations. Contrasting to MAb 1C4-B3, the MAbs 1A10-D2 and 4F5-G6 reacted specifically with certain strains (isolates), similar to MAb 7H8-G2 of CIP. Possibly due to the fact that both MAb lines were produced against the same isolate, MAbs 1A10-D2 and 4F5-G6 gave positive reactions with a similar range of SPFMV isolates, whereas MAb 7H8-G2 (CIP) gave positive reactions also with some 'outlying' isolates like KY46b and Zambia, both infecting *N. benthamiana,* and SPFMV isolates from Brazil. However, the results do not indicate any correlation between the reactions of the MAbs and their geographical, biological or phylogenetic groupings. Whilst extremely valuable as a tool for achieving highly sensitive and specific serological detection of SPFMV, the initial aim of obtaining a MAb which could be used to differentiate strain

groups of SPFMV was not achieved. However, as MAbs 1A10-D2 and 4F5-G6 differentiated between the genetically much closer non-C strains, further attempts to raise C or non-C strain-specific MAbs seem promising. Possibly the CP of a purified virus preparation with native protein folding and epitope presentation may serve as a more suitable immunogen than bacterially expressed CP, which was unsuccessful in this study.

The characterisation of a broad range of diverse isolates in this study confirmed the two main groups of C and non-C strains of SPFMV with various subgroups. These groups and subgroups are widespread, biologically diverse and do not follow any geographical pattern. However, certain strains may be less frequent or even absent in certain geographical regions, like RC in East Africa and possibly all non-C strains in South Africa. This shows that it may be difficult to develop control measures against SPFMV based on resistance breeding and transformation of sweet potato. The precondition for the success of the latter is low sequence variability between a virus within a geographical region and the transgene (Bateson *et al.,* 1994; Tennant *et al.,* 1994). For example, transgenic tobacco plants expressing the CP of *Cucumber mosaic virus,* sub-group I were resistant to infection by CMV-C but susceptible to sub-group II of CMV-WL (Hull and Davies, 1992). Since there are apparently many distinct pathotypes/strains of SPFMV (the most distinct strain groups share ca. 80% aa sequence similarity), it is critical to consider strain variability and the geographical distribution of the various strains when selecting transgenes in biotechnology programmes aimed at generating resistance to SPFMV in sweet potato.

SPFMV strains are widespread in sweet potato fields throughout Africa and elsewhere in the world, which is a significant finding. The differential reactions of different indicator plants to strains of SPFMV were demonstrated although the significance of this with regard to sweet potato germplasm is unknown. These results present the first sequence information of SPFMV in Europe (Spain and Portugal), the Canary Islands, and some countries in Africa namely Kenya, Zambia, and South Africa. Present and future studies focusing on conventional and pathogen-derived resistance should consider strain diversification in breeding programmes. Also, studies to determine if there are any differences in yield effects due to infection f sweet potato by different SPFMV strains.

8.0 CHAPTER EIGHT

ANALYSIS OF SEQUENCE DIVERSITY IN THE P1, HC-Pro AND P3 GENES OF SPFMV AND SPVY ISOLATES

8.1 INTRODUCTION

Sweet potato feathery mottle virus (SPFMV) is the most widespread potyvirus infecting sweet potato (Moyer and Salazar, 1989). Like other potyviruses, the positive-sense RNA genome of SPFMV contains one large open reading frame that encodes a polyprotein, which is processed into ten mature gene products by three virus-encoded proteinases (P1, HC-Pro and NIa) (Riechmann *et al.*, 1995). Although several isolates of SPFMV have been characterized, most of the traditional and molecular studies aimed at determining SPFMV diversity have been confined to the CP and 3' NTR sequences (Abad *et al.*, 1992; Kreuze *et al.*, 2000; Mukasa *et al.*, 2003c). Sweet potato virus Y (SPVY) has only recently been described as a potyvirus closely related to, but distinct, from SPFMV. The discrimination of SPVY from SPFMV was based on biological and serological properties and particularly on 3' terminal sequences. Thus, unlike SPFMV, no other genomic component of SPVY has been determined.

Several comparisons of potyvirus sequences have shown that the genome parts encoding the first protein (P1), third protein (P3) and the CP N-terminus are the most variable regions of the potyviral genome (Ward and Shukla, 1991). Analysis of the P1 gene sequences of *Potato virus V* and *Potato virus Y* grouped isolates according to their geographic origin, symptoms and host range (Tordo *et al.*, 1995; Igor *et al.*, 2001; Lin *et al.*, 2001; Ohshima *et al.*, 2002). It has further been suggested that the diversity among the P1 proteins could be more useful for the taxonomy of potyviruses (Lin *et al.*, 2001).

Although the complete genome of SPFMV has been determined (Sakai *et al.*, 1997), information concerning the variability of its 5' terminal region is not available. In order to better understand the

genetic diversity within SPFMV, the P1, HC-Pro and P3 nucleotide sequences of SPFMV isolates representing different strains were determined and used for multiple sequence alignments and phylogenetic analyses. Since SPVY is closely related to SPFMV but has a much wider host range than SPFMV, the P1, HC-Pro and P3 genes of SPVY were also sequenced.

8.2 MATERIALS AND METHODS

8.2.1 Isolates and total RNA extraction

Isolates of SPFMV and SPVY for which the P1, HC-Pro and/or P3 sequences were determined are listed in table 1. Total nucleic acid was extracted using the Nucleospin Plant Extract kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

8.2.2 Analysis of the P1 gene

Based on preliminary host range studies and considerable differences in their CP sequences, four isolates of SPFMV namely KY46b, KY115/1S, KY25/4A and Zambia as well as a SPVY isolate Thomas 1.6A were selected for P1 sequence analysis. Synthesis of cDNA was carried out as described in section 4.2.7. However, the cDNA primer was HC-pro 5'-SAGYTGRTTGTCACACAT-3' and a random hexamer (Promega). Amplification was achieved using the degenerate upstream primer SPFMV-P1up 5'-ACRCCKTCMATGAAGAAG-3' and the downstream primer SPFMV-HC-prolo 5'-SAGYTGRTTGTCACACAT-3' and the following thermocycling scheme: 94°C for 3 min followed by 30 cycles of 30 s at 94°C, 1 min at 55°C, extension for 3 min at 72°C and a final extension step at 72°C for 10 min. Amplification products were gel purified, cloned in the pGMET vector (Promega) and transformed into *E. coli* (DH5α).

