RESPONSE OF SWEETPOTATO GERMPLASM TO SWEETPOTATO VIRUS DISEASE AND MOLECULAR CHARACTERIZATION OF SWEETPOTATO MILD MOTTLE VIRUS

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Response of sweetpotato germplasm to sweetpotato virus disease and molecular characterization of sweetpotato mild mottle virus

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This work is dedicated to all my family members

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ABBREVIATIONS

ANOVA	Analysis of Variance
AAP	Acquisition access period
AEZ	Agro-ecological zone
CBSV	Cassava Brown Streak Virus
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
CIP	International Potato Centre
CRD	Completely Randomized Design
CP	Coat protein
CTAB	Cetyltrimethyl ammonium bromide
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DMF	N, N- dimethylformamide
FAO	Food Agricultural Organization of the United Nations
GAR	Goat anti-rabbit
IAP	Inoculation access period
ILRI	International Livestock Research Iinstitute
KALRO	Kenya Agricultural & Livestock Research Organization

MoA	Ministry of Agriculture
M-MLV	Moloney Murine Leukaemia Virus
NCBI	National Center for Biotechnology Information
NCM-ELISA	Nitrocellulose membrane enzyme linked immunosorbent assay
NARL	National Agricultural Research Laboratories
NBT	4-Nitroblue Tetrazolium Chloride
nt	Nucleotide
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
RdRp	RNA dependent RNA polymerases
RCBD	Randomized Complete Block Design
+ssRNA	Single-stranded positive-sense RNA
SPVD	Sweet potato virus disease
SPCSV	*Sweet potato chlorotic stunt virus*
SPFMV	*Sweet potato feathery mottle virus*
SPMMV	*Sweet potato mild mottle virus*
TBS	Tris buffered saline
UTR	Un-translated region

ABSTRACT

Sweetpotato virus disease (SPVD) is the most economically important disease affecting sweetpotato production in Kenya, causing yield reduction of up to 90%. Sweetpotato mild mottle virus (SPMMV) also increases the severity of symptoms expressed in SPVD-infected plants. To address this challenge, twenty sweetpotato genotypes were evaluated for it in this study. Two field trials were set up in Kenya Agricultural and Livestock Research Organization (KALRO) Kakamega and Yala swamp. The genotypes were planted in 5m x 3m plots in rows spaced at 100 cm and intra row spacing of 30cm. the experiments were laid out in a randomized complete block design (RCBD) with three replications. Disease severity was scored every month for a period of five months by visually assessing the severity of symptoms on a scale of 1 to 5. To determine genetic variability of SPMMV, PCR products were sequenced and compared with sequences of 14 East African SPMMV isolates from the National Center for Biotechnology Information (NCBI) gene bank using the DNAMAN version 4.02 software. To determine the vector for SPMMV, white flies (*Bemisia tabaci*) were allowed six access acquisition periods (AAP) of 0, 6, 12, 24, 48 and 72 hours of feeding and a uniform inoculation access period (IAP) of 48 hours. After six weeks, leaves from the inoculated *I. setosa* plants were evaluated for presence of SPMMV using an Enzyme Linked Immunosorbent Assay on Nitro cellulose membranes (NCM-ELISA). The virus transmission was calculated by expressing the number of infected recipient *I. setosa* plants as a percentage of the total number of recipient plants used. The test sweetpotato genotypes exhibited significant differences ($P<0.05$) in disease severity. The results indicated that four genotypes, KKFS 56682-03-1, Marooko-3, YS Sopalla and YS Kemb 10 were tolerant to SPVD. Nucleotide sequence similarity of the SPMMV isolates sequenced ranged between 71% and 97% indicating a high genetic variability among the SPMMV isolates. Transmission of SPMMV was observed at the 48 and 72 hours AAP.

CHAPTER ONE

INTRODUCTION

1.1 General introduction

Sweetpotato (*Ipomoea batatas L.*) is one of the most important crops worldwide with an annual production of more than 126 million metric tons (FAO, 2007). It is the third most important root crop after potato (*Solanum tuberosum*) and cassava *(Manihot esculenta)* and is ranked seventh in global food crop production (Kays, 2004). Seventy five percent of sweetpotato production in East Africa is concentrated around the Lake Victoria region (Karyeija, Gibson, Valkonen, 1998). In 2011, Tanzania had the highest sweet potato production in Africa with approximately 3.5 million metric tons, followed by Nigeria (2.7 million metric tons), Uganda (2.5 million metric tons) and Kenya (0.75 million metric tons) (FAOSTAT, 2011). Sweetpotato is an important food security crop in Kenya. The crop is grown in most of the Counties and agro-ecological zones of the country (MOA, 2013).

Production constraints include disease infection by fungi, bacteria, nematodes, and viruses (Clark *et al.*, 2003). The important sweetpotato viruses belong to the families, Potyviridae and Closteroviridae, and up to 20 different ones have been identified. In East Africa, sweet potato feathery mottle virus (SPFMV), sweet potato chlorotic stunt virus (SPCSV) and sweetpotato mild mottle virus (SPMMV) are the most commonly occurring (Tairo , Kullaya, Valkonen, 2004). Virus diseases not only cause reduction in yields but also affect quality of stored roots (Clark & Hoy 2006).

Sweetpotato is vegetatively propagated and the one year-round production common in Western and Nyanza regions in Kenya provides a reservoir of virus-infected plants from which insect vectors can transmit the viruses to newly planted sweetpotato (Clark *et al.*, 2006; Kapinga, Ewell, Jeremiah, Kileo 1995). The crop incurs heavy yield losses as a result of infestation by the sweetpotato virus disease (Miano, 2008). The identification

of sweetpotato clones with resistance or tolerance to sweetpotato virus disease and the distribution of the virus would be desirable for the region. In the current study, twenty sweetpotato genotypes were evaluated for tolerance to sweetpotato virus disease (SPVD). A virus, SPMMV that increases severity of SPVD was also studied.

1.2 Sweetpotato Virus Disease (SPVD)

Sweetpotato virus disease is the major virus constraint for sweetpotato production worldwide causing yield reductions of up to 90 % (Gutierrez, Fuentes, Salazar 2003; Loebenstein, Fuentes, Cohen, Salazar, 2004). The disease is caused by the synergistic interaction between SPFMV and SPCSV (Karyeija *et al.,* 1998a ; Gibson *et al.,* 2002).When the viruses infect singly, sweet potato cultivars exhibit mild or no symptoms (Karyeija *et al.,* 2000; Untiveros, Fuentes, Salazar, 2007). However, when the viruses occur together, infected plants exhibit severe symptoms characterized by overall plant stunting, leaf narrowing and distortion, chlorosis, mosaic or vein-clearing (Aritua, Bua, Barg , Vetten , Adipala, Gibson, 2007; Gasura E., Mashingaidze., Mukasa,2008). Efforts to develop a transgenic sweetpotato with resistance to SPVD through resistance to SPFMV have been attempted in Kenya and in other parts of the world (Okada *et al.,* 2000, Wambugu, 2003). However, the problem of SPVD is not SPFMV but SPCSV which synergizes with different unrelated viruses (Kokkinos 2006), therefore resistance to SPFMV may not hold in the presence of SPCSV (Mukasa., Rubaihayo , Valkonen, 2006).

In Kenya, sweetpotato is often grown continuously with one planting cycle overlapping with another (Bashaasha B., Mwanga R.O.M., Ocitti p'Obwoya C., Ewell P.T. 1995). This one year-round production is especially common in Western and Nyanza provinces. This practice provides a reservoir of virus-infected plants from which insect vectors can transmit the viruses to newly planted sweetpotato leading to high incidences of virus infection in these provinces. In a survey carried out in the main sweetpotato growing areas of Kenya, the incidence of virus infection was highest (18%) in Kisii District of

Nyanza Province and lowest (1%) in Kilifi and Malindi districts of Coast province (Ateka, Njeru, Kibaru, Kimenju , Barg, Gibson, Vetten, 2004). In another survey, virus incidence was found to be 52% in Western province and Nyanza provinces (Opiyo, 2010).

1.3 Genetic Variability and Transmission of Spmmv

Genetic variation is caused by errors occurring during the replication of genomes especially encountered by single stranded RNA viruses such as SPMMV. High genetic variability (82-100% amino acids) in the sequence of the coat protein (CP)-encoding region of SPMMV isolates from East Africa has been reported by Mukasa *et al.* (2003a). Genetic variability can occur through base substitutions, insertions, deletions, inversions and recombination (Paalme, Gammelgåard, Järvekulg, Valkonen, 2004; Bousalem, Douzery, Fargette, 2000; Moonan, Molina, Mirkov, 2000).

A plant virus is entirely dependent upon plant cells for its survival and multiplication. It must also have the ability to move between plant cells and between plants. To develop relevant strategies to control viral infections in plants, it is important to establish the vectors that transmit them. In East Africa, SPMMV is the third most prevalent virus of sweet potato after SPFMV and SPCSV and is known to occur in complexes with these two viruses (Nyaboga, Ateka, Bulimo, 2008). There are reports that Ipomoviruses such as cassava brown streak virus (CBSV) squash vein yellowing virus (SqVYV) are transmitted by the whitefly (Jones, 2003; Maruthi *et al.,* 2005; Stansly, McKenzie, 2008). SPMMV is also an ipomovirus which can be transmitted by grafting but there is limited knowledge on its transmission by vectors. Although Hollings *et al.* (1976) described it as a virus transmitted by the whitefly (*Bemisia tabaci*), later studies did not confirmed this. Better understanding of the virus and the vector is useful in developing management strategies for sweetpotato mild mottle virus.

1.4 Statement of the Problem and Justification

Sweetpotato is adaptable to different agro ecological zones and is an important food security crop for subsistence farmers around Lake Victoria in East Africa (Gibson, Aritua, 2002; Mutuura *et al.,* 1992). Sweetpotato virus disease is the major virus constraint for sweetpotato production and the only way to adequately protect the crops of subsistence farmers from SPVD is by using host plant resistance (Valverde, Clark, Valkonen, 2007). Some genotypes are more readily infected by SPVD than others when exposed to similar amounts of inoculum (Mwanga., Odongo, Ocitti p'Obwoya, Gibson, Smit, Carey, 2001) but little has been done to identify such genotypes. This study was conducted to evaluate selected Kenyan genotypes for tolerance to SPVD.

Most of the viruses which have single-stranded positive-sense RNA (+ssRNA) genomes such as SPMMV are prone to genetic variability (Hull, 2002). Genetic variability is an important aspect in adaptation of viruses to environments and viruses employ several mechanisms to generate sequence variation (Roossinck, 1997). Sweetpotato mild mottle virus increases severity of SPVD (Untiveros *et al.*, 2007) hence the need to determine the genetic variability of this virus. One objective of this research was to determine genetic variability of East African SPMMV isolates. A plant virus must have the ability to move between plant cells and between plants.

To develop relevant strategies to control viral infections in plants, it is important to establish the vectors that transmit them. To control SPMMV infections in sweetpotato plants, it is necessary to determine the vector that transmits the virus.

1.5 OBJECTIVES

1.5.1 Broad objective

To contribute to better management of sweetpotato viruses and study sweetpotato mild mottle virus.

1.5.2 Specific objectives

1. To evaluate selected Kenyan sweetpotato genotypes for resistance to sweetpotato virus disease under field conditions
2. To determine the genetic variability of East African sweetpotato mild mottle virus
3. To determine the vector involved in the transmission of sweetpotato mild mottle virus

1.5.3 Null hypotheses

1. Kenyan sweetpotato genotypes do not differ in resistance to sweet potato virus disease under field conditions
2. There is no genetic variability in sweetpotato mild mottle virus
3. *Bemisia tabaci* does not transmit sweetpotato mild mottle virus

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin of Sweetpotato

Sweetpotato (*Ipomoea batatas*) is thought to have originated in Mexico and possibly Central America (Zhang & Corke, 2001). The discovery of actual remains of cultivated sweetpotato from Casma valley of Peru, provided archaeological evidence for the origin of the crop (Ugent, Pozorski, Pozorski, 1982). Austin (1988) suggested the origin of sweetpotato in the region surrounded by the Yucatan Peninsula of Mexico to the north and Orinoco River to the south, and also Guatemala and southern Peru as secondary centers of origin with high diversity. The crop had already existed in the Central or South America before Europeans first arrived, which was followed by spread of sweetpotato in other areas of the world (Woolfe, 1992a). In 1492, during his first voyage, Columbus discovered sweetpotato and introduced it to Western Europe, from where it was further spread to Africa and Asia (Woolfe, 1992b; Yen, 1982). It was introduced to the tropical areas of Africa, Europe, China, India, and Indonesia during the 16th century (Janssens, 2001).

2.2 Botany

The sweetpotato belongs to the family Convolvulaceae or morning glory and the genus, *Ipomoea* (Austin *et al.*, 1996). It is a dicotyledonous, herbaceous plant whose roots develop into edible storage roots (Jones *et al*., 1986). It is a perennial plant, although it is typically cultivated as an annual crop (Janssens, 2001). It is the only *Ipomoea* species out of the 500 species known that produces edible tuberous roots for which it is grown (Onwueme *et al.,* 1994). The stem and leaf colour of different sweet potato genotypes vary from green to purple due to the presence of anthocyanin pigmentation. The storage root skin range from white to dark purple and flesh colour vary from white to orange

(laurie & Niederwieser, 2004). Sweetpotato is an indeterminate plant without a defined physiological maturity, and as such, storage roots may continue to enlarge for a long time. The crop has great capacity of improvement because of its high level of diversity (Zhang *et al*., 2000; 2004).

