ASSESMENT OF RESPONSES TO VIRUS INFECTIONS, BETA-CAROTENE, ZINC AND IRON IN ORANGE FLESHED SWEET POTATO (*Ipomoea batatas*, Lam).

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A thesis submitted to the Graduate School in partial fulfillment for the requirements of the Master of Science Degree in Biochemistry of Egerton University

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been submitted wholly or in part to any institution for award of any degree.

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DEDICATION

This work is dedicated to the children of Kenya and Africa as a whole.

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ABSTRACT

Sweet potato an ideal tuber crop due to its hardy nature and consistent performance even in stressful growing conditions. Viral diseases such as sweet potato virus disease (SPVD) complex caused by synergistic interaction of Sweet Potato Feathery Mottle Virus and Sweet Potato Chlorotic Stunt Virus are a major cause of yield losses due to lack of resistant varieties. Breeding, promotion and adoption of orange fleshed sweet potato (OFSPs) varieties can be enhanced by identification of virus tolerant clones. However, prerequisite information on changes in quantitative contents such as beta carotene, dry matter, iron and zinc due to virus infection is not well documented. This study aimed at screening ninety sweet potato clones for responses to sweet potato viruses, dry matter, micronutrients levels. Nitrocellulose membrane ELISA and Reverse transcriptase-PCR were used to detect sweet potato viruses and validate results obtained NCM-ELISA respectively. The virus free clones were selected and planted in a field with high SPVD pressure in Randomized Complete Block Design (RCBD) with three replications. Disease progression scores was scored on a scale of 1-9 after two months interval. Root dry matter, beta carotene zinc and iron were quantified. One-way ANOVA was used to compare the mean data in Genstat Version 2014.1. Thirteen clones from five families and four other genotypes were screened using conventional PCR for presence of Simple Sequence Repeats linked to SPVD resistance. Neighbor joining tree was generated using DARwin version 6.0.010 using Unweighted Pair Group Method with Arithmetic Means (UPGMA). XLSTAT 2015 version was used to generate Principal Component Analysis (PCA) while Genetic distance was computed using GenAIEx version 6.5. The test clones clustered in two groups separate from virus susceptible genotypes. Significant differences in beta-carotene, dry matter and iron among families ($P < 0.05$). There was negative correlation ($r=-.296^{*}$). A total of 18 alleles were detected with an average of 3.0 alleles per locus. Mean genetic diversity of the markers was 0.41. Pearson’s correlation coefficient revealed an average similarity of 0.54 among sweet potato genotypes. Further evaluation of SSR markers is proposed in order to identify markers that can be suitably used to analyze germplasm for various traits such as dry matter, micronutrients and weevil resistance. The study has identified two clones (F1C7 and F4C15) with dry matter, carotene, iron and zinc traits ideal for sweet potato improvement.

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ABBREVIATIONS AND ACRONYMS

AACC	American Association of Cereal Chemists
AAS	Atomic Absorption Spectrophotomer
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of variance
BCIP	5 Bromo-4-chloro-3-indolyl phosphate
CIAT	International Centre for Tropical Agriculture
CIP	International Potato Center
CMV	Cucumber mosaic virus
CTAB	Cetyltrimethylammonium bromide
dNTPs	Deoxyribonucleoside triphospate
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and Agricultural Organization of the United Nations
FAOSTATS	Food and Agricultural Organization statistics
IVMV	Ipomoea vein mosaic virus.
LSD	Least significant difference
NBT	Nitro blue tetrazolium
NCM-ELISA	Nitrocellulose membrane enzyme linked immunosorbent assay
NIR	Near infra red spectrophotometer
OFSP	Orange fleshed sweet potato
PTGS	Post Transcriptional Gene Silencing
PVP	Polyvinylpyrolidone
RC	Russet crack
RE	Retinol equivalents
RT-PCR	Reverse transcriptase polymerase chain reaction.
siRNAs	Small interfering Ribonucleic acid
SPCaLV	Sweet potato caulimo-like virus
SPCFV	Sweet potato chlorotic fleck virus
SPCSV	Sweet potato chlorotic stunt virus
SPFMV	Sweet potato feathery mottle virus
SPLV	Sweet potato latent virus
SPMMV	Sweet potato mild mottle virus
SPMSV	Sweet potato mild speckling virus
SPVD	Sweet potato virus disease

SPVY	Sweet potato virus Y
TBE	Tris borate ethylene diamine tetra acetic acid
UPGMA	Unweighted pair group method with arithmetic averaging
VAD	Vitamin A deficiency
WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background information.

Sweet potato ranks first as a key staple crop with the largest rates of production per unit area in a given period. Global annual production exceeds more than 133 million tons; sweet potato now ranks as the seventh most important food crop on a fresh-weight basis. The crop is ranked fifth in importance in over 50 developing countries after rice, wheat, maize, and cassava. Sweet potato is the third most economically important root crop after potatoes and cassava. Sweet potato is ranked fourth as a food crop in economic importance in the developing world after rice, wheat, and maize (Kays, 2005). The crop plays an important role in food security particularly during periods of drought in Africa (Karyeija *et al*., 1998a,b). In East and Central Africa, adverse effects of pests and diseases on production of maize, cassava and banana has increased importance of sweet potato (Otim- Nape *et al*., 2000; Tushemereiwe *et al*., 2004). Maximum utilization of this crop is yet to be realized and the International Potato Center (CIP, Lima, Peru) has been leading worldwide efforts to realize its full potential. The potential utility of sweet potato includes use as food, feed, processed products, and a source of income for millions of resource-poor farmers in developing countries (Mukasa, 2004). Some of the food utilization options include *chapatis* and *mandazi*, flour for porridge and making of sweet potato chips.

Sweet potato is referred to as a subsistence crop, food security or famine relief crop and its utilization has diversified significantly in the developing countries (Scott and Maldinado, 1999). It is particularly an important food security in the East Africa region and more so in Tanzania (Ndunguru *et al.,* 2000). Its cultivation and production is mainly carried out within the Lake Victoria basin where it is an important household food security item (Ndunguru, 2000). In this region, farmers grow different sweet potato cultivars such as the white, yellow, cream and light to deep orange fleshed types.

The most nutritious of cultivars grown in the Lake Basin region are orange-fleshed cultivars (OFSPs) that contain beta–carotene (Kapinga *et al*., 2009). The yellow and orange-fleshed sweet potato varieties provide a source of the vitamin A that has been reported to be deficient in many developing countries (CIP, 1999a,b).

1.2 Statement of the problem

In the past, little importance was placed on the Orange fleshed sweet potato (OFSP) varieties since sweet potato farmers had no prior exposure to these varieties. Significant and robust efforts in promotion of OFSPs to contribute to reduced Vitamin A deficiency coupled with change in farmers perception on the varieties has now led to improved acceptance amongst farmers. However, a major hindrance to the successful adoption of these new varieties is viral infections that infect these varieties coupled by low levels of resistance among existing varieties. Viral diseases impact negatively on production of sweet potato. Yield losses associated with viral diseases are estimated to be between 50-98%. The pathological effect of viral infection and the subsequent plant response to SPVD and the levels beta-carotene, iron, zinc and dry matter content has not been fully determined. This information is vital for development of new virus tolerant varieties with acceptable beta-carotene levels, micronutrients and dry matter content and subsequent propagation of these varieties with good agronomical and nutritional traits.

1.3 Justification

Despite its importance, sweet potato cultivation is constrained by several factors. Besides sweet potato weevils, the other main constraint in sweet potato production and marketing, improved breeding programs and safe movement of germplasm are viruses infecting the crop. Viral diseases occur in areas where sweet potato is cultivated. Since the crop is vegatatively propagated, accumulation, perpetuation and maintenance of viruses has become a major constraint for production.

The effect of sweet potato viruses on critical production and quality parameters such as root dry matter content, beta carotene, iron and zinc is not well understood.

In addition, whereas dry matter content has been studied on white Kenyan sweet potato genotypes after virus challenge with SPVD, there is no information on the effect of viruses on accumulation of dry matter and micronutrients content in orange fleshed sweet potato (OFSP). Orange fleshed sweet potato varieties such as Zapallo, Constanero and Naspot 5 among others are characterized by low dry matter coupled with low virus disease resistance; therefore, there is urgent need to deliberately identify other OFSPs with acceptable agronomic and root quality characteristics.

To alleviate Vitamin A deficiency which is a major health problem in most of the countries of sub- Saharan Africa; the pursuit for high beta carotene varieties with tolerance to viruses, acceptable dry matter and micronutrients levels is core. Beta- carotene content in OFSP varieties however varies significantly among genotypes and hence the need to evaluate clones from different families for this trait. The findings of the study will significantly contribute to understanding of the effects of sweet potato viruses on quality and quantity in OFSPs and contribute to alleviate micronutrient malnutrition.

1.4. Objectives

1.4.1 General objective

To contribute to increased adoption of orange fleshed sweet potato and reduce yield losses due to virus diseases infecting sweet potato.

1.4.2 Specific objectives

1. To screen OFSP clones for multiple virus infections reactions, root dry matter and micronutrients levels.
2. To determine possible correlation between virus disease severity score, beta-carotene levels, dry matter content , zinc and iron levels.
3. To determine presence of putative resistance traits by characterizing OFSP clones using SSR markers.
4. To determine molecular diversity of OFSP clones with putative resistance traits based on SSR markers.

1.5. Hypotheses

1. There is no difference in OFSP clones response to multiple virus infections reactions, dry matter and micronutrients levels.
2. There is no possible correlation between virus disease severity scores, beta-carotene levels, dry matter content and micronutrients.
3. There are no putative resistance traits among OFSP clones using SSR markers.

4. There is no genetic diversity among OFSP clones with putative resistance traits based on SSR markers.

1.6 Outputs

The following outputs are expected from this study.

1. Information on the effect of sweet potato viruses' on root β-carotene levels, dry matter content and levels micronutrients in OFSPs will be determined. At least one sweet potato clone with high beta carotene levels, dry matter content and micronutrients levels and virus tolerance identified.
2. At least two scientific manuscripts published in refereed journals.
3. Presentation of findings in scientific conferences.
4. A Master of Science degree in Biochemistry thesis.

CHAPTER TWO
LITERATURE REVIEW

2.1 Botany of sweet potato

In 1791, Lamarck classified sweet potato species within the genus *Ipomoea* on the basis of the stigma shape and the surface of the pollen grains**.** Sweet potato belongs to species *Ipomoea batatas* (L.) Lam. The sweet potato and strongly associated species are classified in the family *Convolvulaceae* (Morning glory), genus *Ipomoea*, sub-genus, *Eriospermum*, section *Eriospermum* (formerly *Batatas*) and series *Batatas* (Austin., 1988). Sweet potato chromosome number is 2n= 6x= 90. It is a hexaploid plant with chromosome number being x =5. Wild species of sweet potato are tetraploids with 2n =4x = 60. Diploid species also exist with 2n =2x = 30. Polyploidy species from *trifida* have 2x, 3x, 4x and 6x (Huamàn and Zhang, 1997). Sweet potato is a perennial dicot, which is however cultivated as an annual crop storage roots or tubers and vines which are used as seed and feed. Sweet potatoes are sensitive to photoperiod of 11.5 hours day length which promotes flowering, while at 13.5 hrs day light, flowering stops. However, the storage root yield is not affected (Kays, 1985). Root development is enhanced by short days with low light intensity. The crop has a complex sporophytic self-and cross-incompatibility which is a major drawback in breeding (Nakanishi and Kobayashi 1979).