C-tailing of cDNA using the RACE procedure. To obtain the 5' terminal nucleotide sequences of the SPFMV and SPVY genomes, the procedure for rapid amplification of cDNA ends (RACE) was

cDNA (20 µl) was mixed with 4U of RNase H (Fermentas) followed by incubation at 37°C for 30 min. The mixture was purified using the Nucleospin extract kit (Macherey-Nagel, Düren, Germany) and eluted in 20 µl of elution buffer. Two µl of 2 mM dCTPs and 6 µl of 5X terminal deoxynucleotidyl transferase (TDT) buffer (Fermentas) were added and the contents incubated at 4°C for 3 min and a further 3 min on ice. Following the addition of 1.5 µl TDT (20 U/µl) (Fermentas) to the mixture, the latter was incubated for 15 min at 37°C, followed by a 10 min incubation at 70°C. PCR was performed using a sequence specific downstream primer SPFMVP1lo2 5'-CCACCA5TTCTA55TTCA-3' ('5' stands for Inosine) and an upstream poly (G) primer 5'-CGGAATTCCCGGGGGGGGGGGGGGG-3'. The PCR programme was as outlined above except that the annealing temperature was 49°C.

Table 1: Isolates of SPFMV and SPVY used in this study

Virus	Isolate	Strain*	Country	Acc. no
SPFMV	KY115/1S	O	Kenya	
SPFMV	KY25/4A	C	Kenya	
SPFMV	KY45/5S	C	Kenya	
SPFMV	SPFMV-S	RC	Japan	D86371
SPFMV	KY46b	EA	Kenya	
SPFMV	Zambia	EA	Zambia	
SPFMV	Rak6e	EA	Uganda	
SPFMV	Ruk55-2	EA	Uganda	
SPVY	Thomas1.6A	-	South Africa	
SPVY	300902	-	Portugal	

*Strain designations are based on capsid protein gene sequences.

Analysis of the P3 gene

The P3 gene sequences of SPFMV and SPVY were amplified using the oligonucleotide pair SPMV3386up 5'-GCACAGGATATCATGTCCTGA-3' (upstream) and SPFMV4520lo 5'-GCCCAATGATTCGTTCGTA-3' (downstream) derived from the SPFMV-S genome (Sakai *et al.*, 1997). PCR reactions were carried out as outlined earlier and PCR products were analysed by electrophoresis in a 1% agarose gel. Bands of the expected size were excised and purified from agarose gels using the Nucleospin Extract kit (Macherey-Nagel, Düren, Germany). Purified fragments were directly transformed onto the pGMET vector (Promega) and subsequently transformed into *E. coli* DH5α competent cells. Recombinant clones were identified by PCR using the SP6/T7 universal primers, and subsequently grown in liquid LB media supplemented with ampicillin. Recombinant plasmid DNA was isolated from overnight cultures using the Macherey-Nagel Plasmid kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

The nucleotide and deduced amino acid sequences were used in pairwise and multiple sequence alignments to determine the similarities between sequences and for generating phylogenetic trees. Phylogenetic relationships were analysed using the neighbour-joining method of the ClustalX software program version 1.8 (Thompson *et al.*, 1997; Jeanmougin *et al.*, 1998). One thousand bootstrapped data sets were generated to estimate the statistical significance of the branching. The consensus tree was visualized using the Treeview programme (Page, 1996).

8.3 RESULTS

The variability of the 5'-proximal region of the SPFMV genome was investigated by sequencing the P1 and P3 genes (variable regions) and about one half of the conserved helper component proteinase (HC-Pro) gene of four SPFMV isolates. In addition, each of these genomic regions was determined for a SPVY isolate. An alignment of the P1 sequence of the isolates KY25/4A, Zambia,

KY46b and KY115/1S with the published P1 sequence of SPFMV-S from Japan (Sakai *et al.*, 1997) revealed that the P1 gene of the latter is 75 nucleotides (25 amino acids) shorter than that of the four African isolates (Fig. 1). The P1 gene of isolates KY115/1S and KY46b spanned 2067 nucleotides (689aa) in contrast to that of SPFMV-S from Japan that is 1992 nucleotides (664 aa) long (Sakai *et al.*, 1997). The N-terminal one third (ca. 210 aa) of the P1 protein was highly conserved between the isolates KY115/1S, KY46b and SPFMV-S, which represent the strain groups O, EA and RC, respectively. Similarly, the C-terminal one third of the P1 was conserved in all four isolates (data not shown). However, there was considerable variation between SPFMV isolates in the P1 aa sequences spanning from aa position 213 to 340. It is within this hypervariable region that the isolates Zambia, KY115/1S, KY 25/4A and KY46b have 75 extra nucleotides (25 aa) (Fig 1).

Pairwise alignment of the entire P1 gene sequences revealed remarkable variability between the SPFMV isolates studied. The SPFMV-S strain shared an amino acid sequence similarity of 82 and 83% with KY46b and KY115/1S, respectively. Based on comparisons of the 190 N-terminal amino acids of the HC-pro gene, the isolates KY46b and KY115/1S shared an amino acid sequence similarity of 91%, whereas SPFMV-S shared an aa sequence similarity of 87 and 85% with KY46b and KY115/1S, respectively (Table 2).

```
SPFMV-S    TCAGACACCT CTTGAGCCTG TGCCGGAAGT ATTAGCAGCA ACCACATTTG 50
KY25/4A    TCAGGCACCT CTTGTGTCTG CGCCAGAAGT GTCAGTAGCA ACCACATTTG
KY46b      TCAGAAACCT CCTGTGCTTA TGTCAGAAGC ACCAGTAGAA ACCACACTTG
KY115/1S   TCAGGCACCT CTTGTGTCTG CGCCAGAAGT ATCAGTAGCA GCCACATTTG
Zambia     TCAGGAACCT CCTGTGCTTA CGCCAAAAGT ATCAGTAGCA ACCACATTTG

SPFMV-S    TGGAGGCAAC TATTGATGGA AAAGATGCAC ---------- ---------- 100
KY25/4A    TGGAGGCTAC TAACAACGGA GAAGATGAAC AACTTCCAAC CCAGGAGCAA
KY46b      TGGAGGCTAC TAACAACGGA GAAGATAAAC AACTTCCAAC CCAGGAGCAA
KY115/1S   TGGAGGCTAC TAACAACGGA GAAGGTGAAC AACTTCCAAC CCAGGAGAAA
Zambia     TGGAGGCTAC TAACAACGGA GAAGATAAAC AACCTCCAAT CCAGGAGCAA

SPFMV-S    ---------- ---------- ---------- ---------- ---------- 150
KY25/4A    GCCACAAAGA ATGCTAATTG CTCGGCAGCT AACCCTATCA CTCTTGGGGA
KY46b      GTCACAAAGA ATGCTGATTG CTCGGTAGCC AACCCTATTA CTCTTGGGAA
KY115/1S   GTCACGAAGA ACGCTAATTG CTCAGCAGCT AACCCTATTA CTCTTGGGAA
Zambia     GTCACAAAGA ATGCTAATTG CTCGGCGGCC GACCCTATTA TTCTTGGGGA

SPFMV-S    -----CAACT GGTAGCATCC AATTCGGCAC AATTGTTTGC GAATTGGAGC 200
KY25/4A    CGTGTCAATT GGCAACATCC AATTCGGCAC AATTTCTTGC GTATTAGGAC
KY46b      CGTGTCAATT GGTGGCATCC AATTCGGCAC AATTTCTTGC GTATTAGGAC
KY115/1S   CGTGTCAATT GGCGACATCC AATTCGGCAC AATTTCTTGC GCATTAGGAC
Zambia     CGTGTCAATT GGCGACATCC AATTCGGCAC AATTTCTTGC GTATTAGGAC
```