2.3 Production and Utilization of Sweetpotato

Sweetpotato is an important starchy tuberous root crop grown in many tropical and subtropical regions of the world. Seventy five percent of African sweetpotato production occurs around Lake Victoria in East Africa, where it is a basic subsistence crop mainly grown by women (Gibson *et al.,* 2002). Sweetpotato is adaptable to different agro ecological zones ranging from 0-2100m above sea level and occasionally is found in altitudes of about 2400m. It thrives at temperatures above 24°C in abundant sunshine, rainfall of 750-1000mm per annum and a moderate soil pH of 6.0 for optimum production. It requires well drained deep sandy to loam and loamy clay soils (MOA, 2013). Lighter soils are also advantageous in that they are easily removed from the roots at harvest time. Sweetpotato has a growing period of 3-4 months depending on the variety (MOA, 2013). Vine-tip cuttings are used as planting material and are usually planted on flat ground, ridges or mounds. Spacing is 60-100 cm between rows and by 30-60 cm at 45º angle into the hills as this promotes even root development (Egbe, 2012).

The harvesting period of sweetpotato tubers is not clearly defined. It varies with cultivar, cultural practices and climate. It is recommended that harvesting should be done promptly to prevent weevil damage. The average yield of sweetpotato in Kenya is 13 tonnes per hectare (MOA, 2011).

Constraints to sweetpotato production include diseases caused by fungi, bacteria, nematodes, and viruses (Clark *et al*., 2002; 2003) and pests such as sweet potato weevils (rough sweetpotato weevil and striped sweet potato weevil) and white grubs. Sweetpotato is vegetatively propagated, providing a reservoir of virus-infected plants (Clark *et al.,* 2006). Among all diseases recorded in this crop, those that are the most

economically important are caused by viruses (Mukasa, 2006a; Gibson, 1998). The cultural practice of vegetative propagation perpetuates dissemination of viruses between cropping seasons and/or growing areas (Salazar & Fuentes, 2001).

Due to its nutritional qualities (rich in carbohydrates, dietary fiber, beta carotene, vitamin C, and vitamin B6), sweetpotato is considered as a crop with great potential not only for humans consumption but also for animal feed and industrial use (Bovell-Benjamin, 2007; Huntrods, 2008). The flour is used as a dough conditioner for bread, biscuit and cake processing as well as in gluten-free pancake preparation (Shih *et al*., 2006). In China the starch is used for making pasta (Singh, Raina, Bawa,, Saxena, 2004) and for producing alcoholic beverages.

2.4 Importance of Sweetpotato in Kenya

In Kenya, sweetpotato (ipomoea batatas l.) Is regarded as a "poor man's" crop as it is often grown and consumed by resource limited households and has the ability to give satisfactory yields under adverse climatic and soil conditions as well as under low or non-use of external inputs (carey et al., 1997; ndolo et al., 1998). The orange-fleshed sweetpotato varieties benefit the health of consumers through their high level of beta carotene, carbohydrates, dietary fibre, vitamin c, and vitamin b6 (bovell-benjamin, 2007a). These varieties can be milled for value added products. As a food security crop, sweetpotato can be harvested piecemeal as needed, thus offering a flexible source of food and income to rural households that are mostly vulnerable to crop failure and fluctuating cash income. The crop grows well under varying agro-ecological conditions in Kenya ranging from the coastal lowlands to altitudes of about 2000m above the sea level in the central highlands. The main sweetpotato production areas in kenya include western, nyanza, central, coast and eastern provinces where hundreds of sweetpotato cultivars (landraces) are grown (gichuki et al., 2003).

2.5 Sweetpotato Viruses

The important viruses of sweet potato belong to two families namely, Potyviridae and Closteroviridae. Worldwide, up to twenty distinct viruses have been isolated, described, and characterized (Valverde *et al*., 2008a and Loebenstein *et al*., 2004a). In East Africa, sweetpotato feathery mottle virus (SPFMV), sweet potato chlorotic stunt virus (SPCSV) and sweetpotato mild mottle virus (SPMMV) are the most commonly occurring (Nyaboga *et al.,* 2008a, Tairo *et al.,* 2004a ; Kreuze, 2000). Other viruses on sweetpotato in other parts of the world include sweetpotato latent virus (Yun *et al.,* 2002), sweetpotato mild speckling virus (Alvarez, Ducasse, Biderbost, Nome, 1997), sweetpotato leaf curl virus (Lotrakul, Valverde, Clark, Sim, De La Torre, 1998), Ipomoea yellow vein virus (Banks, Bedford, Beitia, Rodriguez-Cerezo, Markham, 1999), sweetpotato virus Y (Ateka *et al.,* 2004a), sweetpotato virus G (Souto, Sim, Chen, Valverde, Clark, 2003) and sweet potato chlorotic fleck virus (Tairo *et al.,* 2004b). Virus diseases not only cause reduction in yields but also affect quality of storage roots (Clark *et al.,* 2006). Often, infection of sweetpotato by more than one virus leads to greater disease severity than when one is involved. This synergism is very clear in sweetpotato virus disease (SPVD), the most important virus disease in the world (Karyeija *et al*., 2000b; Gutierrez *et al*., 2003; Miano, 2008a).

2.5.1 Sweetpotato feathery mottle virus (SPFMV)

SPFMV belongs to the genus Potyvirus and the Potyviridae family. It is the most common and widespread virus infecting sweetpotatoes worldwide (Untiveros *et al.*, 2008 and Valverde *et al*., 2007a). Although sweetpotato is the main host of SPFMV, the virus occurs in wild *Ipomoea* species (Tugume *et al.*, 2008). SPFMV is transmitted from infected to healthy plants in a non-persistent manner (Clark *et al.,* 1988) by several aphid species, including *Aphis gossypii*, *A. craccivora*, *Lipaphis erysimi*, and *Myzus persicae*. It can be transmitted mechanically to various *Ipomoea* species (Loebenstein *et al*., 2004c) but is not seedborne. Symptoms of SPFMV on leaves include irregular

chlorotic patterns (feathering) along leaf veins and chlorotic spots with or without purple margins in some cultivars. Symptom visibility on foliage is influenced by cultivar susceptibility, degree of stress, growth stage, and virus strain virulence. Increased stress can lead to symptom expression, whereas rapid growth may result in symptom remission of disease (Clark *et al.*, 1988). Two serotypes of sweetpotato feathery mottle virus have been reported in Uganda (Karyeija, *et al.*, 2000c).

2.5.2 Sweetpotato Chlorotic Stunt Virus (SPCSV)

SPCSV belongs to the genus *Crinivirus* (family *Closteroviridae*), and is widespread in different sweetpotato growing regions of the world (Kreuze *et al.*, 2002 and Gibson *et al.*, 1998) and infects several *Ipomoea* species (Loebenstein *et al.*, 2004d). It is transmitted in a semi-persistent, non-circulative manner by whiteflies, *Bemisia tabaci* and *Trialeurodes abutilonea* (Sim, Valverde, Clark, 2000; Cohen Franck, Vetten, Lesemann, Loebenstein 1992). It is also graft transmissible. Symptoms associated with SPCSV include mild stunting, interveinal chlorosis, and interveinal purpling of older leaves (Gutierrez *et al.*, 2003; Gibson *et al.*, 1998). Infections by SPCSV can cause significant yield reductions in sweetpotato (Untiveros *et al.*, 2007a and Gutierrez *et al.*, 2003). However, the virus is most significant in synergizing the multiplication of SPFMV (Karyeija *et al.*, 2000d) thereby increasing the severity of SPVD.

2.5.3 Sweetpotato Mild Mottle Virus (SPMMV)

Sweetpotato mild mottle virus belongs to the genus *Ipomovirus* (Van Regenmortel *et al.*, 2000) and the family Potyviridae. Symptoms of SPMMV are leaf mottling vein chlorosis, dwarfing and poor growth (Mukasa, 2004), in intolerant cultivars, and it is symptomless in others. SPMMV has a wide host range and infections have been reported in many plant families such as; *Amaranthaceae, Chenopodiaceae, Compositae, Convolvulaceae, Leguminosae, Portulacaceae, Primulaceae and Solanaceae* (Hollings *et al.*, 1976). It is the third most prevalent sweetpotato virus in East Africa after SPFMV and SPCSV (Ateka *et al.*, 2004b; Tairo *et al.*, 2004c). It also occurs in complexes with

SPCSV and SPFMV (Nyaboga *et al.,* 2008b), and its presence in SPVD increases disease severity (Untiveros *et al*., 2007b).

SPMMV has flexuous (+)ssRNA filamentous particles with a length of between 830-850 nm. It contains a single positive stranded RNA genome of about 10.8 kb which is comparable to that of SPFMV. Its morphology and size, cytoplasmic inclusions in infected cells and genome organization are similar to those of potyviruses but sequences of the coat protein (CP) core region show only limited similarity with other members of the Potyviridae (Colinet, Kummert, Lepoivre, 1996; Moyer, Jackson, Frison, 1989). The SPMMV genomic sequence is 10 818 nucleotides in length with a polyadenylated tract at the 3′ terminus. The structure and organization of the SPMMV genome has a 5′ untranslated region, rich in adenine (A) and uracil (U) between nucleotides 1 and 139. An initiation codon, at nucleotides 140–142, marks the beginning of a large open reading frame (ORF) which ends in UAA at positions 10 508–10 510. A 308-nucleotide untranslated region is present between the termination codon of the ORF and the beginning of the 3′ polyadenylated region (Colinet *et al.,* 1998).

2.6 Sweetpotato Virus Disease (SPVD)

SPVD is the most damaging disease of sweetpotato in many parts of Africa, in particular in East Africa (Geddes, 1990). It is caused by dual infection of sweetpotato with SPCSV and SPFMV (Nyaboga *et al.,* 2008c and Gibson *et al.,* 1998) with the former synergizing the multiplication of the latter (Karyeija *et al.,* 2000e). SPVD can reduce yields by up to 90 % (Miano, 2008b and Carey *et al*., 1997). It is characterized by overall plant stunting, leaf narrowing and distortion, chlorosis, mosaic or vein-clearing leaf strapping (Gasura *et al.,* 2010; Aritua *et al.,* 2006; Gibson *et al.,* 1998).

The high incidence of viruses in sweetpotato is attributed to utilization of infected stem cuttings as planting materials (Clark *et al.,* 2006; Salazar *et al.,* 2001) and the presence of insect vectors (aphids and whiteflies). SPVD is severe and widespread in Kenya (Nyaboga *et al.,* 2008d) with the highest incidences reported in Nyanza and Western

provinces (Ateka *et al*., 2004c). A cheap and effective means of controlling SPVD is the use of resistant varieties (Mwanga *et al.,* 2001) and several studies have been conducted to identify sweetpotato genotypes that are resistant to the disease. The Kenyan sweetpotato genotype ‘‘Marooko’’ showed transient chlorotic spots only when inoculated with US strains of SPVD-causing viruses and was thus considered resistant (Miano, 2008c).

SPVD can be controlled through rouging of infected plants, isolation of sweetpotato fields from SPVD-affected ones, use of disease free planting materials, crop rotation and use of resistant varieties (Nelson & Elevitch 2010). Use of tissue culture regenerated plants has been tried but no significant differences in yield between the conventional propagation and tissue culture regenerated plants were observed although virus titre of SPFMV was lower in tissue culture regenerated plants (Oggema, Kinyua, Ouma, Owuoche, 2007). Host resistance is an attractive option for disease management as it is generally cheaper to the grower and is therefore the best and certainly the most convenient means of controlling SPVD (Valverde *et al.,* 2007b ; Mwanga *et al.,* 2001).

2.7 Host-Virus Interaction

Plants have a passive defense against pathogens based on the presence of existing barriers like the rigid cell wall. However they also exhibit active defense mechanisms upon recognition of pathogens. Some of these mechanisms act as physical and chemical barriers that prevent infection by pathogens. The most common active defense mechanism is the hypersensitive response (HR), a local reaction characterized by programmed cell death at the infection site which results in a visible necrotic local lesion (Goldbach, Bucher, Prins, 2003 ; Heath, 2000). The HR response is induced by the plants’ recognition of the pathogen. It is unfavorable for the development of the virus cycle, and avoids massive and systemic virus dissemination in the host plant.

2.8 Transmission of Viruses

A plant virus is a micro-parasite, a nucleoprotein that is entirely dependent upon plant cells for its survival and multiplication. Additionally, it must have the ability to move between plant cells and ultimately between-plants. The mechanism of transmission of viruses between plants determines the rate and extent of disease development in plant populations (Seal, VandenBosch, Jeger, 2006; Power, 2000). Viruses are transmitted through seed, pollen, vegetative-propagation, mechanical damage and vectors. The most common vectors of plant viruses are aphids, whiteflies, leafhoppers, thrips, beetles, mealybugs, and mites (Spence, 2001).

2.8.1 Insect Vectors of Plant Viruses

Most of the known plant viruses are transmitted by insect vectors and they are entirely dependent on the behaviour and dispersal capacity of their vectors to spread from plant to plant (Fereres & Moreno, 2009). Aphids and whiteflies are very well adapted for virus transmission. They have high rates of population increase, short life cycle and high dispersal potential. Their stylets frequently pass between cells to reach the target tissue, the phloem. Aphids transmit more than 50% of the plant viruses vectored by insects (Nault, 1997). Four whitefly species, *Bemisia tabaci*, *Trialeurodes vaporariorum*, *T. abutilonea* and *T. ricini* are known to transmit plant viruses (Jones, 2003b).