2.2 Diversity of Sweet potato Germplasm

Sweet potato has enormous phenotypic and genotypic diversity which is evident in the size and shape of roots, leaves and branches, color of skin or flesh of the root, (Bhagsari and Brown., 1986). Differences among sweet potato are further found in the resistance to pests and diseases, depth of rooting, time span to maturity and even the flavor and texture of cooked roots (Huaman., 1992). Over 8,000 accessions of sweet potato are maintained in gene banks globally (Nissiläa *et al*. 1999). It is speculated that the accessions represent only a fraction of the existing diversity in the crop species (Chang., 1992). A total of 1157 wild accessions of series *Batatas* and 5,526 accessions of *I. batatas* are also maintained at the International Potato Center (CIP), Lima, Peru (Huaman and Zhang., 1997). Genetic diversity in sweet potato has been determined by using different morpho-physiological traits and molecular markers such as Simple Sequence Repeats (SSRs) (Gichuru *et al*., 2006).

2.3 Sweet potato production

Sweet potato production in Kenya has been on the increase. Area under cassava production in the year 2012 was 66,971 Ha compared to 58,509 Ha recorded in 2013, a decrease of 13%. Area under production recorded in 2014 was 61,067 Ha an increase of 4% compared to the year 2013 (AFA., 2014). Kenya ranks fifth in Africa after Uganda, Rwanda, Burundi and Tanzania in sweet potato production. Uganda is second in the world after China. The tubers store better when left in the ground making the crop ideal for piece meal harvesting (Tairo *et al*., 2005). Sweet potato yields satisfactorily in unfavorable climatic and soil conditions as well as little or no use of soil inputs (Carey *et al.,* 1999a,b). The crop matures in at least four months and provides food security by acting as a famine reserve crop (Mukasa *et al*., 2003). 1.4 Constraints of sweet potato production

Virus diseases infecting sweet potato have been reported wherever the crop is grown. Since the crop is vegetatively propagated, the viruses tend to accumulate leading to further perpetuation of the sweet potato virus disease (SPVD) which is currently the most important constraint in production (Valverde *et al.*, 2007). Sweet potato weevils (*Cylas puncticollis* and *C. brunneus)* in Africa, and *C. formicarius* in the US and other parts of the globe) and viruses have between associated with low yields in the crop (Clark *et al*., 1997; Fuglie, 2007). Yield losses of between 50-90% are realized due to viral infections (Gutierrez *et al*., 2003, Gibson *et al.,* 1997). Major sweet potato infecting viruses include: family *ipomoviridae* to which Sweet potato mild mottle virus (SPMMV) is a member; family *Closteroviridae* to which Sweet potato chlorotic stunt virus (SPCSV) belongs; family *Potyviridae*: a potyvirus Sweet potato feathery mottle virus (SPFMV) and the Sweet potato chlorotic fleck virus (SPCFV). Low yielding cultivars, short shelf-life, lack or shortage of high quality planting materials, limited processing and utilization methods and limited market outlets have been cited as other major production constraints.

2.4 Orange fleshed sweet potato

Sweet potato tuber colors range from orange, yellow, cream, white and purple fleshed. Orange-fleshed sweet potato varieties are rich in beta-carotene (Low *et al.*, 1996). Varieties introduced in Kenya include SPK 004 and Zapallo among others (Ndolo *et al*, 2007). The genotypes have the potential to contribute significantly to vitamin A in human diet. Micronutrient deficiency is common in many places in sub-Saharan Africa where sweet potato is grown (Carey *et al*, 1999). However, most sweet potato varieties currently grown in the region have either white or yellow flesh and contain low levels of beta-carotene

(Carey *et al.*, 1999). OFSP varieties which contain high levels of beta-carotene can be used to address vitamin A deficiency (Low *et al*., 1997).

Orange fleshed sweet potatoes arise from breeding populations which are selected for high β-carotene. Most breeding programmes for cultivars rich in β-carotene have used a classical approach of mass selection. Vitamin A deficiency (VAD) predisposes children to respiratory and diarrheal diseases, measles and malaria (SanJoaquin *et al*., 2009). The deficiency also impairs physical and mental development, vision and immune systems. Severe cases results in blindness and death (Ruel., 2001).

2.4.1 Vitamin A deficiency

At least 250 million children in the developing world have). In Kenya, the prevalence of moderate and acute VAD is 14.7% and 61.2% among children VAD. 230 million preschool children are estimated to be vitamin A deficient (IAEA, 2000). Vitamin A deficiency affects an estimated 127 million children worldwide. (World Bank, 2007 and 9.1% and 29.6% among mothers respectively (Hongo, 2003). Xerophthalmia, is the clinical form of vitamin A deficiency. The disease is characterized by eyes becoming severely affected resulting in night blindness or in most severe cases total, irreversible blindness take place. Xerophthalmia is reported to have affected about 3.1million children globally in 1995 (IVACG., 1995). Sub-clinical vitamin A deficiency affects more people and it is estimated that 227.6 million in 1995 were affected by this form of VAD (IVACG, 1995). Increased sickness and death rates are attributed to diseases such as diarrhea and measles among vitamin A deficient persons. HIV/AIDS is associated with vitamin A deficiency in developing countries (Semba *et al*., 1993; Courtsoudis., 2001).

Encouraging response has also been reported on the complications associated with HIV after vitamin A supplementation is administered (Gerawal *et al*., 1992).

In women VAD increases the risk of death during pregnancy, as well as giving birth to low weight infants. Vitamin A deficiency is a major public health setback in many developing countries, including most of the countries of sub-Saharan Africa (WHO., 1995). Vitamin A in foods is measured using retinol equivalents (RE) as a means of making comparisons among foods. Dietary requirements of vitamin A in humans vary with age and sex. Recommended daily allowance (RDAs) based on age and sex for 1 to 3 years is 400 μg RE, ages 4-6 years, 500 μg RE; 7-10 years, 700 μg RE; non-pregnant female over 10 years, 800

μg RE; and males over 10 years, 1000 μg RE (Williams and Worthington-Roberts., 1988). Orange-fleshed sweet potatoes are estimated to contain from 300 μg RE, to over 3,000 μg RE per 100g fresh weight which can sufficiently provide required RDAs while also providing a rich source of other vitamins and micronutrients (Woolfe., 1992).

2.4.2 Iron and Zinc malnutrition

'Africa is the only continent where child malnutrition is getting worse, rather than better', and that 'halving hunger in Africa by 2015, under international anti-poverty goals seems a distant fantasy' (Fuglie., 2005). Malnutrition is a significant factor accounting to 50% of deaths of young children in Sub Saharan Africa (Müller *et al.*, 2005).Vitamin A deficiency (VAD), iron deficiency anaemia (IDA) are key health concerns. Africa leads in iron deficiency at 65% followed by Asia (47.7%) and Latin America (39.5%) iron deficiency anaemia (McLean *et al.,* 2009). Four general categories of proteins contain iron: (1)mononuclear iron proteins (e.g., superoxide dismutase), (2) diiron-carboxylate proteins (e.g., ribonucleotide reductase, ferritin), (3) iron-sulfur proteins (e.g., aconitase), and (4) heme proteins (e.g., hemoglobin).

Of these four categories, the first three protein groups are typically found in lower levels than hemoglobin, but they are functionally essential. Hemoglobin is the richest iron-containing protein in humans with more than one-half of total-body iron is contained within hemoglobin. Based on the location of hemoglobin in erythrocytes, anemia is a characteristic trait of iron deficiency and iodine deficiency disorders. Erythrocytes and their precursors require large amounts of iron for the production of heme and hemoglobin. Iron is central to hemoglobin structure and its ultimate function (Perutz., 1982).

The most instant iron source for erythroblasts is mono- or diferric transferrin, found in high concentrations in the plasma. Iron deficiency anemia (IDA) is usually associated with low iron saturation of available transferrin. Iron is loaded onto diferric transferrin from three sources: the gut through dietary sources, macrophages as recycled iron, and the liver as stored ferritin iron. Generally, iron stores become reduced or lost before the person develops anemia. This implies that dietary and erythrocyte-recycled iron must meet the demands for erythrocyte production. When iron losses continue, the newly produced erythrocytes are associated with decreased hemoglobin, leading to the amount of iron provided by the same number of senescent erythrocytes to be reduced in number. In

contrast to thalassemia trait, increased numbers of erythrocytes are not produced in the iron-deficient state to compensate for the reduction in intracellular hemoglobin content. For this reason, reticulocytosis is generally not present. Without major hemorrhage, iron deficiency anemia generally develops slowly over the course of months or years. Resolution of iron deficiency (IDA) are key public health concerns globally (ILSI/FAO., 1997).

On the other hand, Zinc is required in most as cellular metabolism reactions (Ruz., 2003); The micronutrient plays major functions among them forming the prosthetic group of various enzymes and as the receptor proteins for steroid and thyroid hormones and vitamins A and D (Bender *et al*.,1999). Since zinc in surplus of short-term metabolic needs in the body, it is either not absorbed or excreted.

Human beings live with very marginal zinc nutrition (Solomons, 2003); it is clearly obvious that insufficient zinc in the diet will result to have undesirable consequences. Zinc malnutrition associated with a number of symptoms, including gastrointestinal problems such as diarrhea and impairment of nutrient absorption, impaired immunity, psychosis, anorexia and possible depression. Other manifestations of zinc deficiency include impaired growth and development; altered reproductive biology; (Solomons, 2003). In children, zinc deficiency leads to slow and stunted growth and the delay in sexual maturity. Zinc deficiency has been reported to contribute to Vitamin A deficiency, since lack of zinc impairs the synthesis of Retinol binding protein (Bender *et al*., 1999). Vitamins and minerals are often adversely lacking in human diets, especially vitamin A, iron and zinc. About 25% of pre-school age children have vitamin A deficiency and a further 37% and 49% of the total world population is affected by low iron and zinc intake, respectively.

Some foodstuffs either interfere with or promote the uptake of zinc and iron in the human diet. Promoters and inhibitors play an important dietary role in areas where micronutrient nutrition is already low. (Raboy, 2002) Two key inhibitors of iron and zinc uptake are phytic acid and oxalic acid. Phytic acid is the key means of phosphorus storage in some food crops including cereal grains, particularly in legumes (Bender *et al*.,1999). Marginal zinc deficiency has been found to be widespread in people who maintain diets rich in legumes (Cichy, *et al.,* 2005). Another inhibitor, oxalic acid, is found in spinach and nuts; in large amounts, it accounts for the toxicity of rhubarb leaves (Bender *et al*., 1999). Two important promoters of iron and zinc are ascorbic acid and beta -carotene. Ascorbic acid, or

vitamin C, is a rich antioxidant commonly found in fresh fruits and vegetables. beta-carotene, a precursor of vitamin A is found in green and orange vegetables (Welch., 2002).

Impact assessment indicated that orange-fleshed sweet potato (OFSP) can be used to contribute in alleviating iron and zinc malnutrition (CIP., 2001). Locally available vitamin A-rich foods that can be grown in home gardens should be promoted as compared to supplementation since it's technically viable and cost-effective. Orange-fleshed sweet potato are an appropriate crop for this approach (Low *et al*., 1997; Woolfe., 1992). While white- to yellow-colored sweet potato have been reported to contain little or no beta-carotene; whereas, orange-fleshed sweet potato contain substantial quantities.

There is a high relationship between color and hue values of orange-fleshed varieties, as measured using color difference meters, and their beta-carotene content (Simonne *et al*., 1993).