Fig. 1: Multiple sequence alignment of partial P1 sequences of 5 SPFMV isolates. Dashes indicate missing nucleotides in the Japanese SPFMV-S (acc. no D86371). The numbering of nucleotides is arbitrary.

Table 2: Percent nucleotide sequence identity (below diagonal) and amino acid sequence similarity (above diagonal) of the N-terminal part (190 aa) of the HC-Pro gene of three SPFMV isolates and one SPVY isolate (Thomas1.6A)

	KY46b	KY115/1S	SPFMV-S	Thomas1.6A
KY46b		92.3	87.2	50.8
KY115/1S	94.9		85.1	51.8
SPFMV-S	85.5	85.5		52.3
Thomas1.6A	56.4	57.7	57.1	

P3 sequence comparisons

A total of 873 nt (~ 83%) of the P3 gene were determined from seven SPFMV isolates. The complete P3 gene sequence was obtained for the SPFMV isolate Zambia, and the SPVY isolate 300902. The P3 genes of the isolates Zambia and 300902 were indistinguishable in size (1056 nt)

ot only from each other but also from that of the SPFMV-S strain (Sakai *et al.*, 1997). Based on airwise P3 alignments, the nucleotide sequence identities and amino acid sequence similarities of e SPFMV isolates ranged from 66.1 to 97.6% and from 64.6 to 98.5%, respectively (Table 3). In e pairwise alignments, the amino acid sequence similarities were slightly lower than the rresponding nt identities (Table 3). Similar results were observed for the P1 gene sequences of *am mosaic virus* (Aleman-Verdaguer *et al.*, 1997). The interspecies (between SPFMV and one PVY isolate), nucleotide sequence identities ranged from 56.1 to 58.7% whereas the percentage mino acid similarity ranged from 47.1 to 49.1%.

The phylogenetic tree resulting from multiple sequence alignments was congruent with the identities/similarities deduced from the pairwise comparisons (Fig. 2 & 3). Isolates Zambia, Ruk 55-2, Rak6e, KY115/1S, KY25/4A and SPFMV-S formed a cluster of closely related sequences. The sequence of isolate KY46b differed from those of the other six SPFMV isolates by forming a separate branch. However, the isolate KY45/3S was clearly deviant, although still phylogenetically closer to the SPFMV isolates than was the SPVY isolate 300902 (Fig. 2). The grouping of the isolates based on the P3 gene sequences did not conform to that based on the CP sequences. Based on the CP sequence analysis, isolate KY115/1S and KY25/4A belong to the strain groups O and C, respectively, whereas isolates Ruk55-2, Rak6e, KY46b and Zambia belong to the strain group EA (Fig 4). In the phylogenetic analysis using the P3 sequences, isolate KY45/3S formed a distinct branch consistent with its assignment to strain group C based on the CP and 3' NTR sequences (Chapter 7). Unexpectedly, the P3 sequence of isolate KY25/4A grouped together with the isolates of strain groups O and EA.

Table 3: Pairwise percent P3 amino acid (above diagonal) and nucleotide sequence identities (below diagonal) between 8 SPFMV and 1 SPVY isolates

Isolate	1	2	3	4	5	6	7	8	9
1. SPFMV-S		96.2	95.5	95.5	94.8	95.5	89.7	64.9	48.5
2. SPFMV Rak6e	93.1		97.9	95.5	98.5	97.3	89.3	65.6	48.8
3. SPFMV Ruk55-2	92.8	97.6		95.5	97.3	97.3	89.7	66.0	49.1
4. SPFMV Zambia	92.6	96.7	96.3		94.8	95.5	87.6	64.9	48.1
5. SPFMV KY115/1S	92.2	97.3	96.6	95.5		96.6	88.7	64.9	48.8
6. SPFMV KY25/4A	93.5	97.6	97.3	97.0	96.4		88.3	66.0	48.8
7. SPFMV KY46b	84.3	83.6	83.2	83.0	83.5	83.2		64.6	47.1
8. SPFMV KY45/3S	66.1	67.8	67.0	67.6	66.8	67.5	66.4		47.4
9. SPVY 300902	58.7	58.0	58.3	58.4	57.6	57.8	56.1	56.6	