2.8.2 Characteristics of A Virus-Vector Interaction

Vector transmission is a specific event in the virus life cycle. Virus-encoded determinants specifically interact with the vector (receptors), thereby facilitating virus transmission. In most cases, viruses of a given genus have a specific type of vector (Ng & Bryce 2006). The transmission of a virus by a vector is often characterized by some degree of specificity. For instance, a virus transmitted by aphids is not transmitted by nematodes. An extreme case of transmission specificity is when a vector transmits one virus or one serologically distinct virus strain, and this virus or virus strain has a single

vector (Andret-Link & Fuchs 2005). A vector needs time to acquire and transmit a virus. Acquisition access period (AAP), is the period of time given for the vector to acquire the virus while inoculation access period (IAP) is the period of time given for the vector to transmit the virus (Ng *et al.*, 2006a).

Transmission of viruses by vectors is dependent on the modes of virus transmission. These modes are classified into three depending on the differences in the virus retention time, sites of retention, and internalization of virions by the vector (Andret-Link *et al.*, 2005b). Plant viruses can be transmitted non-persistently, semi persistently and persistently, depending on the period the vector can harbor infectious particles (Hohn, 2007). Non-persistently transmitted, stylet-borne viruses are transmitted into the plant during short durations of feeding. Virus acquisition is brief, often just a few seconds of feeding. There is no latent period (the time that passes between when the virus is acquired and when it can be transmitted to a plant). Since these types of viruses usually bind to the insect's stylets for only a brief period of time, the virus is retained by the vector for only a few minutes (Ng *et al.*, 2004; Nebreda, Moreno, Pérez, Palacios, Seco-Fernández, Fereres, 2004). Usually, virus transmissibility is lost after a few minutes of feeding on a non-infected plant. Aphids transmit the majority of non-persistently transmitted viruses. The ability of viruses to bind to the insect's stylets is aided by a helper component (a virus encoded, non-structural protein produced only in infected plants). During subsequent periods of feeding the virus is released, or washed from the stylets, thus depositing virus into the plant tissues (Andret-Link *et al.*, 2005c; Gray & Banerjee, 1999).

Semi-persistently transmitted, foregut-born viruses are transmitted into the plant during longer durations of feeding (minutes). Virus acquisition increases with increased time spent feeding (minutes to hours), and the virus stays in association with the insect for several hours, being able to be transmitted into other plants. The virus is thought to be binding in the anterior areas of the alimentary tract, along the stylets to the foregut, and a few virus particles are released during each act of feeding (Hogenhout, Ammar,

Whitfield, Redinbaugh 2008). Viruses which are persistently and semi-persistently transmitted do not require a time interval between acquisition and transmission (latent period), nor do they replicate in the vector. They are specifically associated with the epicuticle that lines the stylets (mouthparts) or the foreguts of their arthropod vectors (Nault, 1997).

Persistent viruses are associated with the vector for the remainder of its lifetime because they enter the haemocoel of vectors and are retained after molting. The vector requires long acquisition (hours to days) and long latent periods (one day to several weeks) (Hogenhout *et al*., 2008). An example of such a virus is the maize streak virus (MSV) (Reynaud & Peterschmitt, 1992). These viruses can be sub-divided further into two groups; propagative viruses that replicate in their vectors and their plant hosts, and non-propagative viruses which replicate only in their plant hosts but not in their vectors (Ng *et al.,* 2006c ; Gray *et al.,* 1999). Passage of persistent viruses through different organs in their insect vectors requires virus and vector specific sites that mediate their interaction (Whitfield, Ullman, German, 2005; Kakani, Reade, Rochon, 2004; Gray *et al.,* 2003).

The whitefly vectors over 100 plant viruses by different modes of transmission (Jones, 2003a). Some of these include SPCSV which is transmitted semi-persistently (Sim *et al.,* 2000) and sweetpotato leaf curl virus *(*SPLCV), transmitted in a persistent manner (Simmons, Ling, Harrison, Jackson, 2009; Valverde *et al*., 2004). It also transmits cassava brown streak virus (CBSV) semi-persistently (Maruthi, 2005a) and transmits the lettuce infectious yellow virus (LIYV) in a semi persistent manner (Duffus, 1986). Squash vein yellowing virus (SqVYV), squash leaf curl virus (SLCV) and cassava mosaic virus (CMV) are also vectored by *B. tabaci* (Hogenhout, *et al.,* 2008).

2.9 Genetic Variability of Plant Viruses

A virus species constitutes a pool of variants termed a quasi-species which are centered on a master sequence (Roossink, 1997 & Eigen, 1996). Plant virus populations often

consist of a few genetic variants (Garcia-Arenal, Fraile, Malpica 2001). More than 90% of all plant viruses have a ribonucleic acid (RNA) genome (Hull, 2002). These viruses are error-prone in their replication. This is thought to be due to lack of proofreading capabilities in the RNA-dependent RNA polymerases (RdRps) which is present in the deoxyribonucleic acid (DNA)-dependent DNA polymerases (Domingo & Holland, 1997). The existence of these genetic variants within a virus population increases the probability of survival and their ability to adapt to different hosts. However, the high potential for genetic variation, through either mutation or genetic exchange by recombination or re-assortment of genomic segments, may not result in high diversity. Selection by factors such as interaction of the virus with host plants and vectors (Albiach-Marti, Guerri, de Mendoza, Laigret, Ballester-Olmos, Moreno, 1999) and random genetic drift (Fraile, Escriu, Aranda, Malpica, Gibbs, Garcia-Arenal, 1997) may in fact reduce genetic diversity in virus populations.

The distribution of genetic variants (population genetic structure) in a population of an organism may change with time, a process called evolution. Evolution may lead to the rise of different taxonomic entities. The effectiveness of control strategies can be compromised by evolution of the pathogens. Previous work shows that virus mutants arise easily upon amplification of biologically or molecularly cloned inocula, giving rise to a heterogeneous population (Kurath & Dodds, 1995; Garcia-Arenal *et al.,* 1984). Serial passaging under different hosts can generate variants and alter viral properties (Kearney, Thomson, Roland, 1999; Yarwood, 1979). Even within the same host species and cropping system, viruses may change in incidence and virulence (Escriu, Fraile, García-Arenal 2003).

Recombination events, which involve exchange of genetic material between two nearly identical or different RNAs, have been reported in various plant viruses (Bousalem *et al.,* 2000; Moonan *et al.,* 2000). Recombinations have also been reported for DNA viruses giving rise to more virulent strains (Paame *et al.,* 2004, Fondong *et al.,* 2000; Chenault & Melcher, 1994).

Other causes of genetic variability are substitution of amino acids as indicated by Mukasa (2004) where in one of the SPMMV sequences, arginine (R) was substituted with lysine (K) or by deletions. These observations suggest that different populations of the same virus could occur in different hosts or crop varieties. This could have implications on sweetpotato cultivation where farmers grow several varieties in the same field. Considering that many natural and artificially engineered mechanisms of virus resistance are strain-specific, introduction of more virus strains should be avoided. Studying genetic variability of the virus is therefore an important step in the development of strategies for its control (Ge, Zhang, Zhou, Li, 2007).

2.9.1 Genetic variability of SPMMV

The genetic variability affects control of viruses and also their detection by both serological and molecular methods. SPMMV has been detected serologically and sequence data are available from some isolates occurring in East Africa (Colinet *et al.*, 1998). Mukasa *et al.* (2003a) found a high genetic variability of 82-100% amino acids (aa) in the sequence of the coat protein (CP)-encoding region of SPMMV isolates. Phylogenetic analysis on the 3′ -proximal genomic sequences of one isolate from Kenya and eight isolates from Uganda revealed no distinguishable strain groups. In a later study, analysis of the CP-encoding region of 12 SPMMV isolates from Uganda indicated a high genetic variability of isolates having nucleotide and amino acid sequence identities of 88.2-100% and 93.0-100%, respectively. However, all isolates were different for the 3' untranslated region (3´-UTR) sequences that showed identities of 91.6-98.1%. This level of intra-specific genetic variability is high.

Translation of the SPMMV nucleotide sequences of the 12 Ugandan isolates revealed an open reading frame (ORF) ending up to a stop codon containing uracil (U) and adenine (A),UAA, in all isolates at position 1504 of the cloned fragment, followed by a non-coding region (Mukasa, 2004). The 3´-UTR was variable in length among the isolates (305-314 nucleotides) due to deletions within the first 42 positions. The SPMMV 3´-

UTR is much longer than that of reported sweet potato infecting potyvirids (Mukasa, 2004). It is also more variable and less common than SPFMV and SPCSV in the sweetpotato growing regions of East Africa (Ateka *et al.,* 2004d and Tairo *et al.*, 2004d). Genetic variability of 16 isolates of SPMMV from East African countries have been studied (Tairo *et al.,* 2005).

Genetic variability is an important aspect in adaptation of viruses to environments and viruses employ several mechanisms to generate sequence variation (Roossinck, 1997). Understanding genetic variability is important in giving some insight on the development and spread of a virus (Power, 2000). Information on the structure of virus populations obtained from the knowledge of genetic variability of sweetpotato viruses is important in the design of appropriate control measures that deploy host resistance in the sweetpotato crop. It helps in the choice of appropriate sources of virus resistance and virus isolates for challenge inoculation in virus resistance breeding programmes. It is also useful in determining effective phytosanitary and cultural control measures (Thresh *et al.,* 2003). Since SPMMV increases the severity of SPVD, there is need to determine the genetic variability of Kenyan isolates of SPMMV. Lack of sufficient molecular information on SPMMV hampers formulation of effective control mechanisms for the virus.

CHAPTER THREE

RESPONSE OF KENYAN SWEETPOTATO GERMPLASM TO SWEETPOTATO VIRUS DISEASE UNDER FIELD CONDITIONS

Abstract

Sweetpotato virus disease can reduce yields by up to 90%. It is widespread in Kenya with the highest disease incidences reported in the Western region. Use of resistant or tolerant genotypes is the cheapest and most effective means of controlling the disease. The objective of this study was to evaluate the response of Kenyan sweetpotato genotypes to sweet potato virus disease. The genotypes were challenged with SPVD at two field trials at KALRO in Kakamega County and Yala swamp in Siaya County of Kenya. A susceptible genotype, "Ejumula'', was also included as a positive control giving a total of twenty one genotypes in the trials. Disease severity was scored every month for a period of five months using a scale of 1-5. The genotypes exhibited significant differences ($P<0.05$) in disease severity in both trials. The genotypes KKFS 56682-03-1, Marooko-3, YS Sopalla and YS Kemb 10 showed high levels of SPVD tolerance with SPVD severity scores below 2 in both trials. The genotype KKFS 56682-03-1 was the least susceptible to SPVD with a severity score of 1.0 in both trials, while Katumani -2 was the most susceptible with severity scores of 4.9 in KALRO Kakamega and 5 in Yala swamp. Eleven genotypes had mild symptoms with severity scores of 2-3. The susceptible control, "Ejumula,'' had severity scores of 4.7 and 5 in KALRO-Kakamega and Yala swamp respectively. The genotypes which showed tolerance to SPVD may be used in breeding programmes to improve the tolerance to SPVD in sweet potato genotypes with desirable agronomic traits.

3.1 Introduction

Sweetpotato virus disease is the major sweetpotato production constraint causing yield reduction of up to 90 % (Miano, 2008d; Loebenstein *et al*., 2004e and Gutierrez *et al*.,

2003). It is caused by the synergistic interaction between the sweetpotato feathery mottle virus (SPFMV) and the sweetpotato chlorotic stunt virus (SPCSV). It is characterized by overall plant stunting, leaf narrowing and distortion, chlorosis, mosaic or vein-clearing and leaf strapping (Gasura *et al.,* 2010 ; Aritua *et al.,* 2007). It is widespread in Kenya with the highest disease incidences reported in the Western region (Nyaboga *et al.,* 2008d). The only way to adequately protect the crops of subsistence farmers from this disease is by using host plant resistance. Some genotypes are more tolerant to SPVD than others when exposed to similar amounts of inoculums (Mwanga *et al.,* 2001), but little has been done to identify such genotypes. The objective of this study was to evaluate the response of twenty Kenyan sweetpotato genotypes to sweet potato virus disease.

3.2 Materials and Methods

Twenty genotypes with known resistance to SPVD in a previous green house experiment were evaluated for resistance to SPVD in two field trials conducted at KALRO Kakamega and Yala swamp in Kakamega and Siaya counties, respectively using the scale in table 3.1

Table 3.1- SPVD severity scale

Score	Symptom severity description
1	No of visible symptoms and no stunting
2	Very mild symptoms on leaves, yellowing or mosaic ,little distortion of leaves, apparent but negligible stunting
3	Moderate symptoms of purpling/yellowing or mosaic leaves , moderate distortion of leaf shape and moderate stunting
4	Severe symptoms of purpling/yellowing or mosaic leaves, severe distortion of leaves with reduced size , plants partially stunted (very short internodes) but apparently still growing
5	Very severe symptoms of purpling /yellowing or mosaic on leaves, severe leaf distortion, reduced leaf size, plant severely stunted (stem extension more or less stopped)

Source: (Njeru *et al.,* 2004)

The genotypes were multiplied under greenhouse conditions to provide sufficient planting material for the field trials. The origin and root colour of the germplasm was determined. (Table 3.2)

Table 3.2: Origin and root colour of the sweetpotato germplasm evaluated for SPVD tolerance in the field trials

S/No.	Sweetpotato genotype[x]	County	Root colour	
			Skin	Flesh
1	WFTC-03-2007	Kwale	Purple red	Cream
2	OP-LNA-006-08	Malindi	Cream	Cream
3	TVT-02-2007	Kilifi	Pink	White
4	SPK004-katumani	Busia	Red	Pale orange
5	Katumani -7	Vihiga	Pink	White
6	YS-Kemb 10	Busia	Cream	Dark cream
7	YS Nyanguyegwo	Busia	Cream	Cream
8	YS Sopalla	Busia	Cream	Dark orange
9	Kikuyu -3	Kakamega	White	Dark cream
10	KKFS 56682-03-1	Kakamega	Purple red	Cream
11	Marooko -3	Busia	Pink	White
12	KAK-04-2007	Homabay	Cream	White
13	Naspot	Migori	Cream	White
14	KKFS Mwavuli	Kisii	Purple red	White
15	Marooko -1	Homabay	Pink	White
16	Katumani -2	Makueni	Cream	White
17	Kikamba - 2	Machakos	White	Pale yellow
18	Kikanda -1	Embu	Cream	Pale yellow
19	MKN-04-2007	Machakos	Pink	Cream
20	Kamau -1	Kirinyaga	White	Dark yellow
21	Ejumula[+]	Kakamega	Cream	Deep yellow

[x] Different genotypes as coded during germplasm collection.