Iron, zinc, and vitamin A are indispensable to the human diet; the large populations globally experience health consequences associated with inadequate intake (Cichy *et al*., 2005). Efforts to overcome these shortages include fortified, processed foods to vitamin supplements. Recent interest is to complement the known caloric and carotenoid contributions of sweet potato to the diet by improving iron and zinc concentrations (Courtney *et al*., 2008). There is growing attention in developing genotypes that are high yielding from indigenous wild plants with resistance to drought and heat, which are rich sources of iron, zinc and beta–carotene (Malaisse and Parent., 1985., Becker., 1986; Kim and Oh., 1996) This is due to ease in cultivation and acceptance by local rural communities (Shrimpton, 1995). Plant-breeding has also been employed to produce new varieties of orange-fleshed, sweet potato, cassava roots with increased contents of iron, zinc and/or beta-carotene (Hagenimana *et al*., 1999). Further efforts such as advances in plant virus diagnostics in sweet potato combined with biochemical analysis of effects of viruses will give additional insights on how to increase bioavailability of beta-carotene, zinc and iron.

2.5 Beta carotene biosynthesis and its importance

Carotenoids are among diverse group of over 600 structures derived from isoprenoids. Carotenoids are 40-carbon isoprenoids, which consist of eight isoprene units. Carotenes are hydrocarbons that occur as linear or cyclized at one or both ends of the molecule. Derivatives of carotenes that are oxygenated are referred to as xanthophylls

(Wurtzel.,1992). The polyene chain consists of conjugated double bonds is responsible for their characteristic colors and photochemical properties associated with carotenoids They are synthesized by all photosynthetic organisms, some bacteria, and fungi as secondary metabolites. In plants, carotenoids are important for growth and development and inhibiting mutations. Carotenoid accumulation have pleiotropic effects on chloroplast biogenesis and seed development (Robertson *et al.*, 1978; Wurtzel.,1992). Carotenoids primarily function as accessory pigments in photosynthesis, preventing photo oxidative damage, and as precursors of abscisic acid (Hirschberg., 2001).

Occurrence of carotenoids in plant endosperm provide additional nutritional value. In humans, carotenoids in diets are essential precursors to vitamin A and to retinoid compounds needed in development (Bendich and Olson, 1989). Non provitamin A carotenoids, such as zeaxanthin, lycopene and lutein play beneficial roles in human health (Sommerburg *et al.*, 1998; Krinsky *et al.*, 2003). Traditionally, carotenoids have been used commercially as food additives, including as colorants, antioxidants, and vitamins (Knekt *et al*, 1991; Rao and Agarwal., 1999). Their ability to protect against oxygen free radicals has resulted in their use in some therapeutic applications as degenerative disease preventives, anti-cancer agents, and immune system stimulators (Ben- Amotz and Avron, 1983; Borowitzka., 1992; Amstrong., 1997).

In plants, the biosynthesis of carotenoids occurs on membranes of chloroplasts, chromoplasts, and amyloplasts. Carotenoids are synthesized within the plastids from the central isoprenoid pathway, which also serves the synthesis of other important compounds, such as terpenes, squalene, gibberellins, sterols and phytol. Isoprenoids arise from a 5-carbon compound isopentenyl diphosphate (IDP). The major source of IDP in plastids is from intermediate and final product of glycolytic pathway: pyruvate and glyceraldehyde-3-phosphate and not from acetyl-CoA via 3- hydroxy-3-methylglutaryl-CoA and mevalonic acid pathway as previously suggested (Litchenthaler *et al*, 1997). IDP isomerase catalyses the IDP isomerization to dimethylallyl diphosphate. The activity of IDP isomerase has been reported to be a rate limiting step in yeast and bacteria. And, therefore, it is also suspected that the enzyme could have the similar activity in plants (Sun *et al.*, 1996; Kajiwara *et al.*, 1997). Addition of three IDP molecules is carried out sequentially into dimethylallyl diphosphate in a reaction catalyzed by geranylgeranyl diphosphate (GGDP) synthase, and produces the 20-carbon molecule GGDP.

Five isoforms of GGDP synthase, each encoded by a different gene exist. The initial committed step to the carotenoid pathway is condensation by head to head of two GGDP molecules to produce phytoene in a reaction catalyzed by the phytoene synthase enzyme. Introduction of four double bonds converts phytoene to lycopene. This is carried out in plants by two enzymes, phytoene desaturase (PDS) and z-carotene desaturase, each catalyzes two symmetric dehydrogenation steps to yield z-carotene and lycopene, respectively. These enzymes bind Flavin adenine dinucleotide (FAD) while plastoquinone acts as an electron acceptor. The cyclization of lycopene is an important branching point in the pathway. One route leads to β-carotene and its derivatives of xanthophylls which include zeaxanthin, violaxanthin and neoxanthin. The alternative pathway leads to carotenoids with one β -ring and one α-ring, such as a-carotene and lutein, which is mainly xanthophylls in the leaves of many higher plants. All carotenoid biosynthesis genes in plants and algae are nuclear encoded and their polypeptide products are imported to the plastids. Concentration and composition of leaf xanthophylls in chloroplasts are affected by light intensity and the accumulation of specific carotenoids in chromoplasts of fruits and flowers is developmentally regulated. In tomato, for example, total carotenoid concentration increases between 10- to 15-fold during fruit ripening, due mainly to a 500-fold increase in the concentration of lycopene (Fraser *et al.*, 1994).

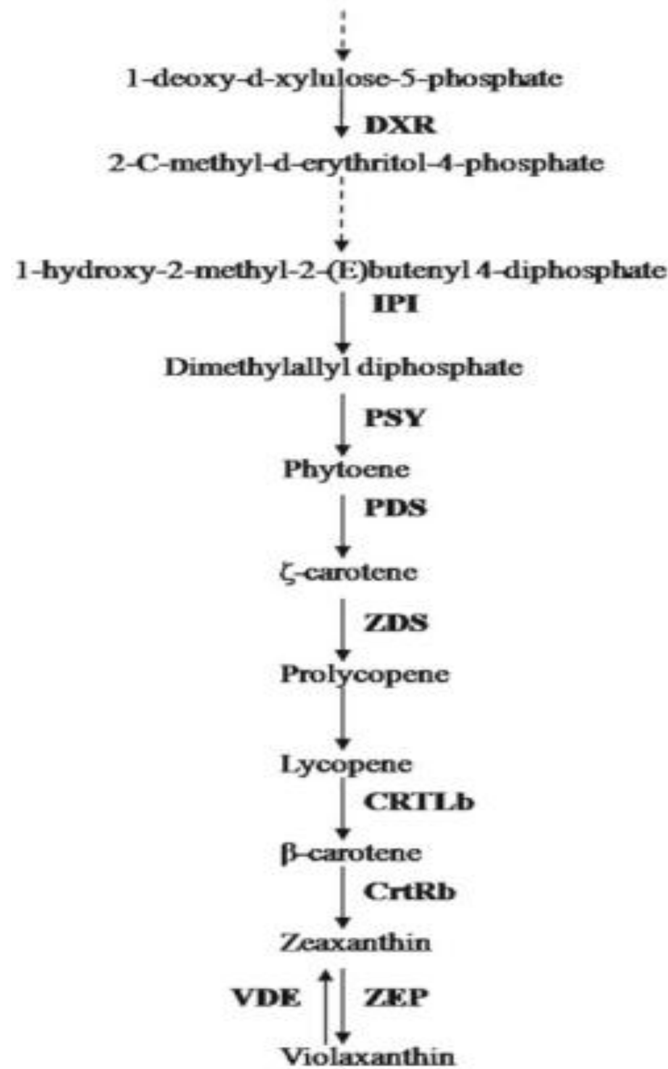

Figure 1: General carotenoid biosynthetic pathway. (Source: Smita *et al*., 2013)

2.6 Dry matter and micronutrients in sweet potato

Sweet potato has higher moisture content when compared to other tuber crops. This results in low dry matter content with an average of 30% (Bradbury and Halloway., 1988). Increase in water stress has been reported to result in an increase in root dry matter content. Dry matter production increases with increasing soil temperatures of between 20 - 30° C and declines in temperatures above 30° C (Woolfe., 1992).

Table 1: The composition of biomolecules in fresh sweet potato in percentage dry matter. (Woolfe 1992).

Constituent	**% dry matter**
Starch	70
Sugars	10
Total proteins	5
Lipids	1
Total fibre (Non-starch polysaccharide and lignin)	10
Vitamins, organic acids and other components in low concentration	<1

Orange fleshed sweet potato varieties with increased β-carotene, dry matter are required to promote their adoption and commercialization (Cervantes-Flores *et al*., 2011; Mwanga *et al*., 2011).

In sub-Sahara Africa, small-scale farmers prefer sweet potato varieties that have a high dry matter content (Cervantes-Flores *et al*., 2011). A dry matter content >25% is an important component for acceptability of newly developed varieties by farmers (Shumbusha *et al*., 2010). Dry matter content has been reported to vary due to factors such as variety, location, climate, incidence of pests and disease severity and soil types (Manrique and Hermann., 2000; Vimala and Hariprakash., 2011). It has been reported that narrow sense of heritability estimates of dry matter content range from 0.65 to 0.92.

In addition, transgressive segregation for dry matter content has been indicated in sweet potato progenies (Cervantes-Flores *et al*., 2008). Genetic studies on the crop and the existence of numerous enzymes involved in starch biosynthesis indicate that dry matter content show quantitative inheritance (Cervantes-Flores *et al*., 2008). Jalal *et al*., (1998) demonstrated that carotene rich orange fleshed sweet potato supplied for 3 weeks to a group of individuals in Indonesia resulted in increased blood serum amount of retinol. The analysis of different mineral elements has become more relevant, predominantly because of the emerging evidence of a synergistic effect between carotene, iron and zinc in diets.

Garc´ıa-Casal *et al*., (1998) demonstrated that vitamin A and beta-carotene increased iron absorption from different sources. Vitamin A has been shown to contribute increasing hemoglobin content (Kolsteren *et al*.,1999, Mwanri *et al*., 2000). Knowledge on nutritional quality attributes of African sweet potato germplasm is inadequate. However, it is known that the average storage root dry matter among the cultivated sweet potato varieties globally is approximately 30% (Woolfe., 1992).

There are two major taste groups have been identified (1) white- and cream-fleshed sweet potatoes usually with dry matter contents ranging from about 25% to 35%; and (2) orange fleshed sweet potatoes (OFSPs) with dry matter of approximated from 20% to 30%. High pro vitamin A content is the major attribute in OFSPs (Gru¨neberg *et al*., 2009). About 80% to 90% of sweet potato storage root dry matter is made up of carbohydrates, largely starch (60% to 70% of dry matter) and sugars (15% to 20% of dry matter with a wide range from 5%to 40% of dry matter), and lesser amounts of hemicelluloses, pectins and cellulose (Woolfe., 1992). Unlike in OFSPs, white- and cream-fleshed varieties have higher starch (50% to 80% of dry matter) and lower sugar contents (5% to 15% of dry matter). OFSP genotypes are characterized by lower starch (45% to 55% of dry matter) and higher sugar contents (10% to 20% of dry matter (Woolfe., 1992). Up to 10 ppm iron and 6.4 ppm zinc in fresh storage roots for North American breeders material has been reported (Courtney, 2007).

Storage root mineral content ranged from 75 to 740 ppm of calcium, 180 to 350 ppm of magnesium, 1.6 to 9.4 ppm iron and 2.7 to 18.9 ppm of zinc in sweet potato accessions has been reported from South Pacific (Bradbury and Holloway., 1988).