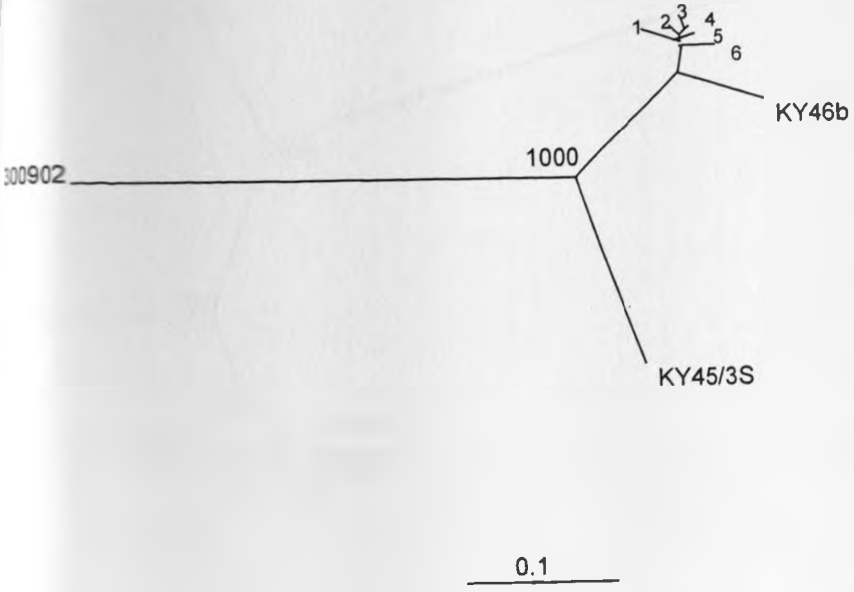

Fig. 2. Bootstrapped phylogenetic tree based on genetic distances calculated using the P3 nucleotide sequences from eight SPFMV isolates and the SPVY isolate 300902. The isolates numbered in the tree are Zambia (1), Ruk55-2 (2), KY115/1S (3), Rak6e (4), KY25/4A (5) and SPFMV-S (6).

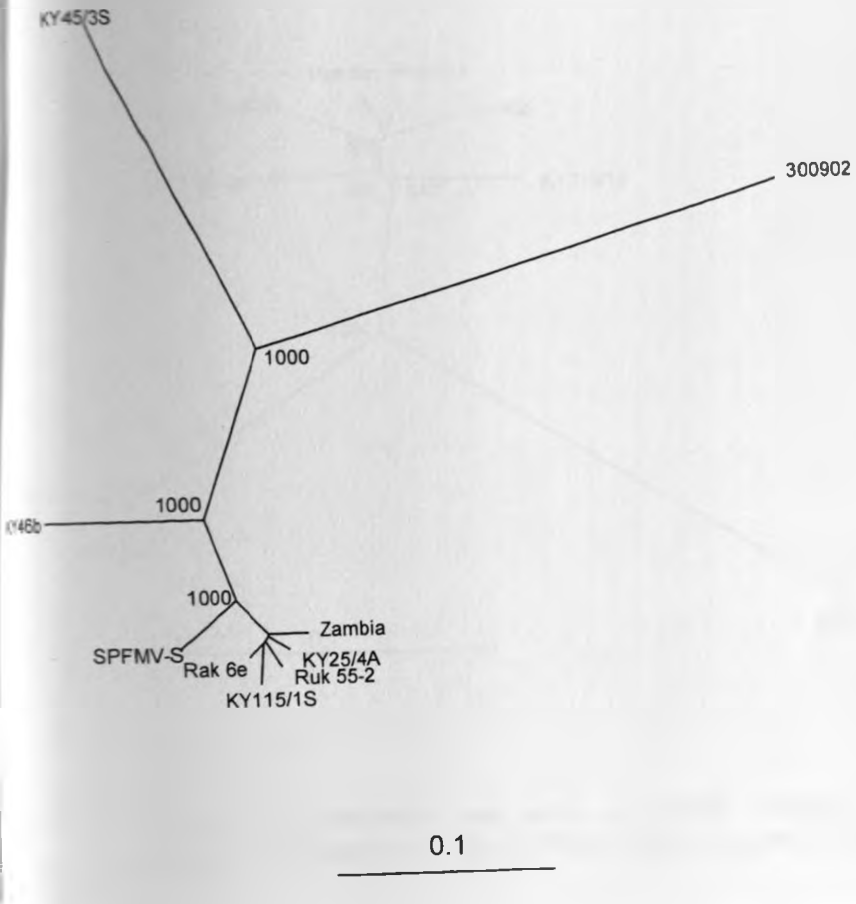

Fig. 3: Bootstrapped phylogenetic tree based on genetic distances calculated using the P3 amino acid sequences from eight SPFMV isolates and the SPVY isolate 300902. The source of the isolates is presented in Table 1.

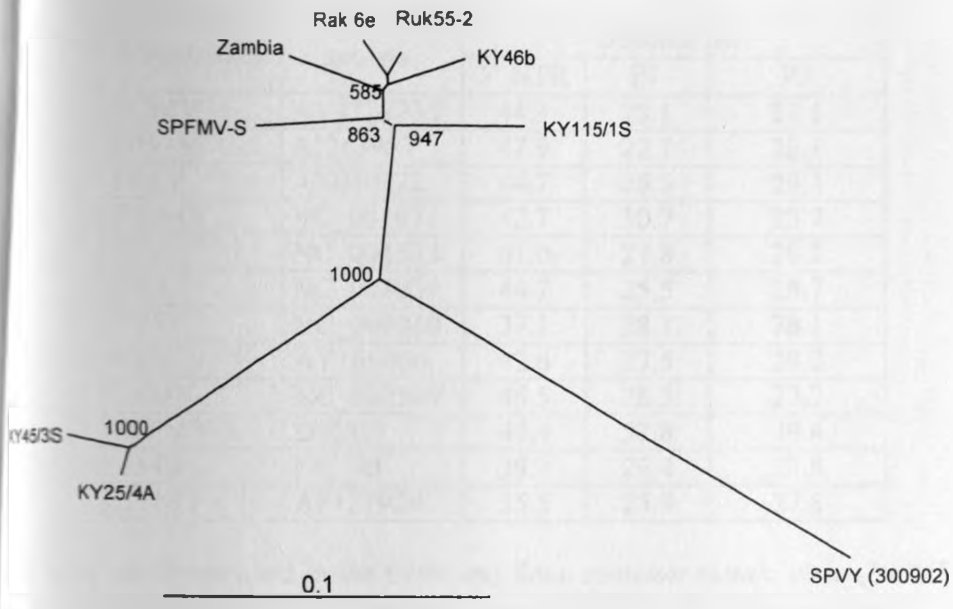

Fig. 4. Bootstrapped phylogenetic tree based on genetic distances calculated using the CP nucleotide sequences from eight SPFMV isolates and the SPVY isolate 300902.