[+] Genotype used in infector rows and as a susceptible control

3.2.2 Field trials

Field trials were conducted in two sites where SPVD and vectors associated with it are prevalent. One site was at the KALRO-Kakamega experimental farm and the other at Yala swamp, Siaya County. The environmental conditions of the two sites are tabulated in Table 3.3

Table 3.3 Environmental conditions of the trial sites

Field trial sites	Agro-ecological zone	Altitude (m) asl	Annual rainfall (mm)	Mean annual temperatures (^{o} C)	Soils
KALRO-Kakamega	Upper midland 1 (UM1)	1554	1730-1929	20.5	Loam
Yala swamp	Lower midland cotton zone (LM3)	1167	1081-1139	22.3	Clay

Source: Farm management handbook of Kenya vol. ii 2nd edition, 2007

The twenty sweetpotato genotypes were planted in 5 x 3m plots at a spacing of 100cm by 30cm in a randomized complete block design (RCBD) with three replications. Symptomless Ejumula, a genotype known to be highly susceptible to SPVD (Mwanga et *al*., 2007) was included as a susceptible control giving a total of twenty one genotypes in each of the two trials. Each plot had three rows of thirty test plants sandwiched between two rows of twenty SPVD infected plants of the variety Ejumula as infector rows. Common agronomic practices such as weeding and hilling were done manually.

3.2.3 Determination of sweetpotato virus disease severity in the field

The field trial in KALRO Kakamega was conducted between October 2009 and March 2010 while Yala swamp trial was from November 2009 to April 2010. The severity of SPVD symptoms was determined every month starting one and half months upto five

months after planting (MAP) using a disease severity scale of 1-5 as described by Njeru *et al.* (2004). The severity score for each of the thirty plants in each plot was determined separately and used to determine the score for the entire plot.

3.2.4 Data analysis

The data on SPVD severity was subjected to analysis of variance (ANOVA) using statistical package for social scientists (PASW Statistics 18) and mean separation done using Student-Newman-Keuls test (S-N-K) at 5% level of significance.

3.3 Results

3.3.1 SPVD severity

The disease was more severe in Kakamega site than in Yala swamp. There were significant ($P<0.05$) differences in disease severity among genotypes in both trials. The SPVD severity scores ranged from 1.0 for KKFS 56682-03-1 to 5 for Ejumula which was included as a susceptible control. Four genotypes namely KKFS 56682-03-1, YS-Kemb 10, Marooko-3 and YS Sopalla had severity scores below 2. The genotype KKFS 56682-03-1 with scores of 1.0 in both trials was the least susceptible (Table 3.3). It was followed by Marooko -3 which had 1.4 in Kakamega and 1.2 in Yala swamp and YS Sopalla with scores 1.7 in Kakamega and 1.1 in Yala swamp. YS-Kemb 10 had 1.8 in Kakamega and 1.5 in Yala swamp. Marooko -1 had 2.1 in Kakamega and 1.6 in Yala swamp. Four genotypes had severity scores ranging between 2.0 and 2.9 in both trials. These were OP-LNA-006-08, Kikamba-2, WFTC-03-2007 and KKFS Mwavuli. Six genotypes had scores ranging between 2.1 and 2.5 in Kakamega. However, in Yala swamp the scores of these genotypes were lower, ranging between 1.1 and 1.5. These included MKN-04-2007, KAK-04-2007, Kamau -1, Kikuyu -3, Kikanda -1 and SPK004-Katumani.

Naspot, Katumani -7 and YS Nyanguyegwo had severity scores ranging between 3.1 and 3.4 in Kakamega and between 1.7 and 2.5 in Yala swamp. Katumani -2 was the most susceptible with scores of 4.9 and 5.0 in Kakamega and Yala swamp, respectively. The susceptible control Ejumula had a severity score of 4.7 in Kakamega and 5.0 in Yala swamp (Table 3.4).

Table 3.4: Severity of SPVD in twenty one sweet potato genotypes evaluated in two trials at KALRO Kakamega and Yala swamp

Genotype [x]	SPVD severity[z]		
	Kakamega (October 2009-March 2010)	Yala swamp (November 2009-April 2010)	Greenhouse [a]
Katumani -2	4.9 [a]	5.0 [a]	1.5
Ejumula	4.7 [a]	5.0 [a]	-
TVT-02-2007	3.7[b]	4.1[b]	1.1
Naspot	3.4[c]	2.5[cd]	1.4
Katumani -7	3.3[c]	1.9[ef]	1.5
YS Nyanguyegwo	3.1[d]	1.7[fg]	1.1
WFTC-03-2007	2.9[de]	2.0[ef]	1.3
OP-LNA-006-08	2.9 [de]	2.7 [c]	1.4
Kikamba -2	2.7[ef]	2.8 [c]	1.0
KKFS Mwavuli	2.6 [ef]	2.2[de]	1.2
MKN-04-2007	2.5[fg]	1.4[ghi]	1.5
KAK-04-2007	2.4[fgh]	1.5[gh]	1.0
Kamau -1	2.4[fgh]	1.2[hi]	1.4
Kikuyu -3	2.4 [fgh]	1.3[ghi]	1.4
Kikanda -1	2.3[gh]	1.1[hi]	1.0
SPK004-Katumani	2.1[h]	1.3[ghi]	1.2
Marooko -1	2.1[h]	1.6[gh]	1.4
YS-Kemb 10	1.8[i]	1.5[ghi]	1.2
YS sopalla	1.7[i]	1.1[hi]	1.4
Marooko -3	1.4[j]	1.2[hi]	1.4
KKFS 56682-03-1	1.0[k]	1.0[i]	1.1

NB: Means followed by the same superscript letter within the column are not significantly different at 5% level.

[x] Different genotypes as coded during germplasm collection.

[a] Data on SPVD severity in the greenhouse obtained from Karuri *et al.,* 2009 showing disease severity of SPVD infected plants in the greenhouse.

[z] SPVD severity was determined following a 1 - 5 scale where; 1 = no visible symptoms, 5 = very severe symptoms (Njeru *et al.,* 2004).

3.3.1.1 SPVD symptoms

The symptoms exhibited by sweetpotato plants infected by SPVD in the field trials were vein clearing, yellowing, leaf mosaic, stunting and leaf mottling (Plate 3.1). The genotypes KKFS 56682-03-1, YS-Kemb 10, Marooko -3 and YS Sopalla had either no visible symptoms or very mild symptoms. Katumani-2, the genotype that was most susceptible to SPVD, had very severe symptoms of yellowing, severe stunting and leaf mottling (Plate 3.1).

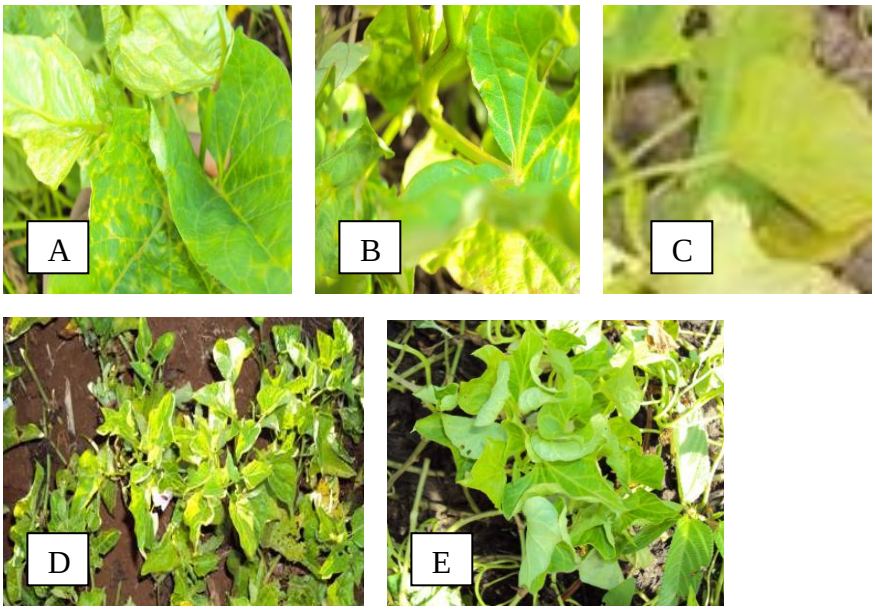

Plate 3.1: Symptoms of SPVD observed in the trials conducted in KALRO Kakamega and Yala swamp

A- Leaf mosaic in genotype OP-LNA, B -Vein clearing in leaves of genotype Ejumula, C-Yellowing of leaves in genotype Ejumula; D-Stunting in genotype Katumani-2, E-Leaf mottling in genotype Katumani-2

3.4 discussion

Sweetpotato genotypes exhibited differences in tolerance to sweetpotato virus disease. The germplasm evaluated in this study included four genotypes with SPVD severity scores below 2. These genotypes were KKFS 56682-03-1, YS-Kemb 10, Marooko -3 and YS sopalla all of which had been obtained from Western and Nyanza provinces of Kenya which are high SPVD pressure zones. These four genotypes which were tolerant to SPVD in this study may be used for breeding purposes as a source of tolerance to SPVD into cultivars with poor SPVD tolerance but possessing other desirable agronomic traits. The possibility of that kind of breeding is supported by Mwanga *et al.* (2002). Among the twenty genotypes tested, the genotype KKFS-56682-03-1 may be very useful for this purpose. Differences in disease severity in the two sites for some genotypes were observed. The differences may be attributed to genotype and environment interaction effects (Mwololo *et al.,* 2009).

Differences between SPVD severity in the field and in the preceding greenhouse experiment were observed. The genotypes showed higher SPVD severity in the field trials as compared to that in the greenhouse experiment. This agrees with the findings of Mwanga (2001) indicating the importance of combining both greenhouse and field screening for SPVD to identify genotypes that are tolerant to SPVD. Four genotypes that had shown high levels of SPVD tolerance in the green house (Karuri *et al.,* 2009) showed comparable levels of resistance in the field. Three genotypes in this study that had been reported to exhibit high dry matter content (Karuri *et al.,* 2009) showed tolerance to SPVD in the field. These were genotypes KKFS-56682-03-1, Marooko-3 and YS-Kemb 10. This is an added advantage on these genotypes in their consideration for selection for further improvement since this trait is an important consideration in farmer's preference for sweetpotato genotypes.

Exposure of the plants to natural inoculum from the vectors of SPVD causing viruses where farmers will eventually grow the crop was more reliable in determination of SPVD tolerant genotypes. Four genotypes namely, KKFS 56682-03-1, Marooko-3, YS sopalla and YS-Kemb 10 showed tolerance to SPVD in two areas that have high levels of SPVD inoculum. This is important because these genotypes can now be multiplied for farmers to use as planting material in these areas. The growing of these genotypes which have low susceptibility to the virus is also likely to reduce SPVD inoculum pressure in these areas. More experiments using these four genotypes in other sweetpotato growing regions known to harbour SPVD causing viruses to further test their tolerance to SPVD in those regions are recommended.

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CHAPTER FOUR

GENETIC VARIABILITY AMONGST EAST AFRICAN ISOLATES OF SWEETPOTATO MILD MOTTLE VIRUS

Abstract

Sweetpotato mild mottle virus enhances the severity of sweetpotato virus disease and there is need to characterize this virus as one way of addressing the challenge of SPVD. This study sought to determine the genetic variability of East African isolates of sweet potato mild mottle virus. SPMMV infected sweetpotato vines were used to graft - inoculate *Ipomoea setosa*, a universal indicator plant for sweet potato viruses. RNA was extracted from symptomatic *I. setosa* plants using CTAB extraction protocol. Complementary DNA (cDNA) was synthesized and the coat protein (CP) gene amplified through polymerase chain reaction (PCR). The phylogenetic analysis clustered the viruses into three major clusters. One cluster had seven isolates; five Ugandan and two Kenyan isolates. The second cluster had nine isolates; three Tanzanian and six Ugandan isolates while the third cluster had two Kenyan isolates. Nucleotide sequence similarity between the Kenyan SPMMV isolates in this study and the East African SPMMV isolates ranged between 71% and 97%. The SPMMV isolate (IE) from Embu had 78% and 74% nucleotide sequence similarity with two isolates (1W and 2W, respectively) whose origin was Kakamega in Western Kenya and 96% nucleotide sequence similarity with a third isolate (3W) from Teso, Western Kenya.

