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Replication of viruses responsible for Sweet potato virus disease in resistant and susceptible sweepotato genotypes and identification of molecular markers linked to resistance

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REPLICATION OF VIRUSES RESPONSIBLE FOR SWEET POTATO VIRUS DISEASE IN RESISTANT AND SUSCEPTIBLE SWEETPOTATO GENOTYPES AND IDENTIFICATION OF MOLECULAR MARKERS LINKED TO RESISTANCE

A Dissertation
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in

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Soil Sciences

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To my brother Nicholas Njoka

and his family.

Your support has in many ways made me

come this far.

May the Almighty God always

order your steps.

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ABSTRACT

Virus diseases are a major constraint to sweetpotato production in East Africa. The most important is the Sweet potato virus disease (SPVD), a result of co-infection of *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV). Studies were done on different aspects of resistance to SPVD, and to determine the presence of Sweet potato virus G (SPVG), Sweet potato virus 2 (SPV2), and *Sweet potato leaf curl virus* (SPLCV), viruses that have not been reported infecting sweetpotato in Kenya. None of the samples reacted to antisera for either SPVG or SPV-2. SPLCV was detected infecting sweetpotato in Kenya for the first time. Some sweetpotato genotypes have irregular distribution or low virus titers, or recover from SPVD. The possibility of using this resistance to obtain virus free cuttings from field-grown sweetpotato vines for propagation was studied. Vines were cut into three pieces (15 cm, 15-30 cm, and >30 cm from the apex) and tested for SPCSV, SPFMV and *Sweet potato mild mottle virus* (SPMMV), the most common viruses in Kenyan fields. The viruses were equally present in all sections of infected vines and no section was any more likely to be virus free. Accumulation patterns of SPCSV and SPFMV in mixed infections were compared in SPVD-susceptible cultivars, 'Beauregard' and 'Namaswakhe', and resistant cultivars, 'Naspot I' and 'Mar Ooko'. Virus titers were estimated using real-time quantitative PCR. Resistance in 'Naspot I' and 'Mar Ooko' was associated with reduced SPCSV and SPFMV multiplication, respectively. Titers of both viruses increase to certain thresholds after which symptoms appear, indicating that both viruses are important in SPVD development. To determine if SPVD resistant genotypes could be identified using molecular markers, sweetpotato genotypes were selected and classified as resistant or susceptible and amplified fragment length polymorphism (AFLP) marker profiles used in association studies. Analysis of molecular variance found significant ($P<0.002$)

differences between the two groups. Discriminant and logistic regression analysis were used to select informative markers, and to develop models to classify the two groups. Four markers, which gave 94% correct classification of a test population, were selected by both statistical methods.

CHAPTER 1: INTRODUCTION

Results presented in this study relate to different aspects of resistance of sweetpotato [*Ipomoea batatas* (L.) Lam] to infection by the causal agents of sweet potato virus disease (SPVD), the main disease limiting sweetpotato production in the sub-Saharan region of Africa and other parts of the world, and to a survey done in Kenya to determine the presence of three viruses that have been reported infecting sweetpotato in other parts of the world but not in East Africa.

Sweetpotato is a dicotyledonous plant, which belongs to the family Convolvulaceae (Morning glory) and is usually considered the only species of *Ipomoea* of major economic importance (Hall and Phatak, 1993). It is a highly heterozygous hexaploid (2n=6x=90) with extensive variation within the species (Jones, 1986). Sweetpotato is ranked 7th among the most important food crops in the world after wheat, rice, maize, potato, barley, and cassava and the third most economically important root crop after potatoes and cassava (FAO, 2005). More than 95% of the global sweetpotato crop is grown in developing countries. In 2005, approximately 130 million metric tons were produced worldwide, of which ~82% was produced in China (FAO, 2005).

In Africa, sweetpotato is the second most important root crop after cassava. Production is concentrated in East Africa, particularly around Lake Victoria (Gibson et al., 1997). Uganda is the largest African producer followed by Tanzania and Rwanda, while Kenya is seventh (FAO, 2005). The main sweetpotato producing regions of Kenya are western, areas around Lake Victoria, eastern region, central and coastal areas (Matin, 1999). In East Africa, the crop is nearly always grown by small – holder farmers with limited land, labor and capital. The crop is grown in a continuous cycle with one season overlapping another. Piecemeal harvesting of storage roots

commonly extends the cropping season (Bashaasha et al., 1995; Kapinga et al., 1995). The crop grows well on soils with limited fertility, is relatively drought tolerant, provides good ground cover, and is usually cultivated without fertilizer or pesticides. The crop is therefore often planted in more marginal fields – poorer soils with limited water supply, making the cost of production minimal. Farmers use only vine cuttings from production fields rather than seed and thus the cost of propagating material is also minimal (Karyeija et al., 1998a). Despite these conditions, sweetpotato produces remarkable amounts of biomass, often producing more edible energy than any other major food crop (CIP, 1996).

1.1 Sweetpotato Viruses in East Africa

Productivity of sweetpotato is greatly constrained by diseases and pests, the most important diseases being caused by viruses (Ngeve, 1990; Geddes, 1990; Gibson et al., 1997; Clark et al., 1997; Fuglie, 2007). Over 50% loss in production is attributed to viruses (Hahn, 1979; Gibson et al., 1997; Gutierrez et al., 2003). In East Africa, over 90% yield reductions have been associated with viruses (Gibson et al., 1997). Being a vegetatively propagated crop, the viruses are spread from mother plants to new plants during the propagation cycle (Clark et al., 1997).

The first report of a suspected virus disease of sweetpotato in Eastern Africa was in Democratic Republic of Congo in the late 1930s and then in Uganda in the early 1940s (Hansford, 1944). Later, viral diseases in sweetpotato were reported in Kenya, Tanzania Rwanda, Burundi, Malawi, and South Africa (Sheffield, 1957). Initial studies indicated the occurrence of two viruses, virus A and virus B, which were aphid- and whitefly-transmitted respectively (Sheffield, 1957). Currently, four sweetpotato viruses have been identified and confirmed to be widely distributed in East Africa (Mukasa et al., 2003; Ateka et al., 2004b; Tairo

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et al., 2004). The four viruses include two that belong to family *Potyviridae*: the potyvirus *Sweet potato feathery mottle virus* (SPFMV) and the ipomovirus *Sweet potato mild mottle virus* (SPMMV); one belongs to the family *Closteroviridae*: *Sweet potato chlorotic stunt virus* (SPCSV) and Sweet potato chlorotic fleck virus (SPCFV) for which the genus *Carlavirus* has been proposed (Aritua et al., 2003). Aritua et al. (2006) reported the occurrence of Sweet potato caulimo-like virus (SPCaLV) in one sample collected in Uganda.

Surveys done in the East African region for virus assessment have mainly used serological techniques, especially nitro-cellulose membrane enzyme-linked immunosorbent assays (NCM-ELISA) using kits supplied by the International Potato Centre (CIP), Lima, Peru (Gibson et al., 1997; Aritua et al., 1998a; IsHak, 2003; Mukasa et al., 2003; Ateka et al., 2004b). The virus assays have also been done directly from the tip cuttings of sweetpotato plants, more often ignoring the older vine parts (Gibson et al., 1997; Mukasa et al., 2003). Kits from CIP contain antisera to detect up to 10 of the over 20 viruses that have been reported infecting sweetpotato in different parts of the world (Valverde et al., 2007). The surveys therefore do not include viruses for which antiserum is not available. Surveys done in Uganda (Mukasa *et al.*, 2003) and Kenya (Ateka et al., 2004b) found sweetpotato plants with symptoms resembling those caused by viruses, but which did not react with any of the antisera used. Such plants could be infected with viruses that have been described but were not tested for in the studies and/or viruses that have not yet been described in sweetpotato. Serological assays are known to be somewhat insensitive in detecting some viruses, especially when done directly from sweetpotato as the plant contains unusually large but variable concentrations of a number of substances such as latex, polyphenols, and polysaccharides that interfere with the assays (Esbenshade and Moyer, 1982; Abad and Moyer, 1992). Also, cuttings from older vine parts may harbor viruses not

present in the apical portion. These reasons may partly explain why some viruses are not documented as existing in East Africa, making it difficult to discern if absence of detection truly reflects absence of the viruses.

Some of the viruses that have not been included in previous surveys in the region include *Sweet potato leaf curl virus* (SPLCV, genus *Begomovirus*), *Sweet potato virus G* (SPVG, genus *Potyvirus*) and Sweet potato virus-2 (SPV-2, genus *Potyvirus*) (Rossel and Thottappilly, 1988; Lotrakul et al., 1998; Souto et al., 2003). SPLCV has been reported in the United States, South America; the Middle East, and South East Asia (Chung et al., 1985; Lotrakul et al., 1998; Banks et al., 1999; Fuentes and Salazar, 2003; Briddon et al., 2006; Luan et al., 2006), indicating that the virus has a world-wide distribution. SPLCV has not been reported in Africa, though Rossel (1981) described symptoms similar to those of SPLCV in *I. aquatica*. SPLCV on its own can significantly impact yield (Clark and Hoy, 2006) and can be confused with other viruses particularly in stressed plants (Lotrakul et al., 1998). SPLCV titers increased in the presence of a potyvirus (Kokkinos, 2006) which may result in easier acquisition and spread by whiteflies, the insect vectors of SPLCV. SPVG has been reported in different parts of the world including China, USA, Egypt, and South Africa (Colinet et al., 1996; Souto et al., 2003; IsHak et al., 2003; Ateka et al., 2007), while SPV-2 (also referred to as Ipomoea vein mosaic virus, IVMV; or Sweet potato virus Y, SPVY) has been reported in Taiwan (Rossel and Thottappilly, 1988; Ateka et al., 2004a), USA (Souto et al., 2003) and probably in Zimbabwe (Chavi et al., 1997). The titers of the two potyviruses are greatly enhanced in the presence SPCSV, resulting in severe symptom development (Kokkinos and Clark, 2006b). Despite SPV-2 and SPVG or their isolates being reported in different parts of the world including some parts of Africa, the two viruses

have not been detected in East Africa. This study included a survey (Chapter 2) to ascertain whether the two potyviruses and the begomovirus are present in Kenya.

Except for SPCSV and SPLCV, most single infections cause mild or no symptoms (Esbenshade and Moyer, 1982; Di Feo et al., 2000; Mukasa et al., 2006; Untiveros et al., 2007) from which plants usually recover and contain very low virus titers (Esbenshade and Moyer, 1982; Abad and Moyer, 1992), and consequently, no significant yield reduction is observed. The most severe symptoms and yield losses are caused by co-infection with viruses from different genera. Invariably, most of these interactions seem to involve SPCSV, which has been observed to have synergistic interactions with SPFMV, SPMMV, *Cucumber mosaic virus* (CMV, genus *Cucumovirus*), *Sweet potato mild speckling virus* (SPMSV, genus *Potyvirus*), *Sweet potato latent virus* (SPLV, genus *Potyvirus*), SPVG, SPV-2 and SPCFV (Cohen & Loebenstein, 1991; Di Feo et al., 2000; Kokkinos, 2006; Mukasa et al., 2006; Untiveros et al., 2007). Sweet potato virus disease (SPVD), caused by simultaneous infection with SPFMV and SPCSV, is the main disease affecting sweetpotato production worldwide, probably because of the wide distribution of the two viruses (Mukiibi, 1977; Geddes, 1990; Gibson et al., 1997; 1998; Karyeija et al. 2000a). The disease is characterized by chlorosis, small deformed leaves, and severe stunting, and can reduce yields of infected plants by up to 90% (Mukiibi, 1977; Hahn, 1979; Gibson et al., 1998; Gutierrez et al., 2003). SPVD is the most serious disease of sweetpotato in East Africa (Geddes, 1990) and a lot of resources are directed towards its management.

1.2 Sweet Potato Feathery Mottle Virus (SPFMV)

SPFMV is the most common sweetpotato virus, occurring virtually everywhere sweetpotato is grown (Brunt et al., 1996; Salazer and Fuentes, 2001). The virus was first described in 1945 in the United States (Doolittle and Harter, 1945). It was first reported in East

Africa in 1957 under the name Sweet potato virus A (Shefield, 1957) and in West Africa under the name sweet potato vein clearing virus (Schaefers and Terry, 1976). The genus *potyvirus* and the family *Potyviridae*, of which the SPFMV is a member, are the largest genus and family of plant viruses, respectively. SPFMV has flexuous filamentous particles between 830-850 nm in length. They contain a single-stranded positive sense RNA genome of about 10.6 kb (Sakai et al., 1997). SPFMV is transmitted by several aphid species (*Aphis gossypii, A. craccivora, Lipaphis erysimi, Myzus persicae*) in a non-persistent manner (Stubbs and McLean, 1958; Moyer and Kennedy, 1978). These aphids do not normally colonize sweetpotato and therefore itinerate alate aphids are presumed to be the means of transmission (Kantack *et al*., 1960; Aritua et al., 1998b). The host range of SPFMV is narrow and mostly limited to plants from the family *Convolvulaceae,* and especially the genus *Ipomoea,* although some strains have been reported to infect *Nicotiana benthamiana* and *Chenopodium* spp. (Campbell et al., 1974; Moyer and Kennedy, 1978; Moyer *et al*., 1980; Nakashima et al., 1993). Symptoms, host range, serology and nucleotide sequences have been used to assign SPFMV to the Common (C), Russet Crack (RC), Ordinary (O) and East African (EA) strain groups (Moyer and Kennedy, 1978; Moyer et al., 1980; Cali and Moyer, 1981; Kreuze et al., 2000). Strains C, O and EA have been detected in East Africa, but the RC strain has not been detected (Krueze et al., 2000; Abubakar et al., 2003; IsHak et al., 2003; Ateka, 2004). The existence of different strains of SPFMV in different regions is important in so far as resistance to the virus is concerned. Karyeija et al., (2000a) reported that a sweetpotato cultivar that was resistant to Peruvian strains of SPFMV and SPFMV-C was not resistant to East African strain of SPFMV. This makes breeding for resistance to the virus (and to SPVD) difficult and resistant materials may need to be developed for particular regions of the world where particular strains are found.

Leaf symptoms caused by SPFMV are mostly mild but may include vein clearing, irregular chlorotic patterns (feathering) along the leaf mid-rib and chlorotic spots which may have purple pigmented borders especially in the older leaves. Depending on sweetpotato cultivar, the storage roots of infected plants may show external necrosis if infected with the RC strain (Moyer and Kennedy, 1978; Campbell et al., 1974; Cali and Moyer, 1981; Moyer and Cali, 1985; Clark and Moyer, 1988). When infecting alone, effects of SPFMV are not conspicuous since its movement and/or replication is restricted (Gibson et al., 1998; Karyeija et al., 2000b). It is difficult to detect the virus in plants artificially infected with SPFMV alone, and aphids do not readily acquire the virus from such plants (Aritua et al., 1998b). This may indicate that there are sweetpotato genotypes that are highly susceptible to SPFMV alone or that there are other viruses that enhance the virus titers in the field that have not been detected (Valverde et al., 2007). However, certain strains can cause qualitative damage due to internal cork or cracking of the tubers (Mori et al., 1995; Ryu et al., 1998). Quantitative losses due to reduced plant vigor associated with chronic infection with SPFMV have been experienced (Esbenshade and Moyer, 1982; Gibson et al., 1997; Njeru et al., 2004). The real importance of SPFMV lies in being a component of a complex with SPCSV, where its titers increase drastically (Karyeija et al., 2000b; Kokkinos and Clark, 2006b). Surveys carried out in East Africa have reported SPFMV as the most predominant virus infecting sweetpotato, with over 50% of the plants showing virus symptoms being reported to have single infections with the virus (Mukasa et al., 2003; Ateka et al., 2004b; Tairo et al., 2004; Aritua et al., 2006).

1.3 Sweet Potato Chlorotic Stunt Virus (SPCSV)

SPCSV is the second most predominant sweetpotato virus in Kenya and in East Africa (Mukasa et al., 2003; Ateka et al., 2004b; Tairo et al., 2004; Aritua et al., 2006), and is also

widespread in different sweetpotato growing regions of the world (Gibson et al., 1998). The virus belongs to the genus *Crinivirus* within the family *Closteroviridae* (Van Regenmortel et al., 2000). The particles of SPCSV are 850 to 950 nm in length and 12 nm in diameter. The size of the major coat protein is 33 kDa, which is similar to other criniviruses (Cohen et al., 1992; Van Regenmortel et al., 2000). The complete genome of SPCSV was sequenced by Kreuze et al., (2002) and consists of two RNA molecules. With a total length of 17630 nucleotides, SPCSV is the second largest single-stranded positive-sense RNA virus infecting plants after *Citrus tristeza virus* (CTV, genus *Closterovirus*). RNA1 has a total length of 9407 nucleotides and contains five putative open reading frames (ORFs). RNA2 contains 8223 nucleotides and has seven putative ORFs. SPCSV is transmitted by whiteflies (e.g. *Bemisia tabaci* and *Trialeurodes abutilonea*) in a semi-persistent, non-circulative manner (Cohen et al., 1992). The host range of SPCSV is limited mainly to family *Convolvulaceae* and the genus *Ipomoea*, although *Nicotiana* spp. and *Amaranthus palmeri* are reportedly susceptible (Cohen et al., 1992). SPCSV has also been reported in Lisianthus (*Eustoma grandiflorum*; Cohen et al., 2001). SPCSV can be serologically divided in two major serotypes, one (designated serotype East Africa) occurs only in East Africa and Peru, while the other serotype is found in United States, South America, West Africa and Egypt (Hoyer et al., 1996; Kreuze et al., 2002; IsHak et al., 2003; Gutierez et al., 2003; Abad et al., 2007). The two serotypes are sufficiently distinct phylogenetically to suggest that they may correspond to two distinct criniviruses (Kreuze et al., 2002; Abad et al., 2007). Abad et al. (2007) further differentiated the East African serotype into two subpopulations (Peru and East Africa) and the West African serotype into three subpopulations (Argentina-Brazil, USA and West Africa). As with SPFMV, the existence of different strains and serotypes of SPCSV has a significant implication in breeding for resistance to SPVD in different regions of the world as it

may require that resistant materials be developed for particular regions of the world where particular strains and/or serotypes are found. Despite the presence of the virus and the vector in the fields of East Africa, and the high incidence of SPFMV, SPCSV is not as common as SPFMV. This suggests that the spread of SPCSV is not as efficient as that of SPFMV.

The symptoms caused by SPCSV alone have been confused with nutritional deficiencies and include such mild symptoms as slight stunting and purpling of older leaves and mild chlorotic mottle in the intermediate aged leaves (Gibson et al., 1998). Although SPCSV can cause yield losses on its own (Gutierrez et al., 2003), its real importance lies in the ability to synergize with different unrelated viruses (Cohen & Loebenstein, 1991; Di Feo et al., 2000; Kokkinos and Clark, 2006b; Mukasa et al., 2006; Untiveros et al., 2007). Understanding how a naturally resistant sweetpotato plant responds to infection by SPCSV will provide fundamental information on the process of virus infection in the plant, paving way for development of varieties with more durable resistance.

1.4 Management of Sweet Potato Virus Disease (SPVD)

Attempts to control SPVD are through the use of host plant resistance or pathogen-tested plants (Valverde et al., 2007). However, use of pathogen tested plants is not considered economically feasible in developing countries, and especially in sub-Saharan Africa where the crop is grown by small-holder farmers with limited land, labor and capital. Developing resistant varieties backed up by farmers selecting disease-free planting stock (apparently free of known viruses) is therefore given the highest priority in the tropics by both international and national institutions. Hundreds of sweetpotato cultivars (landraces) are grown in East Africa, many of which show some resistance to SPVD (Gichuki et al., 2003; Aritua et al., 1998b), and the impact of SPVD is normally mitigated. Since SPVD induces dramatic symptoms, it is possible to readily

identify affected plants in the field. In a field trial in Uganda, Mwanga et al. (2002b) observed

that some genotypes tested negative to both SPFMV and SPCSV, showing that they were either

difficult to infect, were able to suppress the rapid multiplication of the virus, or had a mechanism

of lowering titer during progress of SPVD. Recovery of such plants from virus-induced

symptoms is reported to be a consequence of RNA silencing, an antiviral defence mechanism

(Jovel et al., 2007). Some severely infected genotypes are also reported to localize the

distribution of SPCSV and SPFMV (Aritua et al., 1998b; Gibson et al., 1998; Mwanga et al.,

2002b), indicating that SPVD may not be fully systemic, and that a proportion of uninfected

cuttings may be obtained from previously infected plants.

      The fact that some genotypes have the ability to recover from SPVD implies that the

lowering of the titre during progress of SPVD is an important mechanism in resistance to SPVD.

While low titer and irregular distribution are problematic in virus detection (Esbenshade and

Moyer, 1982; Abad and Moyer, 1992), sweetpotato genotypes with such qualities are expected to

show more rapid recovery compared to plants with fully systemic and high virus titres. Indeed,

farmers in East Africa are reported to reduce losses by utilizing a 30-cm apical portion of

symptomless vines (Gibson et al., 1997). However, no studies have been done to determine if the

absence of the virus(es) was limited to the apical portion, or whether any other section of the

vine could as well be used for propagation purposes. A part of this study (Chapter 3) was to

investigate if there is a scientific basis to decide that the apical portion of the vine has a higher

chance of being virus free compared to other portions of the vine, and therefore form a strong

base to advise farmers to use such a section for propagation purposes.

      Prior efforts at managing SPVD were focused on developing cultivars with resistance to

SPFMV given its universal distribution. However, many sweetpotato cultivars are naturally

resistant to SPFMV, showing no or only mild initial symptoms, from which they usually recover, and containing very low virus titers (Esbenshade and Moyer, 1982; Green et al., 1988; Abad and Moyer, 1992; Gibson et al. 1998; Mwanga et al. 2002b; Dje and Diallo, 2005; Kokkinos and Clark, 2006b). Resistance is lost when the varieties are co-infected with SPCSV (Karyeija et al. 1998a; Mwanga et al., 2002b). As pointed out previously, SPCSV on its own can cause significant yield losses (Gutierrez et al., 2003), and efforts should therefore be directed towards developing varieties resistant to SPCSV (Kreuze, 2002). However, the safest way seems to develop resistance to both viruses simultaneously (Kreuze, 2002). Recent efforts to develop genetically modified sweetpotato to control the disease are yet to attain the anticipated potential.

1.5 Reaction of Sweetpotato Genotypes to SPVD and Infection by SPFMV and SPCSV

Genotypes have been described as resistant to SPFMV, SPCSV, or SPVD if they fail to develop symptoms under natural inoculum pressure or following graft inoculation. However, these two approaches may measure different aspects of resistance. Historically, resistance to diseases of plants has generally been divided into two major categories: non-host resistance and host resistance (Fraser, 1990). Non-host resistance (also referred to as immunity) encompasses the case where all genotypes within a plant species show resistance or fail to be infected by a particular virus. Most plant species are therefore resistant to most plant viruses and susceptibility is the exception to the more general condition of resistance or failure to infect. As viruses are completely dependent on host factors for their replication, incompatibility between viral and host proteins probably accounts for non-host resistance to viruses. Such type of resistance confers immunity and is not expected in sweetpotato resistant to SPVD since many genotypes are susceptible.

Host resistance (also termed as specific resistance, genotypic resistance, or cultivar resistance) occurs when genetic differences in susceptibility are observed within a plant taxon, i.e., some genotypes show heritable resistance to a particular virus whereas other genotypes in the same taxon are susceptible. The resistance may be conferred by a single gene (monogenic resistance) or by a combination of several genes (oligogenic or multigenic resistance). Monogenic resistance genes usually confer strong resistance, but often only against a limited number of pathogen strains or races. Multigenic resistance is mediated through combined effects of many genes that each by themselves may confer only a rather insignificant resistance effect towards the pathogen in question. Multigenic resistance is generally not as strong as monogenic resistance, but it has equivalent effectiveness against all strains or races of the pathogen (Agrios, 2005). In resistant individuals, the virus multiplication may be reduced or inhibited to some extent, or the spread of the pathogen through the plant is demonstrably restricted relative to susceptible hosts, and disease symptoms are highly localized or not evident. It is also important to distinguish between resistance to a disease (tolerance) and resistance to a pathogen. In case of resistance to disease symptoms or tolerance to the disease, the virus may move through the host in a manner that is indistinguishable from that in susceptible host, but disease symptoms are not observed. Kang et al. (2005) noted that resistance to a pathogen typically leads to resistance to the disease. There is therefore a need to differentiate the type of resistance exhibited by different sweetpotato genotypes described as resistant to SPVD, i.e., whether true resistance or tolerance.

Previous studies have shown that plants infected with SPFMV and SPCSV contain higher titers of SPFMV, more severe symptoms, and are a better source of aphid acquisition than plants infected with SPFMV alone (Schaefers and Terry, 1976; Rossel and Thottappilly, 1988; Kokkinos and Clark, 2006b; Mukasa et al., 2006). Karyeija et al. (2000b) studied the aetiology of

SPVD using nucleic acid hybridization, bioassays, tissue printing and thin section immunohistochemistry in the sweetpotato cultivar 'Tanzania' and found that resistance to SPFMV was due to inhibition of virus replication rather than movement and resistance was suppressed by infection with SPCSV, resulting in a ca. 600-fold increase in titers of SPFMV. Karyeija et al. (2000b) also reported that SPCSV was limited to the phloem tissues while SPFMV was not limited to any particular tissue. Similar increase in titer levels of SPFMV in the presence of SPCSV has been confirmed by other workers using real-time quantitative polymerase chain reaction (PCR) (Kokkinos and Clark, 2006a; 2006b; Mukasa et al., 2006). The mechanism(s) by which SPCSV synergizes SPFMV is not yet known, but have been associated with suppression of RNA silencing of plants by SPCSV (Kreuze et al., 2005). However, titres of the phloem-limited SPCSV remain the same or slightly decrease in the SPVD-affected plants as compared to plants infected with SPCSV alone (Gibson et al., 1998; Karyeija et al., 2000b; Kokkinos and Clark, 2006b).