***SPVY sequence comparisons*:** The 5' NTR of the SPVY isolate Thomas1.6A was 210 nucleotides long, which was significantly longer than that of SPFMV (117 nucleotides). The entire P1 protein of isolate Thomas1.6A was 930 nucleotides (310 aa) making it less than half that of SPFMV (2067 nt). The cleavage site at the P1/HC-Pro junction of Thomas1.6A was QY/S, which is similar to that of SPFMV-S (Sakai *et al.*, 1997). The closest relatives of SPVY based on the 5' NTR sequences were *Plum pox virus* (48% identity) and *Turnip mosaic virus* (47%), followed by 10 other potyviruses including SPFMV-S (37-45%) (Table 4). However, SPVY was most closely related to SPFMV in both the P1 and P3 proteins in which they shared aa sequence identities of 53% and 49%, respectively. In contrast, the P1 and P3 aa sequence identities of SPVY to potyviruses other than SPFMV ranged only from 23 to 31% (Table 3).

e 4: Pairwise percent nucleotide sequence identities in the 5' NTR and amino acid sequence identities in the P1 and P3 proteins between SPVY and other members of the genus *Potyvirus*

Virus/strain	acc. no.	Genome part		
		5' NTR	P1	P3
BCMV	AY1122735	44.3	28.1	27.1
PPV-M	AJ243957	47.9	22.7	28.1
PRSV	AY010722	44.7	26.6	29.4
PSbMV	NC_001671	42.7	30.7	25.7
PTV	NC_004573	41.0	27.8	28.2
PVA	NC_004039	44.7	25.5	28.7
PVV	NC_004010	37.1	28.1	28.1
PVY-NTN	AY166866	42.6	27.5	29.2
TuMV	NC_002509	46.5	28.3	27.7
SPFMV-S	D86371	44.4	52.8	49.4
YMV	NC_00	39.2	26.4	27.8
ZYMV	AF127929	35.5	25.9	27.8

he virus acronyms used in the table are; *Bean common mosaic virus* (BCMV), *Papaya ringspot irus* (PRSV), *Pea seed-borne mosaic virus* (PSbMV), *Peru tomato virus* (PTV), *Plum pox virus* PPV), *Potato virus A* (PVA), *Potato virus V* (PVV), *Potato virus Y* (PVY), *Turnip mosaic virus* TuMV), *Yam mosaic virus* (YMV), and *Zucchini yellow mosaic virus* (ZYMV).

8.4 DISCUSSION

The P1 coding region of the SPFMV-S isolate is the largest ever reported for known potyviruses (Sakai *et al.*, 1997). However, the P1 gene of four African SPFMV isolates from Africa had a 75 nt insertion that was absent in the P1 sequence of the isolate earlier reported from Japan making the SPFMV genome even larger. The SPFMV-S is known to cause very severe symptoms on sweet potato in Japan (Sakai *et al.*, 1997), whereas in Africa generally and East Africa in particular, the strains of SPFMV on their own hardly cause symptoms on sweet potato (Gibson *et al.*, 1998; Karyeija *et al.*, 2000). It is not known whether the African isolates sequenced in this study acquired the extra 75 nt fragment or if a similar stretch of nucleotides was deleted from the SPFMV-S RNA during the evolution process. It can only be speculated that this striking difference is either an adaptive feature of the virus in the diverse geographical locations, and that it is responsible for differences in disease phenotype, or both. To be conclusive, more isolates particularly from Asia (Japan) need to be sequenced to find out which of the two P1 gene sizes is typical of SPFMV.

ther analysis of the P1 nucleotide sequences showed that the SPFMV-S isolate (Sakai *et al.*, 97) was not only shorter, but also significantly different from the other four sequences. combination has been reported in the 5' region of the potyviruses leading to considerable ation (Ohshima *et al.*, 2002). However, the number of available P1 sequences of SPFMV ilable is currently too small to allow a thorough examination of the possibility of recombination.

om the alignment, the P3 gene sequences of the SPFMV appeared to be highly variable between olates with amino acid sequence identities ranging from 65 to 99%. A possible criticism of this udy is that the analysis considered only partial sequences of the P3 gene. However, since >80% of e entire P3 gene sequence was used, the results are deemed representative. The sequence of olate KY45/3S shared nt sequence identities ranging from 66 to 68% with other SPFMV strains nalyzed, and was the most divergent isolate. Based on the CP and 3' NTR sequence analyses (Chapter 7), this isolate and KY25/4A were both assigned to strain group C. The P3 sequence of the latter was, however, closely related (94-97% aa sequence similarity) to sequences of isolates in strain groups S, O and EA. These results demonstrated that the P3 gene may be the most variable gene product of the SPFMV genome. Since the P3 aa sequence of isolate KY45/3S has an identity of only 68% with that of SPFMV isolates belonging to other strain groups, the C strain of SPFMV should perhaps be considered a distinct potyvirus species as already suggested by Lyerly *et al.* (2003) and Mukasa *et al.*(2003b) on the basis of CP sequences.

The CP sequence of isolate KY45/3S is about 82% similar to the RC, O and EA strains but is about 68% similar to these strains in the P3 gene. Surprisingly, the P3 sequence of the C-like isolate KY25/4A was highly similar to that of the other three SPFMV strain groups S, O, and EA. This perhaps resulted from a possible recombination event involving the 5' terminal portion of the

genome. Using a rapid approach similar to the one that allowed the demonstration of occurrence of recombination in *Cauliflower mosaic virus* (Chenault and Melcher, 1994), the CP and P3 sequences were analyzed and the trees obtained compared separately. The general topologies of the trees differed in the location of the branches. It can therefore be inferred from this observation that recombination has possibly been taking place between SPFMV strains. Recombination has previously been shown to occur frequently in potyviruses (Cervera *et al.*, 1993; Revers *et al.*, 1996).

In a comparative host range study (Chapter 7), isolate KY46b and Zambia (both belonging to the EA strain group exhibited similar and wide host ranges. The two isolates infected test plants in the genera *Chenopodium, Ipomoea* and *Nicotiana*. The isolates KY115/1S and Rak6e, which belonged to the group O and EA, respectively, only infected *Chenopodium* and *Ipomoea* species whereas the C strains were observed to only infect *Ipomoea*. Thus, the P3 sequences of the isolates KY46b and KY45/3S appear consistent with their biological properties, whereas those of isolates Zambia and KY24/4A were inconsistent. However, it was not possible to identify the possible host range determinants in either the P1 or the P3 sequences obtained. Similarly, it was not possible to study whether these differences are associated with symptoms since SPFMV-S strain was not available.