4.1 Introduction

Genetic variability is in part caused by errors occurring during replication of genomes. For viruses, the two main types of errors are mutations and recombination (Bousalem *et al.,* 2000). The distribution of genetic variants in the population of an organism, also referred to as the genetic structure of the population, may change with time (Fernando Aurora, Jos´e, 2001; Stange 2006). Differences within a virus population within a period of time indicate that the virus has been diverging by nucleotide substitutions (Stenger Seifers, French, 2002). Plant viruses can mutate and evolve quickly. This may be favored by the presence of several viral genomes in each infected plant cell and by the short replication cycles. In recent times, there have been massive global changes in the ecology of plants, their viruses and vectors. Agriculture has greatly increased the opportunity for encounters between wild and cultivated plant species, their pathogens and vectors (Jones, 2009). These new encounters favour the selection and emergence of plant viruses suited to the new conditions.

Most of the viruses which have single-stranded positive-sense RNA (+ssRNA) genomes, and a few with single-stranded DNA genomes encounter genetic variability. SPMMV has a +ssRNA genome, hence is prone to mutations because RNA dependent RNA polymerases (*RdRp*) lack proof reading ability during replication. Genetic variants within a virus population increase its ability to adapt to different hosts and probability of survival through changes in incidence and virulence (Chenault *et al.,* 1994; Fondong *et al.,* 2000; Escriu *et al.,* 2003). The study of genetic variability is therefore important in development of strategies for the control of sweet potato viral diseases.

Sweetpotato mild mottle virus is the third most prevalent sweet potato virus in East Africa after SPFMV and SPCSV occurring in complexes with SPCSV and SPFMV (Nyaboga *et al.,* 2008f), and its presence in SPVD increases disease severity (Untiveros

et al., 2007c). Use of virus-resistant crops offers one of the most cost-effective strategies for the management of plant viral diseases such as SPVD. However, the dynamic nature of virus populations permits the evolution of new strains that can adapt to increasingly changing agricultural practices (Ge *et al.,* 2007). Such variability enables viruses to overcome crop resistance to viral diseases (Mansoor *et al.,* 2003). The effective use of virus-resistant cultivars and management of viral diseases requires a better understanding of the genetic variability of viruses. Since SPMMV increases the severity of SPVD, there is need to determine its genetic variability, as an important step in developing effective and efficient strategies for controlling SPVD.

In this study, the coat protein (CP) gene of SPMMV isolates was the target for sequencing to determine genetic variability.

4.2 MATERIALS AND METHODS

4.2.1 Source of SPMMV isolates

Sweetpotato vines with SPMMV symptoms together with asymptomatic vines from sweet potato growing areas of Central, Eastern, Western, Nyanza and Coast provinces were collected and multiplied at the National Agricultural Research Laboratories, Nairobi. Symptomatic samples were assayed for sweet potato mild mottle virus (SPMMV) by nitrocellulose membrane enzyme linked immunosorbent assay (NCM-ELIZA). This was done using polyclonal antibodies provided in an NCM-ELISA kit from the International Potato Centre (CIP, Lima, Peru).

4.2.1.1 Nitrocellulose membrane enzyme linked immunosorbent assay (NCM-ELISA)

Three leaf discs (1cm diameter) from a composite sample of three leaves from different points (top, middle and bottom) of a sweet potato plant were clutched in 3mls of extraction buffer. The ground samples were allowed to stand for 20-30 minutes 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 membranes were then transferred onto dry pieces of towel paper and allowed to dry at room temperature for about 15-20 minutes.

Once dry, the membranes together with the respective positive control membrane strips were immersed in 30 ml blocking solution in a container and incubated for 1 hour. Meanwhile, each primary antibody solution to be reacted with each virus was prepared by mixing 0.1ml of each antibody with 30ml of antibody buffer in separate beakers. The blocking solution was discarded and the membranes rapidly washed once with TBS pH 7.5. Each primary antibody solution was added to its respective container containing each labeled membrane. The containers were covered and membranes incubated overnight at room temperature with a constant agitation on an orbital shaker (50rpm). The primary antibody solution was discarded and unbound antibodies removed from the membranes by washing with constant agitation in 30ml of T-TBS four times for three minutes each time. Meanwhile the conjugate solution was prepared by mixing 1ml of conjugate GAR with 300ml of conjugate buffer. After discarding the T-TBS in the last wash, the membranes were placed between towel papers on a flat surface and pressed gently to remove excess solution. The membranes were then placed in containers and 30ml of conjugate solution added to each of the membranes and incubated for 1 hour.

The conjugate solution was discarded by washing with constant agitation in 30ml of T-TBS four times for three minutes each time. During the final washing step, the NBT/BCIP substrate solution (colour development solution) was prepared. One ml of the solvent DMF (70%) was added to NBT and 1ml of solvent DMF (100%) to BCIP. The contents were agitated until all the reagents were completely dissolved. All the contents (1ml) of the new dissolved substrates were drawn using clean pipettes and added to 250ml of substrate buffer in a container. NBT was added first, followed by BCIP. The final washing solution of T-TBS was discarded and excess solution removed by placing the membranes between towel papers and pressing gently. The membranes

were then placed in dry containers and 25ml of colour development solution (substrate solution) added to each one. The reaction was allowed to proceed for 30 minutes and up to 60-90 minutes for SPCSV. Purple colour on the nitrocellulose membranes indicated the presence of each virus. The substrate solution was discarded after 30 minutes (or 60 to 90 for SPCSV) incubation and membranes washed twice with distilled water to stop the reaction. The germplasm found to be infected with SPMMV was used as scions to graft inoculate seven day old *I. setosa* sweet potato virus indicator plants in an insect proof cage to increase the concentration of the virus (titre). After three weeks the indicator plants were symptomatic and they were used for RT-PCR.

4.2.2 RNA extraction

Total RNA was extracted from SPMMV-infected *I. setosa* plants using the CTAB RNA protocol. The plant tissue (0.1-0.3g) was ground in liquid nitrogen. Before thawing occurred, 450µl pre-heated CTAB (cetyltrimethyl ammonium bromide (3% (w/v), 1.4 M NaCl, 0.1 M Tris-HCl pH 8.0, 17% β-mercaptoethanol, 20mM EDTA) buffer was added, mixed thoroughly and incubated for 10 minutes at 65^{o}C, with occasional mixing. An amount of 450µl of chloroform: isoamyl alcohol (24:1) was added, inverted twice to mix and centrifuged at 12 000 rpm for 10 minutes. Out of the aqueous phase, 400µl was transferred to a fresh eppendorf tube and 700µl of chilled isopropanol added, inverted once to mix and centrifuged at 12000rpm for 15 minutes. The supernatant was decanted and the pellet air-dried for 30 minutes.

The pellet was washed with 70% ethanol and spinned for 1 minute and dried. The pellet was re-suspended in double distilled water.

4.2.3 Synthesis of complementary DNA (cDNA) and amplification of SPMMV coat protein gene

Complementary DNA (cDNA) was synthesized by reverse transcription using a SPMMV specific reverse primer PV2 (GGC TTT TAG GTA GGC AAC AAG TTA C), and M-MLV reverse transcriptase from invitrogen according to the manufacturer's instructions. Alignment of the SPMMV coat protein nucleotide sequences enabled the designing of JN1 (CAG CAA GAA ATG GAG GAT TTG GAC T) and PV2 (GGC TTT TAG GTA GGC AAC AAG TTA C) forward and reverse primers respectively. The coat protein gene of SPMMV was amplified in a thermocycler using the JN1 (CAG CAA GAA ATG GAG GAT TTG GAC T) SPMMV specific forward and the PV2 (GGC TTT TAG GTA GGC AAC AAG TTA C) SPMMV specific reverse primer. The PCR mix contained 2.5 µl of 10X PCR buffer, 1.5 µl of 25mM $MgCl_2$, 1 µl of 10 mM dNTPs, 0.5 µl of 10 pmoles PV2 reverse and JN1 forward primers , 2 µl of cDNA , 0.25 µl of T*aq* DNA polymerase and 17.25 µl deionized sterile water to reach a final volume of 25 µl. The cDNA was first denatured at 94^{o}C for 2 min and then amplified through 35 thermal cycles of 94^{o}C for 1 minute, 61^{o}C for 1 minute and 72^{o}C for 1 minute followed by a final extension step at 72^{o}C for 10 minutes. The PCR products were separated by electrophoresis on a 1% agarose gel that contained ethidium bromide, and visualized under UV light.

4.2.4 Agarose gel electrophoresis

A 1% agarose gel was prepared and allowed to cool before adding 3µl of ethidium bromide. It was then poured into a horizontal gel tray fitted with an appropriate comb. After about 30 minutes of gel polymerization, the comb was gently removed and the tray immersed in an electrophoresis tank containing electrophoresis buffer. An amount of 5µl PCR products was added to 3µl of PCR sample loading buffer and mixed by pipetting before loading the resulting mixture in the pre-formed sample slots on the gel. The gel

was run at a constant speed of 5v/cm until the bromophenol blue migrated to about 1cm from the bottom end of the gel before viewing it under UV light.

4.2.5 Purification of PCR products, sequencing and sequence analysis

The PCR products obtained in section 4.2.3 were purified using a QIAquick PCR purification kit (Qiagen Inc, USA), following the manufacturer's recommendations. Purified DNA was bi-directionally sequenced at the Segolip laboratory at International Livestock Research Institute (ILRI) Kenya using the PCR primers PV2 and JN1 as sequencing primers in an automated ABI 3700 sequencer. Sequences were manually edited using Chromas version 2.33, Technelysium Pty. Ltd. A BLAST search was done in the NCBI gene bank in order to establish whether there was similarity between the four SPMMV coat protein sequences obtained under this study and other East African SPMMV isolates in the gene bank. Known East African coat protein SPMMV sequences available in the NCBI gene bank databases were used for comparison with the Kenyan SPMMV sequences in this study. The SPMMV isolates obtained from the gene bank together with their accession numbers are listed in Table 4.1. The Kenyan SPMMV nucleotide sequences and the sequences of the East African SPMMV isolates and CBSV (used as an out group) from the NCBI gene bank were subjected to multiple sequence alignments using the DNAMAN version 4.02 software (Lynnon Biosoft, 1998) and a phylogenetic tree constructed using the DNAMAN fast alignment 11 algorithm.

Table 4.1: SPMMV sequences obtained from the gene bank for phylogenetic analysis

S/No	Name of isolate	Accession No	Country
1	SPMMV isolate KAM	AJ459317.1	Uganda
2	SPMMV isolate RUK	AJ459315.1	Uganda
3	SPMMV isolate TOR	AJ459312.1	Uganda
4	SPMMV isolate RUK2	AJ459316.1	Uganda
5	SPMMV isolate KUM	AJ459313.1	Uganda
6	SPMMV isolate ISH	AJ459318.1	Uganda
7	SPMMV isolate BUS	AJ459319.1	Uganda
8	SPMMV isolate Kam2	AJ717732.1	Uganda
9	SPMMV isolate Tor2	AJ717733.1	Uganda
10	SPMMV isolate Kum2	AJ717734.1	Uganda
11	SPMMV isolate BNY	AJ459314.1	Uganda
12	SPMMV isolate Bkb3	AJ783452	Tanzania
13	SPMMV isolate Mis2	AJ783453	Tanzania
14	SPMMV isolate Tar3	AJ783450	Tanzania
15	CBSV isolate Naliendele 2*	FN423418.1	Tanzania

*CBSV was used as an out group

4.3 Results

4.3.1 Amplification of SPMMV CP gene in polymerase chain reaction

A PCR fragment of the CP gene (~1000 bp) was obtained for each sample examined using the primer pair JN1 and PV2. The bands indicated the presence of SPMMV (Plate 4.1).