2.7 Sweet potato virus disease (SPVD)

Sweet potato virus disease is caused by dual infection of the aphid transmitted Sweet potato feathery mottle virus (SPFMV genus *Potyvirus*, family *potyviridae*) and whitefly transmitted Sweet potato chlorotic stunt virus (SPCSV genus *Crinivirus*, family *Clesteoviridae*). SPVD is the most destructive disease infecting sweet potato globally particularly in Eastern Africa region (Gutierrez *et al.*, 2003). Sweet potato mild mottle virus (SPMMV genus *Ipomovirus* family *Potyviridae)* also infects sweet potato. Plant infectivity studies carried out in East Africa described occurrence of two viruses (Virus A and B) which were aphid and whitefly transmitted respectively (Sheffield., 1957). The suspected

virus diseases were then reported in Uganda in 1944 (Hansford., 1944). Sweet potato viral diseases were later reported in Tanzania, Kenya, Burundi, Rwanda, Malawi and South Africa (Sheffield., 1957). Viral synergism involving SPFMV and SPCSV cause severe sweet potato virus disease (SPVD) in which potyvirus titers increase by 600 fold whereas no rise is observed in titers of SPCSV (Karyeija *et al*., 2000a).

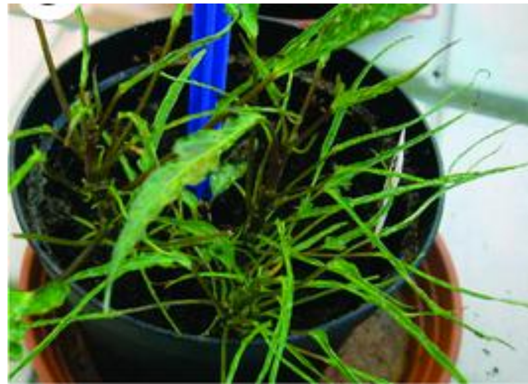

Plate 2: SPVD infected sweet potato (A). (Source: Settumba Mukasa).

2.8. Specific viruses causing SPVD in sweet potato in Kenya.

2.8.1 Sweet Potato Feathery Mottle Virus

Sweet potato feathery mottle virus (SPFMV genus Potyvirus, family potyviridae) is the most prevalent among twenty viruses infecting sweet potato. The virus is transmitted semi-persistently by aphids (Loebenstein *et al*., 2003). Sweet potato feathery mottle virus occurs in all sweet potato growing areas and is reported to be the most widespread in Eastern Africa region (Tairo *et al*., 2004). Foliar symptoms include vein clearing, vein feathering and chlorotic spots primarily on older leaves (Karyeija *et al*., 1998). Sweet potato plants with SPFMV may lead to mild or no symptoms although the infection may cause cracking and necrosis of the tubers (Kreuze, 2002). SPFMV contains a single positive stranded RNA genome estimated to be 10.8 kb (Sakai *et al*., 1997) which is larger than 9.7 kb of a potyvirus genome (Shukla *et al*., 1994). SPFMV CP is also remarkably large (about 38 kDa) compared to other potyviruses (Figure 1). This is attributed to the insertion of a contiguous sequence at the 5´-end of the CP cistron (Abad *et al*., 1992).

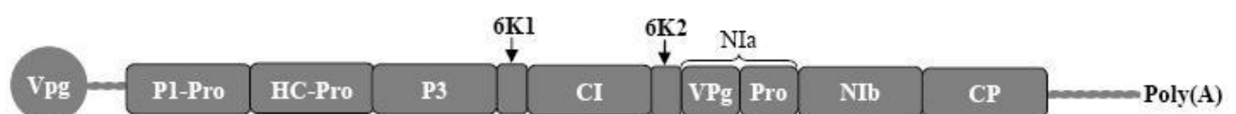

Figure 1: Genome organization of Sweet potato feathery mottle virus

The genome of potyviruses including SPFMV contains one open reading frame. The final protein products (boxes labelled) in Figure 1 are separated by border lines which indicate the putative cleavage sites of the polyprotein are shown. The 5'- and 3'-untranslated regions are also indicated (single thick lines at both ends) and encodes a large polyprotein that is processed into ten mature proteins by virus-encoded proteases including: P1, HC-Pro and NIa-Pro (Reichmann *et al*., 1992). The fully processed proteins are P1 proteinase (P1-Pro), helper component proteinase (Hc-Pro), third protein (P3), 6kDa protein 1 (6K1), cylindrical inclusion protein that is an RNA helicase (CI), 6kDa protein 2 (6K2), nuclear inclusion protein a (NIa), which can be further processed into the viral protein genome-linked (VPg) and NIa proteinase (Pro). The last two proteins are the nuclear inclusion protein b (NIb) which acts as the RNA-dependent RNA polymerase (replicase) and the (CP)

Two strains of SPFMV have been described based on serological and distinct symptoms. Russet crack (RC) strain causes distinctive symptoms in tubers and the common (C) strain have been reported (Moyer *et al*., 1989). RC and C strains have 80 % sequence similarity based on coat protein (CP) and amino acid sequences of SPFMV isolates. Four strain groups of SPFMV were later described based on phylogenetic analysis of coat protein sequences (Kreuze *et al*., 2000). Russet crack has not been identified in East Africa (IsHak *et al*., 2003; Ateka., 2004). Strain C contains SPFMV isolates that are most different with 75.8% – 78.3% nucleotide identity compared to other strains. Group O strains have been described in Niger, Nigeria, Japan and China and Argentina (Kreuze *et al*., 2000). Strain East Africa (EA) was recently described to be of distinguishable lineage phylogenetically and is not distributed far off the region (Tairo *et al*., 2005). SPVD symptoms include necrosis, vein-clearing, mosaic, leaf stunting, purpling and leaf distortion (Plate 3a).East Africa is rich in Ipomea wild type species with at least 89 known to occur (Blundell, 1992), Ipomea wild types are also infected by SPFMV in East Africa (Karyeija *et al*., 1998 a,b). Vector transmission studies on wild Ipomea in Uganda showed SPFMV detection in *Ipomoea* (Banks *et al.,* 1999). It is believed that SPFMV EA strain evolved in wild *Ipomoea* species prior to introduction of sweet potato in Africa and caused infection in the crop. The hypothesis is reliable due to occurrence of native Ipomoea species in East Africa which were resistant to SPFMV (Thresh and Fargette., 2003).

Plate 3: Sweet potato infected with SPFMV (A) and Virus free sweet potato (B)

2.8.2 Sweet Potato Chlorotic Stunt Virus (SPCSV)

Sweet Potato Chlorotic Stunt Virus (genus *Crinivirus*) is the lone member of the family *Closteroviridae* that has been reported to infect sweet potato (Hoyer *et al*., 1996; Alicai *et al*., 1999*). Clesteoviridae* family is characterized by long particles of 950 nm to up to 2000 nm in length and 10-13 nm in diameter (Figure 2). The particles are also very flexuous (Koonin and Dolja.,1993).

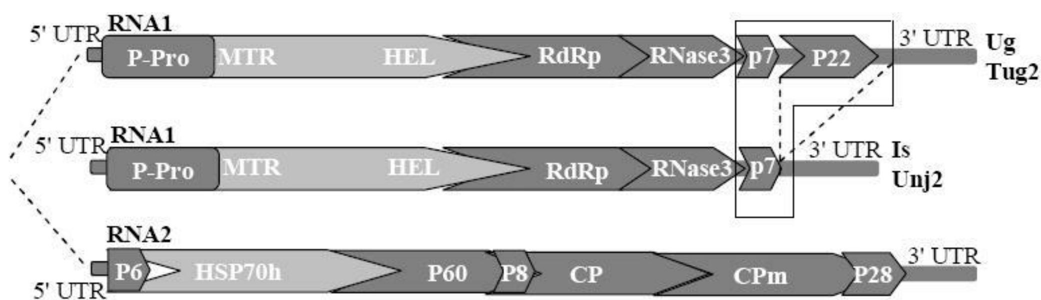

Figure 2: Schematic representation of the genome organisation of SPCSV RNA1 and RNA2

Genetic variation in RNA1 is shown in the middle lane exemplified by isolates Is and Unj2 which lack the *p22* gene (Cuellar *et al*., 2008, Kreuze *et al*., 2002)). The boxes and arrows correspond to ORFs and putativeproteins and their functions: (RNA1) P-Pro, putative papain-like cysteine proteinase; MTR, methyltransferase domain; HEL, helicase domain; RdRp, RNA-dependent RNA polymerase; (RNA2) Hsp 70h: heat shock 70 family protein homologue; CP: coat protein, CPm: coat protein minor. Additional two ORFs absent from other members of *crinivirus*: RNAseIII; dsRNA specific endonuclease; and p22, a silencing suppressor protein (Figure 2).

SPCSV has been reported to be the second most widespread virus infecting sweet potato in Kenya and in East Africa after SPFMV (Ateka *et al*., 2004; Aritua *et al*., 2006), the virus is also widespread in other regions of the world where sweet potato is grown (Gibson *et al*., 1998). SPCSV is transmitted by whiteflies (*Bemisia tabaci* and *Trialeurodes abutilonia*) in a non –circulative, semi persistent manner (Sim *et al*., 2000). *Nicotiana* species and *Amaranthus palmeri* have been reported to be susceptible to SPCSV; however the host range is limited mainly to genus *Ipomoea* (Cohen *et al*., 1992) and Lisianthus (*Eustoma grandiflorum*; Cohen *et al*., 2001).Plant infectivity assays on SPCSV strains have not been reported. Serological assays using polyclonal and monoclonal antibodies on SPCSV East Africa isolates have been distinguished from isolates from West Africa, Asia and America (Hoyer *et al*., 1996).

The heat shock protein-70 homologue (Hsp70h) is used more commonly than the coat protein sequence for phylogenetic analysis of the SPCSV isolates due to its highly conserved nature. A 446-nucleotide fragment of the SPCSV Hsp70h gene corresponding to the 5′-proximal ATPase domain is used as a genetic marker (Tairo *et al*., 2005). Family *Closteroviridae* viruses' heat shock protein 70 family homologue (Hsp70h) is highly conserved and is unique to the virus family (Agranovsky *et al*., 1997). Resistance to SPCSV has been found in sweet potato germplasm screening programs and the resistance to the virus seems to be conferred by a recessive gene which occurs in low frequency in the sweet potato gene pool (Andrade *et al*., 2009).

2.8.3 Other viruses infecting sweet potato

Several other viruses infecting sweet potato include Sweet potato mild mottle virus (SPMMV genus *Ipomovirus*. Plant infectivity studies carried out in East Africa described occurrence of two viruses (Virus A and B) which were aphid and whitefly transmitted respectively (Sheffield, 1957). The virus was closely described to resemble virus B while Virus A description resemble SPFMV. Occurrence of SPMMV has not been reported outside East Africa (Colinet *et al.*, 1996).

Other viruses include sweet potato leaf curl virus (SPLCV, family; *Begomoviridae*, genus *Geminiviridae*) has been reported in South America, the Middle East, and South East Asia and the United States (Briddon *et al*., 2006; Luan *et al*., 2006). Sweet Potato Leaf Curl

Virus has not been reported in Africa despite description of symptoms similar to those of SPLCV in *I aquatica* (Rossel., 1981).

Two potyviruses; Sweet potato virus G (SPVG, genus *Potyvirus*) has been reported in China, Egypt, South Africa and USA (Souto *et al*., 2003; IsHak *et al*., 2003; Ateka *et al*., 2007). Sweet potato virus-2 (SPV-2, genus *Potyvirus*) also known as Ipomoea vein mosaic virus, (IVMV); or Sweet potato virus Y (SPVY) has been reported in Zimbabwe (Chavi *et al*., 1997); in USA (Souto *et al*., 2003),Taiwan (Rossel and Thottappilly,1988; Ateka *et al*., 2004a). 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 these viruses have been reported (Cohen and Loebenstein, 1991; Kokkinos, 2006).