In plants inoculated with both SPCSV and SPFMV, the symptoms of SPVD first develop in the newly emerging leaves (Karyeija et al., 2000b). Karyeija et al. (2000b) hypothesized that the phloem of the youngest leaves provide only limited support for SPCSV replication and/or movement, in contrast to the mature phloem in the veins of the more developed leaves. Consequently, viral RNA accumulation at earlier and later phases of infection could be compared by assaying the extracts of leaves harvested at different developmental stages. Karyeija et al. (2000b) suggested that efficient genome replication of SPCSV requires mature phloem cells present in the leaf veins of the well-developed leaves. Karyeija et al. (2000b) concluded that because the titers of SPCSV were not significantly increased in doubly infected plants, and because the plants infected with SPCSV alone were mostly symptomless or displayed symptoms

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different from SPCSV, the symptoms of SPVD are presumably caused solely or largely by SPFMV. This was supported by the low undetectable titres of SPCSV in the young leaves with severe symptoms and by the correlation of the SPFMV titers with the severity of the symptoms. The symptoms caused by SPVD also resemble those of potyviruses (Shukla et al., 1994). Kreuze (2002) observed that the viral RNA amounts determined by Karyeija et al. (2000b) were done using RNA probes spanning the coat protein (CP) gene, or antibodies detecting the CP. The sub-genomic RNAs (sgRNAs) corresponding to the genes encoded by SPCSV RNA2, including the CP gene, have been found to accumulate later in infection than sgRNAs of SPCSV RNA1 in *I. setosa* (Kreuze at al., 2002). The RNA1 sgRNA were reported to be present even in the youngest leaves of *I. setosa*, implying that the same may apply to sweetpotato (Kreuze, 2002). The early accumulation of RNA1 sgRNA is important in SPVD development in that it contains the genes encoding RNase III and another protein (P22) both of which are important in RNA-silencing suppression activity of SPCSV (Kreuze et al., 2005). Studies involving interactions of SPFMV and SPCSV were done using SPVD-susceptible hosts. No studies have been carried out to compare the behaviour of the two viruses in genotypes showing differential reaction to SPVD. To determine if delayed or mild symptom development observed in SPVD-resistant genotypes is due to resistance or tolerance, the distribution and multiplication of SPFMV and SPCSV in resistant and susceptible genotypes were quantified in a time course experiment using real-time quantitative PCR (Chapter 4).

1.6 Real-time Quantitative PCR

Since its invention, the polymerase chain reaction (PCR) technique has greatly influenced applied sciences especially disease diagnostics (Schaad and Frederick, 2002). It has made it possible to detect, amplify and analyze even trace elements of nucleic acids faster and easier.

The introduction of real-time quantitative PCR made the detection even easier as it allowed direct monitoring of a PCR reaction as it occurs, thus allowing for the detection of PCR amplification during the early phases of the reaction (Zubritsky, 1999). This is a distinct advantage over traditional PCR detection, which is done at the end of the reaction using agarose gels. All real-time PCR systems rely upon the detection and quantification of a fluorescent reporter, the signal which increases in direct proportion to the amount of PCR product in a reaction (AppliedBiosystems, Foster City, CA.). In the simplest and most economical format, that reporter is the double-strand DNA specific dye SYBR® Green. This format has a disadvantage in that SYBR® Green will bind to any double stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration (Schaad and Frederick, 2002). The two most popular alternatives are the Taqman® and molecular beacons, both of which are hybridization probes relying on fluorescent resonance energy transfer (FRET) for quantification (Schaad and Frederick, 2002). In all cases, the amount of fluorescence is monitored during each amplification cycle and is proportional to the amount of PCR product generated. By plotting the increase in fluorescence, versus the PCR cycle number, the system produces plots that provide a more complete picture of the PCR process (Bulletin #2, AppliedBiosystems, Foster City, CA). The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. The use of a pre-designed internal positive control reagent (a 'household' gene such as 18S rRNA) in parallel reactions to the target reactions allows the normalization of DNA/RNA extraction variations between samples (Schaad and Frederick, 2002).

Real-time PCR has many important advantages over classical PCR in that it eliminates the need to do a Southern blot to confirm identification of a PCR product, it is a more contained system and less prone to cross contamination, less labor is required, it is more user friendly, it provides data useful for selecting primers, optimization of PCR protocol is quicker, and it can be used for multiplex PCR. Using TaqMan real time PCR and the ABI 7700 detection system (AppliedBiosystems, Foster City, CA.), the most robust primers and probes can be quickly selected. Also, since the results are quantitative, the concentration of the target can be determined (Bulletin #2, AppliedBiosystems, Foster City, CA).

Real-time quantitative PCR has been used in studying various causal agents of plant diseases including bacteria, fungi, and viruses (Schoen et al., 1996; Schaad et al., 1999; Weller et al., 2000; Frederic et al., 2000; Weller and stead, 2002; Kokkinos and Clark, 2006a; 2006b). Kokkinos and Clark (2006a; 2006b) were the first to use real-time quantitative PCR to quantify different viruses infecting sweetpotato. They utilized the TaqMan® Chemistry to develop assays for the detection and quantification of three potyviruses (SPFMV, SPVG, SPV-2), the crinivirus SPCSV and the begomovirus SPLCV. In the Taqman® system, an oligonucleotide probe sequence of approximately 25-30 nucleotides in length is labeled at the 5' end with a fluorescent dye, usually a 6-carboxyfluorescein (6-FAM) and a quencher dye, usually 6-carboxytetramethyl-rhodamine (TAMRA), at the 3' end (Schaad and Frederick, 2002). The Taqman® probe is degraded by the 5-3' exonuclease activity of the *Taq polymerase* as it extends the primer during each PCR amplification cycle and the fluorescent dye is released. The protocol by Kokkinos and Clark (2006a) was used to study the virus multiplication and movement in various sweetpotato genotypes used in this study.

1.7 Trait-linked Molecular Markers

Although many sweetpotato cultivars and landraces resistant to SPVD exist in East Africa, resistance has been associated with relatively late maturing, low-yielding genotypes (Aritua et al. 1998b), and there is need to incorporate resistance to high yielding, early maturing, β-carotene rich cultivars. Selection for resistance to SPVD is based on symptom development after field exposure in disease prone areas and by graft-inoculations (Hahn et al. 1981; Mihovilovich et al., 2000; Mwanga et al., 2002b), processes requiring considerable time and resources. Recent advances in molecular techniques are offering novel techniques that may greatly reduce costs and time. Attention is focused on molecular markers to accelerate breeding through early selection. DNA markers such as randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellites or simple sequence length polymorphism (SSLP), and amplified fragment length polymorphism (AFLP) have been associated with simply inherited and complex traits (quantitative trait loci, QTL) in different crops (Pongam et al., 1998; Brandshaw et al., 1998; Jin et al., 1998). Mwanga et al. (2002a) found an AFLP marker (*spcsv1*) which explained 70% of the variation in resistance to SPCSV and one RAPD marker (*spfmv1*) which explained 72% of the variation in SPFMV resistance in sweetpotato. However, Mwanga et al. (2002a) did not find markers associated with resistance to SPVD. Mcharo (2005) used AFLP and various multivariate statistical techniques to analyze for morphological, quantitative and molecular marker variation in sweetpotato and in the ornamental liriopogon. This study used the AFLP technique together with discriminant analysis and logistic regression to link molecular markers with resistance to SPVD in order to identify markers of interest from a population of unrelated genotypes selected mainly from a Kenyan germplasm

collection. The AFLP technique (Vos et al., 1995) was chosen because it provides highly polymorphic markers combining both RFLP and PCR strategies.

1.8 Use of Statistical Techniques to Identify Important Trait-linked Molecular Markers

Multivariate statistics have been used to mine through molecular marker information to identify any underlying relationships between various phenotypic groups and DNA markers. Mcharo (2005) noted that there are two approaches to integrating molecular markers and phenotypic traits, namely i) Analysis of Variance using molecular data to test difference among groups defined according to phenotypic data using analysis of molecular variance (AMOVA) based algorithm, and ii) selection of informative markers and computation of a classification model for the phenotypic group membership using discriminant and logistic regression. AMOVA is used to detect statistical differences among pre-defined groups (Excoiffer et al., 1992). Groups that are significantly different are subsequently analyzed using discriminant analysis to determine which markers discriminate between phenotypic groups using molecular marker information. Application of discriminant analysis to a molecular marker data set enables one to determine which markers contribute most to discriminate between groups and then use the information to predict group membership. The identified markers can then be used to test the validity of groups based on actual data, to test groups that have been created, or to assign lines into groups. Phenotypic grouping for subsequent AMOVA, QTL, discriminant and logistic regression analysis may be based on morphological observations or statistical techniques such as cluster analysis (Mcharo 2005). In our study, we used symptom development after graft inoculations to identify two groups consisting of genotypes resistant and susceptible to SPVD.

Discriminant analysis and logistic regression represent novel approaches in marker-assisted selection (Cruz-Castillo et al. 1994; Ebdon et al., 1998; Capdevielle et al. 2000; Fahima

et al. 2002; Aluko 2003; Mcharo 2005). Discriminant analysis is a multivariate statistical technique that can identify differences among groups of individuals (or treatments) and improve the understanding of relationships among the variables measured within those groups (Cruz-Castillo et al., 1994). The technique determines how best to separate or discriminate two or more groups of individuals, given quantitative measurements of several individuals, through simultaneous analysis of several variables (Rencher, 1992). Discriminant analysis is used to find linear functions of variables that maximally separate two or more groups of individuals while keeping variation within groups as small as possible. Discriminant analysis may be used when it is important to separate known groups or *a priori* groupings, and to identify major sources of difference between groups. Genotypes possessing trait extremes, e.g., highly resistant versus highly susceptible are used exclusively, and those with intermediate reactions ignored. Logistic regression is a specialized form of regression that is formulated to predict and explain a binary (two-group) categorical variable rather than a metric dependent measure (Hair et al., 1998). The method analyzes proportions based on the binary event and the proportions are then transformed into odds ratios (Mcharo, 2005). Odds is the ratio of a probability of an event occurring to a probability of the event not occurring while odds ratio is a ratio of two odds. The event may group into either one or the other phenotypic group. During the analysis the odds undergo a logit transformation to obtain log odds for a given variable such as a DNA marker. The log odds for each variable selected by logistic regression are then included in the group classification model. The result of logistic regression analysis is the probability of inclusion into a phenotypic group. Discriminant and logistic regression analysis are especially useful in dealing with populations of unrelated clones such as landrace genotypes since quantitative trait loci (QTL) analysis is not

suited for such populations with no progeny-parent combinations (Mcharo, 2005). These methods were used to identify molecular markers associated with SPVD resistance (Chapter 5).

1.9 Objectives

The objectives of this study were:

1. To survey for SPVG, SPV-2, and begomoviruses infecting sweetpotato in Kenya and determine their relationship with others characterized from different parts of the world.

2. To document the prevalence of three common viruses (SPFMV, SPMMV, and SPCSV) in field-grown sweetpotato genotypes with varying degrees of observable virus symptoms, and to determine if there are significant differences in virus presence between different vine sections.

3. Determine the response of sweetpotato genotypes to dual infection by SPFMV and SPCSV by studying the distribution and multiplication of the two viruses in resistant and susceptible genotypes using real-time quantitative PCR.

4. Identify and classify unrelated sweetpotato genotypes selected from East African germplasm collections into phenotypic groups as resistant and susceptible to SPVD, and to identify the most important AFLP markers contributing to variation among the phenotypic groups using discriminant and logistic regression analysis.

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CHAPTER 2: SURVEYING FOR TWO POTYVIRUSES AND A BEGOMOVIRUS INFECTING SWEETPOTATO IN KENYA*

2.1 Introduction

Sweetpotato is an important subsistence food crop grown in almost all agroecological zones of Kenya. Different studies have been carried out to determine the viruses infecting the crop in the country (Wambugu, 1991; Ateka et al., 2004). Wambugu (1991) reported the presence of seven viruses infecting sweetpotato in Kenya and Uganda using serology. However, only four viruses have been identified and confirmed to be widely distributed in the region (Mukasa et al., 2003; Ateka et al., 2004b; Tairo et al., 2004), of which two belong to family *Potyviridae*: the potyvirus *Sweet potato feathery mottle virus* (SPFMV) and the ipomovirus *Sweet potato mild mottle virus* (SPMMV); one belongs to the family *Closteroviridae*: the crinivirus *Sweet potato chlorotic stunt virus* (SPCSV) as well as Sweet potato chlorotic fleck virus (SPCFV) for which the genus *Carlavirus* has been proposed (Aritua et al., 2003). Aritua et al. (2006) reported on the occurrence of Sweet potato caulimo-like virus (SPCaLV) in Uganda, though the incidence was extremely low (one sample from 1473 samples). There have been reports of viruslike symptoms for which the causal virus(es) was not known (Mukasa et al., 2003; Ateka et al., 2004b; Tairo et al., 2004; Aritua et al., 2006), indicating the likely presence of unknown viruses. Many more viruses have been reported to infect sweetpotato in different parts of the world (Clark and Moyer, 1988; Salazar and Fuentes, 2001). Three such viruses are Sweet potato virus G (SPVG) and Sweet potato virus 2 (SPV-2) (Souto et al., 2003), and *Sweet potato leaf curl virus* (SPLCV) (Lotrakul et al., 1998).

SPVG has been reported in different parts of the world including China, USA, Egypt, and South Africa (Colinet et al., 1994; Souto et al., 2003; IsHak et al., 2003; Ateka, 2004b). SPV-2

was first reported in Taiwan (Rossel and Thottappilly, 1988) and named as SPV-2. It was later found in the US (Souto et al., 2003). Ateka et al. (2004a) characterized the isolate from Taiwan and renamed it Sweet potato virus Y (SPVY). The unique strain of SPFMV found in Zimbabwe (Chavi et al., 1997), may also represent SPV-2. Valverde et al. (2007) concluded that the name Sweet potato virus 2 takes precedence and should be used for the viruses previously referred to as IVMV or SPVY. Although SPV-2 and SPVG have been reported in different parts of Africa, they have not been detected in East Africa.

Different geminiviruses, including *Sweet potato leaf curl virus* (SPLCV), Ipomoea crinkle leaf curl virus (ICLCV), and *Sweet potato leaf curl Georgia Virus* (SPLCGV, previously called Ipomoea leaf curl virus, ILCV) have been reported to infect sweetpotato (Chung et al., 1985; Cohen et al., 1997; Lotrakul et al., 1998). *Ipomoea yellow vein virus* (IYVV) has also been isolated from *I. indica* (Banks et al., 1999). The viruses are currently placed in the Begomovirus genus of the family Geminiviridae. Even though the viruses have been reported in different parts of the world, previous surveys for viruses in sweetpotatoes in Africa did not assay for the presence of geminiviruses.

The objective of this study was to survey for SPVG, SPV-2, and begomoviruses infecting sweetpotato in Kenya and determine their relationship with others that have been characterized from different parts of the world.

2.2 Materials and Methods

2.2.1 Survey for SPVG and SPV-2

A survey was conducted in sweetpotato fields in Nyanza, Western, Central, Coast and Eastern provinces of Kenya to determine the presence of SPVG and SPV-2. Sweetpotato cuttings with virus disease symptoms similar to those exhibited by potyviruses (including chlorotic spots,

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vein clearing, vein banding, purple spots, mottling and mosaic) were collected. Each cutting was grafted to a one-month-old *I. setosa* test plant. The grafts were assessed for 3 weeks for graft establishment and also monitored for symptom development. Different symptoms observed on the *I. setosa* were recorded for a period of 2 months. Symptomatic leaves from the *I. setosa* test plants were then assayed for the presence of specific viruses.

Presence of SPVG and SPV-2 were assayed using nitro-cellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA) as described by Gutierrez et al. (2003) using polyclonal antibodies supplied in kits provided by International Potato Center (CIP), Lima, Peru and/or from C. Clark's lab (Department of Plant Pathology and Crop Physiology, Louisiana State University). NCM strips spotted with sap from virus-positive control plants provided by C. Clark were included as positive controls.

Two leaf disks (about 1cm in diameter) were taken from two leaves (symptomatic leaves when available) of each *I. setosa*, placed in a plastic bag, and homogenized in 2 ml of Tris-buffered saline (TBS) pH 7.5 containing 0.2% of sodium sulfite (Na_2SO_3). The tissue was completely ground using a test tube, and then the plastic bag was allowed to stand for 20-30 min at room temperature. The clarified sap extract (15μl) was blotted onto a TBS buffer-saturated nitrocellulose membrane and air-dried for 20-30 min. All the incubations and washings were done at room temperature in a shaker with gentle agitation (50 rpm for incubations and 100 for washings). The membranes were blocked with TBS containing 2% powdered milk and 2% Triton X-100 and incubated for one hour. The blocking solution was discarded and the membranes rinsed with TBS. The first antibody (polyclonal specific to SPVG or SPV-2), diluted in TBS containing 2% powdered milk, was added to the membranes and incubated overnight.

The membranes were washed in T-TBS (TBS containing 0.05% Tween 20) four times for 3 min each. The second antibody (goat anti-rabbit) conjugated with alkaline phosphatase and diluted in TBS containing 2% powdered milk, was added to the membranes and incubated for one hour. The membranes were washed as before and the presence of bound antibody was visualized by the addition of substrate: BCIP/NBT color development solution. The color reaction was stopped after 10-20 min using deionized water. Positive reactions were determined by visual assessment, and purple color reaction was recorded as positive.

2.2.2 Survey for SPLCV

Symptomatic sweetpotato plants with leaf curling, leaf rolling, and/or vein clearing symptoms similar to those observed in SPLCV-infected sweetpotato plants (Lotrakul et al., 1998), were collected for further assay. The collected sweetpotato plants were grafted onto *I. setosa*, and symptoms observed for 2 months after grafting. Total DNA was isolated from 100 mg of foliar tissue obtained from grafted *I. setosa* using the GenElute[TM] plant Genomic DNA Kit (Sigma-Aldrich Inc., St. Louis, Mo.).

Sweetpotato cuttings from 38 clones, which had been selected from the Kenyan germplasm collection plots for their resistance or susceptibility to sweetpotato virus disease (SPVD), were sent to the Plant Germplasm Quarantine Office of USDA-ARS for virus indexing and therapy. Though the plants were not being collected for virus detection, and they did not have obvious symptoms associated with geminiviruses, the viruses were assayed as a routine. The cuttings were planted in a greenhouse and total DNA extracted from sweetpotato leaves one month later using a cetyltrimethylammoniumbromide (CTAB) extraction method (Li et al., 2004). The extracted DNA was used to assay for the presence of geminiviruses using PCR.

2.2.3 Polymerase Chain Reaction (PCR) for Geminiviruses

Degenerate primers SPG1/SPG2 described by Li et al. (2004) to anneal to regions of open reading frame (ORF) AC2 and ORF AC1 were used. The two regions are highly conserved in geminiviruses infecting sweetpotato and other begomoviruses. PCR was performed using a Genius thermocycler (Techne, Cambridge, UK). The amplification was performed in 50µl reaction volumes containing 1µl of DNA extract, 1µl of primer SPG1/SPG2 (10µM), 4µl of 2.5 mM dNTP mix, 5µl 10x *Taq* DNA polymerase reaction buffer, 2.5µl of 50 mM MgCl$_2$, 0.5µl (5 U) of Taq DNA polymerase (Sigma-Aldrich^{TM}) and 35µl water. The amplification conditions were 1 cycle at 94°C for 90 s; 35 cycles of 94°C for 40 s, 56°C for 40 s, 72°C for 90 s; and 72°C for 10 min. PCR products were assessed by electrophoresis in 1.2 % agarose gels in TBE buffer, stained with ethidium bromide, and viewed under ultraviolet light.

SPLCV-specific primers PW285-1/PW285-2 described by Lotrakul and Valverde (1999) were used to amplify a 512 bp fragment of the conserved sequences within ORF AC1. The reaction volumes were as described above. The amplification conditions were 1 cycle at 94°C for 60 s; 45 cycles of 94°C for 60 s, 55°C for 60 s, 72°C for 3 min; and 72°C for 10 min. PCR products were assessed as above.

2.2.4 Cloning and Sequencing

Bands amplified using primers PW285-1/PW285-2 were excised from agarose gels and purified using MinElute^{TM} Gel Extraction kit (QIAGEN Inc., Valencia, CA). The purified PCR products were ligated into pGEM-T Easy vector (Promega Corp., Madison, WI) according to manufacturer's directions. A ligation mix containing 1µl of the cloning vector, 1µl of the T4 DNA ligase and 1µl of deionized water were made in 5µl of 2X rapid ligation buffer. Two µl of

the PCR product was added into the ligation mix and mixed by pipetting. The ligation mix was incubated at 4ºC overnight.

The ligation reactions were briefly centrifuged to collect the contents at the bottom of the tube. Two µl of the ligation reaction was put into a sterile 1.5 ml microcentrifuge tube on ice. A tube containing *Escherichia coli* strain JM 109 High efficiency Competent cells (Promega Corp[TM]) were transferred from -70ºC into an ice bath until just thawed (ca. 5 min). Competent cells (50µl) were carefully transferred into the tube containing ligation reactions and gently flicked to mix the contents. The tubes were incubated on ice for 20 min. The cells were heat-shocked for 45-50 s in a water bath at exactly 42ºC and the tubes immediately placed on ice for 2 min. LB medium (950µl) was added to the tubes containing transformed cells. The tubes were incubated for 1.5 h at 37ºC with shaking (150 rpm). 100µl of each transformation culture was plated onto duplicate LB/ampicillin/PTG/X-Gal plates. The plates were incubated overnight at 37ºC. Colonies with inserts were identified by blue/white screening. Single white colonies were picked, suspended in 3 ml of LB broth media containing 6µl ampicillin and incubated overnight at 37ºC with shaking at 150 rpm.

Recombinant plasmids were recovered from 1.5 ml of the overnight culture of competent cells using Flexiprep[TM] kit (Pharmacia Biotech Inc., Piscataway, NJ), according to the manufacturer's directions. To establish if the insert of interest was cloned, restriction enzyme EcoRI was used to digest the recovered plasmids. The restriction reaction was performed in 20µl reaction volumes containing 4µl of plasmid DNA, 2µl of the appropriate 10X digestion buffer, 1 unit of EcoRI restriction enzyme, and 13µl water. The reaction mixture was incubated at 37ºC for 60 min. Five µl of bromophenol/glycerol mix was added to each sample, and the reaction products assessed by electrophoresis in 1.2% agarose gel in TBE buffer, stained with ethidium

bromide, and viewed under ultraviolet light. Plasmid minipreps containing the correct size of DNA fragment were selected for sequence analysis.

Nucleotide sequences were determined commercially by automated sequence analysis at Genomic Technology Support Facility of Michigan State University, East Lansing using a Perkin Elmer/Applied Biosystems 3100 capillary sequencer (Perkin Elmer, Foster City, CA). The sequences obtained comprising a 512 nucleotide fragment from ORF AC1 were compared with corresponding sequences of other geminiviruses infecting sweetpotato.

Relationships between the Kenyan begomovirus isolates and other begomoviruses reported infecting sweetpotato from other parts of the world were analyzed based on a 457 nucleotide fragment from ORF AC1 nucleotide sequences and derived amino acid sequences from the same region. The sequences and their accession numbers are listed in Table 2.1. Percent nucleotide and amino acid identities were determined using pairwise global alignment. The alignments were used to determine the percent nucleotide sequence identity using ClustalX version 1.83 procedure (Jeanmougin et al., 1998). Multiple sequence alignments and phylogenetic analysis using neighbor-joining and bootstrap option (1000 replicates) were carried out using version 1.83 of ClustalX program (Jeanmougin et al., 1998).

2.3 Results

2.3.1 Survey for Viruses

A total of 146 samples with virus disease symptoms similar to those exhibited by potyviruses were collected. The samples analyzed included 55 samples from Western, 22 from Nyanza, 20 from Central, 31 from Coast and 18 from Eastern Provinces of Kenya. Although all the 146 samples induced symptoms in *I. setosa* test plants, none of the samples was positive to SPVG or SPV-2 using NCM-ELISA.

Ten symptomatic sweetpotato plants with leaf curling, leaf rolling, and vein clearing symptoms (Figure 2. 1A, B), were collected from a germplasm collection plot at Kakamega Research Station in Western Kenya during February 2005. Whiteflies, the vectors for begomoviruses, were observed in the same plots. When *I. setosa* test plants were graft-inoculated

Table 2. 1. Designation, geographic origin, and GenBank accession numbers of sweetpotato begomovirus isolates used for phylogenetic analysis.

Virus isolate	Origin	GenBank accession number
Sequenced in this study		
Kenya-4	Kenya	DQ361004
Kenya-43	Kenya	DQ361005
Kenya-46	Kenya	Not submitted
Kenya-79	Kenya	Not submitted
Kenya-80	Kenya	Not submitted
Kenya-84	Kenya	Not submitted
Obtained from GenBank		
SPLCV-US[x]	USA	AF104036
SPLCGV[y]	USA	AF326775
IYVV[z]	Spain	AJ586885
Korea	USA	AY679765
W-361	USA	DQ361003
Puerto Rico I (P Rico I)	USA	AY679766
Puerto Rico II (P Rico II)	USA	AY679767
Alabama	USA	AY769766
China Isolate (China)	China	DQ512731

[x]US isolate of *Sweet potato leaf curl virus*, [y]*Sweet potato leaf curl Georgia virus*, [z]*Ipomoea yellow vein virus*

Figure 2. 1. Leaf curling (A), leaf rolling (B), and vein mottle (C) symptoms observed on sweetpotato in Kenyan fields.

with scions from the symptomatic sweetpotato plants, some test plants developed symptoms of leaf curling, leaf rolling, interveinal chlorosis and stunting, similar to those caused by infection with SPLCV or SPLCV with SPFMV.