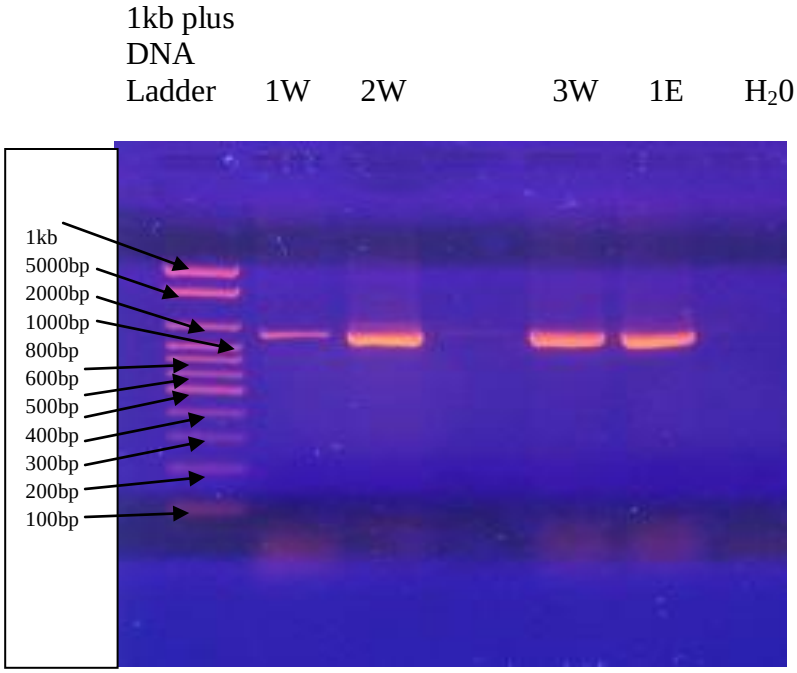

Plate 4.1 : Bands of SPMMV samples:1W and 2W from

Kakamega, 3W from Teso and 1E from Embu

4.3.2 Phylogenetic analysis of SPMMV sequences

The four SPMMV sequences were analyzed and a Phylogenetic tree constructed (Fig. 4.1)

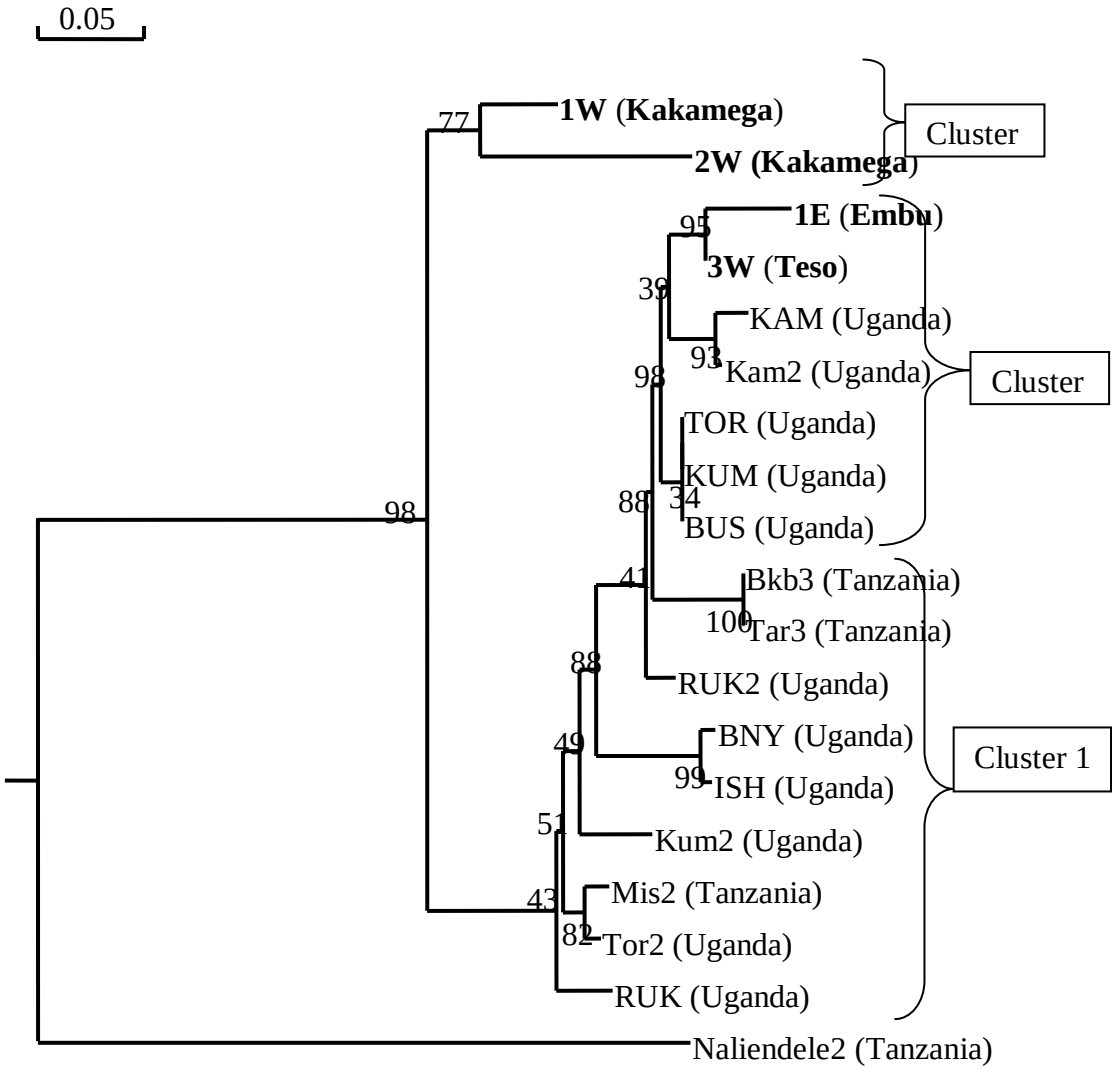

Figure. 4.1: A phylogenetic tree based on the nucleotide sequences of the coat protein gene of SPMMV showing the relationship among SPMMV infecting sweetpotato in Western and Eastern Kenya and that of the other East African countries.

The phylogenetic analysis clustered the viruses into three clusters. The first cluster had three Tanzanian isolates: Bkb3, Tar3, Mis2 and six Ugandan isolates: RUK2, BNY, ISH, Kum2, Tor2 and RUK. The second cluster had five Ugandan isolates: KAM, Kam2, TOR, KUM, BUS and two Kenyan isolates: 1E (Embu) and 3W (Teso). The third cluster had two Kenyan isolates: 1W and 2W. Some Kenyan isolates were different from other East African isolates. They were separately clustered (cluster 3). Some Ugandan and Kenyan isolates showed close relationships as they were clustered together (cluster 2). No Tanzanian isolate showed close relationship with any Kenyan isolate (cluster 1)

4.3.1 Comparison between nucleotide Sequences of the Kenyan SPMMV isolates with other East African SPMMV isolates from the gene bank

Seven samples yielded positive results for presence of the virus using NCM-ELIZA. PCR fragments of the coat protein gene from four of those samples were sequenced and used to study the genetic variability of SPMMV. Sequence comparisons between the SPMMV sequences in this study and the sequences of the East African SPMMV isolates in the gene bank indicated 71–97 % nucleotide sequence identity. Table 4.2 displays the nucleotide sequence similarity between the East African sequences in this study and those in the gene bank. Nucleotide sequence similarity between the Kenyan SPMMV isolates in this study and the East African SPMMV isolates ranged from 71% to 97%. The most closely related East African and Kenyan SPMMV isolates sharing genetic similarity of 97% was KUM, BUS and TOR from Uganda and 3W from Teso in Western Kenya. The least related isolates sharing genetic similarity of 71% were 2W from Kakamega in Western Kenya and KAM from Uganda (Table 4.2).

SPMMV isolates	**1E***	**1W***	**2W***	**3W***	**Bkb3**	**BNY**	**BUS**	**ISH**	**KAM**	**Kam2**	**KUM**	**Kum2**	**Mis2**	**RUK**	**RUK2**	**Tar3**	**TOR**	**Tor2**	**Naliendele 2****
1E*(K)	100																		
1W*(K)	78	100																	
2W*(K)	74	86	100																
3W*(K)	96	80	75	100															
Bkb3(T)	88	80	73	93	100														
BNY(U	83	81	74	88	88	100													
BUS(U)	93	81	74	97	94	91	100												
ISH(U)	83	81	74	88	88	99	91	100											
KAM(U	90	79	71	94	92	86	94	88	100										
Kam2(U	92	79	74	96	91	87	96	88	98	100									
KUM(U	93	81	74	97	94	91	100	91	94	96	100								
Kum2 (U)	85	81	75	90	90	90	91	90	88	89	91	100							
Mis2(T)	86	86	78	90	90	90	91	90	88	89	91	95	100						
RUK(U)	87	85	78	91	88	89	91	89	87	89	91	91	95	100					
RUK2(U)	91	83	76	95	93	90	97	90	95	95	97	92	93	92	100				
Tar3(T)	88	80	73	93	100	88	94	88	92	93	94	90	90	88	93	100			
TOR(U)	93	81	74	97	94	91	100	91	94	96	100	91	91	91	97	94	100		
Tor2 (U)	86	87	79	91	90	91	92	91	89	90	92	94	98	95	94	90	92	100	
Naliendele 2**	37	43	40	37	38	39	37	39	38	36	37	41	42	42	38	38	37	40	100

Table 4.2: Percent coat protein (CP) nucleotide sequence similarity among 18 Eas African SPMMV sequences

* Isolates sequenced in this study.

** Naliendele 2 is a CBSV isolate used as an out group.

K: Kenya

U: Uganda

T: Tanzania

Two Kenyan isolates originating from different geographical locations had the highest genetic similarity with a percent nucleotide (% nt) identity of 96%. The isolates 1E and 2W had the lowest % nt identity between them.

Three (3) Ugandan isolates: KUM, BUS, TOR and a Kenyan isolate: 3W originating from Kakamega had the highest genetic similarity with a percent nucleotide (% nt) of 96%. The isolates with the lowest genetic similarity was a Kenyan isolate: 2W and the Ugandan Isolate: KAM with sequence identity of 71%.The most similar Kenyan and Tanzanian isolates were the Kenyan isolate: 3W and two Tanzanian isolates: Bkb3 and Tar3 (Table 4.3).

Table 4.3: Percent nucleotide sequence identities of the CP gene in multiple comparisons amongst Kenyan and other East African SPMMVsequences

SPMMV isolates	1E (Kenyan)	1W (Kenyan)	2W (Kenyan)	3W (Kenyan)
BNY (Ugandan)	83	81	74	88
BUS (Ugandan)	93	81	74	97**
ISH (Ugandan)	83	81	74	88
KAM (Ugandan)	90	79	71*	94
Kam2 (Ugandan)	92	79	74	96
KUM (Ugandan)	93	81	74	97**
Kum2 (Ugandan)	85	81	75	90
RUK (Ugandan)	87	85	78	91
RUK2 (Ugandan)	91	83	76	95
TOR (Ugandan)	93	81	74	97**
Tor2 (Ugandan)	86	87	79	91
Bkb3 (Tanzanian)	88	80	73[z]	93[y]
Mis2 (Tanzanian)	86	86	78	90
Tar3 (Tanzanian)	88	80	73 [z]	93 [y]

**The highest genetic similarity between Kenyan and Ugandan isolates

* The lowest genetic similarity between Kenyan and Ugandan isolates

[y] The most similar Kenyan and Tanzanian isolates

[z] The least similar Kenyan and Tanzanian isolates

4.4 Discussion

A phylogenetic analysis based on the nucleotide sequence of the CP gene of SPMMV isolates in this study and other East African SPMMV isolates in the gene bank clustered the viruses into three major clusters. Clustering of the Kenyan SPMMV isolates in this study was not correlated with their geographical origin. An example is isolate 1E from the eastern region of Kenya that was placed in the same cluster with isolate 3W from Teso in western Kenya. Isolates 1W and 2W, both from the western region were placed in their own cluster and not placed together with isolate 3W which also originated from the western region of Kenya (Fig 4.1). This is consistent with previous findings by Mukasa (2003b) where isolate EAK from Kenya, eastern side of Uganda, was grouped with isolates BNY, ISH and RUK from western Uganda. This indicates existence of distinguishable sequence variants or strains of SPMMV in Kenya.

Most RNA viruses have an error-prone nature in their replication (Hull, 2002), leading to a high potential for genetic variability and existence of genetic variants within a virus population. Genetic variability is an important aspect in adaptation of viruses to environments and viruses employ several mechanisms to generate sequence variation (Roossinck, 1997). Percent coat protein (CP) nucleotide sequence similarity among the 18 East African SPMMV isolates analyzed ranged between 71% and 97%. The most closely related East African and Kenyan SPMMV isolates sharing genetic similarity of 97% was KUM, BUS and TOR from Uganda and 3W from Teso in Western Kenya. The least related isolates sharing genetic similarity of 71% was 2W from Kakamega in Western Kenya and KAM from Uganda. Genetic similarity of the isolates sequenced in this study ranged between 74% and 96%. This indicates a high genetic variability among the SPMMV isolates which is consistent with the findings of Mukasa (2003a) who reported high genetic variability of SPMMV. Similarly, this is consistent with previous findings by Tairo (2005) after analyzing the CP nucleotide sequences of 16 African SPMMV isolates which revealed no phylogenetically distinguishable groups of SPMMV isolates but indicated high genetic variability. High nucleotide diversity in the P1 region

of SPMMV has also been reported by Adams *et al.*, (2005). This high genetic variability is probably because RNA viruses have very high mutation rates because viral RNA polymerases lack the proof-reading ability (Domingo *et al.,* 1997).

The existence of genetic variants within a virus population increases the probability of survival and their ability to adapt to different hosts. Recombination, a process by which segments of genetic information are switched between the nucleotide strands of different genetic variants during the process of replication (Garćia-Arenal *et al.,* 2001) has been reported in a wide range of viruses (Tugume *et al.*,2010 ; Bousalem *et al.,* 2000). In the family *Potyviridae*, where *Ipomovirus* SPMMV belongs, frequent occurrence of recombination events within and between species of this family has been reported. The P1 of SPMMV may be a result of recombination between *Ipomovirus* SPMMV and an unknown *Potyvirus* that is closely related to the *Potyvirus* SPFMV (Untiveros *et al.,* 2008a). High genetic diversity of the P1 of viruses in the family *Potyviridae* attributed to recombination has been shown to be significant for adaptation to hosts (Valli, Lopez-Moya, Garcia, 2007). These observations suggest that different populations of the same virus could occur in different hosts or crop varieties. This could have implications on sweetpotato cultivation where farmers grow several varieties in the same field. Sweet potato mild mottle virus has a wide host range that includes species in 14 plant families (Hollings *et al.,* 1976) and this is important in designing appropriate control mechanisms for this virus (Ge, *et al.,* 2007).

Many mechanisms of virus resistance are strain-specific hence the need to characterize local virus strains. Introduction of other different virus strains should be avoided through effective quarantine procedures and deployment of reliable virus diagnostic protocols. Sequence identity levels obtained indicated that all the virus isolates in this study were SPMMV. The results of genetic variability in this study provide estimates on the level of genetic variability among East African SPMMV isolates. This knowledge is important in understanding the epidemiology of this virus, which will assist in developing effective control measures.