9.0 CHAPTER NINE

CONCLUSIONS AND RECOMMENDATIONS

A survey of viruses infecting sweet potato in Kenya revealed that SPVD is widely distributed. The areas with high virus disease incidence were mainly Nyanza, Western province and Kwale district of coastal Kenya. Since SPFMV and SPCSV were the most commonly detected viruses and are known to interact synergistically varieties resistant to these two viruses should be bred.

Further characterisation of SPV2 (=SPVY) allowed its assignment to the genus *Potyvirus*. Co-infection with SPVY and SPCSV showed very severe and conspicuous symptoms, enhanced virus distribution within infected plants and elevated SPVY titres. More studies need to be conducted in order to gain a better understanding of the range of variability in SPVY, and to determine if certain divergent strains escape detection.

Three strains of SPVG were identified on the basis of their molecular characteristics. Work on the biological variability among SPVG strains will improve the understanding of this potyvirus, which appears to have a very restricted host range. Since SPCSV synergizes the infection by potyviruses such as SPFMV and SPVY, it most probably also synergizes the infection by SPVG and this needs to be elucidated

Serological tests, sequences (P3, CP, and 3'-UTR) and host range studies provided evidence for the occurrence of strain group EA, C and O isolates of SPFMV in East Africa. Further work needs to be done to elucidate if the different strains cause different disease symptoms and their effects on yield.

A broad-reacting and highly specific monoclonal antibody (MAb1C4) was raised and recommended for routine diagnosis of SPFMV. However, more work is required to obtain MAbs or develop nucleic acid-based techniques that can be used for the discrimination of SPFMV strains that strikingly differ in biological and/or molecular properties.

The transformation of sweet potato cultivars using the CP gene of SPFMV is one of the approaches being used to manage the infection of SPFMV in Kenya. Since the success of such approaches depends on high sequence identity between the virus in a targeted region and the transgene (Bateson *et al.*, 1994; Tennant *et al.*, 1994), it is likely that such approaches will face challenges especially in East Africa where high genetic diversity (*ca.* 20%) of SPFMV was observed. Such attempts therefore should consider strain diversity when preparing the transgenes for the management of SPFMV.

The potyviruses SPFMV, SPVY and SPVG were detected in sweet potato plants originating from southern Africa. Unlike SPVY and SPVG, SPFMV occurs everywhere sweet potato is grown. The fact that SPVY and SPVG were not detected in East African sweet potato suggests that quarantine measures should be instituted to prevent further spread of these viruses. Since sweet potato viruses may be moved across borders by vectors, tubers and vines, resistant cultivars need to be developed ahead of virus introduction into areas where the viruses and/or strains do not exist currently. This can only be effective if quarantine measures are approached on a regional rather than a national basis.

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10.0 APPENDICES

APPENDIX 1: SWEET POTATO PRODUCTION STATISTICS

Table1: Production statistics for sweet potato major producers in Africa

Country	Yield (Hg/Ha	Area harvested (Ha)	Production (MT)
Africa	44,093	2,532,312	11,165,757
Uganda	44,123	570,000	2,515,000
Nigeria	48,982	511,000	2,503,000
Twanda	66,149	195,370	1,292,361
Tanzania	18,191	522,300	950,100
Burundi	65,072	120,000	780,859
Kenya	82,090	67,000	550,000
Madagascar	55,630	94,500	525,700
Angola	44,375	80,000	355,000
Egypt	266,543	11,807	314,707
DR Congo	50,110	43,889	219,926
Ethiopia	80,000	25,000	200,000
Cameroon	22,436	78,000	175,000
Ghana	13,846	65,000	90,000
Guinea	59,684	14,500	86,542
Benin	54,581	13,652	74,514
Mali	158,037	4,713	74,483
Mozambique	73,333	9,000	66,000
Chad	25,600	25,000	64,000
Zambia	147,222	3,600	53,000
Cote dIvoire	21,500	20,000	43,000
Burkina Faso	61,702	5,996	37,000
Equatorial Guinea	25,714	14,000	36,000
South Africa	34,054	10,000	34,054
Sierra Leon	27,273	11,000	30,000
Niger	142,398	2,068	29,448
Liberia	100,000	1,800	18,000
Sudan	133,846	650	8,700
Somalia	100,000	650	6,500
Morocco	136,703	455	6,220
Comoros	27,273	2,200	6,000
Rep Congo	64,477	842	5,429
Cape Verde	52,778	720	3,800
Gabon	17,500	1,600	2,800
Swaziland	17,692	1,300	2,300

(FAOSTAT, 2003)

Table 2: Sweet potato production in six Kenyan provinces (1996-1998 averages)

Province	Area (ha)	Production (t)	Yield (t/ha)
Nyanza	35950	362378	10.1
Western	17953	180971	10.1
Central	3558	31449	8.8
Eastern	12414	109744	8.8
Coast	1117	9871	8.8

Source: Adapted from Matin (1999).

APPENDIX 2: QUESTIONNAIRE

SELECTION OF TARGET AREAS FOR EXTENSIVE SURVEY IN KENYA

Sampling will favour localities where sweet production is greatest while ensuring that a wide range of agro-ecologies and socio-economic types of farms are included. A minimum of 100 fields will be visited. The number of fields will be sub-divided amongst 5 provinces depending on the size and the density of sweet potato production in each division. Selection of locations in each division will be done at random. Field selection within locations will be done at 5-km intervals along a road. On arrival at each point, the nearest sweet potato crop (3-5 months old) will be sampled.

The following data will be collected:

(a) A field data sheet will be filled out to give details in each field.
(b) A FARMER X SWEET POTATO VIRUSES information sheet will also be filled.

FIELD ASSESSMENT OF DISEASE INCIDENCE AND SEVERITY

Incidence: Fifty plants will be sampled by making two diagonal transects across the field. In the form of an 'X' (25 plants along each diagonal). Intervals between sampled plants will depend on the size of the field. The interval will be estimated before sampling begins and when sampling, plants will be selected directly at these interval points. In overgrown crops, sampling will be done by pushing into the foliage with a forked stick to select a shoot and thereby a plant. For sampled plants, the following data will be recorded.

a. Symptom type. The symptom type should be recorded under: P/Y (purpling or yellowing), M (mosaic), D (leaf distortion) or S (stunting).
b. Disease severity: the scores are as follows:
    i. No symptoms. No stunting etc
    ii. Mild symptoms on leaves, little distortion of leaf shape, apparent but negligible stunting.
    iii. Moderate symptoms on leaf, moderate distortion of leaf shape, moderate stunting.

iv. Severe symptoms on leaf, severe leaf distortion with reduced size, plant partially stunted (very short internodes) but still apparently growing.

v. Very severe symptoms on leaf, severe leaf distortion, reduced size, plant severely stunted (stem extension more or less stopped).