2.9 Effects of viruses on sweet potato

Various studies have concluded that virus diseases infecting sweet potato are an important factor in cultivar decline which varies with the cultivar and environment where sweet potato is grown (Carey *et al*., 1999b) due to sweet potato viruses and somatic mutations (La Bonte *et al*., 2001). These two factors initiate detrimental effects that are aggravated by the vegetative propagation system (Lewthwaite *et al*., 2011). Symptoms severity which initially occurs in the young leaves is associated with the remarkable yield reductions in the fields (Salazar and Fuentes., 2001). Duration from initial infection to the emergence of symptoms vary due to the age and size of the plant and symptoms take longer to be manifested on older and bigger plants (Karyeija *et al*., 2000). Viruses require plant proteins for accumulation and movement during their life cycle. The host plant act in response to an infection by activating pathways which are specific or general resistance (Whitham *et al*., 2003).

Kokkinos., (2006) determined differential gene expression after virus infection. The decrease in expression levels of genes that are either directly or indirectly involved in the general photosynthetic pathway, observed in the SPVD-affected plants is a phenomenon normally observed in yellowing diseases and plant leaves showing characteristic chlorotic and/or mosaic symptoms (Hull, 2002). Reduction in photosynthetic activity can also be attributed to irreversible damage to photosystem II (Van Kooten *et al*., 1990). Plant resistance genes (R genes) are capable of recognizing pathogens carrying the analogous avirulence genes (gene-for-gene resistance). This recognition mechanism triggers the

hypersensitive reaction (HR), which includes programmed cell death (PCD). The hypersensitive reaction often occurs after the accumulation and production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2). Several genes are differentially expressed only in plants affected by SPVD, identified as resistance-related or stress-induced genes. Interestingly, some of these genes were down-regulated whereas others were up regulated (Kokkinos., 2006). SPCSV, whose titers are considerably greater than those of SPFMV in single infections, are unaltered during SPVD (Kokkinos and Clark, 2004). Sweet Potato Chlorotic Stunt Virus has the potential to encode and "supply" a large amount of its protein products throughout the plant.

Most of the genes identified as stress-induced or resistance-related are not differentially expressed in plants infected with SPFMV or SPCSV alone, imply that the most probable mechanism of SPVD involves interaction between the two viruses, leading to enhancement of SPFMV and SPCSV that suppresses the host resistance mechanism for SPFMV to achieve high titers and lead SPVD (Kokkinos, 2006). . Muqit. *et al.*, 2007 conducted research to determine the changes in ash gourd (*Benincasa hispida*) due infections of *Watermelon mosaic virus 2* (WMV2) *Bottlegourd mosaic virus* (BgMV), and *Papaya ringspot virus* (PRSV).Their findings indicated that chlorophyll, beta-carotene, organic carbon and DNA were found to be reduced in the leaves of infected plants as compared to virus free ones. Total phosphorus and RNA contents were elevated in the infected leaves. Ratios of chlorophyll a to b and RNA to DNA were higher in the virus infected leaves. Total nitrogen and protein levels was decreased due to BgMV and WMV2 infection while it was increased in case of PRSV. Similarly, according to Nejla *et al.*, (2006) macro- and microelements were assayed in plants infected with Alfalfa mosaic virus to determine the effects of the virus on the nutritional content of alfalfa. The quantities of phosphorous, iron, copper, zinc and manganese decreased and nitrogen increased in the infected alfalfa leaves as compared to the healthy plant samples, while Phosphorous did not change.

This result is similar to observations made by Elegba *et al.*, (2013) which reported a negative correlation between starch yield and cassava mosaic disease incidence after evaluating genotypes for the effect of cassava mosaic diseases on dry matter and starch content. Kapinga *et al.,* (2009) used high beta-carotene content variety but highly susceptible to SPVD called Resisto to determine the effects of SPVD on total carotenoids content and root yield. Compared with plants without SPVD, they recorded a reduction of 43%, 16%, and 37% of the total carotenoids content in the variety were observed in plants

infected with SPCSV, SPFMV, and co-infection of both viruses. Storage root fresh weight was significantly ($P<0.001$) reduced due to virus infection. Highest reduction was recorded for SPFMV infection followed by infection of SPVD. Some of the clones evaluated have potential to contribute greatly to alleviation of vitamin A deficiency and meaningful, desired mineral intakes. Introduction of evaluated clones with different traits program in the breeding programs is essential in improving existing varieties.

Development of SPVD tolerant genotypes is important for sweet potato breeding programs and subsequent release of the varieties will contribute greatly to improved sweet potato production. Furthermore, there is a constant need to evaluate more germplasm in order to identify virus tolerant varieties since SPVD is a major production constraint after sweet potato weevil. SPVD is important economically since the diseased plants produce almost usable yield (Gibson *et al.*, 1998b). Therefore a concerted effort by pathologists and sweet potato breeders is key to reduction of negative impacts of SPVD and develop genotypes with desired qualitative and quantitative traits.

2.9.1 Virus resistance in sweet potato

Sweet potato viruses' resistance is a good option for management of SPVD since it does not require expenditure to develop resistance by the farmers (Valverde., 2007). Post transcriptional gene silencing (PTGS) or Ribonucleic acid silencing is induced by plant virus RNA. It is a powerful defense tool against plant viruses (Matzke *et al.*, 2001; Vaucheret and Fagard., 2001). Small interfering RNAs (siRNAs) have also been associated with RNA silencing (Waterhouse *et al.*, 2001. Post transcriptional gene silencing system recognizes and specifically degrades RNA it deems as foreign, unusual or abnormal and sends a systemic signal, which induces degradation of homologous RNA in distal parts of the plant (Waterhouse *et al.,* 2001b).

Ribonucleic acid silencing is however not full proof as viruses manage to infect plants due to their ability to counter RNA silencing. One of the mechanisms is by encoding to RNA silencing suppressors (Voinnet *et al.*, 1999). Suppressors of RNA silencing have been identified among the virus-encoded proteins which are determinants of viral pathogenicity. Host resistance mechanisms target suppressors of RNA silencing (Li *et al.*, 1999). Suppressors of RNA silencing include HC-Pro in *Potyviruses* (Kasschau and Carrington, 1998), 2b in *Cucumoviruses* (Brigneti *et al.,* 1998). Development of virus resistance in sweet potato first focused on SPFMV due to its distribution worldwide. An additional gene

action is important in resistance to SPFMV is suggested to confer resistance (Karyeija *et al.*, 1998a; Mwanga *et al.*, 2002b). It has also been demonstrated that infection with SPCSV overcomes resistance to SPFMV due to its influence in its titre(Karyeija *et al.*, 2000a).

Resistance to SPFMV strains in most sweet potato cultivars is apparently innate showing with only mild initial symptoms being manifested. Recovery follows after low virus titers are detected (Gibson *et al.*, 1998). SPVD even in the most resistant clones of sweet potato is caused by co-infection of SPFMV with SPCSV which interferes with the plant recovery (Karyeija *et al.*, 2000b; Mwanga *et al.*, 2002).

2.9.2 Detection of viruses infecting sweet potato

Viruses infecting sweet potato are predominantly Ribonucleic acid (RNA) with few DNA viruses such as begomoviruses such as Sweet potato leaf curl virus. Nucleic acid based detection methods such as ordinary polymerase chain reaction and rolling circle amplification has been used to detect DNA viruses. RNA viruses are detected using reverse transcription step that precedes polymerase chain reaction (Colinet *et al.*, 1998). Immunocapture RT-PCR has been used for detection of sweet potato viruses (Nolasco *et al.*, 1993). Real-time PCR has also been used to detect both DNA and RNA viruses. Real-time polymerase chain reaction (PCR) assays have been developed for the detection and comparative quantification in singleplex reactions of Ipomoea vein mosaic virus (IVMV) (Kokkinos et al., 2006). The crinivirus Sweet potato chlorotic stunt virus (SPCSV); the begomovirus Sweet potato leaf curl virus(SPLCV) and potyviruses Sweet potato feathery mottle virus (SPFMV), Sweet potato virus G (SPVG) have been detected from infected sweet potato plants. However, molecular diagnostic methods are expensive despite their high reliability and sensitivity. Serological detection of sweet potato viruses is possible with antisera available from the International Potato Center (CIP), Lima, Peru using nitrocellulose membrane –enzyme linked immunosorbent assay (ELISA) method (Guttierez *et al.*, 2003).

2.9.3 Molecular characterization of sweet potato using Simple Sequence Repeats.

Various molecular marker techniques have been developed for identifying of polymorphisms in plants and animals. First to be developed and widely used molecular markers were Restriction Fragment Length Polymorphisms (RFLPs) which are very ideal in polymorphisms identification (Tanksley *et al.*, 1989). Inability to automate the

genotyping process and the large amounts of DNA required has reduced the use and suitability of the technique.

Amplified Fragment Length Polymorphisms (AFLPs) which is a Polymerase Chain Reaction (PCR)-based tool for detecting and identification of polymorphisms has also been used for diversity studies due to the ability of automation of the technique (Kochert., 1994). Simple Sequence Repeats (SSRs) have been most frequently used, particularly in maize, sweet potato, cassava, soybean, barley, wheat and sunflower among other crops. Simple sequence repeats have a number of advantages among them being ease in automation process, a large number of public resources providing sequences for SSRs and cost-effectiveness developed (McCouch *et al.*,1997). Simple sequence repeats (SSRs) which are also referred to as microsatellites, are short sequences of about 1-6 base pairs that are tandemly repeated and widely dispersed in eukaryotic genomes.

Comparative studies in plants have shown that SSR markers, which have multiple alleles in a single locus, are more reliable than other markers since they are an effective means for determining differences between genotypes (Powell *et al.*, 1996). Simple sequence repeats have been applied in a number of areas such as cultivar identification, analysis of genetic diversity, evolutionary studies, construction of molecular maps and to obtain patents and intellectual property rights for plant varieties (Buteler *et al.*, 2002).

Simple sequence repeats markers exhibit high levels of polymorphism and have since been developed for sweet potato diversity studies (Hu *et al.*, 2004). High levels of polymorphism has been reported in the genetic diversity of white and orange-fleshed sweet potato farmer varieties from East Africa (Tumwegamire *et al*, 2011a,b) The results obtained from various studies reveal the excellent discriminatory capacity of SSR markers. Buteler *et al.*, (2002) reported only a few useful microsatellite loci have been identified for sweet potato at present. These markers have been used in the assessment of genetic diversity and DNA fingerprinting (Zhang *et al.*, 2001). Evaluation of sweet potato genetic diversity and their relatedness among genotypes is of key not only for germplasm conservation and breeding purposes but also it is application in the selection of parents resistant and/or tolerant to diseases infecting the crop.

The use of high yielding cultivars in breeding and selection of disease resistant genotypes has led to cultivar decline and erosion of genetic diversity during the past decades (Hawkes., 1987).

Genetic pool of available variation for disease resistance traits within cultivars has become inadequate, therefore there is increasing need to find novel sources of resistance (Callow *et al*., 1997),

In sweet potato, molecular markers have been applied in phylogenetic analysis and germplasm evaluation to study the origin of sweet potato and its dissemination into the Pacific and Asia (Zhang *et al*., 2004). African germplasm has been studied by Gichuki *et al*., 2003 using Restriction fragment length polymorphism (RFLP). A number of SSRs have already been developed in sweet potato analysis (Jarret and Bowen., 1994). Application of DNA markers have been employed in identification of economically important traits such as resistance to SPVD (Miano *et al*., 2008), root knot nematodes resistance (Mcharo *et al*., 2005b), dry-matter content, yield and beta-carotenoid content (Cervantes-Flores, 2006). These have further been screened and applied in paternity analysis in sweet potato and its wild relative species (Buteler *et al*., 1997; 1999).