2.3.2 PCR

Degenerate primers SPG1/SPG2 amplified a 912 bp DNA fragment from three out of ten total DNA extracts from *I. setosa* (Figure 2.2), and five out of 39 sweetpotato samples sent to USDA. This is the expected size for sweetpotato geminiviruses (Li et al., 2004).

SPLCV-specific primers PW285-1/PW285-2 amplified the expected 512 bp DNA fragment from 7 of 8 samples that were positive to geminiviruses using degenerate primers (Figure 2.3). However, one of the 7 amplified samples had a very weak band and was not successfully sequenced. One sample, which had amplified with the degenerate primers, was not amplified with the specific primers, suggesting that this might be a different strain or isolate.

2.3.3 Comparison of the Nucleotide and Amino Acid Sequences of the Partial ORF AC1

To test for variation between the Kenyan isolates and geographically diverse begomovirus isolates infecting sweetpotato, the nucleotide and derived amino acid sequences of the partial ORF AC1 from the 6 Kenyan isolates were compared within themselves and with others from different countries (7 from US, 1 from Spain and 1 from China). Pairwise comparisons of nucleotide and amino acid sequences from the 6 Kenyan isolates gave identities ranging from 92 to 100% and 96 to 100%, respectively (Table 2.2), indicating that they are isolates of the same virus. Comparisons with nucleotide and amino acid sequences from other parts of the world gave 82 to 100% and 79 to 100%, respectively. The Kenyan isolates were most closely related to SPLCV-US, with over 94% nucleotide sequence identity and over 97%

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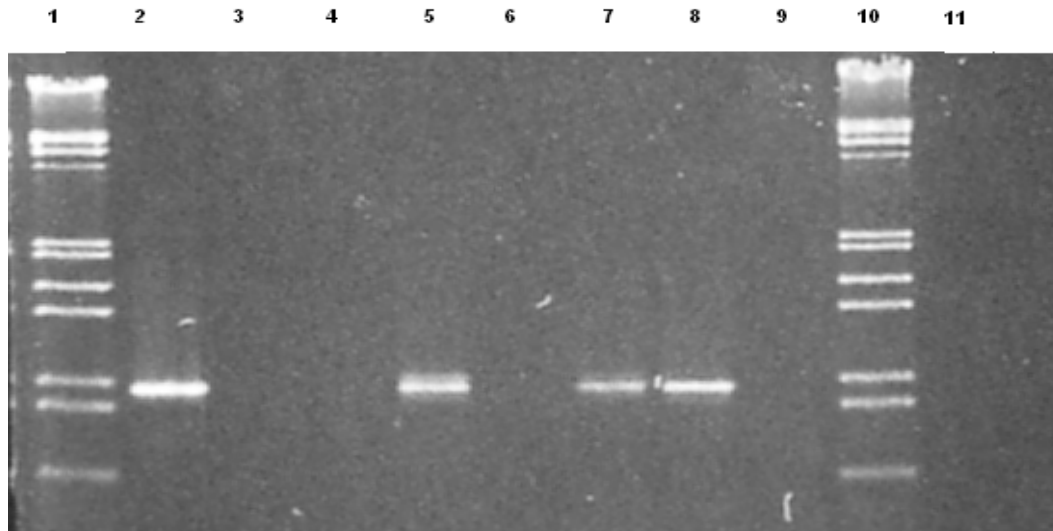
Figure 2. 2. Detection of sweetpotato geminivirus isolates from Kenya by PCR using degenerate primers SPG1/SPG2. Agarose gel (1.2%) containing DNA from: Lane 1 and 10, 1-kb DNA ladder; lanes 2 – 7, DNA extracts from *I. setosa* grafted with Kenyan sweetpotato suspected to be infected with geminiviruses; lane 8, SPLCV-US isolate (positive control); lane 9, DNA from healthy *I. setosa* (control); lane 11, water. The primers amplified a 912-bp product from infected plants (lanes 2, 5 and 7).
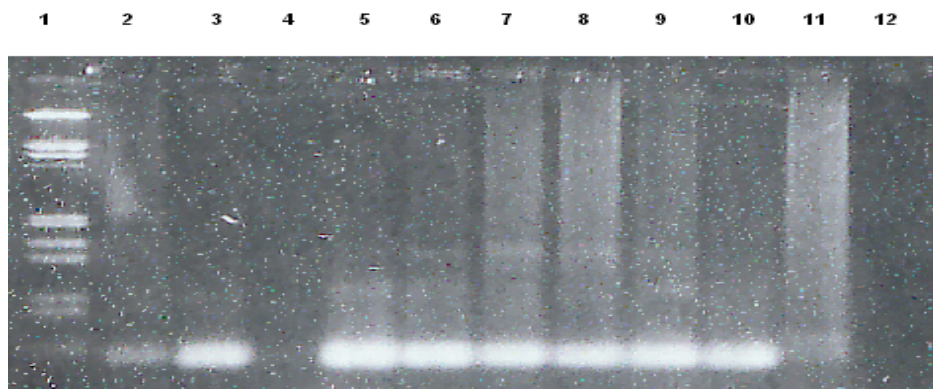
Figure 2. 3. Detection of the Kenyan sweetpotato isolates of geminiviruses by PCR using specific primers PW285-1/PW285-2. Agarose gel (1.2%) containg DNA from: Lane 1, 1-kb DNA ladder; lanes 2 – 9, DNA extracts from Kenyan sweetpotato samples detected to be infected with geminiviruses using degenerate primers; lane 10, US isolate of *Sweet potato leaf curl virus* (SPLCV-US, positive control); lane 11, DNA from uninoculated *I. setosa* (healthy control); lane 12, water. The primers amplified a 512-bp product from infected plants (lanes 2, 3, 5-9).

Table 2. 2. Pairwise percent nucleotide sequence identity (upper diagonal) and amino acid sequence identity (lower diagonal) among the partial ORF AC1 of begomovirus isolates as determined using ClustalX software.

Virus Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Kenya-4		93	94	92	92	93	94	87	92	93	89	89	88	88	82
2. Kenya-43	96		97	96	96	96	96	85	94	94	86	88	86	86	81
3. Kenya-46	97	97		96	96	97	96	85	94	95	87	89	87	87	83
4. Kenya-79	97	96	98		100	96	96	86	93	94	87	88	86	86	82
5. Kenya-80	97	96	98	100		96	96	86	93	94	87	88	86	86	82
6. Kenya-84	96	97	98	98	98		96	84	93	94	86	88	86	86	82
7. SPLCV-US[x]	98	97	98	99	99	98		87	96	96	88	90	87	87	83
8. SPLCGV[y]	94	92	94	93	93	92	94		85	86	92	89	90	91	85
9. IYVV[z]	86	85	88	88	88	86	88	83		95	86	89	86	86	83
10. Korea	95	94	96	96	96	95	96	92	87		87	90	87	87	83
11. W-361	94	93	95	94	94	94	94	96	84	92		91	98	99	87
12. Porto Rico I	94	92	94	94	94	93	94	94	84	94	94		90	91	86
13. Porto Rico II	92	91	93	92	92	92	92	94	82	90	97	92		98	85
14. Alabama	94	92	94	93	93	93	94	95	83	92	99	94	96		87
15. China Isolate	81	79	82	80	80	80	81	80	88	79	84	82	81	83	

[x]US isolate of *Sweet potato leaf curl virus*, [y]*Sweet potato leaf curl Georgia virus*, [z]*Ipomoea yellow vein virus*

amino acid sequence similarity. Phylogenetic analysis using nucleotide sequences (Fig. 2.4), resulted in three groups of isolates being visually distinguished: (a) a single isolate from China; (b) four isolates from US which included SPLCGV; and (c) a large clade consisting of all isolates from Kenya, one isolate from Spain and 3 isolates from US including SPLCV-US. These three groupings were confirmed when amino acid sequences were used (Fig. 2.5). Here, however, IYVV (isolate from Spain) diverged and formed its own group, mainly because IYVV sequence from the GenBank had 20 nucleotides missing.

2.4 Discussion

SPVG and SPV-2 were not detected in any of the samples collected. An earlier survey did not detect SPV-2 (also referred to as SPVY; Ateka et al., 2004a) in Kenya (Ateka et al., 2004b) and Uganda (Aritua et al. 2006), despite being detected in South Africa. SPVG has been detected in Egypt (IsHak et al., 2003). As previously noted by Ateka et al. (2007), it is not clear why SPVG
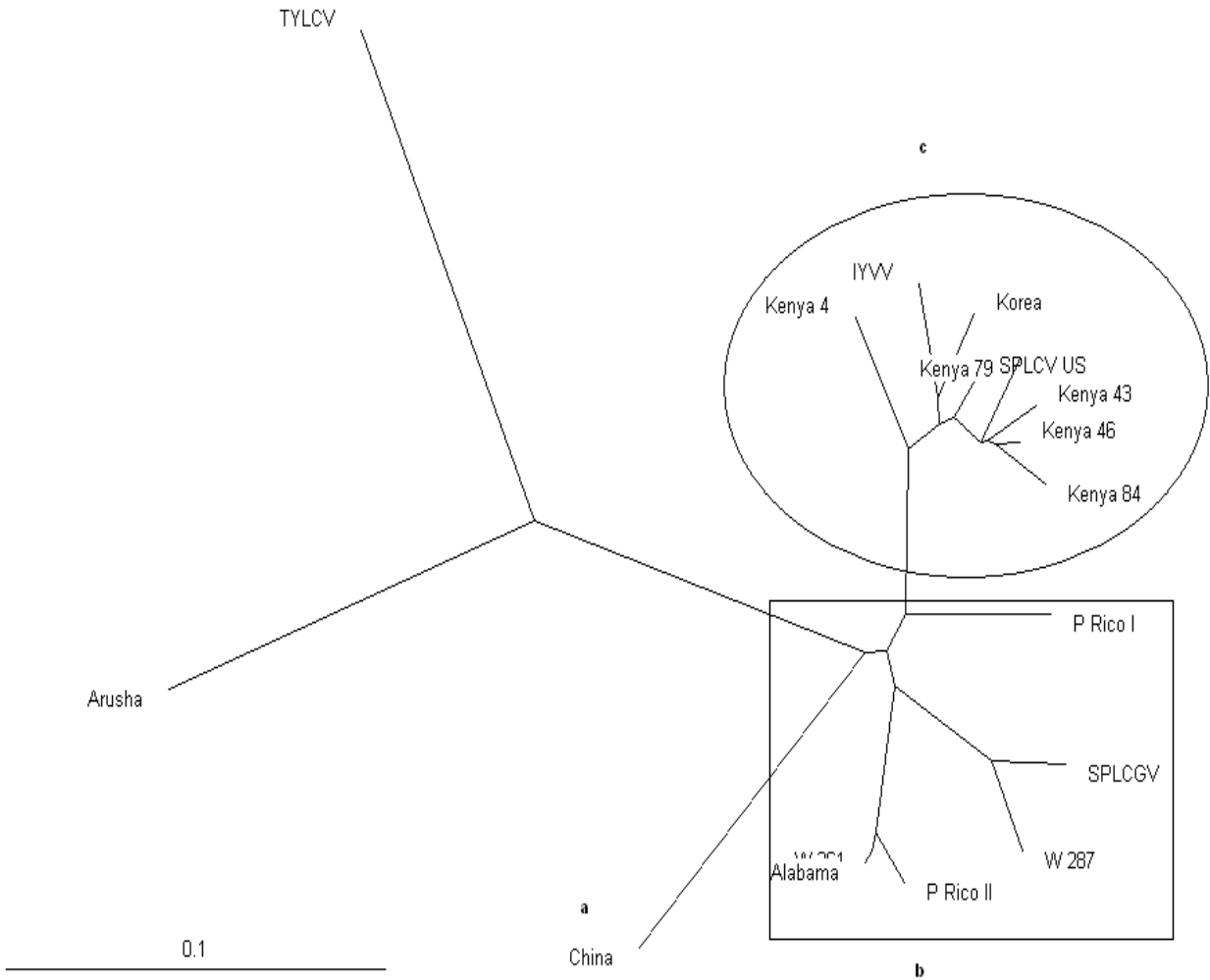
Figure 2. 4. Unrooted neighbor-joining tree obtained from the alignment of the partial ORF AC1 nucleotide sequences of begomovirus isolates infecting sweetpotato. AC1 nucleotide sequences from the Arusha isolate of *Tomato leaf curl virus* (GenBank accession no. EF194760) and of *Tomato yellow leaf curl virus* (isolate from Egypt, GenBank accession no. AY594174) were used as outgroup sequences. Trees were constructed with ClustalX v1.83 and drawn with Tree View v1.66.
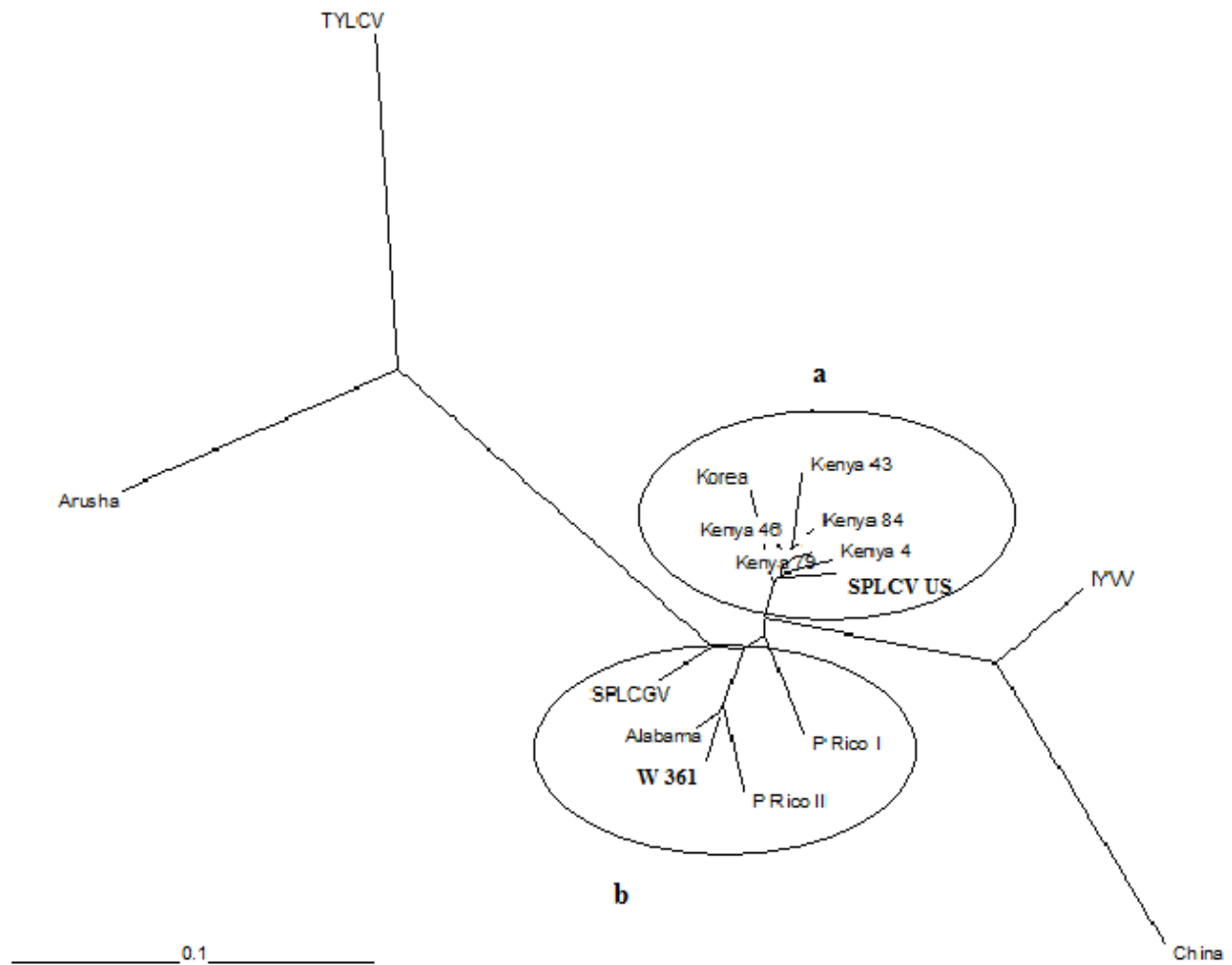
Figure 2. 5. Unrooted neighbor-joining tree obtained from the alignment of the partial ORF AC1 amino acid sequences of begomovirus isolates infecting sweetpotato. AC1 amino acid sequences from the Arusha isolate of *Tomato leaf curl virus* (GenBank accession no. EF194760) and of *Tomato yellow leaf curl virus* (isolate from Egypt, GenBank accession no. AY594174) were used as outgroup sequences. Trees were constructed with ClustalX v1.83 and drawn with Tree View v1.66.

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and SPV-2 are not detected in Kenya and Uganda but are found in South Africa and Egypt. However, it would be important to be careful when exchanging germplasm from these and other parts of the world to ensure that the viruses are not introduced in East Africa.

As part of this study, the occurrence of a begomovirus infecting sweetpotato in Kenya was reported for the first time (Miano et al., 2006). Previous studies in the region and in Africa did not specifically survey for geminiviruses in sweetpotato. However, there have been indications that more viruses may be present in the region than previously reported especially from the symptoms that could not be associated with any of the viruses already detected (Mukasa *et al.*, 2003; Ateka et al., 2004b; Aritua et al., 2006). When cuttings from these apparently virus-free plants were propagated in a greenhouse, some had upward curling of leaves (Aritua et al., 2006), similar to those induced by SPLCV, although no geminivirus was detected with the primers used. Together with reports from other parts of the world including US, China, Spain, Japan and Peru (Lotrakul et al., 1998; Banks et al. 1999; Fuentes and Salazar, 2003; Briddon et al. 2006; Luan et al. 2006), it is now evident that begomoviruses are associated with sweetpotato in almost all geographic regions where sweetpotatoes are grown.

Three out of 10 (30%) plants collected with characteristic leaf curl symptoms from the germplasm collection plots tested positive for the begomovirus using degenerate primers. Of the 38 sweetpotato clones that were sent to the Plant Germplasm Quarantine Office of USDA-ARS for virus indexing and therapy, 5 (~13%) tested positive for the begomovirus. The 38 genotypes had been selected from the Kenyan germplasm collection plots for their resistance or susceptibility to sweetpotato virus disease (SPVD), and were therefore not necessarily being collected for detection of begomoviruses. The percent of plants infected with a begomovirus is

therefore quite high and efforts should be made to evaluate the economic importance of the virus in the region.

When the eight samples that were positive to begomoviruses using degenerate primers were tested using primers specific to sweetpotato begomoviruses, one isolate was not amplified, which suggests that a different specie or strain of the virus could be present. Also, in one of the field visits, sweetpotato plants with yellow vein mottle symptoms (Figure 1c) similar to those observed in *I. aquatica* infected with SPLCV (Lotrakul et al., 1998) were observed, though DNA was not extracted from these plants. Such symptoms are not common in sweetpotato, and there is need to determine if these symptoms are actually caused by begomoviruses. This may indicate that there might be a number of distinct isolates present in the region, or that different sweetpotato genotypes react differently to begomovirus infection.

The partial ORF AC1 sequences were used to compare the relationship between the begomovirus isolates used in this study and others from different parts of the world. The AC1 region has a high percentage of similarity among geminiviruses, and has previously been used to analyze relationships between different geminiviruses (Lotrakul and Valverde, 1999; Lotrakul et al., 2002; Lotrakul et al., 2003). Pairwise alignments revealed that the Kenyan isolates were highly similar and closely related to SPLCV-US. The phylogenetic analysis clustered the viruses into different groups, of which the two main groups consisted of a) four isolates from USA including SPLCGV, and b) a group in which all isolates from Kenya belonged and which included SPLCV-US. The isolate from China did not cluster with the other isolates, while IYVV clustered away from the Kenyan group only when amino acid sequences were considered, mainly because the sequences in the GenBank were less by 20 nucleotides, may be due to a possible deletion. SPLCGV (previously called ILCV) has been classified as a different species

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apart from SPLCV-US (Lotrakul et al., 2003). These results indicated that all six of the isolates from Kenya were more closely related to each other and to SPLCV-US, than to SPLCGV. Lotrakul and Valverde (1999) proposed that SPLCV-US may have originated from the old world (though named US), and it seems the Kenyan isolates used in this study have a similar origin.

Although the sequences from isolates used for comparisons come from four continents (Africa, America, Asia and Europe), it is important to note that only one isolate from Europe and one isolate from China were used, and are therefore not a true representation of the diversity in these regions. Further, the Kenyan isolates were from a collection plot located in the Western region of Kenya. The germplasm consisted of genotypes from different parts of Kenya, and included some genotypes from neighbouring East African countries as well as others from other parts of the world. If it were known that the infections originated in the countries of origin of the accessions, it would provide a good representation of the regions from where the germplasm was collected. However, it is not known if the materials were brought in when infected or if they became infected at the site by whitefly transmission from one or few infected accessions. Also, there was a surprising absence of sequences from the GenBank of isolates from Japan, Taiwan, Israel and isolates recently reported from Peru. The prevalence, distribution and diversity of the viruses in these regions need to be studied, and a comprehensive phylogenetic relationship developed.

Virus diseases are the major disease constraints affecting sweet potato production in the East Africa region. The major disease is the Sweet potato virus disease (SPVD) caused by the dual infection of SPFMV and SPCSV. Efforts are being made to develop and/or select for resistance to this disease. Selecting symptomless vine cuttings for propagation from resistant genotypes have been an effective means of managing SPVD (Gibson et al. 1997). The presence

of a geminivirus infecting sweetpotato in the region complicates the efforts to develop

sweetpotato resistant to viruses in that the begomoviruses may cause significant yield reductions

without inducing symptoms. Clark and Hoy (2006) reported that yields of 'Beauregard' infected

with SPLCV alone were reduced by 25-30% even when no symptoms were observed on the

plants. SPLCV may also interact with other viruses synergistically. Experiments with single and

mixed infections with russet crack strain of SPFMV and SPLCV indicated that SPLCV DNA

titer increased in mixed infections, while that of SPFMV remained the same (Kokkinos and

Clark, 2006), but the plants remained asymptomatic. However, preliminary results on *I. setosa*

indicate that mixed infections of SPFMV and SPLCV cause more severe symptoms than either

alone (Kokkinos and Clark, 2006). Higher virus titers mean that whiteflies, the vectors of

SPLCV, will more readily acquire and spread the virus to uninfected plants. Potyviruses

infecting sweetpotato are widespread in East Africa (Ateka et al., 2004b; Aritua et al. 2006). The

fact that SPLCV may not show symptoms, even when the virus titers are increased by the

presence of other viruses poses a challenge to the dissemination of planting materials in the

region, now that farmers use symptoms to select for virus free planting materials. There is

therefore a need to incorporate specific begomovirus test procedures into sweetpotato virus

indexing protocols.

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CHAPTER 3: IRREGULAR DISTRIBUTION OF VIRUSES IN FIELD-GROWN SWEETPOTATO AND ITS SIGNIFICANCE IN PROPAGATION BY SMALL-HOLDER FARMERS IN KENYA

3.1 Introduction

Viruses cause the most important diseases of sweetpotato crops in the field in East Africa (Ngeve, 1990; Geddes, 1990; Gibson *et al.*, 1997). Major sweetpotato viruses reported in the region include the aphid transmitted *Sweet potato feathery mottle virus* (SPFMV, genus *Potyvirus,* family *Potyviridae*), the whitefly transmitted *Sweet potato chlorotic stunt virus* (SPCSV, genus *Crinivirus,* family *Closteroviridae*), the possibly whitefly transmitted *Sweet potato mild mottle virus* (SPMMV, genus *Ipomovirus*, family *Potyviridae*), Sweet potato caulimo - like virus (SPCaLV), and Sweet potato chlorotic fleck virus (SPCFV), (Mukiibi, 1977; Geddes, 1990; Gibson et al, 1998; Mukasa et al., 2003; Ateka et al., 2004; Aritua et al., 2006). The most severe symptoms are caused by co-infection with SPCSV and SPFMV, which result in the synergistic sweet potato virus disease (SPVD) (Mukiibi, 1977; Geddes, 1990; Gibson et al., 1998; Karyeija et al. 2000). The disease is characterized by chlorosis, small deformed leaves and severe stunting (Mukiibi, 1977), leading to production losses of over 90% (Gibson et al., 1998; Karyeija et al., 1998). SPMMV has also been reported to synergize with SPCSV resulting in sweetpotato severe mosaic disease (SPSMD), with associated 80% yield loss (Mukasa et al., 2006).

Despite the continuous presence of the viruses, their vectors and favorable environment for disease development, the farmers in the region have continued to successfully grow sweetpotatoes, though the yields are normally low. The fact that the crop continues to be grown has been attributed to a number of factors. Farmers in the region grow different sweetpotato varieties and/or landraces in the same plots. The genotypes have large differences in

susceptibility to virus diseases, and good sources of resistance are present in local germplasm (Aritua et al. 1998a). In an attempt to control the virus diseases, the farmers select for symptomless apical cuttings of approximately 30 cm long for propagation (Aritua et al., 1998a; Karyeija et al., 1998; Mukasa et al., 2003). Gibson et al. (1997), using nitro-cellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA) and grafting to indicator plants, observed an apparent absence of viruses in most symptomless sweetpotato cuttings obtained from farmers' fields in Uganda and suggested that the use of such cuttings has helped the farmers minimize yield losses from virus infections. However, Gibson et al. (1997) did not test the different vine section to confirm if absence of viruses was limited to a particular section.