The results provide a starting point in sweetpotato germplasm management against SPMMV infection. However, since these isolates were from only two Provinces, more information on isolates from geographically diverse locations could lead to a better understanding of genetic variability in SPMMV. Accordingly, more surveys should be conducted in other sweetpotato growing regions of Kenya to obtain more SPMMV isolates for further genetic characterization of the virus.

CHAPTER FIVE

DETERMINATION OF THE VECTOR OF SWEETPOTATO MILD MOTTLE VIRUS

Abstract

White flies were allowed varying periods of time to feed on virus infected *Ipomoea setosa* plants in order to acquire the virus. Six acquisition access periods (AAP) of 0, 6, 12, 24, 48 and 72 hours were tested. After each AAP whiteflies were allowed to inoculate healthy *I. setosa* by feeding on them for a uniform inoculation access period (IAP) of 48 hours. The experiment was arranged in a completely randomized design (CRD) and treatments replicated 8 times. After six weeks, leaves were removed the previously healthy plants and evaluated for presence of SPMMV by enzyme linked immunosorbent assay on Nitro cellulose membrane (NCM-ELISA). The rate of virus transmission at each AAP was expressed as the percentage of infected *I. setosa* plants out of the total number inoculated. The highest transmission was 50% at 72 hour AAP, followed by 31.3% at 48 hour AAP. Whiteflies subjected to lower AAP of 24 hours, 12 hours, 6 hours and 0 hours did not transmit the virus. It was concluded that SPMMV is transmissible by *B. tabaci* at 48 hour IAP and AAP longer than 24 hours. The determination of the whitefly as the vector of SPMMV in this study gives a starting point to address the challenge of SPMMV by choosing the best control method for the whiteflies thus reducing the incidence of SPMMV. SPMMV increases severity of SPVD leading to reduction in sweetpotato production. These findings are therefore important in reduction of the impact of SPVD infection in sweetpotato farming.

5.1 Introduction

Arthropod vectors that transmit most plant viruses include aphids, whiteflies, leafhoppers, thrips, beetles, mealybugs, and mites (Spence, 2001). The sweet potato whitefly vectors many plant viruses in the genera *Begomovirus* (Geminiviridae), *Crinivirus* (Closteroviridae) and *Ipomovirus* (Potyviridae) (Jones, 2003b). Some of these include sweet potato chlorotic stunt virus (SPCSV), transmitted semi-persistently (Sim *et al.*, 2000) and sweet potato leaf curl virus (SPLCV), a member of the genus *Begomovirus* of the family *Geminiviridae*, which is transmitted in a persistent manner (Hogenhout, *et al.*, 2008; Valverde *et al*., 2004a). Squash vein yellowing virus (SqVYV), squash leaf curl virus (SLCV), cassava mosaic virus (CMV) and cassava brown streak virus (CBSV) are also vectored by *B. tabaci* (Hogenhout, *et al.*, 2008; Mware *et al*., 2009a). Most of the known plant viruses are transmitted by insect vectors and are entirely dependent on the behaviour and dispersal capacity of their vectors to spread from plant to plant (Fereres & Moreno, 2009). The mode of transmission of viruses between plants determines the rate and extent of disease spread in plant populations (Power, 2000; Seal *et al.*, 2006).

It is therefore important to establish the vector responsible for the transmission of a virus in order to develop effective strategies to control infections in plants.

Sweetpotato mild mottle virus belongs to the genus Ipomovirus and the family Potyviridae (van Regenmortel *et al.*, 2000). The presence of SPMMV in SPVD-infected plants increases the severity of symptoms expressed in infected plants and may cause a significant loss in tuber yield in some varieties (Njeru *et al.*, 2004). A better understanding of SPMMV and its vector is useful in the development of effective management and control strategies. Experiments have been conducted on other Ipomoviruses and are reported to be transmitted by whiteflies. These include CBSV as reported by Maruthi *et al.* (2005b) and squash vein yellowing virus (Jones, 2003c).

Very little has been done to determine the vector for SPMMV. This study was conducted to determine the vector that transmits this virus.

5.2 Materials nd Methods

An experiment was conducted twice in the greenhouse to determine the vector of sweet potato mild mottle virus using *Ipomoea setosa* for both virus acquisition feeding and inoculation feeding by *Bemisia tabaci*. White flies (*Bemisia tabaci*) were allowed varying periods of time to feed on virus infected *Ipomoea setosa* plants in order to acquire the virus and given 48 hours inoculation access period (IAP) on 48 recipient *I. setosa* test plants.

5.2.1 Rearing of whiteflies

Whiteflies were reared on field bean (*Phaseolus vulgaris*) plants planted in pots in an insect proof cage. When the beans were at the 6-8 leaf stage, whiteflies (*Bemisia tabaci*) biotype B obtained from *Vitex duranta* were introduced and allowed to multiply until they were approximately two thousand in total. These whiteflies were then fed on the SPMMV graft-inoculated *I. setosa* plants to acquire SPMMV.

5.2.2 Growing *Ipomoea setosa*

Brazilian morning glory (*Ipomoea setosa*) exhibits prominent visible symptoms to several sweetpotato viruses, thus is commonly used to detect viruses in sweet potato through grafting (Moyer *et al.,* 1989). *Ipomoea setosa* was graft inoculated with SPMMV and used to feed non-virulent whiteflies to acquire the virus to inoculate virus free *I. setosa* in this study. The seeds were obtained from Kenya Plant health Inspectorate Services (KEPHIS) Muguga, Kenya. Twenty of these seeds were scarified using concentrated sulphuric acid ($H_2SO_{4)}$ in the Plant pathology laboratory in NARL. The seeds were placed in a 5ml beaker and concentrated H_2SO_4 added until all the seeds were covered by the acid. The beaker was then covered with foil paper and allowed to

stand for 15 minutes. The H_2SO_4 was then poured and seeds rinsed thoroughly. The seeds were planted in petri dishes containing wet filter papers to keep the seeds moist and incubated in a growth chamber at 25^{o}C for 7 days to germinate. The germinated seeds were then planted in sterilized pots using sterilized soil media obtained from KEPHIS Muguga which consisted of forest soil, manure and 1/6 inch size gravel in the ratio of 6.5: 2.5: 1 respectively. They were kept in insect proof cages in an insect proof greenhouse and allowed to grow for 7 days.

5.2.3 Grafting of SPMMV-infected sweetpotato vines onto I. setosa

Known sweetpotato mild mottle virus infected sweet potato plants were used to graft inoculate with SPMMV, the 7-day old *I. setosa* plants grown as described in section 5.2.2. This was done to increase the concentration of SPMMV (virus titre). Wedge grafting was done as described by CIP (2010). One wedge graft was made per plant by inserting a scion from the source SPMMV infected sweetpotato plant into a slit on the side and near the base of the stock plant (7-day old *I. setosa*). The plants were observed until they became symptomatic and this occurred between 2-3 weeks after grafting (Plate 5.1B). The symptoms observed were vein clearing and rugosity.

A

A

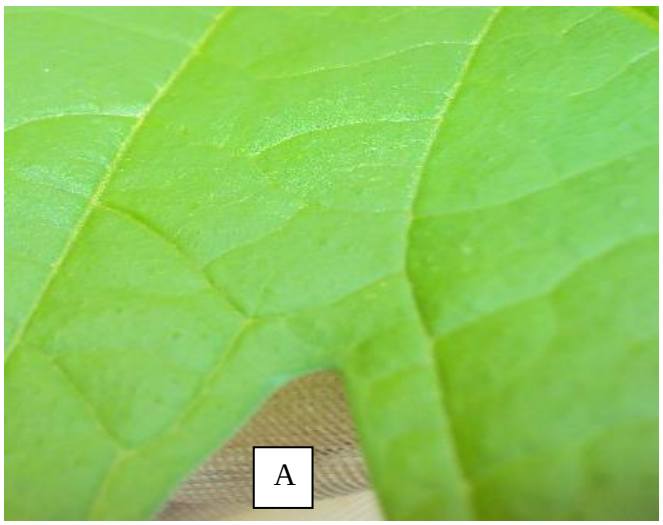

Plate.5.1: *Ipomoea setosa* leaves; A: Healthy B: Vein chlorosis as a result of graft inoculation with SPMMV; three weeks after inoculation

The graft inoculated indicator plants were used for feeding whiteflies for inoculation of *I. setosa* plants free of SPMMV.

5.2.4 Acquisition access feeding

Whiteflies were transferred from the whitefly rearing cage to insect proof cages in batches of about 150 per cage using an aspirator. The cages were then clipped to the SPMMV - infected I. setosa plants with the I. setosa leaves inside the cages. Each group of 300 whiteflies was allowed to feed on these plants for different periods to acquire the virus, referred to as the acquisition access period (AAP) of 72hr, 48hr, 24hr, 12hr, 6hr and 0hrs. One group of whiteflies was not fed on the SPMMV - infected I. setosa plants and was used for inoculation feeding on the control test plants in the experiment.

5.2.5 Inoculation access feeding

The whiteflies used for each AAP were removed after expiry of time designated and used to inoculate 8-day old SPMMV free *I. setosa* plants. For each AAP, about 15-20 of the viruliferous whiteflies were transferred to 8 insect proof cages using an aspirator. Each of eight, 8-day old *I. setosa* plants to be inoculated was clipped inside a cage containing the whiteflies. The viruliferous whiteflies were allowed to feed on the *I. setosa* plants for 48 hours to acquire the virus. After 48 hours, the whiteflies were removed and plants sprayed with an insecticide whose active ingredient (a.i.) is 250g/kg Thiamethoxam with a trade name, Actara®. Plants were evaluated for SPMMV infection by NCM-ELISA, four weeks after inoculation.

5.2.6 Layout of the experiment in the greenhouse

The experiment was conducted in insect proof green houses in KALRO-NARL-Kabete and repeated in the same station. Sweetpotato vines infected by SPMMV were obtained from Western, Nyanza, Eastern, Coast and Central regions of Kenya. A universal indicator plant, *Ipomoea setosa,* was used for acquisition feeding and inoculation feeding by whiteflies. Treatments comprised six virus acquisition access periods (AAP);

0hr, 6hr, 12hr, 24hr, 48hr, and 72hr followed by inoculation access period (IAP) of 48 hours. Each treatment had 8 replications, each consisting of one plant and arranged in a completely randomized design (CRD).

5.2.7: Data analysis

The number of *I. setosa* plants infected by SPMMV was obtained through NCM-ELISA. The percentage of transmission was calculated as the number of infected recipient *I. setosa* plants expressed as a percentage of the total number of recipient plants used.

5.3 Results

5.3.1 Ncm- Elisa

Results of NCM-ELISA showed that in the first experiment, there was transmission in 7 out of 48 samples tested for SPMMV indicated by a purple color on the nitrocellulose membrane (Plate 5.2). In the second experiment 6 out of the 48 samples tested were positive for SPMMV.

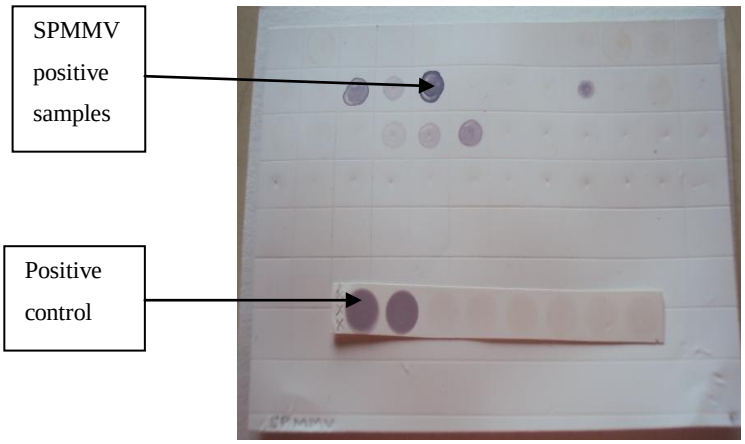

Plate 5.2: Serological test using NCM-ELISA with purple coloured spots on nitrocellulose membranes indicating presence of SPMMV on inoculated I. setosa test plants

5.3.2 Transmission of SPMMV

Those test plants inoculated using whiteflies exposed to 48hr and 72hr AAP showed infection to SPMMV in the first experiment. There was transmission in three out of eight test plants at the 48hr AAP and four out of eight test plants at the 72hr AAP. Transmission, calculated as the number of infected recipient *I. setosa* plants expressed as a percentage of the total number of recipient plants used, was 37.5% and 50% at the 48hr and 72hr AAP respectively in the first experiment. All the test plants exposed to the 24hr AAP, 48hr IAP; 12hr AAP, 48hr IAP; 6hr AAP, 48hr IAP and 0hr AAP, 48hr IAP were not infected with SPMMV. This may probably suggest that the virus was not acquired by the whiteflies. In the repeat experiment, there was transmission in two out of eight test plants at the 48hr AAP and four out of eight test plants at the 72hr AAP. Transmission was 25% and 50% at the 48hr and 72hr AAP respectively. Similar to the first experiment, there was no transmission on all the test plants subjected to the 24hr AAP, 48hr IAP; 12hr AAP, 48hr IAP; 6hr AAP, 48hr IAP and 0hr AAP, 48hr IAP. In both experiments, there was transmission in 31.3% of the sixteen test plants subjected to 48hr AAP, 48hr IAP and 50% of the sixteen test plants exposed to the 72hr AAP, 48hr IAP (Table 5.1).