Microsatellite markers have been used to investigate genetic diversity of a wide range of species such as wheat with number of alleles amplified per primer pair ranged from3-16, (Plaschke *et al*., 1995), in maize number of alleles amplified per primer pair ranged from 2-23 (Senior *et al*., 1998) and in rice with number of alleles amplified per primer pair ranging from 3 to 25 (Yang *et al*., 1994).

SSR have been touted to be the most efficient and reliable markers for genetic diversity studies in a range of plants (Rakoczy-Trojanowska and Bolibok., 2004) including sweet potato (Zhang *et al*., 2000). High levels of allelic variation and their co-dominant ability which implying that offer more information content per unit assay than any other markers (Rakoczy-Trojanowska and Bolibok., 2004). Successful conservation of any available gene pool is largely reliant on understanding the diversity and its distribution in a given geographical region (Zhang *et al*., 1998).

With the current accessibility of genome sequences resources, Single Nucleotide Polymorphisms (SNPs) are becoming more popular in genomics studies, research and breeding programs (Koopaee *et al* .,2014)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The experiment was planted at a field adjacent to sweet potato breeding blocks as source of natural infection at the Kenya Agricultural and Livestock Research Organization–KALRO Njoro, in a randomized complete block design with three replicates..

3.2 Source of sweet potato seeds, sweet potato viruses and their detection

Sweet potato germplasm from six families ware obtained from the International Potato Centre (CIP) in Mozambique (Table 2). Seeds from the 6 families were acid scarified using concentrated sulphuric acid. Breaking of dormancy in sweet potato clones seeds was carried out by counting 400 seeds per family and put in a 500 milliliter beaker previously cleaned and rinsed with distilled water. Seeds from each family were put in separate labeled beakers. Concentrated sulphuric acid was added to fully submerge the seeds. Beakers were placed in a shaker and gentle shaking was done for 40 minutes. Sulphuric acid was poured through a sieve in order not to lose seeds. Seeds were washed using distilled water four times with standing for three minutes in each wash. The seeds were put in a petridish with pre-wetted filter paper to enable germination to occur. Pre-germinated seeds were planted on forest soil and sand which was sieved and mixed at the ratio of 3:1 respectively. The mix was put in small perforated potting bags to near fill and arranged on a bench. Water was put on the soil to pre wet the soil before introducing seeds. Controlled watering was carried out avoiding excessive watering which would cause rotting of seeds. Germination counts were taken after three weeks.

Control of SPVD vectors was carried out by spraying insecticides Actara® and Dynamec® from Syngenta in two weeks intervals as recommended by the manufacturer. Detection of sweet potato viruses was carried out using Nitrocellulose membrane ELISA (NCM-ELISA). The kit was obtained from CIP in Peru and used to detect the following viruses; Sweet potato chlorotic stunt virus (SPCSV), Sweet potato feathery mottle virus (SPFMV), Sweet potato mild mottle virus (SPMMV), sweet potato chlorotic fleck virus (SPCFV), Sweet potato latent virus (SPLV), Sweet potato mild speckling virus (SPMSV), sweet potato caulimo-like virus (SPCaLV), C-6 virus and Cucumber mosaic virus (CMV). RT-PCR was used to validate results obtained NCM-ELISA. Only virus free clones were planted in the field. The virus

free clones were selected and planted in a field with high SPVD pressure using Random Complete Block Design (RCBD) with three replications. Each plot had eight plants.

Disease progression scores were taken using a scale of 1-9 where 1= no symptoms and 9 =very severe symptoms after two months interval. Scores range as follows: 1 -no virus infestation, 2 - unclear virus symptoms 3- clear virus symptoms 5% of plants affected 4-clear virus symptoms 6-15% of planted affected 5-clear virus symptoms 16-33% of plants affected 6-clear virus symptoms 34-66% of plants affected 7-clear virus symptoms 67-99% of plants affected 8- clear virus symptoms all plants affected 9--clear virus symptoms, all plants affected and stunted, almost dead plants.

Table 2. Orange fleshed sweet potato clones from six families and two checks (Kenspot 4 and Kenspot 5) used in the study.

Family	Family identity	Number of clones tested	Number of clones used in the field experiment	Response to viruses challenge
1	MUSG 0622-58	24	15	Unknown
2	MUSG 0608-22	24	15	Unknown
3	108196G07-06	24	15	Unknown
4	UW 11906-289	24	15	Unknown
5	MUSG 0608-22	24	15	Unknown
6	MUSG 0704-16	24	15	Unknown
7	Kenspot 4	1	1	Medium tolerance
8	Kenspot 5	1	1	Medium tolerance

3.1.2 Nitrocellulose membrane ELISA (NCM- ELISA) for detection of sweet potato viruses

Detection of sweet potato viruses was carried out at the Plant Diagnostics Laboratory using plants maintained in a greenhouse at the Kenya Agricultural and Livestock Research Organization (KALRO) in Njoro, Kenya.

Test samples were collected and identity of the samples indicated on the plastic bags. Sample collection was carried out on the same day the tests were performed. Composite samples from each plant to be tested were collected by taking one leaf from the top, middle and bottom levels. From each leaf sample, a disc of about 1 cm in diameter was cut by positioning each leaf within the upper part of the plastic bag and using a small test tube, leaf discs were cut by exerting pressure on the outside of the plastic bag. Remaining part of the leaves were discarded and the discs ground in 3 ml of extraction buffer (each 1 ml of extraction buffer composed of 1 gm Sodium Sulphite in 500 ml TRIS buffered saline 500 ml) per leaf disk. The disks were ground completely using a medium sized test tube. Bags were allowed to stand in an upright position for 30-45 minutes at room temperature until the plant sap phased out.

Nitrocellulose membranes were cut to the required size prior to sample application. The membranes were identified by writing the name of the virus on the top and followed by pre-wetting the membranes in TRIS buffered saline (TBS) which comprised of 0.02M TRIS and 0.50 M Sodium Chloride for at least 5 minutes prior to use. Pre-wet nitrocellulose membranes were placed over a piece of pre wet No. 4 Whatman paper. Plant sap (30 µl) was pippeted into each square grid on the nitrocellulose membrane. Care was observed not to pipette plant tissue. A clean tip for each sample was used as the process was repeated until all the samples were spotted. Nitrocellulose membranes were transferred to a dry piece of filter paper and allowed to dry for 15-30 minutes. Samples were recorded as they were spotted onto the membrane on the NCM-ELISA record sheet.

The nitrocellulose membranes spotted with samples were dipped in blocking solution (2% skimmed milk) for one hour at room temperature with gentle shaking at 50 revolutions per minute. Primary specific antibodies to test viruses were diluted in TBS + 2 % skimmed milk and sufficient aliquots added in a container with the membrane. Incubation was done overnight at room temperature with gentle shaking (50 rpm). The nitrocellulose membranes

were washed thrice in Tween TRIS buffered saline (T-TBS) composed of 1.0 ml Tween in 2 litres of TBS with rapid shaking (100 rpm). Conjugated antibody or secondary antibody for test viruses which is Goat anti Rabbit (GAR), was added in TBS + 2 % milk after dilution, followed by incubation for 1 hour at room temperature with gentle shaking (50 rpm).

The nitrocellulose membranes were washed four times in T-TBS with rapid shaking (100 rpm) before air drying. Nitro blue tetrazolium (NBT) and 5 Bromo-4-chloro-3-indolyl phosphate (BCIP) substrate solutions were prepared immediately before use. The membranes were incubated in NBT/BCIP substrate at room temperature for colour development. This was done for 30 minutes for other viruses and 1 hour for SPCSV. Colour development was stopped by discarding the NBT/BCIP substrate solution and immersing the nitrocellulose membranes in distilled water (Appendix 2). The nitrocellulose membranes were washed in tap water three times for 3 minutes each. Membranes were dried before recording reactions on the NCM-ELISA record sheet. Clones infected with any of the ten viruses tested were not advanced to subsequent experiments since virus free material was required

3.2.2 Genomic DNA extraction from sweet potato leaves

The CTAB method by Doyle and Doyle (1990) was used to extract genomic DNA from the test sweet potato genotypes. Leaf tissue (0.4 gm) was weighed and ground in 3 ml of extraction buffer (20 g CTAB 100ml 1M TRIS-HCL pH 8.0, 100ml 0.5M EDTA pH 8.0, 81.76 g Sodium Chloride, 10 g Sodium Sulphite and 20g Polyvinylpyrolidone (PVP-40) dissolved to make up one litre (Appendix 1)) in a mortar and pestle with aid of acid washed sand. The slurry was then transferred to 1.5 ml microfuge tubes and incubated at 65°C for 20 minutes in a water bath with mixing after every 5 minutes interval. Centrifugation was carried out at 13000 rpm for ten minutes before transferring 750µl of supernatant to a fresh tube. About 750µl of chloroform: isoamylalcohol (24:1) was added to the tubes. Tubes were well shaken and centrifuged at 13000 rpm for ten minutes. About 600 µl of the aqueous supernatant phase was transferred to a fresh tube and mixed with an equal volume of Chloroform: Isoamylalcohol (24:1). This step was repeated twice. Aqueous supernatant phase (450 µl) was added to a fresh tube while avoiding the interphase. DNA was precipitated using ice cold isopropanol stored in a freezer (-20ºC) for 20 minutes before centrifuging at 6500 rpm for ten minutes. Isopropanol was decanted carefully leaving the DNA pellet at the bottom of the tube. The pellet was washed with 500µL of 70% ethanol before air-drying. The pellets were dissolved in 100µl sterile distilled water. DNA samples were treated with 2 µl of

RNase A by incubating them for one hour at 37°C. The isolated DNA samples were then stored at -20°C until used.

3.2.3 DNA quantification and quality determination

Agarose powder was dissolved in Tris Borate EDTA (1X TBE buffer (1% w/v) (89mM Tris HCL, pH 8.3;Boric acid ,2.5mM EDTA) by boiling under medium heat in a microwave oven. The dissolved agarose was allowed to cool to about 50º C before ethidium bromide was added to the gel at a concentration of 1 mg/ml. Before the agarose cooled, the gel casting tray was prepared by sealing the open edges with masking tape, dry glass tray so as to form a mold to avoid leakage and so that the tray could accommodate the preferred thickness of the gel. The warm agarose solution was then poured into the gel tray in which a comb was inserted to form wells where samples would be loaded after 30 minutes to solidify. The gel was allowed to set for 30 minutes before removing the autoclave tape, and immersing the gel in the electrophoresis tank containing 1X TBE buffer. The combs were removed and 7µl of each DNA sample containing 2 µl of loading solution (Biolabs England). Molecular weight marker (100 base pairs ladder) was loaded to one of the wells of the gel alongside the sweet potato DNA samples.

The gel was run at a constant voltage of 100 volts until the tracking dye migrated almost towards the end of the gel. The gel was then removed from the gel tank and placed in a UV trans-illuminator (UVP Photo Doc It™ Imaging system) and photograph taken at a wavelength of 300 nm. DNA quantities were estimated using a 100 base pair molecular weight marker (Hyperladder ™ 100 bp from Bioline). DNA samples were then diluted to give a concentration of about 10 ng/µl.