Various studies have shown that the distribution of SPFMV along the length of sweetpotato vines is irregular particularly in terms of titers or presence or absence in different parts (Nielson and Pope, 1960; Green et al., 1988; Abad and Moyer, 1992; Gibb and Padovan, 1993; Aritua et al., 1998b). Some local East African sweetpotato genotypes showing field resistance to SPVD have also been reported to recover from virus infections, have the ability to localize the distribution of SPVD-causing viruses, or some branches within a plant may show no symptoms (Aritua et al., 1998a; Gibson et al., 1998; Mwanga et al., 2002). These observations have led to the suggestions that uninfected cuttings may be obtained from previously infected plants (Mwanga et al., 2002), and that the gains made by farmers in managing SPVD in East Africa was due to the use of such cuttings (Gibson et al., 1997). This study was done to document the prevalence of three common viruses (SPFMV, SPMMV, and SPCSV) in field-grown genotypes with varying degrees of observable virus symptoms, and to determine if there are significant differences in virus presence between different sections. The ultimate aim was to

establish if this variability can be a basis to advise farmers on which section to use for propagation.

3.2 Materials and Methods

3.2.1 Plant Material

Twenty-three accessions (Table 3.1) were selected from a Kenyan germplasm collection of sweetpotatoes located at the University of Nairobi, Kabete Field Station Farm in Central Kenya. Selection was based on the reaction of the plants to natural virus infections in the field plots, and genotypes with different types of symptoms were selected. One vine from each of the 23 accessions was taken and divided into three sections; the growing shoot-tip (about 15 cm long), the mid-section (15 - 30 cm) and greater than 30 cm from the apex. The 3 vine sections of each accession were planted in the screenhouse and allowed to grow for about 2 weeks. The sweetpotato vines were then grafted to one-month old Brazilian morning glory (*I. setosa*), a nearly universal indicator plant for sweetpotato viruses (Schaefers and Terry, 1976). Two grafts, an apical and a side graft, were done on each *I. setosa*. Graft establishment was assessed and only those on which a scion survived for at least 3 weeks were included in the study. Symptom development was observed for 2 months. Symptomatic leaves were used to assay for three viruses (SPFMV, SPCSV and SPMMV) that have been reported to be the most common in the region (Mukasa et al., 2003; Ateka et al., 2004; Aritua et al., 2006).

Nine accessions were selected out of the 23 for replicated tests based on the presence or absence of either or all of the three viruses in the three sections. Ten cuttings from different plants in the same plot were taken twice over a period of two months (five cuttings per time) from each of the nine accessions in the field and divided into three pieces as described above.

Table 3. 1. Distribution of viruses in three sections of 23 sweetpotato genotypes from field plots in Kenya (preliminary test).

Accession[x]	Symptoms observed in the field	Plant section[y]		
		15 cm	15-30 cm	>30 cm
K/KA/2002/10	Ps	SPFMV	SPFMV	-
K/KA/2002/12[z]	Cs	-	SPFMV	SPFMV
K/KA/2002/64[z]	Vc, Cs, Ps, Mt, Gc, Ld	SPFMV, SPCSV	SPFMV, SPCSV	SPFMV, SPCSV
K/KA/2002/70	Vc, Cs	SPFMV, SPCSV	SPFMV	SPFMV
K/KA/2002/80	Ps	-	SPFMV	SPFMV
K/KA/2002/82[z]	-	-	-	-
K/KA/2002/88	Ps, Vc	SPFMV	-	SPFMV
K/KA/2002/90	-	SPFMV	SPFMV, SPMMV	SPFMV, SPMMV
K/KA/2002/91[z]	-	SPFMV	-	SPFMV, SPMMV
K/KA/2002/95	Ps	SPFMV	SPFMV	SPFMV
K/KA/2002/96[z]	-	-	-	-
K/KA/2002/99[z]	-	-	SPFMV	SPFMV
K/KA/2002/100	-	SPFMV	SPFMV	SPFMV
K/KA/2002/101	Vc, Ps	SPFMV, SPCSV	SPFMV, SPCSV	Plant died
K/KA/2002/110	Ps	SPFMV	SPFMV	SPFMV
K/KA/2002/122	-	SPFMV	SPFMV	-
K/KA/2002/149	-	SPFMV, SPMMV	SPFMV, SPMMV	SPFMV, SPMMV
K/KA/2002/150	Ps	SPFMV	SPFMV	SPFMV
K/KA/2002/154[z]	-	SPFMV	SPFMV	-
K/KA/2002/174	Ps	SPFMV	SPFMV	SPFMV
K/KA/2002/180[z]	Ps	-	-	SPFMV
K/KA/2002/195	Ps, Cs, Vc	SPMMV	SPFMV, SPCSV, SPMMV	SPFMV, SPCSV, SPMMV
K/KA/2002/207[z]	-	-	-	-

[x]Different genotypes as coded during germplasm collection.
[y]Cuttings in each genotype were divided into 3 sections (15 cm, 15-30 cm, and greater than 30 cm from the apex). The cuttings were grafted onto *I. setosa* and leaves from *I. setosa* tested for *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato mild mottle virus* (SPMMV) 2 months later.
[z] The genotypes were selected for further, replicated tests
Cs=chlorotic spots, Gc=general chlorosis, Ld=leaf distortion, Ps=purple spots, Vc=Vein clearing, - = no symptoms observed.

The cuttings were grown in the screenhouse for about 2 weeks before grafting on to one-month-old *I. setosa*, as described. Assays were again done on *I. setosa*.

3.2.2 Virus Detection

Presence of SPFMV and SPMMV were assayed using nitro-cellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA) as described by Gutierrez et al. (2003) using kits and antisera provided by the International Potato Center (CIP), Lima, Peru. The kit contains polyclonal antibodies for different sweetpotato viruses and NCM strips spotted with sap from virus-positive and noninfected control plants. The kit also contains alkaline phophatase-labelled goat anti-rabbit (GAR-AP) IgG and the substrate (NBT/BCIP) nitro blue tetrazolium Chloride / 5-bromo-4-chloro-indolyl phosphate, toluidine salt.

Presence of SPCSV was assayed by NCM-ELISA using a monoclonal antibody (Mix 1) provided by E. M. Ateka of KARI Biotechnology Centre (the antibody mix originated from J. H. Vetten of Federal Biological Research Centre for Agriculture and Forestry, Braunscheig, Germany) and alkaline phophatase-labelled goat anti-mouse (GAM) IgG supplied by Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD, US). NCM strips spotted with SPCSV-positive and a noninfected control from the CIP kit were included as controls.

Two leaf disks (about 1cm in diameter) were taken from each of two leaves (preferably with symptoms) of each *I. setosa*, placed in a plastic bag, and homogenized in 2ml of Tris-buffered saline (TBS) pH 7.5 containing 0.2% of sodium sulfite (Na_2SO_3). The tissue was completely ground using a test tube, and the plastic bag was let to stand for 20-30 min at room temperature. The clarified sap extract (15µl) was blotted onto a TBS buffer-saturated nitro-cellulose membrane and air-dried for 20-30 min. All the incubations and washings were done at room temperature in a shaker with gentle agitation (50 rpm for incubations and 100 for

washings) The membranes were blocked with TBS containing 2% powdered milk and 2%

Triton X-100 and incubated for one hour. The blocking solution was discarded and the

membranes rinsed with TBS. The first antibody (polyclonal specific to SPFMV and SPMMV,

monoclonal specific to SPCSV), diluted in TBS containing 2% powdered milk, was added to the

membranes and incubated overnight. The membranes were then washed in T-TBS (TBS

containing 0.05% Tween 20) four times for 3 min each. The second antibody (goat anti-rabbit for

SPFMV and SPMMV, goat anti-mouse for SPCSV) conjugated with alkaline phosphatase and

diluted in TBS containing 2% powdered milk, was added to the membranes and incubated for

one hour. The membranes were washed as before and the presence of bound antibody was

visualized by the addition of a substrate: BCIP/NBT color development solution. The color

reaction was stopped after 10-20 min using deionized water. Positive reactions were determined

by visual assessment, and purple color reaction was recorded as positive.

3.2.3 Data Analysis

Data were analyzed in SAS (2001) using analysis of variance procedure and least

significant difference tests used for mean comparisons.

3.3 Results

The 23 initial genotypes were selected based on wide ranging virus symptoms on plants

growing in the field, which included chlorotic spots on leaves, general leaf chlorosis, leaf

distortion, purple spots, vein clearing, and some with no observable symptoms (Table 3.1).

NCM-ELISA results showed 10 out of 23 had one or all of the viruses assayed (SPFMV, SPCSV

and SPMMV) in all three sections. No viruses were detected in any section of the genotypes

K/KA/2002/82, K/KA/2002/96 and K/KA/2002/207. Virus was detected in the other genotypes

except on the oldest vine section (over 30 cm from apex) in K/KA/2002/12, K/KA/2002/80,

K/KA/2002/99 and K/KA/2002/180; or in the youngest vine sections (15 cm from apex) of

K/KA/2002/10, K/KA/2002/122 and K/KA/2002/154; or in the middle section of two genotypes,

K/KA/2002/88 and K/KA/2002/. Based on the distribution pattern observed using the 23

genotypes, 9 genotypes were selected for further tests (Table 3.1).

From the 9 genotypes, 10 cuttings were taken from different plants in the same plot (not

necessarily including the plant on which the previous cutting was taken) during a two month

period, and assays carried out for the 3 viruses. Significant differences ($P<0.0001$) were observed

among genotypes but no differences existed within vine sections for all the 3 viruses (Table 3.2).

Out of the 9 accessions studied, 8 were infected with SPFMV in at least 1 out of the 10

cuttings (Table 3.3). K/KA/2002/64 was highly susceptible to SPVD and all cuttings had severe

symptoms both in the field and in the screenhouse and SPFMV was detected in all three sections

of each of the ten vines. K/KA/2002/207 had no symptoms in the field or in the screenhouse, and

no virus was detected in any of the three sections in any of the 10 vines. All the other genotypes

had at least one out of the 10 vines showing variable distribution of SPFMV. Accession

K/KA/2002/82 had developed severe symptoms in the initial 3 weeks after planting the cuttings

in the screenhouse, but the symptoms disappeared thereafter. However, only SPFMV was

detected in this genotype. Accession K/KA/2002/91 showed purple chlorotic spots in the field

but no symptoms in the screenhouse. Almost all the 10 vines had SPFMV detected but not

SPCSV. In a few cases, no virus was detected in the mid-section (15-30cm) of the vine, but at

least one of the viruses was detected in the top and the lower region of the vine.

SPCSV was only detected in 2 genotypes, K/KA/2002/64 and K/KA/2002/154 (Table

3.4). Accession K/KA/2002/64 had all the vines infected with SPCSV. In K/KA/2002/154,

SPSCV was detected in 2 vines, one of which had the virus only at the basal part. Again there

Table 3. 2. Analysis of variance table of vine sections and genotypes infected with *Sweet potato feathery mottle virus virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato mild mottle virus* (SPMMV).

Virus	Source of variation	DF	MS	F Value	Pr>F
SPFMV	Section	2	0.037	0.04	0.9564
	Genotype	8	36.204	43.69	<0.0001
SPCSV	Section	2	0.0000	0.000	1.000
	Genotype	8	154.667	19.33	<0.0001
SPMMV	Section	2	0.778	2.15	0.1485
	Genotype	8	8.417	23.31	<0.0001

Table 3. 3. Number of sweetpotato vine sections from different genotypes infected with *Sweet potato feathery mottle virus* (SPFMV).

Accession[x]	Vine section[y]			Means
	15 cm	15-30 cm	>30cm	
K/KA/2002/12	3	4	2	3.0 cd
K/KA/2002/64	10	10	10	10.0 a
K/KA/2002/82	5	6	6	5.7 b
K/KA/2002/91	10	9	8	9.0 a
K/KA/2002/96	3	2	5	3.3 c
K/KA/2002/99	3	1	1	1.7 de
K/KA/2002/154	2	2	3	2.3 cde
K/KA/2002/180	1	2	1	1.3 ef
K/KA/2002/207	0	0	0	0.0 f
Means	4.1 a	4.0 a	4.0 a	

[x]Different genotypes as coded during germplasm collection.
[y]A total of 10 cuttings in each genotype were divided into 3 sections (15 cm, 15-30 cm, and greater than 30 cm from the apex) and tested for SPFMV.
Column means (or row means) followed by same letter(s) are not significantly different according to least significant difference procedure (SAS Institute) at P=0.005.

Table 3. 4. Number of sweetpotato vine sections from different genotypes infected with *Sweet potato chlorotic stunt virus* (SPCSV).

Accession[x]	Vine section[y]			Means
	15 cm	15-30 cm	>30cm	
K/KA/2002/12	0	0	0	0.0 c
K/KA/2002/64	8	8	7	7.7 a
K/KA/2002/82	0	0	0	0.0 c
K/KA/2002/91	0	0	0	0.0 c
K/KA/2002/96	0	0	0	0.0 c
K/KA/2002/99	0	0	0	0.0 c
K/KA/2002/154	1	1	2	1.3 b
K/KA/2002/180	0	0	0	0.0 c
K/KA/2002/207	0	0	0	0.0 c
Means	1.0a	1.0a	1.0a	

[x]Different genotypes as coded during germplasm collection.
[y]A total of 10 cuttings in each genotype were divided into 3 sections (15 cm, 15-30 cm, and greater than 30 cm from the apex) and tested for SPCSV.
Column means (or row means) followed by same letter(s) are not significantly different according to least significant difference procedure (SAS Institute) at P=0.005.

were no significant differences within sections infected with SPCSV.

SPMMV was found in 4 out of the 9 genotypes, with significant differences (P<0.0001) only being observed among genotypes but not within the sections (Table 3.5). The number of vines in each section infected with at least one virus is recorded in Table 3.6. Again, significant differences were only found among genotypes and not within sections.

3.4 Discussion

The lower-than-expected incidence of viral diseases in the East African sweetpotato farms has been associated with use of resistant landraces, use of symptomless 30 cm-long cuttings for planting and the uneven distribution of viruses in sweetpotato vines. We investigated if there are differences in levels of detection of three of the most common viruses detected in the East African fields. Though farmers normally use 30-cm long cuttings, we used a 15 cm-long cutting to determine if a smaller cutting (but long enough for routine field propagation) would

Table 3. 5. Number of sweetpotato vine sections from different genotypes infected with *Sweet potato mild mottle virus* (SPMMV).

Accession[x]	Vine section[y]			Means
	15 cm	15-30 cm	>30cm	
K/KA/2002/12	0	0	1	0.3 bc
K/KA/2002/64	2	2	1	1.7 b
K/KA/2002/82	0	0	0	0.0 c
K/KA/2002/91	5	3	4	4.0 a
K/KA/2002/96	0	0	0	0.0 c
K/KA/2002/99	2	0	1	1.0 bc
K/KA/2002/154	5	4	3	4.0 a
K/KA/2002/180	0	0	0	0.0 c
K/KA/2002/207	0	0	0	0.0 c
Means	1.6 a	1.0 a	1.1 a	

[x]Different genotypes as coded during germplasm collection.
[y]A total of 10 cuttings in each genotype were divided into 3 sections (15 cm, 15-30 cm, and greater than 30 cm from the apex) and tested for SPMMV.
Column means (or row means) followed by same letter(s) are not significantly different according to least significant difference procedure (SAS Institute) at P=0.005.

Table 3. 6. Number of sweetpotato vine sections from different genotypes infected with at least one of the three viruses (SPFMV, SPCSV and/or SPMMV).

Accession[x]	Vine section[y]			Means
	15 cm	30 cm	>30cm	
K/KA/2002/12	3	4	2	3.0 de
K/KA/2002/64	10	10	10	10.0 a
K/KA/2002/82	5	6	7	6.0 b
K/KA/2002/91	10	9	9	9.3 a
K/KA/2002/96	3	2	5	3.3 cd
K/KA/2002/99	4	1	1	2.0 de
K/KA/2002/154	5	5	5	5.0 bc
K/KA/2002/180	1	2	1	1.3 ef
K/KA/2002/207	0	0	0	0.0 f
Means	4.6 a	4.3 a	4.4 a	

[x]Different genotypes as coded during germplasm collection.
[y]A total of 10 cuttings in each genotype were divided into 3 sections (15 cm, 15-30 cm, and greater than 30 cm from the apex) and tested for *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato mild mottle virus* (SPMMV).
Column means (or row means) followed by same letter(s) are not significantly different according to least significant difference procedure (SAS Institute) at P=0.005.

increase the number of healthy cuttings obtained from the fields. No significant differences were observed when the different sections within the vines were compared, indicating that the probability of obtaining a cutting apparently free from virus infection from an already infected vine is very low. More cuttings were infected with SPFMV than with either SPCSV or SPMMV. SPFMV is more common than both SPCSV and SPMMV in Kenya (Ateka et al., 2004) and is detected even in plants showing mild or no symptoms (Green et al., 1988). Different accessions used in this study had different numbers of vines infected with at least one of the viruses, possibly indicating differences in susceptibility and/or resistance to the viruses.

Detection of SPFMV and SPMMV directly from sweetpotato infected with either virus alone is not reliable, especially using serology (Esbenshade and Moyer, 1982; Abad and Moyer, 1992; Mukasa et al., 2006). The rate of detection is increased when infected sweetpotato plants are grafted on to *I. setosa*, a host in which most viruses, including SPFMV, SPCSV, and SPMMV induce symptoms and attain a sufficient titer to be detected serologically (Kokkinos and Clark, 2006; Schaefers and Terry, 1976). SPFMV has uneven distribution in infected sweetpotato plants both in terms of titer levels and in terms of its presence and absence (Nielsen and Pope, 1960; Frison and Ng, 1981; Green et al., 1988; Abad and Moyer, 1992; Gibb and Padova, 1993; Aritual et al., 1998b; Gibson et al., 1998; Mwanga et al., 2002; Dje and Diallo, 2005). Aritua et al. (1998b) observed that plants initially infected with SPFMV alone recover with time, that the uneven distribution of SPFMV in infected plants can lead to an absence of SPFMV in an increasing proportion of new shoots, and that the proportion of new shoots in which SPFMV was not detected increased more rapidly in the more resistant variety. SPVD-resistant sweetpotato plants graft-inoculated with SPFMV and subsequently planted in a field in Uganda all became virus free over time (Aritua et al., 1998b). However, even in such cases

where recovery from symptom development was observed, grafting of the plants to *I. setosa* often resulted in infections, showing that the virus may at times be present in the plant, though in low titers. The virus titers of both SPFMV and SPMMV increase drastically in the presence of SPCSV, and detection is much more reliable from mixed infections (Kokkinos and Clark, 2006; Mukasa et al., 2006). Some sweetpotato genotypes have also been reported to localize the distribution of SPVD, meaning that the disease may not be fully systemic in resistant genotypes, or some branches within a plant may show no symptoms (Gibson et al., 1998). Gibson et al. (1997) and Aritua et al. (1998b) compared this phenomena to the recovery observed in cassava infected with *Cassava mosaic virus* (CMV). In this study, we investigated whether this variability may be useful to farmers by looking for a section of the vine that is continually free from virus infection. We expected the cuttings from older vine parts to have more viruses present than in the terminal portion. We report that though the distribution was variable in some vines, the three viruses were equally present in the three sections. This is supported by the fact that it is generally not easy to clean sweetpotatoes from virus infections even when using meristem tip cultures. Aritua et al. (1998b) also noted that the likelihood of SPFMV being absent was similar for all portions of sweet potato stem. Dje and Diallo (2005) observed that SPFMV was present in all the leaves sampled from susceptible plants, even in the youngest growing leaves, indicating that the virus might only be absent in the emerging leaf. Green et al. (1988) observed that no obvious spatial pattern of virus distribution was evident in leaves sampled from an individual plant. It is therefore improbable that a healthy cutting long enough for propagation can be obtained from an already infected vine.

An interesting observation was noted where the midsection of some vines had no virus(es) while the basal- and tip-sections were infected. Dje and Diallo (2005) explain that

SPFMV colonizes a zone before moving to a higher zone. However, they did not indicate if a section within the plant could remain uninfected for sometime before the virus spreads to the other regions. Dje and Diallo (2005) also noted that the leaves located between the grafting point and the apex had the highest virus concentrations both in susceptible and tolerant clones. Green et al. (1988) reported that a few SPFMV-infected leaves were interspersed with non-infected leaves, but also observed that such a situation may not be the same for all virus-infected cultivars. An explanation for such an occurrence is needed.

We conclude that it may not be easy for the farmers to predict the section of the vine that is not infected with viruses. However, an important aspect that should be included in further studies in this area (that was not included in this study) is the comparison of the field-derived symptomless cuttings with pathogen-tested cuttings of the same genotypes. Previous research implied that unique environmental conditions may aid recovery, though no data exists to support the hypothesis. Such environmental and/or climatic conditions favoring recovery of previously virus infected sweetpotato genotypes should be investigated. If identified, particular localities and periods of the year when such conditions occur can be utilized to generate cuttings for propagation.

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CHAPTER 4: RESPONSE OF SWEETPOTATO TO INFECTION BY CAUSAL AGENTS OF SWEET POTATO VIRUS DISEASE

4.1 Introduction

The most important diseases affecting sweetpotato production in the world are caused by viruses (Carey et al., 1999; Fuglie, 2007). Currently, more than 20 viruses infect sweetpotato (Valverde et al., 2007). With the exception of a few viruses like *Sweet potato leaf curl virus* (SPLCV, genus *Begomovirus*) and *Sweet potato chlorotic stunt virus* (SPCSV, genus *Crinivirus*), most single infections cause mild or no symptoms, and consequently, no significant yield reduction is observed (Clark and Hoy, 2006). In nature, co-infection of two or more viruses in sweetpotato is common (Gutierrez et al., 2003; Mukasa et al., 2003; Ateka et al., 2004; Mukasa 2006; Untiveros et al., 2007), resulting in enhanced symptom development and significant yield reductions. Novel synergistic interactions have been reported involving SPCSV with viruses belonging to different genera, including *Sweet potato feathery mottle virus* (SPFMV), Sweet potato virus G (SPVG), Sweet potato virus 2 (SPV 2), *Sweet potato mild speckling virus* (SPMSV) (each in the genus *Potyvirus*), *Sweet potato mild mottle virus* (SPMMV, genus *Ipomovirus*), *Cucumber mosaic virus* (CMV, genus *Cucumovirus*), Sweetpotato chlorotic fleck virus (SPCFV, a putative *Carlavirus*), and C-6 (also a putative *Carlavirus*) (Cohen and Loebenstein, 1991; Di Feo et al., 2000; Mukasa et al., 2006; Kokkinos and Clark, 2006b; Untiveros et al., 2007).

Sweet potato virus disease (SPVD), a result of dual infection by SPFMV and SPCSV, is the main disease limiting sweetpotato production worldwide (Geddes, 1990; Gibson et al., 1997; Carey et al., 1999; Karyeija et al., 2000; Gutierrez et al., 2003), especially because of the broad geographic distribution of the two viruses. Since over 90% of the world sweetpotato production is concentrated in the developing countries where most farmers have limited resources (Fuglie,

2007), the most viable method of SPVD control has been the use of resistant cultivars. Selection and breeding for resistance to SPVD is therefore a priority in different sweetpotato breeding programs all over the world. Selection for SPVD resistance has mainly been based on the absence (or attenuation) of symptoms after field exposure in disease prone areas, by graft-inoculations and by use of serology (Hahn et al., 1981; Mihovilovich et al., 2000; Mwanga et al., 2002b). However, none of these methods is effective in giving relative virus concentrations in different sweetpotato genotypes, and therefore, cannot differentiate whether the plants are tolerant (i.e., lack of, or reduced symptom development) or resistant (i.e., reduction in or a lack of virus accumulation) to SPCSV and/or SPFMV. Also, no studies have been done to compare how resistant and susceptible sweetpotato genotypes react to infection by the two viruses. Kokkinos and Clark (2006a) developed the use of real-time quantitative PCR assays for detection and relative quantification of different potyviruses (including SPFMV), SPCSV and *Sweet potato leaf curl virus* (SPLCV, genus *Begomovirus*) in sweetpotato. The technique can be used to differentiate tolerant genotypes from resistant ones in that concentration levels of viruses between different genotypes are determined, therefore providing a better understanding of virus titer characteristics associated with resistance.

In a previous study (Miano et al., 2007), we identified a group of genotypes as resistant to SPVD using mild or delayed symptom development after natural and/or graft inoculation with SPFMV and the East African strain of SPCSV. However, it was not determined if the delayed or mild symptom development was related to resistance to symptom expression or resistance to SPFMV and/or SPCSV multiplication and/or spread in the plant. The work presented in this report was aimed at understanding this aspect and analyzing the mechanism of disease resistance

or tolerance of sweetpotato to SPVD by studying the distribution and multiplication of SPFMV and SPCSV in resistant and susceptible genotypes using real-time quantitative PCR.

4.2 Materials and Methods

4.2.1 Plant Materials

The four sweetpotato genotypes used in this study were 'Beauregard', 'Naspot I', 'Mar Ooko' and 'Namaswakhe'. 'Beauregard' is the main cultivar grown in the United States. This cultivar was developed by the Louisiana Agricultural Experiment Station to combine resistance to diseases and insects of local importance with good horticultural and culinary characteristics. Though high yielding and rich in β-carotene, the cultivar is highly susceptible to SPVD. The characteristics of the cultivar are described by Rolston et al. (1987). 'Naspot I', 'Mar Ooko' and 'Namaswakhe' were obtained from the Kenya Agricultural Research Institute (KARI, Nairobi, Kenya) germplasm collection, and were among a group of 38 selected genotypes that were sent to Plant Germplasm Quarantine office of USDA-ARS, Beltsville, MD for virus indexing and therapy (Miano et al., 2007). 'Naspot I' was originally released in Uganda and has been described as resistance to SPVD (Mwanga et al., 2003; Miano et al., 2007). When infected with the US strains of SPVD-causing viruses, the genotype only showed mild mottling, and was thus considered resistant for this study. 'Mar Ooko', a popular landrace with western Kenya farmers, has not been described. However, when inoculated with the US strains of SPVD-causing viruses, 'Mar Ooko' only showed transient chlorotic spots, and was thus considered resistant. 'Namaswakhe' is a popular variety grown mainly in western Kenya (Njuguna, 2005), and is being evaluated in the national yield performance trials by breeders. The genotype showed severe SPVD symptoms both in Kenya and in the greenhouse in USA. It was included as a susceptible genotype in this study.