Thus a symptomless plant would score 1 for P/Y, 1 for M, 1 for D and 1 for S.

c. *Symptom description*

Sample collection: At least 1000 diseased cuttings and 500 Asymptomatic cuttings in total spread uniformly across all fields sampled in the country for further laboratory/screen house diagnosis. Cuttings will be planted in an insect-free environment, numbered and maintained alive until samples are known to contain only duplicate/unwanted viruses.

FIELD DATA SHEET

Basic Field Data

1. Field Number _____ 2. Date_____ 3. Collector (s) _____

_____

4. District _____ 5. Division _____ 6. Location _____

___

7. Latitude: _____ 8. Longitude _____9. Altitude __

_____

10. Producers name: _____ 11. Male ___ Female _____

12. Age of respondent (observe) (a) < 40 yr____ (b) 40-60 yr _____ (c) >60 yr. __

13. Age of the crop _____

P/Y= Purpling / Yellowing, M= Mosaic, D= leaf distortion, S= stunting (SPCSV alone typically causes P/Y + S; SPCSV + SPFMV (=SPVD) typically also includes M and D.

Plant No	Severity score Virus type					Describe symptoms	Other diseases	Variety
	P/Y	M	D	S	Sample collected			

Any other comment _____

_____

_____

Photographs taken? Yes _ No _____ If yes, describe photos: _____

___Observations _____

_____

QUESTIONNAIRE FOR FARMERS ON SWEET POTATO VIRUS DISEASE

14. Field Number: _____
15. What variety of sweet potato do you plant on this farm (Greatest to least area)?
    Var. 1 _____
    Var. 2 _____
    Var. 3 _____
    Var. 4_____
    Var. 5 _____
16. Where do you usually obtain planting material? _____
    _____
    ___
17. Interviewer shows sweet potato plant with virus symptoms, and asks them what causes the disease. If there are no virus-diseased plants present, show them a picture.
    (a) Can the producer recognize the disease? Yes _____ (01), No _____ (02)
    (b) What do you call the disease (local name) _____
    (c) What causes/ spreads it? _____
    _____
18. Is there a disease problem in your farm? Yes _____ (01), No _____ (02) Don't know ___ (03)
19. Do these problems appear every year? Yes _____ (01), No _____ (02)
20. What months is this disease severe? _____
    ___
21. List the methods you use to control the disease problem in order of importance (1= the most important): Give scores for their effectiveness in controlling the problem: (1) very effective; (2) Partly effective: (3) not effective (4) damaging.

Method		Effectiveness
1.	_____	_____
2.	_____	_____
3.	_____	_____
4.	_____	_____
5.	_____	_____

22. Do you ever plant these (diseased) plants? Yes _____ (01), No _____ (02)
    10 Do you ever pull these (diseased) plants out? Yes _____ (01), No _____ (02)
    If yes, when _____
    11. Are some varieties more severely damaged than others? Yes _____ (01), No _____ (02)
14. (a). If you have noticed differences, why do you grow the ones that are more diseased? _____
    _____
    (b) Why do you think some varieties are more diseased than others? _____
    _____
    13. Do you have any really good resistant varieties? Yes _____ (01), No _____ (02) If yes, give names _____
14. Which other good traits does the variety have? _____
    _____
    _____
15. Would you be interested in receiving a new variety? Yes _____ (01), No _____ (02)
    If yes, what characteristics would want in the new variety? (List them in order of importance)
    (a) _____
    (b) _____
    (c) _____
    (d) _____

APPENDIX 3: Buffers and solutions

Buffers for microplate ELISA (DAS and TAS-ELISA)

(i) Wash buffer (PST -T) 10X stock solution

- NaCl 80.0g
- $KH2_2PO_4$ 2.0g
- $Na_3HPO_4.2H_2O$ 14.4g
- KCl 2.0g
- Tween 20 5.0ml
- NaN_3 2.0g
- 1L, pH 7.4

(ii) Coating buffer

- Na_2CO_3 1.59g
- NaH CO_3 2.93g
- NaN_3 0.2g
- 1L, pH 9.6

(iii) Sample buffer

- PBS-T 1L
- PVP 20g
- 1L, pH 7.4

(iV) Conjugate buffer

- PBS-T 1L
- PVP 20g
- Egg albumin 2g
- Brought to 1L, pH 7.4

(v) Substrate buffer

- Diethanolamine 97ml
- H_2O 600ml
- NaN_3 0.2 g
- 1L, pH 9.8

Buffers for NCM-ELISA

(i) TBS pH 7.5

- 0.02 M Tris base
- 0.5M NaCl

(ii) T-TBS

- TBS
- 0.05%, Tween-20

(iii) Substrate buffer pH 9.5 (0.5l)

- Tris base 6.05 g (0.1 M)
- NaCl 2.92 g (0.1 M)
- Mg $Cl_2.6H_2O$ 0.51 g (0.005M)

(iv) Substrate solution for NCM-ELISA

(a) NBT stock solution
- NBT 40mg
- N, N-dimethylformamide (70%) 1.2 ml
- Mix well and store at 4°C protected from light

(c) BCIP stock solution
- BCIP 20mg
- N, N-dimethylformamide (70%) 1.2 ml
- Mix well and store at 4°C protected from light

Preparation of the substrate solution

- Substrate buffer	30 ml
- NBT stock solution	90µl
- BCIP stock solution	90µl

Dissolve NBT in 30 ml of substrate buffer then add BCIP drop wise.