3.2.4 Simple sequence repeats (SSRs) analysis for SPVD resistance

Ten primer pairs (Table 3) for SSR loci linked to known SPVD resistance genes sourced from (Buteler *et al*, 1999, Tseng *et al.*, 2002, Yanez, 2002 and Yada *et al.*, 2010. (Table 4) were used for PCR amplification of the sweet potato genomic DNA test samples (test clones). Two checks (Kenspot 4 and Kenspot 5) known to have moderate virus resistance and two virus susceptible varieties (Ejumula and Carrot C) (Mwanga and Ssemakula., 2011) were included in SSR analysis.

Each 20 µl Polymerase Chain Reaction (PCR) reaction contained 0.5µl of 10 pmol/µl of each primer, 1µl of 2.5 mM $MgCl_2$, 1µl of 10 mM dNTPs, 0.2µl of 0.1Units of Taq DNA polymerase (AmpliTaq™DNA polymerase from ThermoFisher USA.) 2.5 µl of 5X PCR buffer and 13.3 µl of PCR grade nuclease free water. Amplifications were carried out using the Gene-Amp PCR system 2720 (Applied Biosystems, USA) under the following Touchdown PCR thermocycling conditions: 1 cycle of 94°C for 2 minute, followed by 15 cycles of 94°C for 1 minute, 60°C for 2 minute, 72°C for 1.5 minute, and 94°C for 1 minute, 50°C for 2 minutes and 72°C for 1.5 minutes for 30 cycles and a final extension step of 10 minutes at 72°C. The PCR products of each sample and a 100 base pair molecular weight marker that was loaded in one of the wells were resolved on a 2% agarose gel using high resolution agarose (UltraPure ™ Agarose 1000 from ThermoFisherscientific) in 1X TBE buffer. SSR bands/alleles were scored for presence as 1 and 0 for absence. Negative controls without template DNA were used in optimization of PCR conditions and evaluation of the samples.

Table 3: List of microsatellite markers used in the study Buteler *et al*, 1999, Tseng *et al*., 2002,Yanez, 2002, Yada *et al*., 2010. Solis *et al* (unpublished) and Benavides (unpublished).

Lab. No.	Name of locus	Motif	Temp.	Ta	Size range	Alleles per locus
1	IB-R03	(GCG)5	58	58	302-312	5
2	IB-S07	(TGTC)7	60	60	193-211	4
3	IB-R12	(CAG)5A	60	60	356-395	5
4	IB242	(CT)3 CA(CT)11	58	58	136-155	6
5	IB297	(CT)13	58	58	150-182	4
6	IB316	(CT)3C(CT)8	58	58	151-167	5
7	IBCIP-1	(ACC)7A	63	63	155-167	4
8	IB324	*	56	56	140-280	4
9	IBJ 522a	(CAC)6-7	57	57	110-190	6
10	JB 1809	(CCT)6 (CCG)6	60	60	225-298	9

* Required information in the fields not available.

3.2.5. RT- PCR validation of NCM- ELISA for detection of SPVD

The CTAB method by Doyle and Doyle (1990) with few modifications was used to extract total RNA from the test sweet potato genotypes.

Leaf tissue (0.4 gm) was weighed and ground in 3ml of extraction buffer (20g CTAB 100ml 1M TRIS-HCL pH 8.0, 100ml 0.5M EDTA pH 8.0, 81.76g Sodium Chloride, 10g Sodium Sulphite and 20g Polyvinylpyrolidone (PVP-40) dissolved to make up one litre (Appendix 1)) in a mortar and pestle with aid of acid washed sand. The slurry was then transferred to 1.5ml microfuge tubes and incubated at 65°C for 20 minutes in a water bath with mixing after every 5 minutes interval. Centrifugation was carried out at 14000 rpm for ten minutes before transferring 750µl of supernatant to a fresh tube. About 750µl of chloroform: isoamylalcohol (24:1) was added to the tubes. Tubes were well shaken and centrifuged at 14000 rpm for ten minutes. About 600µl of the aqueous supernatant phase was transferred to a fresh tube and mixed with an equal volume of Chloroform: Isoamylalcohol (24:1). This step was repeated thrice. Aqueous supernatant phase (450 µl) was added to a fresh tube while avoiding the interphase. Nucleic acids were precipitated using ice cold isopropanol stored in a freezer (-20ºC) before centrifuging at 10000 rpm for ten minutes. Isopropanol was decanted carefully leaving the RNA pellet at the bottom of the tube. The pellet was washed with 500µL of 70% ethanol before air-drying. The pellets were dissolved in 100µl sterile distilled water. The total nucleic acids were treated with 2 µl of RNase A by incubating them for one hour at 37°C. The isolated total nucleic acids samples were then stored at -20°C until used.

Total RNA isolated from previously virus tested plants using NCM- ELISA were tested for presence or absence of SPCSV, SPFMV and SPMMV. Specific primers were used. The sequences were as follows. SPFMV-F: 5'-GGA CGA GAC ACT AGC AA-3', SPFMV-R 5'-TTC TTC TTG CGT GGA GAC GT-3' for SPCSV-F: 5'-ACG TTG GTT GGC GTT GA-3'SPCSV-R5'-ATC TGC GGG AAC TGA CAC G-3' and for SPMMV-A- ACC GGG AGA TGG CGA TGA A, SPMMV-B2 –CAC GTG ATA CAT RGC GCT TCT TA.

cDNA synthesis and RT-PCR was carried out using specific primers for SPCSV and SPFMV and SPMMV in separate reactions. Complimentary DNA (cDNA) was synthesized using SuperScript™ III Reverse Transcriptase 1st-Strand cDNAkit (Invitrogen, Carlsbad, USA) was used the following manufacturer's instructions for a two-step RT-PCR.

In a 25-µl reaction mix, 5 ng of total RNA, 1µM of specific reverse primers, 250 µM dNTP mixtures, 4µl of 5x cDNA synthesis buffer and 15 units of Thermoscript Reverse

transcriptase (ThermoFisher Scientific, UK) was added to a final volume of 25 µl with sterile distilled water. The RNA and specific primers were denatured at 65°C for 5 minutes and after adding the master mix, incubated in a thermocycler preheated to 60°C for1 hour. The reaction was terminated by incubating at 85°C for 5 minutes. The first strand cDNA product was used for PCR immediately or stored at -20°C. PCR amplifications for cDNA were carried out using the Gene-Amp PCR system 2720 (Applied Biosystems, USA) under the following thermocycling conditions: 1 step of 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minutes, 58°C for 1 minutes, 72°C for 2 minutes, and a final extension step of 10 min at 72°C.

Positive and negative controls for SPCSV, SPFMV and SPMMV were used. 20 µl of samples were then resolved in 1.5 % agarose stained with 2 µl of ethidium bromide. Samples were loaded on the solidified gel in Tris borate EDTA buffer (TBE). Samples were run for 1 hour using 100 volts. Expected fragments in positive controls were used to score for presence or absence of the three viruses in the test samples. Positive controls used had previously been detected to be infected with SPCSV, SPFMV and SPMMV. Negative controls (Non template control and virus free plants RNA) were used in the detection experiments.

3.2.6 Collection of disease progression scores

Disease severity was determined based on a score of 1-9 where 1 = no visible symptoms and 9 represents very severe symptoms including purpling /yellowing or mosaic on leaves, severe leaf distortion, reduced leaf size, severe stunting and chlorosis. Disease scores were taken after every two months.

3.2.7. Selection of sweet potato clones for dry matter and micronutrient analysis.

Most OFSPs are characterized by low dry matter and low resistance to SPVD and fungal diseases such as *Alternaria spp*. Pursuit for identification of sweet potato weevil resistance, high yielding and disease resistant genotypes with good flavor, texture that is influenced by dry matter offer the best selection criterion for identification of potential clones for further evaluation and release of suitable clones. Therefore, selection of clones for dry matter and micronutrient analysis was primarily based on viruses' response data.

Clones with low to medium scores of 1-6 were selected in addition to other criteria such as beta-carotene content, low weevil damage and harvestable roots. Clones with higher virus scores were not candidates for selection due to virus susceptibility.

3.2.8 Dry matter analysis

Sweet potato clones were maintained in the field until maturity. Tubers from virus tolerant clones were harvested, cleaned with tap water and cut into cuboid or rectangular blocks weighing a100 grams and labeled appropriately.

The dry matter content was determined by drying the samples in an oven at105°C for 16 hours. Dry matter was the resultant weight after drying in the oven (AACC, 1983).

3.2.9 Iron and zinc analysis in clones with viral tolerance traits

Iron and zinc were determined in the clones after maturity using a method described by Okalebo *et al* (2002). The tubers were washed with tap water to remove dirt and soil. The tubers were cut to thin slices of about 0.5 cm^3 using a stainless steel knife. The samples were then dried in an air oven at 60°C for 4 days, and then cooled to room temperature. Milling was carried out using a Teflon coated hammer mill. Samples were sieved through a 1 mm diameter mesh sieve. Samples were then stored in air tight container until analysis was carried out. Dried samples (0.3 g) were put in a dry digestion tube. A mixture of Selenium-Sulphuric acid (4ml) was added and allowed to react for at least two hours at room temperature. Samples in the digestion tubes were heated at 200°C in a block digester, allowed to cool before adding 1 ml hydrogen peroxide thrice after 10 seconds. Samples were returned to the digestion block before temperatures were adjusted to 330°C. Digestion was deemed complete after samples became light yellow or colourless. Tubes were removed from the digester and allowed to cool to room temperature; contents were transferred into volumetric flask and filled up to 50 ml mark. Samples were then analyzed for iron and zinc in an Atomic Absorption Spectrophotometer (AAS) (Shimadzu Corporation, Model AA- 6300, Japan). Standard serial dilutions of Iron and Zinc (1.25 ppm, 2.5 ppm, 5 ppm, 7.5 ppm and 10 ppm.) were used to calibrate the AAS. A calibration curve was drawn using the standards. Iron and zinc concentrations were determined by atomic absorption as they absorbed radiations from element-specific hollow cathode lamps at wavelengths of 248.3nm and 213nm, respectively. Absorbencies obtained were used to estimate iron and zinc concentrations in the test samples.

The formula for calculation of zinc and iron is given below in Equation 1.

Zn/Fe (mg kg-1) = (a-b) × v × f ×1000 1000 × w

where a = concentration of Zn/Fe in the solution; b = concentration of Zn/Fe in the mean values of the blanks; v = final volume of the digestion process; w = weight of the sample; f = the dilution factor (Okalebo *et al*., 2002).

3.3.1 Beta carotene analysis in virus tolerant clones

Beta carotene content in was determined as follows. The roots were cut longitudinally through the middle, with one half being divided into five transverse sections. A small portion of each section was cut off and weighed up to 2 g. This was done for all the samples in triplicates. The weighed samples were placed in a mortar and ground. To extract the beta carotene, 5ml of acetone was added to the mortar and grinding carried out until color was released. This extract was filtered through glass wool in a funnel to obtain 50ml in a volumetric flask. More acetone was added to the mortar and grinding done till color was fully extracted; the volume of extract was approximately 50ml. The flask was labeled and covered with aluminum foil.

The filtrate collected in the volumetric flask was transferred into a well labeled round bottom flask and placed in a water bath at 60°C. Acetone was allowed to evaporate to full dryness leaving behind the colour. Silica gel (20 g) was weighed, put in a dry beaker and activated using 1ml of absolute alcohol. Petroleum ether was then added to the mixture with constant stirring as the silica was poured continuously into the column with a small amount of glass wool at the bottom. More petroleum ether was added to the beaker and the silica packed into the column to completion. Care was taken to ensure that the column did not crack by ensuring that there was constant supply of petroleum ether to prevent drying. Anhydrous Sodium sulphate (2 g) was added at the top of the column to prevent entry of atmospheric moisture. The dried beta carotene in the round bottom flask was dissolved using petroleum ether and added at the top of the column. Beta carotene was migrated through the gel as a colored band separated from the other carotenoids. Beta carotene was collected in a 25 ml volumetric flask, labeled and covered with foil paper. The levels of beta carotene for each sample were determined using UV light absorbance spectrophotometry at a wavelength of 450nm using the formula in Equation 2 (CIAT, 2007).

$$\text{Beta carotene}(\mu g) = \frac{a \times f}{w}$$

where a = is absorbance, w = weight of the sample and f = the dilution factor.