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4.2.2 Virus Inoculation and Quantification

Sweetpotato cv. 'Beauregard' that was previously graft inoculated with SPFMV (russet crack strain of SPFMV, isolate 95-2) and SPCSV (isolate BWFT-3 from USA) were used to generate the scions to graft-inoculate virus tested, clonally propagated 'Beauregard', 'Naspot I', 'Mar Ooko' and 'Namaswakhe'. Apical cuttings from each genotype were grown under standard greenhouse conditions in 17-cm-diameter ITML Elite Azalea pots (ITLM Horticultural Products Inc., Brantford, ON, Canada) containing autoclaved soil mix consisting of 1 part river silt, 1 part sand, 1 part Red-earth® Plug and Seedling Mix Series (Sun Gro Horticulture Distribution Inc., Bellevue, WA) and 3.5g per pot of Osmocote® 14-14-14 (Scotts-Sierra Horticultural Products Company, Marysille, OH). The plants were graft-inoculated 10 days after planting. One wedge graft was made per plant by inserting a scion from the source plant into a slit on the side and near the base of the stock plant. Only those plants on which the scion survived for 3 weeks were used. Each treatment was replicated four times. A weekly insecticide spray program was applied to control aphids and whiteflies. Lateral branches were removed to allow only one vine to grow. Three 1-cm-diameter disks were cut from different parts of the same leaves every 5 days post inoculation (DPI) for a period of 25 days. Leaves were selected for sampling beginning with the first leaf above the graft and progressing up the plant as the plant grew. The leaf above the point of inoculation was considered leaf number 1 and leaves were numbered sequentially from base to growing tip. The plants were cut back twice (at 26 and 48 DPI), each time the plants were allowed to regrow from axillary buds and a composite sample taken three weeks after cut back. The samples were immediately frozen in liquid nitrogen and stored at -80 ℃ until extraction.

4.2.3 Total RNA Extraction

Frozen leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle and total RNA extracted from about 50 mg of the finely ground leaf tissue using Qiagen's

RNeasy Plant Mini Kit® (Qiagen Inc, Valencia, CA). To eliminate possible residual DNA contamination, the RNA samples were treated on-column with DNase I using RNase –Free DNase Set (Qiagen Inc™) as an optional step during the RNA extraction. The RNA was then eluted from the column using RNase-free water. Total RNA was then quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE) to establish the concentration and purity of the extracted RNA. RNA concentrations were then diluted to approximately 15 ng/µl.

4.2.4 Real-time Quantitative PCR

The relative quantification, at transcriptional level, of the target virus (SPCSV or SPFMV) was performed using the ABI PRISM® 7000 Sequence Detection System (AppliedBiosystems, Foster City, CA). For the amplification and relative quantification of the target, a set of primers (including a forward and a reverse primer) and a fluorogenic probe (MGB Taqman® probe) described by Kokkinos and Clark (2006a) were used.

Real-time quantitative PCR was performed in 25µl reaction mixtures with 2.5µl template, 450nM of each primer, 100nM of the Taqman® probe, 12.5µl of the 2x Master Mix, and 0.625µl of the 40x MultiScribe™ and RNase inhibitor mix (AppliedBiosystems™). The 2x and 40x mixes were the components of the Taqman® One Step PCR Master Mix Reagent kit (AppliedBiosystems™). The following real-time quantitative PCR thermal cycler conditions were used: 48ºC for 30 min (cDNA synthesis), 95ºC for 10 min (AmpliTaq Gold® activation), followed by 40 cycles of denaturation at 95ºC for 15 s and annealing/extension at 60ºC for 1 min. These thermal cycling parameters were optimized for use with the Taqman® One Step PCR Master Mix Reagent kit (AppliedBiosystems™). All reactions were performed in MicroAmp® optical 96-well reaction plates that were sealed with Optical® Caps and placed on a MicroAmp® 96-well tray/retainer set (AppliedBiosystems™). To minimize any errors due to pipetting

differences between samples, duplicates of each sample were performed on each plate, and the C_T (threshold cycle) values obtained were averaged during data analysis. A standard curve was generated to establish if the target RNA and the endogenous control were transcribed with the same efficiency. Virus quantifications were then calculated using the $\Delta\Delta$ct method (User Bulletin #2, AppliedBiosystems, Foster, CA).

To normalize for possible variations in extraction efficiency, the samples assayed for the target virus were also used in reactions for an endogenous RNA on the same plate using a pre-developed Taqman® primer/probe internal positive control. Eukaryotic 18S rRNA endogenous control in the form of 20x primer/probe mix (AppliedBiosystems™) was used as an endogenous control for the normalization process. The same protocol as described above was used for the endogenous control reactions, except for the substitution of the primers and probe designed for the target with 1.25µl of the 18S rRNA pre-developed primer/probe mix. Every plate also included a non-template water control (NTC), negative control (total RNA from virus-tested [healthy] plants) as well as positive control (total RNA from virus infected source plant). The same extract of the positive control was used in all plates in order to compare the results between different plates. The $\Delta\Delta$ct method, which eliminates the use of standard curves on every plate, was implemented for the normalization of samples.

4.2.5 Data Analysis

Data were analyzed in SAS (2003) using the General Linear Model and MIXED procedures and least significant difference tests used for mean comparisons.

4.3 Results

The kinetics of SPFMV and SPCSV accumulation in the four graft-inoculated sweetpotato genotypes was studied in a time course experiment. The pattern of accumulation and

distribution of SPCSV and SPFMV in different leaves along vines of the different genotypes are presented in Figures 4.2 to 4.5, and are described below.

Beauregard: Mottling, vein banding and clearing, and chlorotic spots symptoms were apparent in 'Beauregard' 8-12 DPI, and most evident in the young leaves (Figure 4.1a,b). The symptoms later developed into mosaics, fan leaf and leaf deformations, ultimately resulting in reduced plant growth compared to uninfected controls. Virus titers of SPCSV and SPFMV in leaves sampled at different positions in a vine are shown in Figure 4.2. SPCSV was detected at 5 DPI in some leaves, though the titer levels were extremely low. The virus was easily detected 10 DPI, and the titers continued to accumulate up to 25 DPI, the last day of sampling before the plants were cut back. The virus was not detected in the leaf immediately above the point of graft inoculation, and the youngest leaf had very low titer levels. Otherwise, the virus was evenly distributed in leaves along the vine, with high titers being recorded in the leaves located in the middle of the vine.

SPFMV was detected 10 DPI, and titers continued to accumulate 25 DPI. The virus was not detected in the leaf immediately above the point of inoculation. However, unlike SPSCV titers which were high throughout the plant, SPFMV titers were greater in the upper portion of the plant, particularly the youngest leaves. The region where SPFMV titers were highest corresponded to the region with severe symptoms, indicating the symptoms observed were closely associated with SPFMV.

Naspot I: Only mild mottling symptoms and chlorotic spots in the older leaves were observed on 'Naspot I', as early as 10 DPI in some plants (Figure 4.1c). The symptoms observed in the old leaves later developed into mosaics, but no observable symptoms were recorded in the young leaves. Relative virus titers of SPCSV and SPFMV in different leaves along the vines of 'Naspot

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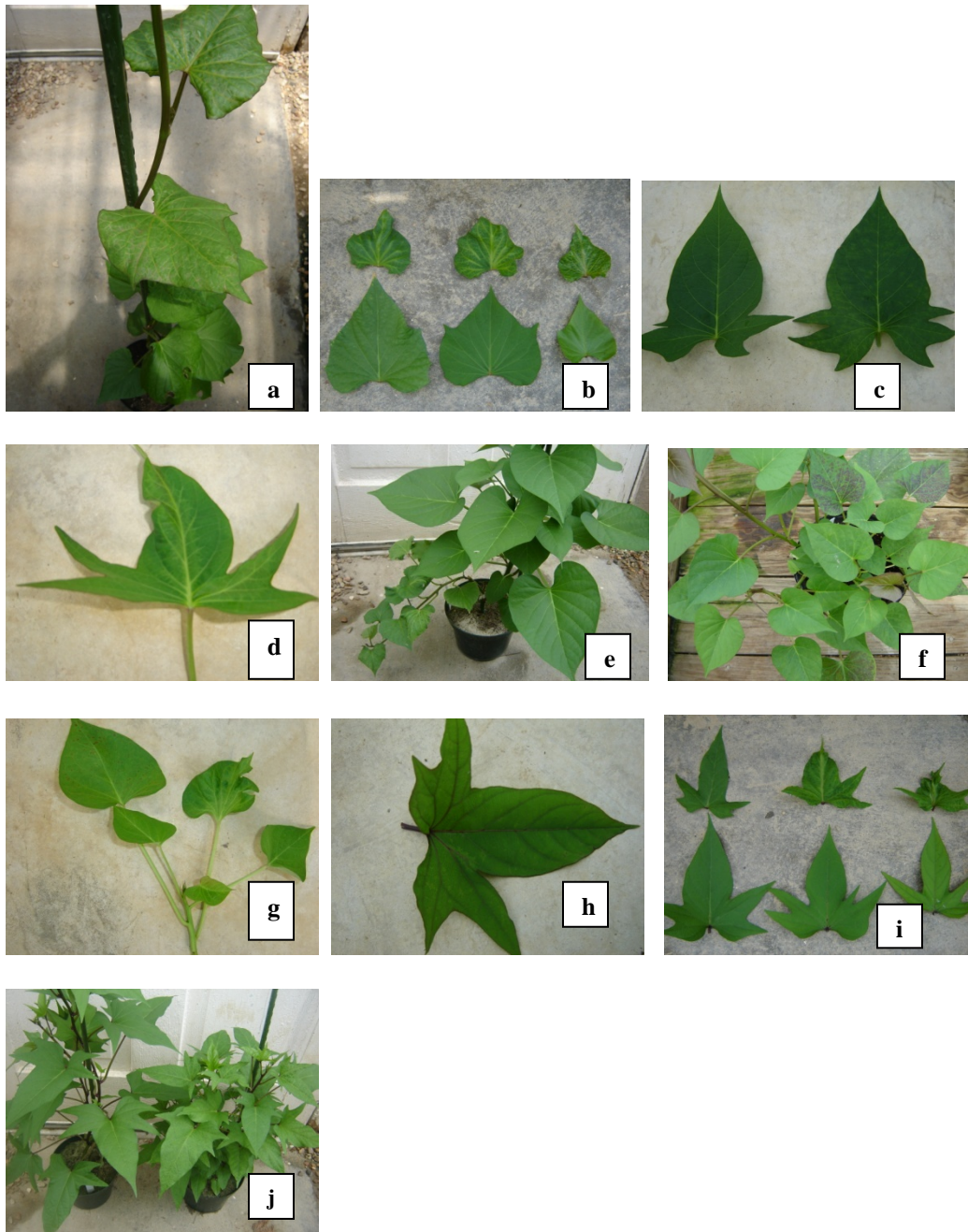
Figure 4. 1. Symptoms observed in different sweetpotato genotypes graft inoculated with *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV). (a) Sweet potato virus disease (SPVD) symptoms in 'Beauregard' 15 DPI. Symptoms were more severe in younger leaves. (b) SPVD symptoms in 'Beauregard' leaves 46 DPI (top row) compared to leaves from a healthy plant (bottom). (c) Chlorotic spots in the older leaf (right) of 'Naspot I' 25 DPI compared to a healthy leaf (left). (d) Leaf deformation in 'Naspot I' 70 DPI. (e) 'Mar Ooko' plant graft inoculated with a SPVD-infected scion. No symptoms were observed 20 DPI. (f) Purpling of older leaves and (g) leaf deformation observed in 'Mar Ooko' 70 DPI. (h) Mild vein banding symptoms in 'Namaswakhe' 15 DPI. (i) Severe SPVD symptoms (top) observed in 'Namaswakhe' 46 DPI compared to leaves from a healthy plant (bottom). (j)Vein clearing and banding and reduced plant growth in 'Namaswakhe' after plants were cut back (46 DPI).

Figure 4. 2. The pattern of accumulation and distribution of *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV) in sweetpotato cultivar 'Beauregard'. Numbers in the horizontal axis represent the relative position of the leaf sampled with respect to the point of inoculation (the leaf above the point of inoculation was considered leaf number 1 and leaves were numbered sequentially from base to growing tip). The virus titers (represented in the vertical axis) are relative quantities estimated using real time quantitative PCR and calculated using ΔΔct method (User Bulletin #2, AppliedBiosystems, Foster City, CA). Each value corresponds to the average titer content of leaf samples from 4 independent plants. Bars equal the standard error for each mean. The vertical axis scale for SPCSV is different from that of SPFMV. Day 5 to Day 25 represent days of sampling post inoculation.

I' are shown in Figure 4.3. SPCSV was detected at very low levels in some leaves 5 DPI, and most were positive 10 DPI, though the titer levels remained low. At 25 DPI, a peak was observed in leaf position 7 above the point of inoculation. The virus was detected both in young and mature leaves throughout the plant, except leaf number one.

SPFMV was detected in 'Naspot I' 10 DPI. The virus titers reached a peak 20 DPI and declined at 25 DPI. There was a continuous increase in SPFMV titers in different leaves sampled until day 25 of sampling when extremely low or no SPFMV was detected in leaf number 19 and above.

Mar Ooko: Mild mottling and chlorotic spots were observed in one plant of cultivar 'Mar Ooko' 20 DPI. Purple spots were also observed in the mature leaves near the base of the plant. Relative virus titers of SPCSV and SPFMV in leaves of 'Mar Ooko' located in different positions are shown in Figure 4.4. SPCSV was detected in one plant 5 DPI and in all the plants 10 DPI. However, a unique feature was observed at 15, 20 and 25 DPI in leaf number 7 in all replicates when a sudden and significant increase in SPCSV titers occurred. Though the virus was detected in the other leaves (except leaf number 1), the titer levels were generally low throughout the plant.

SPFMV in 'Mar Ooko' was detected in only one plant 10 DPI, two plants at 15 DPI and in all the plants 20 DPI. The titers continued to increase even at 25 DPI, though there were fluctuations between different sampling times. One notable observation was lack of virus detection in some leaves located in the middle section of the plant even though being detected in leaves located in the upper and lower sections of the plant.

Namaswakhe: Mild vein clearing and banding was observed in 'Namaswakhe' 9-15 DPI (Figure 4.1h). However, the symptoms did not become severe and the plants seemed to recover

Figure 4. 3. The pattern of accumulation and distribution of *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV) in sweetpotato cultivar 'Naspot I'. Numbers in the horizontal axis represent the relative position of the leaf sampled with respect to the point of inoculation (the leaf above the point of inoculation was considered leaf number 1 and leaves were numbered sequentially from base to growing tip). The virus titers (represented in the vertical axis) are relative quantities estimated using real time quantitative PCR and calculated using ΔΔct method (User Bulletin #2, AppliedBiosystems, Foster City, CA). Each value corresponds to the average titer content of leaf samples from 4 independent plants. Bars equal the standard error for each mean. The vertical axis scale for SPCSV is different from that of SPFMV. Day 5 to Day 25 represent days of sampling post inoculation.

Figure 4. 4. The pattern of accumulation and distribution of *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV) in sweetpotato cultivar 'Mar Ooko'. Numbers in the horizontal axis represent the relative position of the leaf sampled with respect to the point of inoculation (the leaf above the point of inoculation was considered leaf number 1 and leaves were numbered sequentially from base to growing tip). The virus titers (represented in the vertical axis) are relative quantities estimated using real time quantitative PCR and calculated using ΔΔct method (User Bulletin #2, AppliedBiosystems, Foster City, CA). Each value corresponds to the average titer content of leaf samples from 4 independent plants. Bars equal the standard error for each mean. The vertical axis scale for SPCSV is different from that of SPFMV. Day 5 to Day 25 represent days of sampling post inoculation.

thereafter. The pattern of distribution of SPCSV and SPFMV in 'Namaswakhe' leaves is shown in Figure 4.5. SPCSV was detected 5 DPI and peaked 20-25 DPI. The virus was detected in all leaves except leaf number 1.

SPFMV was also detected 5 DPI and peaked after 15 DPI in most of the leaves except leaf number 7 where titers declined by 25 DPI. Except for leaf number 1, the virus was detected in all other leaves.

4.3.1 Comparison of the Four Genotypes

In order to observe and compare the differences in reaction of the four genotypes to the dual infection of SPFMV and SPCSV, the virus titers of the four genotypes were analyzed together, and graphed using similar scales for each virus. The overall pattern of accumulation of SPCSV and SPFMV are shown in figure 4.6. The titers of SPCSV differed among the 4 genotypes ($P=0.0011$), among leaf positions ($P<0.0001$) and sampling times ($P<0.0001$). Titer levels of SPCSV had differences of up to 500-fold among the genotypes during the observation period. For example, 'Beauregard' and 'Mar Ooko' had maximum relative SPCSV titers 25 DPI at around 0.326 and 0.422, respectively, whereas 'Naspot I' had maximum SPCSV titers at the same time at 0.00056. Though SPCSV was detected in different leaves along the vines of 'Naspot I', the titer levels were very low compared to 'Beauregard', and were at the threshold of detection, while 'Namaswakhe' had SPCSV titers being significantly lower than in 'Beauregard' and 'Mar Ooko', but higher than in 'Naspot I'.