Buffers and solutions used for electron microscopy

(i) 0.1M Na/K phosphate buffer, pH 7.0 (Extraction buffer)

(ii) $NaSO_3$ buffer, 0.1M Na/K phosphate buffer, pH 7.0, containing 2% PVP (W/V)

(iii) 0.1M Na/K phosphate buffer, pH 7.0, containing 0.05% NaN_3

(iv) Staining solution: 1% Uranyl acetate (Uac) in distilled water, pH 4.3

Buffers used in the purification of SPFMV

(a) Extraction buffer (0.5L)

- K_2HPO_4	0.5M	43.54g
- Na_2SO_3	0.02M	01.26g
- DIECA	0.01M	01.126g
- EDTA	0.05M	0.93g

- Dissolve in 0.5 L distilled water, pH 8.5

(b) 0.5M Na-citrate buffer (pH 7.5)
- 0.5M Na-citrate
- 0.5M Urea
- 0.1% Mercapto-ethanol

Buffers for agarose gel electrophoresis

(i) TAE

0.04mM Tris acetate, pH 8.0
1mM EDTA

(ii) Electrophoresis gel loading solution
50 % (v/v) glycerol, 0.01 M NaH_2PO_4, pH 7.0, 0.04 % (w/v) Bromophenol blue in 5x TAE buffer.

Buffers and solutions for SDS-PAGE

Solutions

(i) 29.2g and 0.8g N'N'-bis-methylen-acrylamide dissolved in 100ml H2Ofiltered and kept in the dark

(ii) Resolving gel buffer (4x) : 1.5M Tris-HCl, pH 8.8 (36.3g/200ml)

(iii) Stacking gel buffer (4x): 0.5M Tris-HCl, pH 6.8 (3g/50ml)

(iv) 10% SDS

(v) 10% ammonium persulfate (APS) solution (0.1g/ml in water)

(vi) sample buffer (5x), pH 6.8

10% SDS	12.5ml
Stacking gel buffer	20ml
Glycerol	10ml
H_2O	50ml
Mercapto ethanol	5ml (added just before use)

(vii) TEMED: N,N,N'N'-tetramethylen diamine

(viii) Electrophoresis buffer: 25mM Tris base; 192mM glycine, 0.1% (w/v) SDS, pH 8.3

(ix) Staining solution
60g of trichloroacetic acid
70ml acetic acid (96%)
200ml methanol
800ml H2O
For gel staining; 2.5ml of 1% (w/v) Coomassie Brilliant Blue R-250 solution in 100ml staining solution was used.

(x) Staining solution
200ml methanol + 65ml acetic acid (96%) in 1 litre of H_2O.

(xi) Antibody dilution buffer
0.01 Tris HCl, pH 7.0, containing 0.85% (w/v) NaCl, 0.05% (w/v) NaN3, 0.05% (v/v) Tween 20 , 0.3% (v/v) Triton X-100 and 1% (w/v) bovine serum albumin (BSA)

Table: SDS-Gel preparation

Solutions	Resolving gel (12%)	Stacking gel (4%)
Acrylamide	6.0ml	1.0ml
distilled water	4.95ml	4.475ml
resolving gel buffer (4x)	3.75ml	-
stacking gel buffer (4x)	-	1.85ml
10% (w/v) APS	150µl	75µl
10% (w/v) SDS	150µ	75µl
TEMED	15µ	7.5µl

Protein purification under denaturing conditions

(i) Lysis buffer
Buffer A: 6 MGu.HCl; 0.1M NaH_2PO_4; 0.0 M Tris.Cl, pH 8.0
Buffer B: 8 M urea; 0.1M NaH_2PO_4; 0.0 M Tris.Cl, pH 8.0

(ii) Wash buffer
Buffer C: 8 M urea; 0.1M NaH_2PO_4; 0.0 M Tris.Cl, pH 6.0

(iii) Elution buffers
Buffer D: 8 M urea; 0.1M NaH_2PO_4; 0.0 M Tris.Cl, pH 5.9
Buffer E: 8 M urea; 0.1M NaH_2PO_4; 0.0 M Tris.Cl, pH 4.5

Buffers and solutions for the modified total plant nucleic acid extraction with silica

(i) Grinding buffer

4.0M guanidine thiocyanate
0.2M NAOAc pH 5.2
25mM EDTA
1.0M KOAc
2.5% (w/v) PVP-40
1% 2-ME (added just before use)

(ii) Wash solution
10.0 mM Tris-HCl, pH 7.5
0.5mM EDTA
50.0 mM NaCl
50% ethanol
Store at 4°C

(iii) NaI
- Dissolve 0.75g Na2SO3 in 40 ml water
- Add 36g NaI (Sigma) stir until completely dissolved
- Store in dark bottle at 4°C

(iv) Preparation of the silica particle solution

- Add 60g to 500ml of water in a cylinder
- Let to settle for 24h
- Discard the upper 470ml supernatant
- Add to 500ml and mix well
- Let settle for 5h
- Discard 440 ml
- Adjust the remaining 60ml slurry to a pH 2.0 with HCl.
- Autoclave and store in a dark bottle at room temperature.

APPENDIX 4: CULTURE MEDIA

All media and solutions were autoclaved (121^0C 1bar, 30 min)

(i) Lauria-Bertani (LB) medium

- Tryptone 10g (Difco)
- Yeast extract 5g (Difco)
- NaCl 10g
- 1 litre of distilled water, pH 7.0

(ii) LB Agar (plates)

- LB medium 1L
- Agar 15g

(iii) LB medium Kanamycin plates

- LB agar
- Kanamycin 30μg/ml

(iii) LB Ampicillin/XGAL plates

- LB agar
- 150μg/ml ampicillin
- 40mg X-gal (5-bromo–4-chloro-3-indoyl-β-galactopyranoside) in 1ml of N,N-dimethylformamide

Ampicillin and X-gal added after autoclaving LB agar.

APPENDIX 5: MEDIA FOR MONOCLONAL ANTIBODY PRODUCTION

Dulbelcco's modified eagle medium (DMEM) 4500mg/L glucose, Sodium pyruvate and $NaHCO_3$, Pyridoxine (GIBCO)

DMEM powder	13.4g
Na-bicarbonate	3.7g
Glutamine (2mM)	0.29g
Na-pyruvate (1mM)	0.11g
Gentamycine sulphate	0.05g
Mercapto-ethanol	5ul

Mix with 1L of 18 mΩ water. Adjust pH to 6.9 with 1M HCl. Filter sterilize (0.2 μm pore size). Store at 4°C.

HAT (DMEM containing 20% FCS, aminopterin and HT)

FCS- Foetal calf serum triple sterile filter 0.1um sterile filtered
HT supplement 50X (Gibco)
Aminopterine 100X

Polyethylene glycol (PEG)
Add 5g PEG 4000 to 4 ml DMEM and heat to dissolve PEG. Add 1ml DSMO and mix. Filter sterilize (0.2 μm pore size).