Table 5.1: Transmission of SPMMV by *B. tabaci* to *I. setosa* indicator plants used as acquisition and inoculation hosts based on virus indexing by NCM-ELISA

		Experiment one		Experiment two		Summary of both experiments	
AAP (hrs)[a]	IAP (hrs)[b]	Infected plants/ total number of recipient plants used	percent of transmission (%) *	Infected plants/total number of recipient plants used	Percent of transmission (%)*	Infected plants/total number of recipient plants used	Overall percent of transmission (%)*
0	48	0/8	0	0/8	0	0/16	0
6	48	0/8	0	0/8	0	0/16	0
12	48	0/8	0	0/8	0	0/16	0
24	48	0/8	0	0/8	0	0/16	0
48	48	3/8	37.5	2/8	25	5/16	31.3
72	48	4/8	50	4/8	50	8/16	50

*****Percent of transmission calculated as the number of infected recipient *I .setosa* plants expressed as a percentage of the total number of recipient plants used.

[a] Acquisition access periods of 0, 6, 12, 24, 48, 72 and 96 hrs was tested in both experiments.

[b] Inoculation access period of 48 hrs was tested in both experiments.

5.4 Discussion

Bemisia tabaci has been reported to be the vector of *Ipomoviruses* such as CBSV (Mware *et al*., 2009b ; Jones, 2003d). Symptoms observed included leaf mottling and chrolosis. The presence of SPMMV was confirmed by a serological test after one and half months after setting the experiment. There was transmission at the 48hr AAP and 72hr AAP giving evidence that *B. tabaci* is the vector responsible for transmission of SPMMV. This virus is an ipomovirus which are known to be vectored by *B. tabaci* and

these findings concur with those of Maruthi *et al.* (2005c) and Mware *et al.* (2009) on transmission of cassava brown streak virus, another ipomovirus. The result pointed to the possibility that this virus is transmitted in a persistent manner since transmission occurred at the 48hr AAP and 72hr AAP and none below 48hr AAP. Persistent viruses require long acquisition times (hours to days) because they are transmitted to plants after injection into the insect hemocoel (Hogenhout *et al*., 2008). Once acquired from infected plants, they are associated with the vector for the remainder of their lifetime. Non-persistent transmission was not evident in this study as there was no transmission in the AAP below 48hrs.

Since non persistent viruses do not require a time interval between acquisition and transmission (latent period), they are acquired from infected plants and inoculated within seconds or minutes to recipient plants (Nault, 1997). Lack of transmission in the 6hr, 12hr and 24 hr AAP may probably mean that the virus was not acquired by the whiteflies. It could also be possible that the virus titres acquired were not sufficient and failed to get into the hemocoel to interact with the vector components if this virus is transmitted persistently. Passage of persistent viruses through different organs in their insect vectors requires specific interactions between virus and vector components (Whitfield *et al.,* 2005, Hogenhout *et al.,* 2003; Power, 2000).

Determination of vectors of plant disease causing viruses is an important aspect in control of its spread.

Since sweetpotatoes are vegetatively propagated, sweet potato viruses are spread through infected planting materials used by farmers. This study gives an insight on the vector of SPMMV and hence a starting point in controlling its spread through the planting materials used by the farmers. The identification of the whitefly *(B. tabaci)* as the vector of SPMMV in this study gives a starting point to address the challenge of SPMMV by choosing the best control method for the whiteflies (*B.tabaci*) thus reducing the incidence of SPMMV.

Sweetpotato mild mottle virus increases severity of SPVD leading to reduction in sweetpotato yields (Untiveros *et al.*, 2007d). These findings are therefore are useful in the search for SPVD control to reduce the impact of SPVD infection in sweet potato farming. The success of controlling virus diseases depend on the availability of effective virus detection tools and epidemiological information regarding virus spread, vector transmission efficiency and the occurrence of alternative hosts for the viruses and the vectors. *Bemisia tabaci* is a major agricultural pest of field and horticultural crops world-wide with over 600 host plant species (Oliveira, Henneberry, Anderson 2001). It is a vector of many plant viruses belonging to various genera, namely; *begomoviruses, criniviruses carlaviruses and ipomoviruses* (Monger *et al.*, 2001, Jones, 2003e and Maruthi *et al.*, 2005d). It also causes damage to plants through direct feeding, which results in chlorosis of leaves and reduction in plant vigour (Legg & Fauquet, 2004). Heavy phloem feeding may also lead to stunting of plants and yield loss (Byrne, 1990). The effective control of the vector, removal of infected plant material, and good cropping practices that may avoid, delay, or lessen the severity of the *Bemisia tabaci* infestation and use of resistant cultivars are some of the measures that can be used to control viral diseases (Gallitelli, 1998). The use of alternative hosts for vectors has also been used to control the spread of diseases. In places where such programmes are lacking, viruses are disseminated widely in infected propagules and also spread by vectors.

Understanding the transmission process and the relationship between a virus and its vector can facilitate the development of effective control strategies against plant viruses. The study therefore gives an insight on the vector of SPMMV and hence a starting point in controlling its spread through the planting materials used by the farmers. A further study using different IAP and AAP above 72hrs to determine the mode of transmission of SPMMV by *B. tabaci* is recommended.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion

The success of controlling virus diseases depend on the availability of information regarding virus spread. Symptoms of SPVD which included vein clearing, yellowing on leaves together with reduced leaf size were observed in this study. KKFS-56682-03-1 which had no visible symptoms together with Marooko -3, YS Sopalla and YS Kemb 10 with very mild symptoms indicated extremely low susceptibility to SPVD. Genotypes which are tolerant to SPVD are important for breeding purposes. These four genotypes identified in this study can be considered for this purpose.

Determination of vectors of plant disease causing viruses is an important aspect in control of its spread. The whitefly *(B. tabaci)* was identified as the vector of SPMMV in this study. The results point to a persistent type of transmission since non-persistent transmission was not evident as there was no transmission in the AAP below 48hrs. This was probably because the virus was not acquired by the whiteflies or the virus titres acquired were not sufficient. Percent coat protein (CP) nucleotide sequence similarity among the 18 East African SPMMV isolates analyzed ranged from 71% to 97%. Genetic similarity of the isolates sequenced in this study ranged between 74% and 96%. This indicates a high genetic variability among the SPMMV isolates.

6.2 Conclusions

The experiments conducted in this study will be important in addressing the challenge of sweetpotato viruses faced by the growers of this crop. This conclusion was arrived at after evaluating the response of Kenyan sweetpotato germplasm to SPVD and conducting a study on SPMMV. This will lead to reduction of production losses thus increasing yields. Four genotypes KKFS 56682-03-1, YS-Kemb 10, Marooko-3 and YS sopalla had low susceptibility to SPVD and could be considered for further improvement. This study identified *B. tabaci* as the vector for SPMMV, an important milestone in dealing with the spread of the virus. The results of genetic variability in this study provide estimates on the level of genetic variation among East African SPMMV isolates.

This knowledge is important in understanding the epidemiology of this virus, which will assist in developing effective control measures.

6.3 Recommendations

Four of the tested sweetpotato genotypes contain SPVD tolerance traits, which can be integrated into genotypes without these traits but have other desirable agronomic traits. More studies are however needed to determine other traits in these genotypes. The knowledge on the genetic variability of the SPMMV isolates in this study provides a starting point in sweetpotato germplasm management against SPMMV infection. However, since these isolates were from only two regions, more information on isolates from geographically diverse locations could lead to a better understanding of genetic variability in SPMMV. The results on the vector for SPMMV indicate that SPMMV virus is transmitted by *B. tabaci*. However, no conclusion could be made on the mode of transmission from this without conducting further studies.

The following recommendations were made after the study;

1. Further study of the four genotypes, that have tolerance to SPVD infection in this study, in other sweetpotato growing areas which have been shown to harbour SPVD causing viruses to further test their resistance to SPVD in those areas is recommended.

2. More surveys should be conducted in all sweetpotato growing regions in Kenya to get more SPMMV isolates for further studies on molecular characterization of SPMMV.

3. Further studies to determine the mode of transmission of SPMMV by *B. tabaci* are recommended.

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APPENDICES

Appendix i- Field layouts of SPVD trials in KALRO Kakamega and Yala swamp

Block 1	Block 2	Block 3
Ejumula	Marooko-1	YS-Nyanguyegwo
Katumani-2	OP-LNA-006-08	Kamau-1
SPK 004 –Katumani	WFTC-03-07	MKN-04-07
Marooko-2	YS-Kemb-10	YS Sopalla
MKN-04-07	Kikamba-2	Kikamba-2
Marooko-1	SPK 004 -Katumani	Kikanda-1
Kamau-1	KKFS-56682-03-1	Ejumula
Kikuyu-3	Marooko-2	WFTC-03-07
Katumani-7	KAK-04-07	YS-Kemb- 10
TVT-02-07	Naspot	Naspot
YS Nyanguyegwo	Ejumula	OP-LNA-006-08
Naspot	Katumani-7	Katumani-7
KKFS-Mwavuli	Kikuyu-3	Katumani-2
YS- Sopalla	TVT-02-07	KKFS-56682-03-1
OP-LNA-006-08	KKFS Mwavuli	SPK 004 -Katumani
YS-Kemb- 10	YS Sopailla	Marooko-2
WFTC-03-07	MKN/04/07	KKFS-Mwavuli
KKFS-56682-03-1	Katumani-2	KAK-04-07
KAK-04-07	Kikanda-1	TVT-02-07
Kikamba-2	YS Nyanguyegwo	Marooko-1
Kikanda-1	Kamau-1	Kikuyu-3

Figure 1: Experimental field layout in KALRO Kakamega trial

BLOCK 1	**BLOCK 2**	**BLOCK 3**

SPK 004 -Katumani	KKFS- Mwavuli	YS -Kemb-10
MKN-04-2007	Kikuyu -3	Kikanda -1
Marooko -3	SPK 004 –Katumani	Kamau -1
Katumani -7	WFTC-03-2007	Marooko -1
Kamau -1	YS Kemb-10	YSNyanguyegwo
YS-Nyanguyegwo	OP-LNA-006-08	Kikuyu -3
WFTC-03-2007	Katumani -7	Ejumula
Ejumula	MKN-04-2007	Katumani -7
YS-sopalla	YS-Nyanguyegwo	OP-LNA-006-08
TVT-02-2007	YS- sopalla	SPK 004 -Katumani
Kikuyu -3	KKFS-56682-03-1	TVT-02-2007
Kikamba -2	TVT-02-2007	Marooko -3
YS-Kemb -10	Kamau -1	Naspot
KKFS- Mwavuli	Katumani -2	Kikamba -2
Naspot	Kikanda -1	MKN-04-2007
Kikanda -1	Naspot	KKFS- Mwavuli
Marooko -1	Marooko -3	KKFS -56682-03 -1
OP-LNA-006-08	Marooko -1	WFTC-03-2007
Katumani -2	Kikamba -2	KAK-04-2007
KAK-04-2007	Ejumula	Katumani -2
KKFS- 56682-03 -1	KAK-04-2007	YS-sopalla

Figure 2: Experimental field layout for Yala swamp trial

- Each plot had 30 of the test plants (three rows, each of 10 plants)
- Ejumula –This is a susceptible genotype obtained from KALRO Kakamega

Appendix ii: SPVD severity analysis of variance (ANOVA) tables

Trial one -SPVD severity ANOVA

Source	Sum of Squares	df	Mean Square	F	P-value
Block	15.696	2	7.848	11.180	.001**
genotype	1651.487	20	82.574	117.627	.001**
Error	1310.637	1867	.702		
Total	2977.820	1889			

**Significant

Trial two -SPVD severity ANOVA

Source	Sum of Squares	df	Mean Square	F	P-value
Block	22.179	2	11.089	10.491	.001**
genotype	2651.633	20	132.582	125.428	.001**
Error	1973.488	1867	1.057		
Total	4647.299	1889			

**Significant

Appendix iii: Results of NCM ELIZA performed on *I.setosa* test plants after inoculation with SPMMV

+ mild; ++ moderately severe; +++ severe

Experiment one			Experiment two		
Sample ID	AAP (hrs)	NCM ELISA results	Sample ID	AAP (hrs)	NCM ELISA results
1	48	0	1	12	0
2	48	0	2	0	0
3	0	0	3	6	0
4	12	0	4	0	0
5	6	0	5	0	0
6	24	0	6	72	+
7	12	0	7	72	+
8	48	0	8	6	0
9	6	0	9	12	0
10	0	0	10	12	0
11	12	0	11	0	0
12	48	0	12	24	0
13	24	0	13	24	0
14	72	0	14	72	0
15	48	++	15	72	0
16	48	+	16	72	0
17	72	+++	17	12	0
18	12	0	18	24	0
19	48	0	19	6	0
20	0	0	20	48	+
21	48	++	21	12	0
22	12	0	22	48	+
23	24	0	23	48	0
24	6	0	24	72	0
25	6	0	25	0	0
26	0	0	26	6	0
27	24	0	27	6	0
28	72	+	28	24	0
29	72	+	29	48	0
30	72	++	30	6	0
31	24	0	31	24	0

32	24	0	32	0	0
33	12	0	33	12	0
34	12	0	34	24	0
35	6	0	35	0	0
36	0	0	36	12	0
37	24	0	37	48	0
38	0	0	38	48	0
39	6	0	39	48	0
40	0	0	40	6	0
41	6	0	41	24	0
42	0	0	42	72	+
43	72	0	43	12	0
44	12	0	44	24	0
45	6	0	45	6	0
46	24	0	46	0	0
47	72	0	47	48	0
48	72	0	48	72	+