The relative titers of SPFMV differed among the 4 genotypes ($P=0.0001$), among leaf positions ($P<0.0001$), and sampling times ($P<0.004$). 'Beauregard' had significantly higher titers of SPFMV than the other three genotypes. 'Mar Ooko' had the lowest SPFMV titers among the four genotypes with a peak of 0.00019 at 25 DPI, this being over three times lower than in
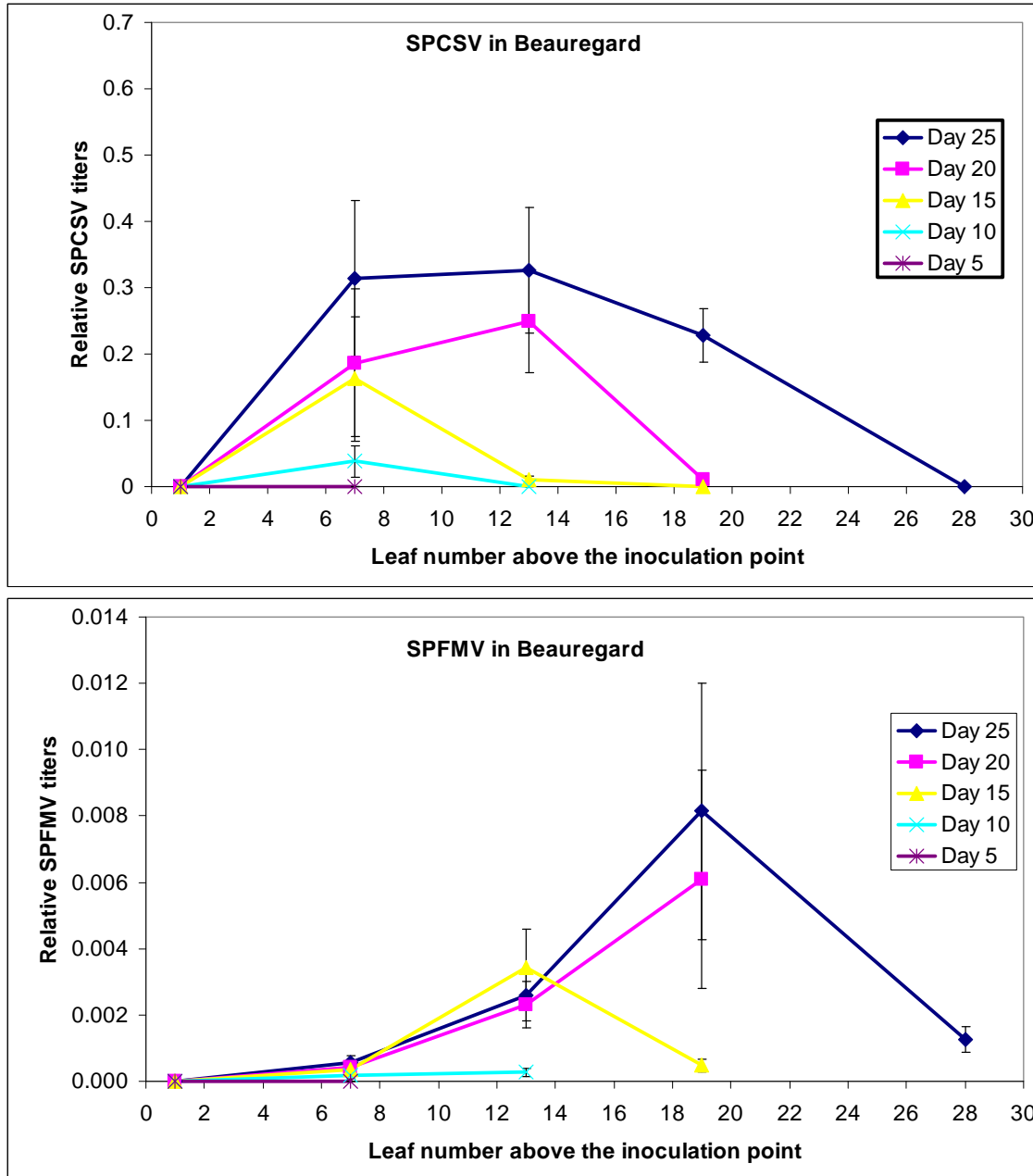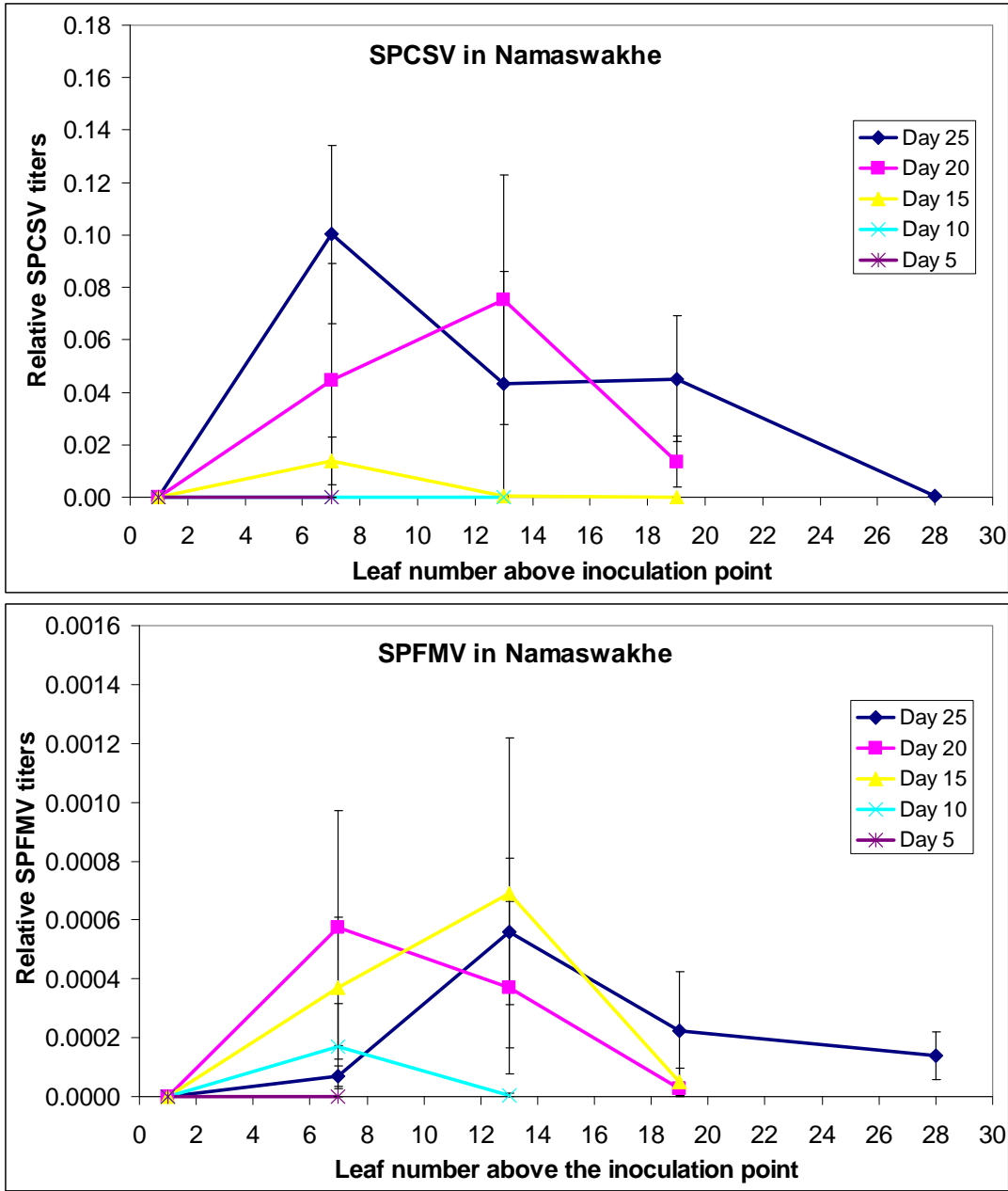
Figure 4. 5. The pattern of accumulation and distribution of *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV) in sweetpotato cultivar 'Namaswakhe'. Numbers in the horizontal axis represent the relative position of the leaf sampled with respect to the point of inoculation (the leaf above the point of inoculation was considered leaf number 1 and leaves were numbered sequentially from base to growing tip). The virus titers (represented in the vertical axis) are relative quantities estimated using real time quantitative PCR and calculated using ΔΔct method (User Bulletin #2, AppliedBiosystems, Foster City, CA). Each value corresponds to the average titer content of leaf samples from 4 independent plants. Bars equal the standard error for each mean. The vertical axis scale for SPCSV is different from that of SPFMV. Day 5 to Day 25 represent days of sampling post inoculation.
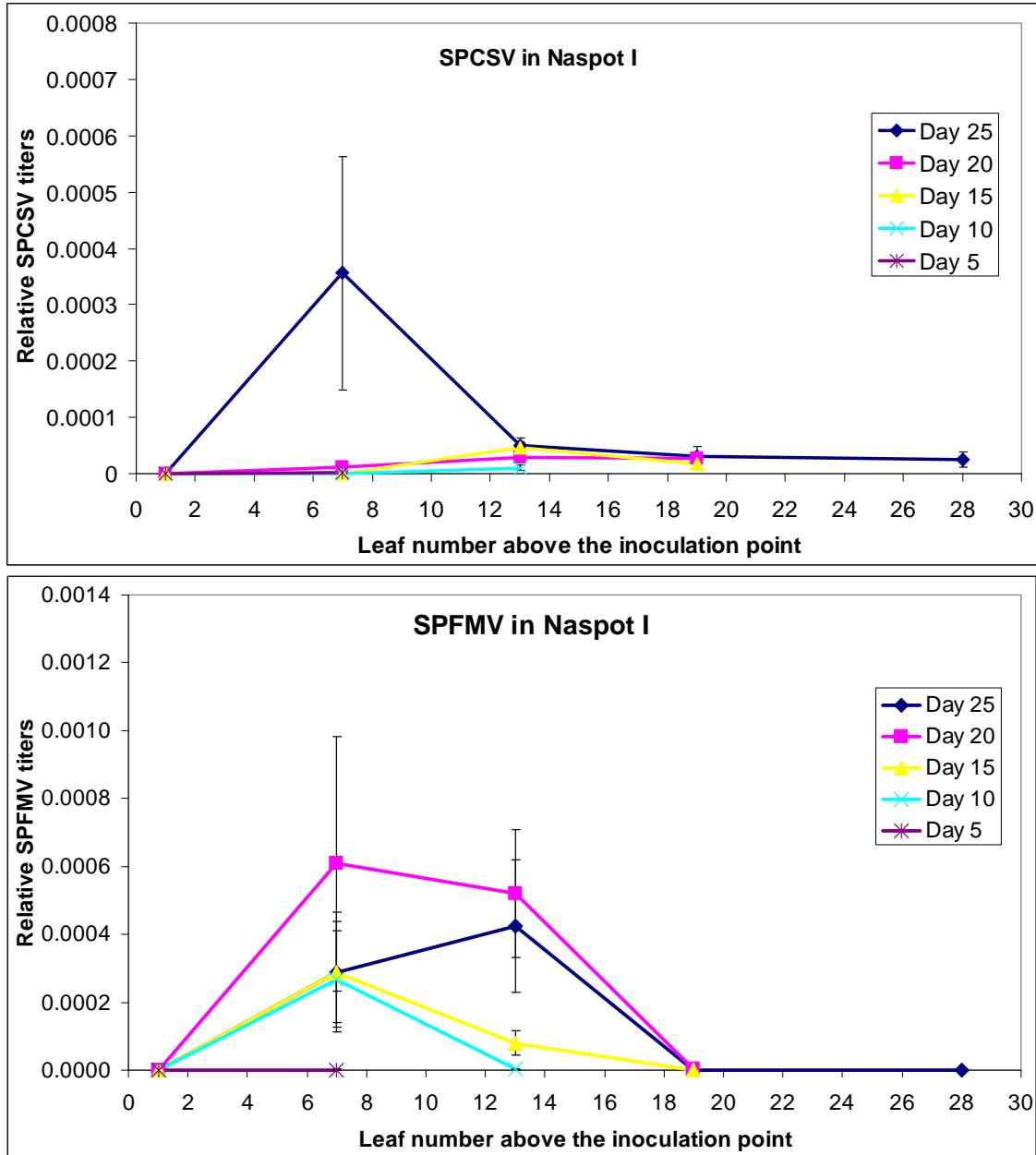
Figure 4. 6. Accumulation of *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV) in different sweetpotato genotypes. Numbers in the horizontal axis represent the relative position of the leaf sampled with respect to the point of inoculation (the leaf above the point of inoculation was considered leaf number 1 and leaves were numbered sequentially from base to growing tip). The virus titers (represented in the vertical axis) are relative quantities estimated using real time quantitative PCR and calculated using ΔΔct method (User Bulletin #2, AppliedBiosystems, Foster City, CA). Each value corresponds to the average titer content of leaf samples from 4 independent plants. Bars equal the standard error for each mean. The vertical axis scale for SPCSV is different from that of SPFMV. Day 5 to Day 25 represent days of sampling post inoculation.
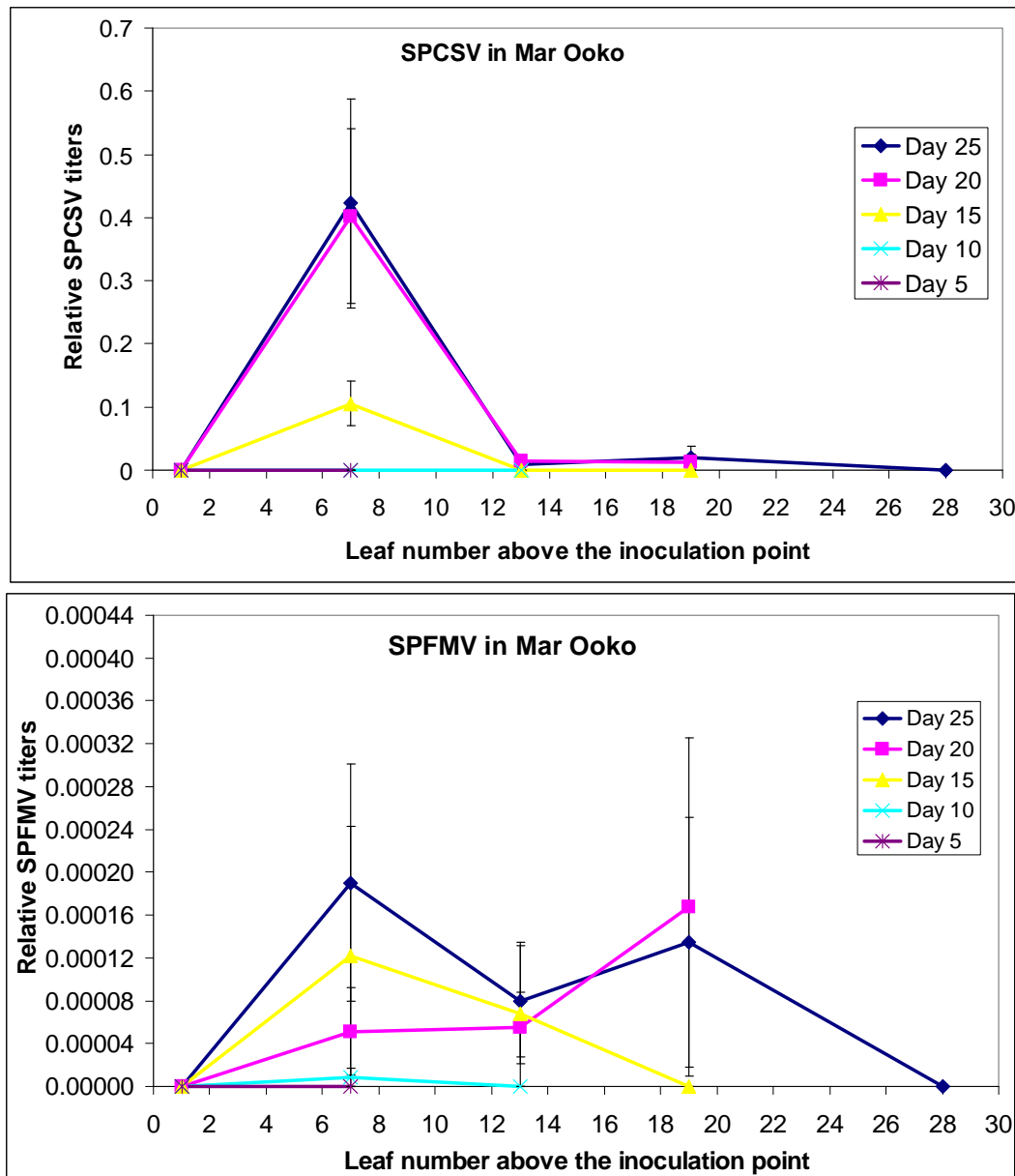
'Naspot I' (0.00061) and 'Namaswakhe' (0.000691), and over 40 times lower than in 'Beauregard' (0.0081).

As expected, there was a general increase in titer levels of both viruses in most of the leaves sampled in all genotypes. However, a few leaves had virus titers decline after sometime as is shown in Figure 4.7 where titer of SPFMV in 'Naspot I' declined after 20 DPI.

4.3.2 Virus Titer after Cut Back

To determine whether there were differences in symptom development and virus titers later in the growing stages of the plant, the plants were allowed to grow up to 70 DPI (with cut backs at 26 and 48 DPI), and sampled at 47 and 70 DPI. 'Beauregard' continued to show severe SPVD symptoms until the end of the experiment. 'Namaswakhe' developed severe symptoms on the shoots on the branches that emerged after the first cut back (Figure 4.1i,j). However, 'Naspot I' and 'Mar Ooko' developed SPVD symptoms only after the plants were cut back for the second time (Figure 4.1d,f.g). By 70 DPI, all the genotypes had clear SPVD symptoms, indicating that resistance to the disease had broken down even in the initially resistant 'Naspot I' and 'Mar Ooko'. Titer levels of SPCSV in 'Beauregard' were ranked highest among the four genotypes 47 DPI, though the differences were not significant (0.516) (Figure 4.8). The titer levels of SPCSV in 'Naspot I' was specifically noted to have drastically increased compared to titer levels in the same genotype at 25 DPI. Titer levels of SPCSV in 'Naspot I', 'Mar Ooko' and 'Namaswakhe' increased significantly (P<0.017) between 47 DPI and 70 DPI, but not in 'Beauregard'. At 70 DPI, SPCSV titers in 'Naspot I', 'Mar Ooko' and 'Namaswakhe' were ranked higher than in 'Beauregard', though the difference was not significant.

Titer levels of SPFMV were significantly greater (P<0.001) in 'Beauregard' and 'Namaswakhe' in comparison to 'Naspot I' at 47 DPI. There was a general but insignificant
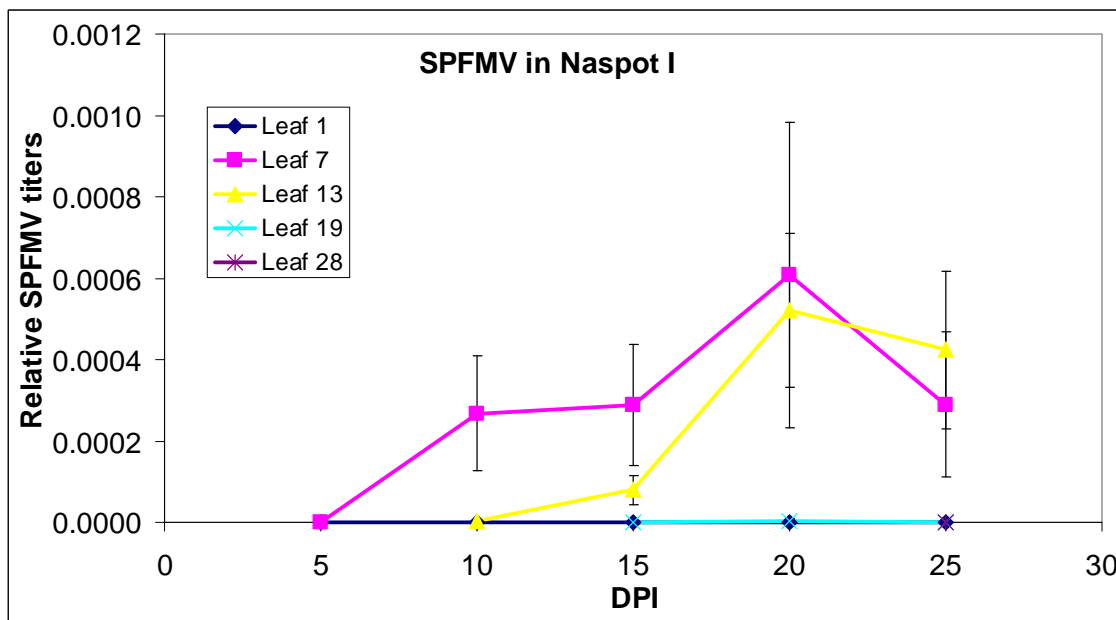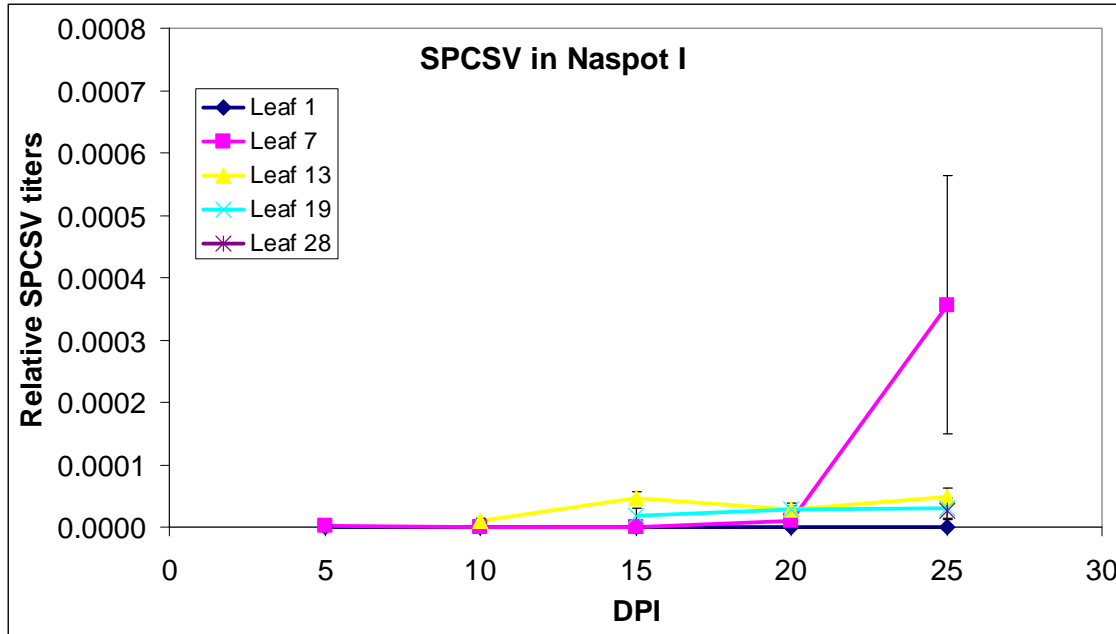
Figure 4. 7. The pattern of accumulation and distribution of *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV) in sweetpotato cultivar 'Naspot I'. Numbers in the horizontal axis represent days after inoculation (DPI) when samples were taken. The virus titers (represented in the vertical axis) are relative quantities estimated using real time quantitative PCR and calculated using $\Delta\Delta$ct method (User Bulletin #2, AppliedBiosystems, Foster City, CA). Each value corresponds to the average titer content of leaf samples from 4 independent plants. Bars equal the standard error for each mean. The vertical axis scale for SPCSV is different from that of SPFMV.
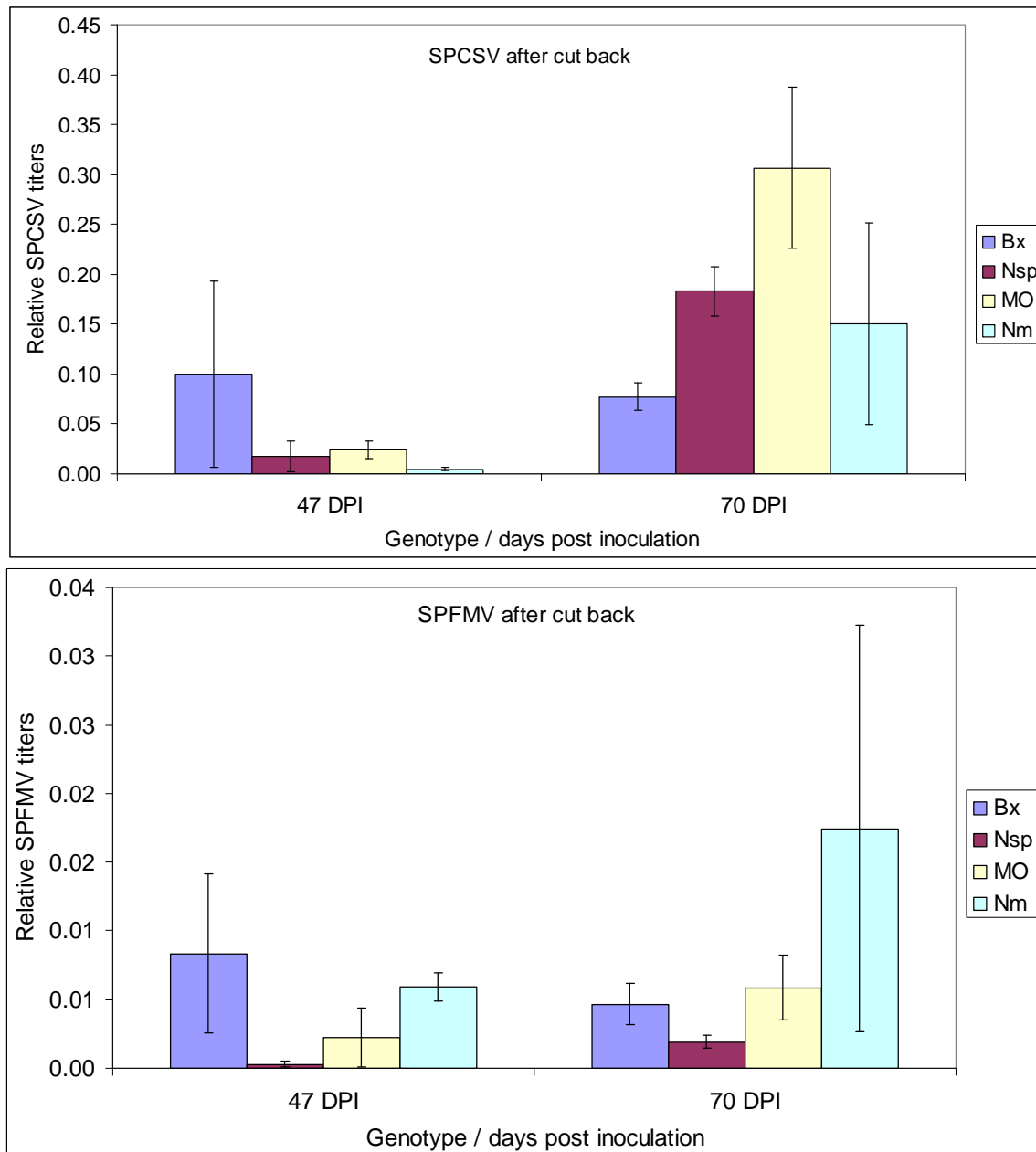
Figure 4. 8. Relative titers of *Sweet potato chlorotic stunt virus* (SPCSV, top) and *Sweet potato feathery mottle virus* (SPFMV, bottom) in sweetpotato cultivars 'Beauregard' (Bx), 'Naspot I' (Nsp), 'Mar Ooko' (MO) and 'Namaswakhe' (Nm) at 47 DPI and 70 DPI. The plants were cut back at 26 and 48 DPI, and each time allowed to re-grow for 3 weeks before samples were taken. The virus titers (represented in the vertical axis) are relative quantities estimated using real time quantitative PCR and calculated using ΔΔct method (User Bulletin #2, AppliedBiosystems, Foster City, CA). Each value corresponds to the average titer content of leaf samples from 4 independent plants. Bars equal the standard error for each value.

increase in SPFMV titers between 47 DPI and 70 DPI in all genotypes except 'Beauregard', until titer levels of SPFMV in 'Mar Ooko' and 'Namaswakhe' surpassed that of 'Beauregard' at 70 DPI.

4.4 Discussion

This study reports on the differences in reaction of resistant and susceptible genotypes to the dual infection of SPFMV and SPCSV. Previous studies identified genotypes resistant to SPVD using symptom observation. However, those results could not explain if delayed or mild symptom development were related to resistance to symptom expression (i.e., tolerance) or resistance to SPFMV and/or SPCSV multiplication and/or spread in the plant. To analyze this we used real-time quantitative PCR to determine the pattern and magnitude of accumulation of the two viruses in genotypes that were either susceptible or that had delayed or mild symptom development.

The highest titers of SPCSV were recorded in 'Beauregard' and 'Mar Ooko'. However, while SPCSV titers in 'Beauregard' were high in almost all leaves sampled, titer levels in 'Mar Ooko' were significantly lower in the leaves located in the upper portion of the plant. Though SPCSV was detected in different leaves along the vines of 'Naspot I', titer levels were very low compared to 'Beauregard' and could barely be detected, indicating limited virus multiplication within the genotype. The results showed that 'Naspot I' was resistant to SPCSV accumulation. However, the fact that the virus was detected in different sections of the plant indicates that there was virus movement within the plant, but multiplication was limited. These results suggest that SPCSV could systematically colonize graft inoculated 'Naspot I' but less efficiently than in susceptible 'Beauregard', at least early on. The higher levels of SPCSV observed in lower sections of 'Mar Ooko' could indicate a different type of resistance mechanism, where cells of

lower (more mature) leaves were more susceptible. This suggests that phloem cells of young leaves of 'Mar Ooko' do not support replication or movement of SPCSV as has been reported in other closteroviruses (Monis and Bestwick, 1996; Kreuze et al., 2002; Marco et al., 2003). 'Namaswakhe' had SPCSV titers significantly lower than in 'Beauregard' and the lower portion of 'Mar Ooko', but higher than in 'Naspot I'. However, as in 'Beauregard', the distribution was fairly even, and the virus could easily be detected in leaves located in almost all sections of the plant. 'Naspot I' was therefore categorized as more resistant to SPCSV than all the other genotypes, 'Namaswakhe' was moderately resistant, 'Beauregard' was susceptible while younger leaves of 'Mar Ooko' were more resistant than older leaves. We could not detect SPCSV in the leaves immediately above the point of inoculation in all the four genotypes. This may be due to the fact that the leaves were mature at the time of inoculation and were therefore able to resist infection, or that the mature leaves were not metabolic sinks and therefore translocation carried the virus past them to younger leaves that were metabolic sinks.

The highest titer levels of SPFMV were recorded in 'Beauregard', and were significantly higher than in the other three genotypes. 'Mar Ooko' recorded the lowest levels, being at the threshold of detection. In 'Beauregard', SPFMV titers were higher in leaves in the upper half of the vine, unlike in single infections with SPFMV where the distribution is uneven or more readily detectable in the older leaves. Younger leaves of different plants susceptible to different viruses have been noted to have higher virus titers early after inoculation (Hull, 2002). In contrast to 'Beauregard', SPFMV titers were higher in the older leaves in 'Naspot I' at 25 DAI. 'Mar Ooko' was therefore highly resistant to SPFMV, 'Naspot I' and 'Namaswakhe' moderately resistant, while 'Beauregard' was highly susceptible. However, these results may only be true when the two viruses co-infect the plant, and the situation may change in single infections.

The 4 genotypes reacted very differently to infection by the two viruses. 'Beauregard' was susceptible to both viruses and severe SPVD symptoms developed soon after inoculation; 'Namaswakhe' was also susceptible to both viruses but the rate of multiplication was slower than in 'Beauregard'. SPVD symptoms in 'Namaswakhe' were observed only after the plants were cut back; 'Mar Ooko' resisted SPFMV multiplication before being cut back, but allowed rapid multiplication of SPCSV in the older leaves. The genotype also had delayed symptom development; 'Naspot I' resisted SPCSV multiplication before being cut back, and had limited multiplication of SPFMV. 'Naspot I' also had delayed symptom development. However, when all the genotypes were allowed to grow for a period of 10 weeks post inoculation, and were cut back twice, the virus titers increased and SPVD symptoms developed even in the resistant genotypes until there were no significant differences in titer levels in the four genotypes for the two viruses.

In resistant individuals, the virus multiplication may be reduced or inhibited to some extent, or the spread of the virus through the plant is demonstrably restricted relative to susceptible hosts, and disease symptoms are localized or not evident (Fraser, 1990; Kang et al., 2005). In some cases, the plant may not develop severe symptoms (tolerant to the disease) even though the virus may multiply and move through the host in a manner that is indistinguishable from that in a susceptible host. In 'Naspot I' and 'Mar Ooko', SPVD symptoms became evident when the virus titers increased. The lack of symptom expression was therefore accompanied by reduced levels of virus multiplication. It may be that the observed low levels of SPCSV and/or SPFMV are not enough to trigger the cascade of events associated with symptom induction (Maule et al., 2000), but the situation changes when titers of both viruses increase beyond a certain threshold where they synergize to cause symptoms. The genotypes can therefore be

classified as resistant to the viruses but not to the disease, at least before the plants are cut back, but the resistance may not be durable. Mwanga et al. (2002a) reported that resistance to SPCSV and SPFMV were mediated by two major (but separate) recessive genes. It is possible that the resistance to the two viruses observed in 'Naspot I' and 'Mar Ooko' are controlled by the same genes, and is associated with molecular markers identified by Miano et al. (2007).

Symptoms were more severe and relative titer levels higher in the leaves that grew after the plants were cut back; indicating that cutting the plant affected its physiology in some way, allowing the two viruses to somehow synergize or resulting in breakdown of resistance. This information is important in so far as propagation of sweetpotato in developing countries is concerned. In tropical environments where some sweetpotato plants can be kept in the field year-round, crop multiplication is done almost exclusively with vine cuttings (Gibson et al., 1997; Carey et al., 1999; Fuglie, 2007). Usually, more than one harvest of cuttings may be obtained from the same field. Any subsequent cuttings obtained after an initial one could have high virus titers, resulting in significant yield reductions.

We used grafting to inoculate the sweetpotatoes with the viruses because SPCSV is not mechanically transmitted. However, when a virus is transmitted by an insect, resistance may be expressed at the insect-plant interface (Vidavsky and Czosnek, 1998) during the short time the insect is feeding, unlike in grafting where the virus is directly delivered in to the vascular system continually for as long as the scion remains viable. Once the virus is in the vascular system, resistance may be lost (Kheyr-Pour et al., 1994). Also important to note is the fact that the graft scion is usually derived from a susceptible host, which provides a reservoir on the inoculated plant in which virus replication might continue regardless of the resistance of the test plant stock. Such conditions do not normally exist in the field especially if insects transmit the viruses, and

success of infection would depend on successful replication and translocation of the few virus particles. 'Naspot I' is reported to be resistant to SPVD in the field in Uganda (Mwanga et al., 2003), where both viruses are transmitted by insects, but resistance breaks down when the plants are graft inoculated with the East African strains of SPVD-causing viruses. Resistance in 'Naspot I' may therefore have been broken down due to the method of inoculation. While insect transmission might give a more natural inoculation, graft inoculation has the advantage that it is less time consuming and the survival of the scion may be used as an indicator of successful inoculation. However, it might be advisable to remove the scion after successful inoculation to reduce the potential virus multiplication in this susceptible tissue. Despite the advantages of using graft inoculation, we recommend that insect transmissions be included in evaluations of genotypes for resistance to viruses that are mainly transmitted by insects such as SPCSV, so that resistant genotypes are not discarded. Regardless of the strong challenge imposed on the genotypes by graft inoculation, we were able to show how the different genotypes react to SPVD and to the causal agents of the disease.

We did not include plants inoculated singly with SPCSV and SPFMV. Previous studies have shown that most sweetpotato genotypes are resistant to SPFMV infection alone with virus levels being very low or below the detection limits (Esbenshade and Moyer, 1982; Abad and Moyer, 1992; Clark and Hoy, 2006; Kokkinos and Clark, 2006b), and that the problem arises when SPFMV synergizes with SPCSV. Similar resistance to SPFMV has been reported in 'Beauregard' (Kokkinos and Clark, 2006b), the most susceptible of the four genotypes used in this study. Single inoculations with SPCSV can cause economic yield losses (Hahn, 1979; Ngeve and Bouwkamp, 1991; Gibson et al., 1998; Gutierrez et al., 2003) and virus titers are often high (Kokkinos and Clark, 2006b). Titers of SPCSV have also been noted to decline or remain

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unaffected in the presence of SPFMV (Karyeija et al., 2000; Kokkinos and Clark, 2006b). Karyeija et al. (2000) concluded that since the titers of SPCSV were not significantly increased in doubly infected plants, and because the plants infected with SPCSV alone were mostly symptomless or displayed symptoms different from SPVD, the SPVD symptoms observed are presumably caused largely by SPFMV. However, their studies were carried out using genotypes that were susceptible to SPVD. In our study, the titers of both viruses increased with time, at least in 'Naspot I', 'Mar Ooko' and 'Namaswakhe', especially after cutback. Also, no symptoms were observed in the different genotypes until there was a significant increase in the titer levels of the two viruses. We therefore conclude that increase in both viruses play an important role in symptom development.

US strains of SPFMV-RC and SPCSV were used in this study. SPFMV-RC has not been detected in East Africa (Kreuze et al., 2000; Ateka, 2004), and the SPCSV isolate used in this study is distinguishable from but most similar to the West African serotype (Vetten et al., 1996; Abad et al., 2007). Even though SPFMV-RC is closely related to the Ordinary and East African strains of SPFMV (SPFMV-O and SPFMV-EA, Ateka, 2004), both of which also synergize with either of the two SPCSV serotypes to cause SPVD (unlike the SPFMV-C which causes mild or no symptoms even in presence of SPCSV), the East African strains may induce different responses from the US strains. Future work should therefore compare how different strains react in genotypes showing resistant and susceptible reaction to SPVD. However, 'Naspot I' has been shown to be resistant to SPVD in East Africa and now in the United States, indicating that it is possible to have cultivars that are resistant to different virus strains and/or serotypes.

In our study, we used SPVD resistant and susceptible genotypes, with sampling being done soon after inoculation and at 5-day intervals. This strategy enabled us to observe the

differences in response of different genotypes to infection by the two viruses with time. Unlike in the previous studies where SPCSV titers remained the same or declined with time, we observed that multiplication of either or both of the viruses was inhibited, and that virus titer increased with time in resistant genotypes. We conclude that mild and/or delayed symptom development observed in resistant genotypes was due to suppression of virus multiplication, that increase in both viruses is important in symptom expression, and that virus titers correlate with symptom development. Further studies should be done to determine the genetic control of the resistance.

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CHAPTER 5: IDENTIFICATION OF MOLECULAR MARKERS ASSOCIATED WITH SWEETPOTATO RESISTANCE TO SWEET POTATO VIRUS DISEASE IN KENYA*

5.1 Introduction

Sweet potato virus disease (SPVD), a result of co-infection of whitefly-transmitted *Sweet potato chlorotic stunt virus* (SPCSV, genus *Crinivirus,* family *Closteroviridae*) and the aphid-transmitted S*weet potato feathery mottle virus* (SPFMV, genus *Potyvirus,* family *Potyviridae*), is the most destructive disease of sweet potato world-wide and especially in East Africa (Geddes, 1990; Gibson et al., 1997; Carey et al., 1999; Karyeija et al., 2000; Gutierrez et al., 2003). The disease is characterized by chlorosis, small, deformed leaves, and severe stunting and can reduce yields of infected plants by over 90% (Gibson et al., 1998; Gutierrez et al., 2003).

Attempts to controlling SPVD are through host-plant resistance backed up by farmers selecting disease-free planting stock (apparently free of known viruses). Studies in Uganda indicate no benefit of using pathogen-tested plants when compared with farm-derived plants of the same cultivars in the region (Gibson et al., 1997; Carey et al., 1999). Thus, plant resistance is important to SPVD control (Karyeija et al., 1998b; Carey et al., 1999). Hundreds of sweetpotato cultivars (landraces) are grown in the region (Gichuki et al., 2003), which have been shared by farmers through generations. The landraces have large differences in susceptibility to SPVD, and good sources of resistance are present in local germplasm (Aritua et al., 1998a). The impact of SPVD has been reduced by the use of resistant cultivars and landraces (Aritua et al., 1998b; Karyeija et al., 1998a). However, resistance has been associated with relatively late maturing, low yielding genotypes (Aritua et al., 1998b). Despite the short comings, unintended gains in the

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development of virus resistance has occurred as farmers and breeders have both selected for high yield, and / or mild symptoms in plantings that were exposed to natural virus infection.

Prior efforts of virus resistance breeding were focused on control of SPFMV given its universal distribution. However, many sweetpotato cultivars are naturally resistant to SPFMV, showing no or only mild initial symptoms, from which they usually recover, and containing very low virus titers (Esbenshade and Moyer, 1982; Abad and Moyer, 1992; Kokkinos and Clark, 2006b). Many East African sweetpotato cultivars are resistant to SPFMV (Gibson et al., 1998; Mwanga et al., 2002b). Resistance is broken when the varieties are co-infected with SPCSV (Karyeija et al., 1998a; Mwanga et al., 2002b). There have been efforts towards developing a transgenic sweetpotato resistant to SPVD through resistance to SPFMV both in Kenya and other parts of the world (Okada et al., 2001; Wambugu, 2003). However, it is becoming increasingly clear that the problem is not SPFMV but SPCSV which synergizes with different unrelated viruses (Cohen and Loebenstein, 1991; Di Feo et al., 2000; Kokkinos and Clark, 2006b; Mukasa et al., 2006), and resistance to SPFMV may not hold in the presence of SPCSV. Although SPCSV can cause yield losses on its own, little effort seems to be directed towards developing varieties resistant to SPCSV. Selection for resistance to SPVD is based on symptom development after field exposure in disease prone areas and by graft-inoculations (Hahn et al., 1981; Mihovilovich et al., 2000; Mwanga et al., 2002b), processes requiring considerable time and resources. Attention is focused on DNA markers to accelerate breeding through early selection. While studying inheritance of resistance to SPCSV, SPFMV and SPVD in sweetpotato, Mwanga et al. (2002a) constructed a preliminary linkage map of sweet potato, and identified markers linked to SPCSV and SPFMV resistance. They found an AFLP marker (*spcsv1*) which explained 70% of the variation in resistance to SPCSV and one RAPD marker

(*spfmv1*) which explained 72% of the variation in SPFMV resistance. Mwanga et al. (2002a) could not determine if the two genes present in a common background would suppress SPVD effectively.

Discriminant analysis and logistic regression represent novel approaches in marker-assisted selection (Cruz-Castillo et al., 1994; Ebdon et al., 1998; Capdevielle et al., 2000; Fahima et al., 2002; Aluko, 2003; Mcharo, 2005). Genotypes possessing trait extremes, e.g. highly resistant vs. highly susceptible are used exclusively, and those with intermediate reactions ignored. Discriminant and logistic regression analysis are especially useful in dealing with populations of unrelated clones such as landrace genotypes since quantitative trait loci (QTL) analysis is not suited for such populations with no progeny-parent combinations (Mcharo, 2005).

The objective of this study was to identify and classify unrelated sweetpotato genotypes selected from East African germplasm collections into phenotypic groups as resistant and susceptible to SPVD, and to identify the most important AFLP markers contributing to variation among the phenotypic groups using discriminant and logistic regression analysis.

5.2 Materials and Methods

5.2.1 Selection of SPVD-resistant and Susceptible Sweetpotato Genotypes

The germplasm pool surveyed consisted of over 400 genotypes collected from the main sweetpotato growing regions of Kenya (Western, South-western, Eastern and Central Provinces), neighboring and non-contiguous countries and maintained *in situ.* The collections are located at the University of Nairobi, Kabete Field Station Farm in Central Kenya and at the Kakamega Research Station in Western Kenya. The plants were assessed for symptoms caused by virus diseases twice in a period of two years. SPVD severity in each genotype was assessed using a subjective 5-point severity rating scale where: 1 = no symptoms observed; 2 = mild symptoms

consisting mainly of chlorotic and /or purple spots; 3 = moderate symptoms which included chlorotic spots, vein clearing, interveinal chlorosis, mottling, and mosaics; 4 = plants were not stunted, but had severe symptoms of vein clearing, interveinal chlorosis, chlorotic spots, mottling, mosaics, and general chlorosis; and 5 = very severe symptoms, which included severe chlorosis, small-deformed leaves (shoestring), and severe plant stunting.

A total of 92 genotypes with SPVD severity rating of 1 or 2 were selected as resistant and used for challenge inoculations. Two apical cuttings from each genotype were planted in a screenhouse in Kenya and allowed to grow for two weeks. The plants were then graft-inoculated with a scion from a sweet potato plant (cultivar 'Marera I') from a Kenyan field, which had previously been confirmed to be infected with both SPFMV and SPCSV (East African serotype) using nitrocellulose membrane enzyme linked immunosorbent assay (NCM-ELISA) (CIP, 2001). The scions were multiplied and maintained in the screenhouse. Three susceptible cultivars from tissue culture, which had tested negative to different viruses using NCM-ELISA were also grafted with Marera I to ensure it induced SPVD. Inoculated plants were monitored for symptom development for two months. Genotypes were again selected as resistant or susceptible based on symptom severity and time (in days) to symptom development. A total of 28 genotypes were selected as resistant to SPVD.

Twenty out of the 92 SPVD graft inoculated clones were randomly selected and used to determine efficiency of inoculation by confirming the presence of SPFMV and SPCSV. The genotypes were grafted onto *I. setosa* indicator plants and NCM ELISA done as described in the text.

Nineteen other genotypes with severe SPVD symptoms in the germplasm collection plots (rated at a scale of 4 or 5) were selected as susceptible, giving a total of 47 genotypes (Table 5.1). Cuttings from 38 of the selected genotypes were sent to Plant Germplasm Quarantine

Table 5. 1 Sweetpotato clones used for marker selection and country of origin.

No.	Clone	SPVD reaction group[w]	Country of origin	No.	Clone	SPVD reaction group[w]	Country of Origin
a) Training group							
1	Nyathi Odiewa	Resistant	Kenya	16	Beauregard	Susceptible	USA
2	Osombo Nyuol	Resistant	Kenya	17	Bungoma	Susceptible	Kenya
3	Unknown-1	Resistant	Kenya	18	Mar Ooko	Susceptible	Kenya
4	Unknown-2	Resistant	Kenya	19	Namaswakhe	Susceptible	Kenya
5	Agriculture	Resistant	Kenya	20	Spoungi	Susceptible	Tanzania
6	Nyar Busia	Resistant	Kenya	21	K37	Susceptible	Kenya
7	Mwavuli	Resistant	Kenya	22	K46	Susceptible	Kenya
8	Maria Angola	Resistant	Peru	23	K207	Susceptible	Kenya
9	Nyar Koyugi Mumbo	Resistant	Kenya	24	SPK 013	Susceptible	Kenya
10	Butso Butso I	Resistant	Kenya	25	Cheglina Mowar	Susceptible	Kenya
11	Tanzania[x]	Resistant	Uganda	26	Marera I[x]	Susceptible	Kenya
12	Unknown-3	Resistant	Kenya	27	Nyaboro	Susceptible	Kenya
13	Ondieki Chilo	Resistant	Kenya	28	Kemb 10	Susceptible	Kenya
14	Nyandere	Resistant	Kenya	29	Salyboro	Susceptible	Peru/CIP[y]
15	Jayalo	Resistant	Kenya	30	W220	Susceptible	USA
b) Validation (test) group							
31	K118	Resistant	Kenya	41	Nemanete	Susceptible	Peru
32	Polista	Resistant	Tanzania	42	Mugande[x]	Susceptible	Rwanda
33	NASPOT I	Resistant	Uganda	43	Merenge	Susceptible	PNG[z]
34	Rateng	Resistant	Kenya	44	Naveto	Susceptible	PNG[z]
35	Ondijo	Resistant	Kenya	45	Webuye	Resistant	Kenya
36	Mary Oketch	Resistant	Kenya	46	Nduma	Resistant	Kenya
37	Nyanduwo	Resistant	Kenya	47	Nyar Buhola	Resistant	Kenya
38	Nyandege	Resistant	Kenya				
39	Tur Ninde	Resistant	Kenya				
40	Unknown -4	Resistant	Kenya				

[w]Genotypes were grouped as resistant or susceptible based on disease severity or days to symptom development in plants following graft inoculation using scions infected with SPFMV and SPCSV (resulting in SPVD) obtained from a Kenyan field.
[x]Cultivar 'Marera I' was classified as resistant when 2 or 3 markers selected using STEPDISC procedure or logistic regression (SAS, 2001) were used during cross-validation, while cultivar 'Tanzania' was classified as susceptible when a 2-marker model was used. 'Mugande' was misclassified using the 4-marker model.
[y]International Potato Center
[z]Papua New Guinea

Office of USDA-ARS, Beltsville, MD for virus indexing and therapy. The remaining 9

genotypes were obtained from the USDA/ARS repository, Griffin, GA.

5.2.2 DNA Extraction

DNA extraction out of the 9 clones obtained from the USDA/ARS repository was done

using the GeneElute Plant Genomic DNA Kit (Sigma-Aldrich Inc., St. Louis, Mo) as described

by Mcharo et al. (2004). The 38 clones sent to the Plant Germplasm Quarantine Office of

USDA-ARS were planted in a greenhouse and total DNA extracted from leaves one month later

using a cetyltrimethylammoniumbromide (CTAB) extraction method (Li et al., 2004). DNA was

further purified using GeneElute DNA binding columns (Sigma-Aldrich Inc., St. Louis, Mo).

5.2.3 AFLP Analysis

AFLP analysis was conducted as previously described (Mcharo et al., 2005). The DNA

samples were amplified in a three-step process using GeneAmp PCR system 9600 thermocycler

(Perkin Elmer, Fulerton, CA). Reagents for AFLP[TM] were obtained from Invitrogen[TM] (AFLP

starter primer kit, Cat No. 10483-014) and LI-COR Inc. (Lincoln, NE, Cat. No. 420032). About

100 ng/µl of total genomic DNA was digested using an EcoRI/*Mse*I restricted enzyme mix in 5X

reaction buffer at 37ºC for 3 h. The enzymes were inactivated by incubating the mix at 70ºC for

10 min. Double stranded adaptors were then ligated to the restricted DNA fragments resulting in

template DNA which was used for pre-amplification.

Diluted template DNA (1.5 µl) was added to 10 µl pre-amp primer mix, 1.25 units Taq

DNA polymerase and 1.25 µl RedTaq[TM] PCR reaction buffer 10X with $MgCl_2$ (Sigma-

Aldrich[TM]) to make a 13 µl reaction volume. The pre-amplification conditions were 20 cycles

each of 94ºC for 30 s, 56ºC for 60 s, 72ºC for 60s and a final hold at 4ºC for 2 h.

The reaction volume for selective amplification consisted of 3.5 µl pre-amplified diluted

DNA, 0.4 µl of EcoRI (fluorescently labeled) primer (AAG), 4.4 µl of *Mse*I (unlabelled) primer,

2.0 µl RedTaq^{TM} PCR reaction buffer 10X with MgCl$_2$ (Sigma-Aldrich^{TM}), 1.4 µl MgCl$_2$

(Sigma-Aldrich^{TM}), 1 unit Taq DNA polymerase (Invitrogen^{TM}), dNTPs (200 uM), and 6.38 µl

double distilled or AFLP grade water. Four selective primer pairs identified by Fajardo et al.

(2002) and also used by Mcharo (2005) were used for selective amplification (CAG, CTA, CTG,

CTT). Blue stop solution (5.0 µl) (LI-COR, Lincoln, NE) was added onto each amplified DNA

sample. The amplified DNA sample was denatured at 94ºC for 4 min, covered in aluminium foil

and placed in a freezer at -20ºC for 10 min to prevent annealing of complementary fragments,

before loading 0.8 µl of the DNA sample onto a 25-cm acrylamide gel. PCR amplification

fragments were separated by 6.5% acrylamide gel electrophoresis using LI-COR Global IR^2

sequencer (LI-COR, Lincoln, NE) for 2 h 45 min. The AFLP fragments were automatically

detected and recorded during electrophoresis using the LI-COR SAGA^{MX} v 3.1.0 software. The

markers were named starting with the three letters coding for the primer followed by the

molecular weight of the marker in base pairs.

5.2.4 Statistical Analysis

Analysis of molecular variance (AMOVA) on the genotypes with the AFLP marker

profiles was used to test genotypic variability based on molecular marker information using

WINAMOVA 1.55 software (Excoffier et al., 1992; Huff et al., 1993). Genetic distances for the

AMOVA analysis were estimated using the Euclidean metric distance of Excoffier et al. (1992).

Discriminant analysis as previously described by Mcharo et al. (2004) was used to select

informative markers that are linked to SPVD resistance in the two populations. The 47 clones

were divided into two populations, the first one consisting of 28 clones classified as resistant to

SPVD and another 19 clones classified as susceptible to SPVD. A training sample consisting of 15 resistant and 15 susceptible clones from the original 47 was used for the development of a phenotypic group prediction model. The training sample proportions were equal (15 resistant and 15 susceptible) for both the susceptible and resistant group, making the prior probabilities of group membership equal at 0.5. A second group consisting of the remaining 4 susceptible and 13 resistant clones was used as a test population to validate the model.

From the original array of AFLP generated molecular markers, the most informative markers were selected using STEPDISC procedure, and logistic regression using PROC LOGISTIC procedure (SAS, 2001). The forward selection option of STEPDISC was used to select markers to be used in the classification model. The forward selection process commences with no markers in the model. Entry-significance levels of $P \leq 0.01$ of the chi-square score for entering an effect or marker into the model to achieve at least 95% prediction accuracy was imposed to choose the most discriminating markers (SAS 1999). The selected markers were then used in a nonparametric discriminant analysis (k=1), DISCRIM option (SAS, 2001), to construct and validate a class prediction function and to predict membership, resistant or susceptible, of the test. A nonparametric method; the k-nearest neighbor method (Rosenblatt, 1956) was used to estimate the group-specific densities that produce a classification criterion because the data was categorical in nature and could not allow assumption of normal distribution. The genotypes comprising the training and test groups were mostly from Kenya, but included genotypes from 7 other countries. We assume that genotypes used in the present study consisted of unrelated clones (mainly landraces) and therefore did not have any population structure. The performance of the discriminant criterion was evaluated by posterior probability error rate and group-specific error count estimates during cross-validation. The error estimator gives the proportion of

misclassified observations in each group. Total error, from which the percent correct classification is derived, is the weighted mean error estimates of the two phenotypic groups.

PROC LOGISTIC (SAS, 2001) was used to perform logistic regression to select markers that accounted for the phenotype variation, with the forward selection option used for marker selection. The phenotype was a binary outcome as either resistant or susceptible to SPVD. Significance level to include a marker was set at $P = 0.01$. The Akaike Information Criterion (AIC) model fit statistics for logistic regression were computed as described in SAS (1999) and by Mcharo (2005), and used to compare models with different variables. The model with the lowest AIC value achieves the best fit.

5.3 Results

5.3.1 Germplasm Screening

All 92 sweetpotato genotypes which initially had mild or no symptoms in the collection plots developed a range of symptoms at different times when inoculated with scions infected with SPVD. Symptoms observed included vein clearing, interveinal chlorosis, chlorotic spots, mottling, mosaics, general chlorosis, rugosity and stunting. Some genotypes developed typical SPVD symptoms consisting of vein clearing, interveinal chlorosis, chlorotic spots, mottling, and / or general chlorosis within one week, others took as long as one month, while some genotypes did not develop the typical severe symptoms characteristic of SPVD. The 20 clones taken from the 92 and grafted on to *I. setosa* were confirmed to be infected with both SPFMV and SPCSV using NCM-ELISA, validating the protocol used. Resistant genotypes were selected as those that showed mild chlorotic spots and/or flecks, mild vein clearing, or those that took longer to show SPVD symptoms. A total of 28 genotypes were grouped as resistant and 19 as susceptible (Table 5.1).

5.3.2 AMOVA, Discriminant and Logistic Regression Analysis

A high level of polymorphism was observed using AFLP markers. A total of 350 markers were generated using the four primer combinations, 206 of which were polymorphic. The number of polymorphic markers generated from each primer combination ranged from 45 to 61 with an average of 51 markers. The analysis of two groups (resistant and susceptible) using AMOVA and all 206 polymorphic markers showed a significant (P<0.002) variation between the two phenotypic groups of the 47-clone population

The STEPDISC procedure identified 9 markers that met the entry-significance level (P≤0.01) to achieve at least 95% prediction accuracy (Table 5.2). The Wilks' lambda and Pr<lambda were used to determine how powerful the selected markers are. No more markers could be selected even when the entry significance levels were changed to P≤0.05. During evaluation by cross-validation to test the predictive power of the selected markers or model, only 4 markers selected by STEPDISC procedure were required to achieve 100% correct classification of the 30 genotypes in the training set. Three markers achieved a 96.7% correct classification rate. Genotype 'Marera I' was misclassified as resistant when 2 or 3 markers selected by STEPDISC procedure were used. When only two markers were included in the classification model, cultivar 'Tanzania' which was initially grouped as resistant, was classified as susceptible, reducing the classification accuracy to 93.3% using two predictor markers.

Four markers, identical to those selected by the STEPDISC procedure, were also selected by logistic regression (Table 5.3). However, the order of importance of marker selection differed between the two procedures. The four markers also gave 100% correct classification of the training population. Increasing the number of markers increased the AIC value, indicating that a model with the four markers could be the most desirable. The rate of correct classification of

Table 5. 2. STEPDISC selection for AFLP DNA markers in sweetpotato associated with resistance to SPVD.

Marker[1]	Entry step	Partial R-square[2]	Wilks' Lambda[3]	Pr<lambda
Cag202	1	0.55	0.45	<.0001
Cta110	2	0.33	0.31	<.0001
Cta168	3	0.36	0.19	<.0001
Cta334	4	0.33	0.13	<.0001
Cta136	5	0.43	0.07	<.0001
Ctg621	6	0.33	0.05	<.0001
Cta195	7	0.29	0.04	<.0001
Cta076	8	0.63	0.01	<.0001
Cag246	9	1.00	0.00	<.0001

[1]Markers are named starting with the three letters coding for the primer followed by the molecular weight of the marker in base pairs.
[2]Partial R-square is the marginal variability accounted for by a variable when all others are already included in the model.
[3]Wilks' lambda is the likelihood ratio measure of a marker's contribution to the discriminatory power of the model.

Table 5. 3. Logistic regression selection for AFLP DNA markers associated with SPVD resistance in sweetpotato.

Marker entry step	Marker[1]	Estimate (β)	χ^2 score	Pr> χ^2
0	Intercept	10.02		
1	Cag202	-17.76	16.43	<.0001
2	Cta168	-18.04	13.00	0.0003
3	Cta110	-17.30	10.58	0.0011
4	Cta334	16.95	15.00	0.0001

[1]Markers are named starting with the three letters coding for the primer followed by the molecular weight of the marker in base pairs.
[2]χ^2 score is the largest significant score for marker not in model to be included in the model.

genotypes into resistant and susceptible groups achieved by the various models using logistic

regression is shown in Table 5.4.

One test clone (Mugande) could not be classified correctly out of the 17 genotypes when

four markers were used, giving a 94.12% correct classification efficiency. Increasing the marker

number above four could not classify the misclassified clone correctly.

5.4 Discussion

In this study, we report on the first systematic effort to evaluate sweetpotato genotypes

grown in Kenya for their reaction to SPVD and identify a core collection of resistant and

susceptible genotypes. Using discriminant and logistic regression analysis, we were able to select

molecular markers associated with SPVD resistance that could be useful to sweetpotato breeders.

When the 92 symptomless, field-grown sweetpotato genotypes were graft-inoculated,

over half of the genotypes developed severe symptoms indicating that field resistance was not

durable and could have been due to escape, resistance to insect vectors or that the susceptible

scion provided a continuous source of virus that overcame resistance. Graft inoculation

represents the most potent, high dosage, continuous supply of virus inoculum to a plant (Wroth

and Jones, 1992; Njeru et al., 1995). Resistance is therefore overcome by graft inoculations even

in sweetpotato genotypes showing high field resistance to SPVD (Mwanga et al., 2003; Mwanga

et al., 2002b). Despite the strong challenge imposed on the genotypes by graft inoculation, 28

genotypes showed mild chlorotic spots and/or flecks, mild vein clearing, or delayed symptom

development, and were thus selected as resistant. Delayed symptom development can be an

important part of resistance (Dasgupta et al., 2003), especially if it is associated with reduced

virus replication and translocation. Reduction in yields may be moderate in such genotypes. It

would be desirable to know the titer of viruses in the genotypes with varying levels of SPVD

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Table 5. 4. Rate of correct classification of 30 training clones of sweetpotato into SPVD resistant (or tolerant) and susceptible groups and the AIC model fit statistic for logistic regression.

Number of predictor markers	Probability level entry	Resistant group error rate	Susceptible group error rate	Total error rate	Akaike Information Criterion
1	<0.01	0.07	0.20	0.13	26.90
2	<0.01	0	0.20	0.10	21.84
3	<0.01	0	0.07	0.03	15.35
4	<0.01	0	0	0	10.01
5	0.95	0	0	0	12.01
6	0.95	0	0	0	14.01
7	0.95	0	0	0	16.01

resistance. Standard ELISA assays are not reliable for this purpose because sweetpotato contains large, variable quantities of interfering substances such as phenols, latex, and polysaccharides (Esbenshade and Moyer, 1982; Abad and Moyer, 1992). The use of real-time quantitative PCR (Kokkinos and Clark, 2006a) will be helpful in determining the concentration levels of viruses between different genotypes and provide a better understanding of virus titer characteristics associated with resistance.

The genotypes were graft inoculated with scions infected with SPFMV and SPCSV (East African serotype) from a Kenyan field. However, there are different strains of SPFMV (Cali and Moyer, 1981; Kreuze et al., 2000) and different serotypes of SPCSV (Alicai et al., 1999; Hoyer et al., 1996; Vetten et al., 1996) in different regions of the world. The different strains and serotypes have biological significance in as much as resistance to SPVD is concerned. Genotypes described as resistant to SPFMV in Peru were susceptible in East Africa (Gibson et al., 1998; Karyeija et al., 1998b). Genotypes resistant to SPVD in Nigeria where the West African serotype of SPCSV predominates were susceptible to SPVD in Uganda where the East African serotype is predominant (Alicai et al., 1999). The reaction of genotypes used in this study may therefore be limited to strains and serotypes common in East Africa.

The aim of our study was to identify a combination of molecular markers that could be used to assign individuals to resistant and susceptible groups, and to verify the predictive power of the selected markers or model. The analysis of molecular variance was significant, indicating statistical differences between the two pre-defined phenotypic groups, and thus we could determine which markers discriminate between the two groups. Application of discriminant and logistic regression analysis to a molecular marker data set enables one to determine which markers discriminate between groups and then use the information to predict group membership. Four markers, which gave 100% correct classification of the two groups in the training population, were identified by both discriminant and logistic regression. While comparing discriminant analysis and logistic regression, Mcharo (2005) reported that the marker variables selected by the two techniques differed, and that logistic regression was more accurate. In our case, the two methods selected the same four critical markers, suggesting that the markers selected have a strong association with the phenotypic traits. There is a likelihood that the markers identified in this study are linked to the genes responsible for resistance and susceptibility to SPVD. Capdevielle (2001), while investigating the linkage between marker assisted classification and differential response to rice sheath blight disease, noted that identified markers are associated with QTLs responsible for expression of this trait. Accuracy was slightly compromised when classification models were based on two or three markers. These results are consistent with previous studies (Mcharo, 2005).

In the present study, 17 genotypes were assigned a resistance/susceptibility rating based on a 4-marker model. Only one genotype (Mugande), which was initially grouped as susceptible was not classified correctly and was instead grouped as resistant. It is possible that the classification criteria may misclassify genotypes at low frequency. Second, the nature of the

resistance is yet to be established, i.e., whether it is due to tolerance or true resistance, due to the effect of different strains of viruses, or the presence of other viruses. Previous reports showed *Sweet potato mild mottle virus* (SPMMV), *Cucumber mosaic virus* (CMV), and *Sweet potato mild speckling virus* (SPMSV) may enhance symptom development of other viruses (Cohen and Loebenstein, 1991; Di Feo et al., 2000; Mukasa et al., 2006). The presence of other viruses could result in reduced or delayed symptom development and remission (C. Clark, unpublished data). Any combination of these factors may result in phenotypic misclassification. Still our classification method was 94% accurate.

Mwanga et al. (2002a) reported that resistance to SPCSV and SPFMV was mediated by two major (but separate) recessive genes. These results were based on QTL analysis of progeny derived from a cross between two SPVD resistant genotypes. They further observed that some QTL might be associated with resistance to both viruses; however, no genes were identified for SPVD resistance given the paucity of SPVD resistant progeny in the population. Mcharo et al. (2004) noted that the power of discriminant and logistic regression analysis is that one doesn't need a parent-progeny population, unlike using QTL analysis, which needs closely related individuals. However, the genes controlling resistance to SPVD associated with markers identified in this study may be working in a similar manner to those identified by Mwanga et al. (2002a).

The fact that only one genotype from the test group could not be correctly classified when using the 4-marker model means that the identified markers are strongly associated with the phenotypic traits and have potential in selection of putative SPVD resistant genotypes in East Africa. Our understanding of host-plant resistance can also be furthered by understanding the behavior of the two viruses in core collection genotypes.

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CHAPTER 6: SUMMARY AND CONCLUSIONS

Sweetpotato has emerged as one of the most important subsistence crops in many parts of the African continent, especially in the countries surrounding Lake Victoria. The crop is mainly grown by female farmers on poor, marginal soil and harvested peace-meal to meet the daily food needs of the household. Sweetpotato is considered an important crop for poverty alleviation in the region and any effort towards improving its production will have a direct effect on hunger, malnutrition, disease, and any other dimension of under-development facing poor and small-scale farmers. Virus diseases are ranked among the greatest challenges to sweetpotato production, and methods to manage the diseases are ranked among the most important crop management needs in East Africa. The research and results described in this study relate to different aspects of resistance to the causal agents of sweet potato virus disease (SPVD) and to a survey carried out in Kenya to detect the presence of three sweetpotato viruses that were not detected before in the region. These have a direct bearing on the control of sweetpotato virus diseases in the region.

An important aspect to the management of virus diseases is detection and identification of viruses endemic in a particular region, and understanding how the complex of viruses interacts with the plant host. Discussions at the International Workshop of Sweetpotato Cultivar Decline in Mijakonojo, Japan in 2002 noted that three major groups of viruses infecting sweetpotato should be given detailed attention. These viruses include the *Potyviridae* family, the *Closteroviridae* family, and the *Geminiviridae* family. Despite the recommendations, previous surveys in Africa did not include geminiviruses in their studies. The geminiviruses reported infecting sweetpotato in different parts of the world belong to the genus begomovirus and are transmitted by whiteflies. Recent changes in whitefly populations and the importance of

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whitefly-transmitted viruses in different crops require that begomoviruses be given more attention. Also, surveys carried out in East Africa report on sweetpotato plants with typical virus symptoms but with no virus identified, an indication that more viruses may be infecting sweetpotato in the region. A systematic study was therefore needed. One objective was to survey for *Sweet potato virus G* (SPVG), Sweet potato virus 2 (SPV-2) and *Sweet potato leaf curl virus* (SPLCV), two potyviruses and a begomovirus reportedly infecting sweetpotato in other parts of the world but not reported in East Africa. An important finding from this study is the report of SPLCV infecting sweetpotato in the region, and that SPV-2 and SPVG have so far not been detected in the East African region. The presence of a geminivirus infecting sweetpotato complicates the efforts to develop sweetpotato resistant to viruses in that the begomoviruses may cause significant yield reductions without inducing symptoms. Considering the number of samples that were collected from the field, the percentage of plants infected with SPLCV was noted to be fairly high. I recommend that efforts be made to evaluate the economic importance of the virus in the region, that specific begomovirus test procedures be incorporated into sweetpotato virus indexing protocols, and that quarantine regulations be followed to avoid the introduction of SPVG and SPV-2 to the East African region.

Different viruses infecting sweetpotato interact resulting in disease complexes that are more severe than when infecting alone. The most severe disease is SPVD, caused by a combination of *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV). Some sweetpotato genotypes are reported to localize the distribution of viruses, have low virus titers, or to recover from infection by SPFMV or by SPVD. Recovery refers to a situation where a plant is initially susceptible to a virus and is systematically infected showing typical symptoms, but the new leaves, which develop later, are symptomless, and may contain

low virus titers or are virus free. Farmers in East Africa routinely select the youngest shoots and may avoid transplanting infected cuttings. It was necessary to establish if this phenomenon can be used as a basis to advise farmers on which section to use for propagation. From my studies, no significant differences were observed between vine sections, and I concluded that the differences may be due to variation in resistance between different genotypes rather than to differences between sections. My data does not support the hypothesis that one can reliably escape virus infection by selecting young, symptomless shoots. However, more studies should be done to compare the symptomless cuttings from the field with pathogen-tested plants. Previous research has implied that unique environmental conditions aided recovery and quick growth of shoots. However, no data substantiates this hypothesis. More should be done to study the behavior of viruses in genotypes showing recovery. One such genotype that I recommend for such studies from the genotypes screened from the Kenyan germplasm is 'Nyandere', a local landrace.

Previous studies used symptom development and serology to identify resistant and susceptible genotypes. These methods could not differentiate if resistance was associated with resistance to symptom development or to reduced virus multiplication. The use of real-time quantitative PCR was helpful in studying the accumulation and distribution of viruses in sweetpotato, making it possible to characterize the response of different genotypes to dual infection by SPCSV and SPFMV. Previous studies have indicated the role of SPCSV as primarily inducing synergism with SPFMV, with the titer levels of SPCSV remaining constant or decreasing with time. The results from this study indicate that multiplication of both viruses is important in disease development, and that resistant genotypes inhibit the multiplication of either or both of the viruses. When both viruses reach a certain threshold, they synergize and symptoms begin to appear even in the previously resistant genotypes. To compliment the results from real-

time PCR, there is a need to study the replication of viruses in individual cells. Green fluorescent protein (GFP) has been used successfully to monitor viral infection in different genomes. Tagging a virus with a reporter gene such as GFP is particularly useful in understanding resistant mechanisms, virus replication, and cell-to-cell and systemic movements of viruses. GFP-tagged recombinants of SPFMV and SPCSV should therefore be developed to assist in further studies involving cellular mechanisms of resistance in sweetpotato.

Considering the economic status of the people involved in sweetpotato production in sub-Saharan Africa, use of resistant genotypes in the management of virus diseases will remain the most viable strategy. There is a high diversity of sweetpotato genotypes grown in the East African region, many of which show different levels of susceptibility to virus diseases. Farmers select for resistant genotypes and thus mitigate the yield losses due to the virus infections. There is need to characterize, conserve and utilize this diversity to improve resistance. Such efforts can be greatly enhanced by marker assisted selection. Previous work identified two different molecular markers associated with SPFMV and SPCSV, respectively. However, synergistic interaction of the two viruses to induce SPVD is the problem, and not single virus infections. Identifying sufficient numbers of SPVD-resistant genotypes from a segregating population is difficult, but necessary using traditional genetic map making approaches. There was therefore a need to identify genotypes resistant to SPVD and their associated molecular markers. A germplasm collection maintained by the Kenya Agricultural Research Institute (KARI), was utilized to identify resistant genotypes. Using discriminant and logistic regression analysis, I was able to select four molecular markers associated with SPVD resistance that could be useful to sweetpotato breeders. I recommend that molecular markers identified in this study be developed

into primers that can be used in quick and easy PCR assays to identify genotypes resistant to SPVD.

The research carried out in this dissertation used grafting to inoculate different genotypes with viruses. The method is less time consuming and it is easy to know when an inoculation is successful through the survival of the graft. However, the challenge to the grafted plant is greater than what occurs in the field, which may result in genotypes with usable field resistance being discarded. Studies should be done to compare insect transmissions with graft inoculations during challenge trials. Also, there has not been a unified way of using graft inoculations in virus studies in sweetpotato, and different people and/or institutions use very different approaches. Some use a side graft; others use a top graft, while some use both. The assumption has been that the movement of different viruses towards the growing shoot or towards the base is the same. There is a possibility that this may not be the case, and a study should be carried out to establish if this is so. Inoculations with different viruses to induce diseases such as SPVD also vary. From our studies, it seems that disease development is dependent on the titers of viruses synchronizing at a certain threshold, and the sequence of infection is therefore an important factor that should be studied further.

Propagation of sweetpotato in sub-Saharan Africa relies on cuttings from previous crops. This system of production has its challenges in that pathogens are transmitted from one crop to another, resulting in great losses. In periods of drought or flooding, the crops in the fields may be lost, meaning that there will be no plant source of propagation material. Findings from my studies indicate that disease symptoms and virus titers increased when plants were cut back. This may be due to changes in the physiology of the plant. The findings are important in the tropics since more than one cutting per crop may be obtained in a field for propagation. One possible

alternative is the use of root beddings to generate cuttings for propagation, a method that is common in the United States and other parts of the world but not in tropical regions. Together with being a good source of propagation material, there is a possibility that the roots may have low virus infections, or that the shoots from roots may 'outgrow' the virus and produce clean planting materials, especially when using genotypes showing resistance or irregular virus distribution. The significance of using roots from SPVD-infected roots compared to vine cuttings in the management of SPVD has not been investigated. Also, the effect of cutting back the plants on the virus multiplication and on symptom development should be further studied.

A recent effort towards managing SPVD is the use of biotechnology to develop a transgenic sweetpotato. Initial work concentrated on resistance to SPFMV though attention is now being directed towards developing genotypes conferring resistance to SPCSV since it is the virus that synergizes with other viruses. Though a promising strategy, resistance by the local communities to the use of genetically modified crops is still high. Genetic resistance will therefore continue to play an important role in virus disease management. From the results reported in this study, and work carried out by other scientists, it is clear that the high diversity of sweetpotato genotypes in East Africa is useful in understanding and developing genotypes with natural resistance to virus diseases and especially SPVD.

The following are specific recommendations for future research based on results obtained from this study:

1. Further studies should be carried out in East Africa to determine the diversity, distribution and economic significance of begomoviruses infecting sweetpotato in the region.

2. Previous characterization of sweetpotato genotypes for resistance to SPVD was not done systematically, and our characterization is in no way comprehensive. As an effort to utilize the diversity in sweetpotato genotypes, a comprehensive characterization and conservation for virus resistance should be initiated. The molecular markers identified in this study should be developed into PCR primers that can be used in rapidly screening germplasm for resistance to SPVD in the early stages of selection.

3. The possibility of tagging SPFMV and SPCSV with reporter genes such as the GFP gene should be explored. The use of such recombinants would greatly complement other techniques such as real-time quantitative PCR and marker assisted selection in screening for resistant genotypes.

4. Environmental and/or climatic conditions favoring recovery of previously virus infected sweetpotato genotypes should be investigated. If identified, particular localities and periods of the year when such conditions occur can be utilized to generate cuttings for propagation.

5. Since farmers in tropical regions use cuttings for propagation, the effect of cutting back the plants on the virus multiplication and on symptom development on different sweetpotato genotypes should be investigated.

6. The value of storage roots from virus resistant genotypes in virus disease management and in generating high quality cuttings should be investigated.

7. Grafting as a method of inoculation should be standardized and compared to more natural means of infection.

APPENDIX 1: DATA ANALYSIS PROTOCOLS

A) Analysis for field data on virus distribution (Chapter 3)

dm'log;clear;output;clear';
TITLE 'Total of sections with at least one virus';
Data one;
Input Genotype $ Section Y @@;
cards;

1	1	3
1	2	4
1	3	2
2	1	10
.		
.		
.		
8	3	1
9	1	0
9	2	0
9	3	0

;

proc GLM; class Genotype Section;
model Y=Section Genotype;
random Genotype Section;
lsmeans Genotype/stderr pdiff;
lsmeans Section/stderr pdiff;
means Genotype/ tukey bon lsd;
means Section/ tukey bon lsd;
run;

B) Analysis for virus response data (Chapter 4)

dm "output;clear;log;clear";
Title1 "Virus Response data Analysis";
Data Virus;
Input Number $ Rep $ Genotype $ Time $ Position $ CSV FMV @@;
Datalines;

1	1	Bx	1	1	0.0000000000	0.000000000
2	1	Bx	1	7	0.000046739	0.000000000
3	1	Bx	1	13	.	.
4	1	Bx	1	19	.	.
5	1	Bx	1	28	.	.
6	2	Bx	1	1	0.000000000	0.000000000
7	2	Bx	1	7	0.000002284	0.000000065

```
8       2       Bx      1       13      .               .
9       2       Bx      1       19      .               .
10      2       Bx      1       28      .               .
11      3       Bx      1       1       0.000000000     0.000000000
.
.
.
399     4       Nm      5       19      0.034915223     0.000080258
400     4       Nm      5       28      0.001094889     0.000282395
;
Run;
/*Proc print data=virus;
run;*/

proc GLM data=virus;
class Genotype Time Position;
model CSV = Genotype Position Time Genotype*Position Genotype*Time Position*Time
Genotype*Position*Time;
means Genotype*Position*Time;
run;

proc Mixed data=virus;
class Genotype Time Position;
model CSV = Genotype Position Time Genotype*Position Genotype*Time Position*Time
Genotype*Position*Time;
random Genotype;
lsmeans Genotype*Position*Time;
run;

Proc GLM data=virus;
Class Genotype Time Position;
Model FMV = Genotype Position Time Genotype*Position Genotype*Time Position*Time
Genotype*Position*Time;
Means Genotype*Position*Time;
run;

proc Mixed data=virus;
class Genotype Time Position;
model FMV = Genotype Position Time Genotype*Position Genotype*Time Position*Time
Genotype*Position*Time;
random Genotype;
lsmeans Genotype*Position*Time;
run;

proc univariate data=virus normal plot;
var CSV;
```

```
run;

proc univariate data=virus normal plot;
var FMV;
run;
quit;
```

Analysis on cutback data

```
dm "output;clear;log;clear";
Title1 "Cutback data Analysis";
Data Cutback;
 Input Number $ Rep $ Genotype $ Cutback $ CSV FMV @;
 Datalines;
1      1      Bx     1         0.038024469  0.025648175
2      2      Bx     1         0.005699298  0.004910208
3      3      Bx     1         0.008257953  0.002282768
.
.
.
30     2      Nm     2         0.049893665  0.003012131
31     3      Nm     2         0.045062523  0.061853541
32     4      Nm     2         0.018262145  0.002991324
;
Run;
Proc print data=cutback;
run;

proc GLM data=cutback;
class Genotype Cutback;
model CSV = Cutback Genotype Genotype*Cutback;
means Cutback Genotype Genotype*Cutback;
run;

proc GLM data=cutback;
class Genotype Cutback;
model FMV = Cutback Genotype Genotype*Cutback;
means Cutback Genotype Genotype*Cutback;
run;

proc Mixed data=cutback;
class Rep Genotype Cutback;
model CSV = Cutback Genotype Genotype*Cutback ;
Random Rep Cutback*Rep;
Lsmeans Cutback Genotype Cutback*Genotype;
run;
```

proc Mixed data=cutback;
class Rep Genotype Cutback;
model FMV = Cutback Genotype Genotype*Cutback ;
Random Rep Cutback*Rep;
Lsmeans Cutback Genotype Cutback*Genotype;
run;

Proc univariate data=cutback normal plot;
var CSV;
run;

Proc univariate data=cutback normal plot;
var FMV;
run;
quit;

C) Analysis for Molecular markers data (Chapter 5)

AMOVA Protocol

1. For analysis of dominant marker data generated by techniques such as AFLPs and RAPDs, AMOVA-PREP and WINAMOVA programs are used.

2. AMOVA-PREP prepares input files for Analysis of Molecular Variance (AMOVA) using WINAMOVA. Use of AMOVA-PREP program is a four step process

a) Load or enter a dominant marker data set into AMOVA-PREP's text editor.
    In cases where a large data set is being analyzed, it may be easier to initially enter the data into a spread sheet program and export the data as ASCII text file that can subsequently be loaded into AMOVA-PREP.

b) From the 'Make Files' menu of the text editor, select either 'Two-level' or 'Three-level', depending on the nature of the data set.
    For example, in a two level data set like the one used in our analysis of molecular markers (analysis of individuals within populations), data for each observation/individual in the data set is placed in a single line, with the first character of each line being a number that identifies the population from which the individual was sampled, as shown below

47 93
 1 0
1, 1,1,1,1,1,1,1,1…….
1, 0,1,1,0,0,1,0…….
2, 1,0,0,0,1,0,0…….
2, 0,0,0,0,1,1,1……..

The first line includes information on the number of rows (observations) and the number of columns (number of markers) in the data set. The second line of the data set gives information on the codes for the presence or absence of a marker.

c) A new window will appear. From within this window, decide on the distance coefficient to be calculated. Three different distance coefficients are supported by AMOVA-PREP: the Euclidean metric, the non-Euclidean metric, or a distance based on the simple matching coefficient.

d) From within the same window, specify the names of the output files that AMOVA-PREP will generate. Provide a name for the distance file (*.dis), the group file (*.grp), and a generic population file name. The files created by program can then be analyzed by WINAMOVA.

3. From WINAMOVA program,
a) Select the files prepared by AMOVA-PREP through a dialogue window activated by the **File/Select Input files** menu.

b) Modify the AMOVA **settings** according to your need (for more details on what settings to use, see the help function of WINAMOVA).
c) Run an analysis by activating the **Go!** menu. Analysis may be interrupted by pressing on the **Stop!** Menu.

d) When the analysis is over, the results appear in a **result window**. The content of this window may be saved by activating the menu **File/Save as**…..

Discriminant and logistic regeression analysis

```
dm'log;clear;output;clear';
data SPVDresis;
input Clone $ cag057 ... ctt706 Group;
cards;
bx     1     0     ...     0
k43    0     1     ...     0
.
.
.
maria  0     1     ...     1
run;

Title1 'Stepwise discriminant analysis on SPVD markers';
proc stepdisc data=SPVDresis method=forward slentry=0.01;
class Group;
var  cag057 cag076 … ctt706;
run;

Title2 'Discriminant analysis on SPVD markers';
proc discrim data=SPVDresis testdata=SPVDresis method=npar k=1 crossvalidate testlist;
testid clone;
```

```
class Group;
var ... selected markers ... ;
run;

Title1 'SPVD resistance in sweetpotatoes';
Proc logistic data=SPVDresis descending outest=SPVDresisone;
class clone;
model Group=cag057 cag076 . . . ctt653 ctt706/ selection=forward slentry=0.01 lackfit
clparm=wald;
output out=SPVDresistwo predprobs=(individual crossvalidate);
run;
quit;
```

APPENDIX 2: PERMISSION LETTERS FROM JOURNAL PUBLISHERS

From: "Joseph Nyachiro" <Joseph.Nyachiro@gov.ab.ca>
To: "Douglas Miano" <dmiano1@lsu.edu>
CC:
Subject: Re: Permission to use published paper
Date: Monday, December 10, 2007 9:47:01 PM

Hello Douglas:

Is this paper published yet? If so, then go ahead and cite per standard operating procedure. If the paper is not yet published, then follow the procedure and proper notation.

Regards,

Joseph M. Nyachiro, PhD
Plant Breeder/Barley
Alberta Agriculture and Food
Field Crop Development Centre
5030 - 50 Street, Lacombe, AB T4L 1W8
Phone (403) 782-8692,   Fax (403) 782-5514
e-mail: joseph.nyachiro@gov.ab.ca
http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/fcd5615

"If you have less than 1 year to invest, plant barley. If you have 10 to 25 years to invest, build a house.
If you have more than 25 years to invest, invest in your children" - Anonymous.

Dear Dr Nyachiro
I wish to request for permission to use the publication cited below as part of my dissertation. I am a Ph.D student in Louisiana State University and i am the senior author of the paper.

Miano DW, LaBonte DR, Clark CA (2007) Identification of molecular markers associated with sweet potato resistance to sweet potato virus disease in Kenya. Euphytica: DOI 10.1007/s10681-007-9495-2

Euphtica will be cited.
Thank you very much.

Douglas Miano

VITA

Douglas Watuku Miano was born in May, 1970, in Murang'a District of the Republic of Kenya. After completing high school, he joined the University of Nairobi and graduated with a Bachelor of Science degree in agriculture. He was offered a scholarship to pursue a master's program in plant pathology in the same institution in 1996. After completing his studies, he started his professional career as a research scientist in the Crop Protection Program of Kenya Agricultural Research Institute (KARI), Kenya. He joined Louisiana State University to pursue a doctoral degree in Fall of 2003. He will receive the degree of Doctor of Philosophy during the Spring Commencement 2008.