3.3.2 Data analysis

Mean virus disease scores, dry matter and Iro and Zinc contents data was analyzed using analysis of variance-ANOVA (SAS Inc.) followed by separation of means using Least Significant Difference (LSD). The SSR data was analyzed using Dissimilarity Analysis Representation for Windows (DARwin version 6.0.010) to generate neighbor joining tree for the 17 genotypes. A dendogram was constructed using the UPGMA (Unweighted Paired Group Method with Averaging with Arithmetic Averaging) method. Pearson's correlation coefficient was used to carry out multiple correlation analysis for virus resistance traits with beta carotene, micronutrients (zinc and iron) and dry matter content. Principal component analysis (PCA) was obtained using XLSTAT 2015 version. Summary statistics of the molecular data were computed using PowerMarker version 3.0. Genetic distance and Identity between the genotypes as well as the level of molecular variance (AMOVA) between and within genotypes was computed using GenAIEx version 6.5.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1. Results

4.1.1 Screening of orange fleshed clones for Sweet potato viruses.

Detection of viruses infecting sweet potato was carried out using Nitrocellulose membrane ELISA (NCM-ELISA). The kit was used to detect the following viruses (Table 4): Sweet potato chlorotic stunt virus, Sweet potato feathery mottle virus (SPFMV), Sweet potato mild mottle virus (SPMMV), sweet potato chlorotic fleck virus (SPCFV), Sweet potato latent virus (SPLV), Sweet potato mild speckling virus (SPMSV), Sweet potato chlorotic fleck virus (SPCFV), sweet potato caulimo-like virus (SPCaLV), C-6 virus and Cucumber mosaic virus (CMV). Presence of the viruses in the samples tested was a clear purple spot where the sample had been applied on the membrane (see example on Plate 4).

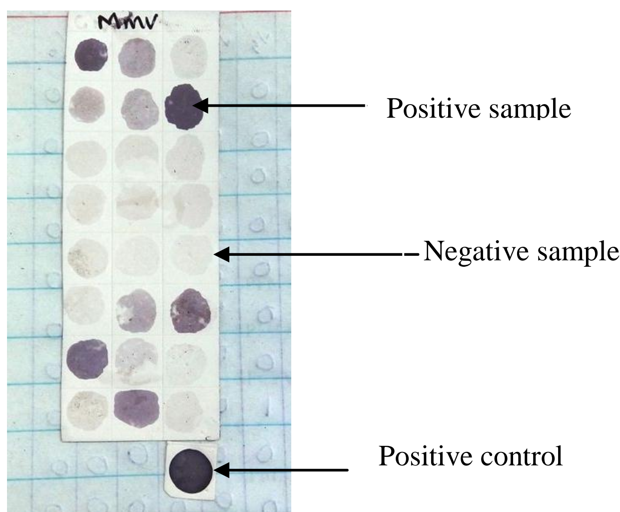

Plate 4: Detection of SPMMV using NCM ELISA

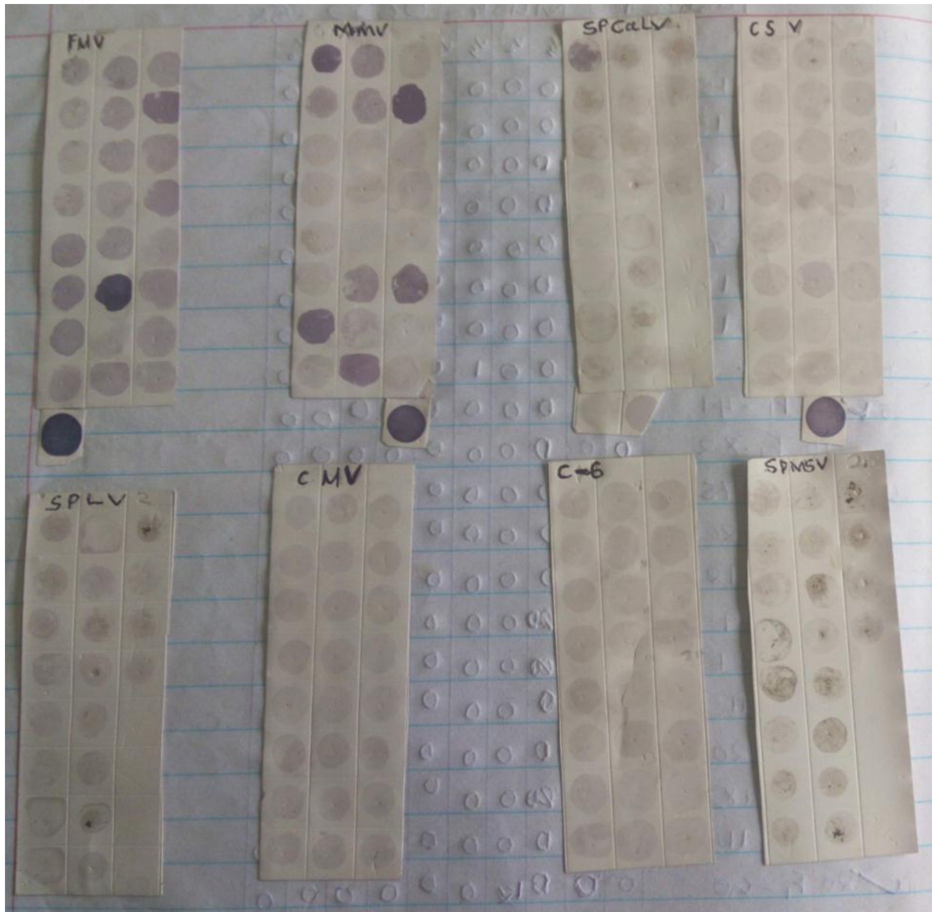

Plate 5: Detection of sweet potato viruses using NCM ELISA among sweet potato test clones.

Table 4. Summary of presence or absence of the nine viruses assayed sweet potato clones using NCM-ELISA kit.

Sample	1	2	3	4	5	6	7	8	9
	SPFMV	**SPMMV**	**CMV**	**SPCSV**	**SPCFV**	**C-6**	**SPCaLV**	**SPLV**	**SPMSV**
1	0	1	1	0	0	0	0	0	0
2	0	1	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	1	0	0	0	0	0	0	0	0
6	1	0	0	0	0	0	0	0	0
7	1	1	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	1	0	0	0	0	0	0	0
10	0	1	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	1	1	0	1	0	0	0	0	0
15	0	1	0	0	0	0	0	0	0
16	0	1	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	1	1	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	1	0	0	0	1	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	1	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
Total	**6**	**10**	**1**	**1**	**1**	**0**	**0**	**0**	**0**

4.1.2. Reverse Transcriptase PCR for validation of results obtained from NCM-ELISA screening of sweet potato clones.

Samples that showed a positive and a negative signal on the NCM-ELISA test were selected and used in the validation experiments. Positive samples had single infections of SPFMV and SPCSV Gel photographs of the RT-PCR products of assayed sweet potato samples are presented in Plates 5 and 6. Up to 94, 90 and 88 percent of the 12 test samples were negative for the SPCSV, SPFMV and SPMMV respectively.

Plate 5: 1.5 % agarose gel showing the detection of SPFMV. Samples 9 and 12 tested positive for the virus. Sample 14 was used as a non template negative control. Sample 15 and 16 are positive controls. M is 100 bp ladder.

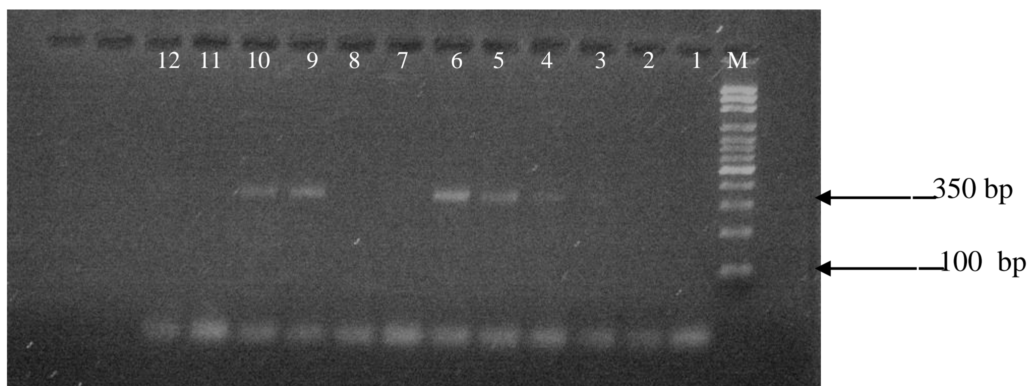

Plate 6. 1.5 % agarose gel showing the detection of SPCSV in a section of the sweet potato samples. Test samples 4, 5, 6 and 9 tested positive. Sample 10 is a positive control. Sample 11 and 12 were negative controls.

4.1.3. Determination of dry matter, zinc, carotene and iron in orange fleshed clones.

A total of thirteen sweet potato clones and two checks were analyzed for root dry matter, Iron, Zinc and content (Table 5). Two way ANOVA of the data obtained revealed significant differences in beta –carotene, dry matter content and iron among sweet potato families (P< 0.05). Beta-carotene content was highest in clones F1C7 (52.00), F4C15 (47.33) and F2C13 (34.00). Lowest carotene levels were found in Clones F4C11 (0.33) and F3C5 (3.67). Levels of Iron varied among test clones. Highest values were recorded in clones F4C9 (5.00), F1C9 (6.00). Lowest Iron levels were found in clone F6C10 (1.67). Clones F4C9 (44.33 and Kenspot 4 (47.67 recorded the highest Zinc content. Lowest Zinc content was recorded in clone F4C15 (3.33) however this clone had high beta-carotene content (47.33). Lowest virus scores were in clones F1C2 (4.00 and F1C9 (4.00). Highest virus score among selected clones was in clones F3C9 (5.67) and in clone F4C9 (5.33).

Table 5. Content of root beta-carotene, dry matter, zinc, iron and average virus scope in OFSP clones.

Family/Clone	ß-Carotene in ppm	Dry matter in gm	Iron in ppm	Zinc in ppm	Average virus score
F1C7	52.28[a]	23.55[abc]	4.03 [abc]	19.67	4.67
F4C15	47.13[ab]	24.96[abc]	4.6 [abc]	3.33	4.00
F2C13	33.67[abc]	18.07 [c]	3.367 [abc]	8.00	4.33
Kenspot 4	30.82[abc]	25.52[abc]	3. 467 [abc]	47.67	4.67
Kenspot 5	19.89[abc]	27.76[abc]	3.233 [bc]	24.67	4.67
F3C9	17.33[abc]	19.72 [bc]	4.233 [abc]	35.00	5.67
F4C9	17.07[abc]	28.45[abc]	4.667 [ab]	44.33	5.33
F6C10	9.81 [bc]	29.18[abc]	1.843[c]	4.67	5.67
F2C14	7.7 [c]	26.28[abc]	4.267 [abc]	5.00	5.33
F1C2	6.86 [c]	29.82[abc]	5.8 [ab]	6.0	4.00
F2C9	6.46[c]	21.39[abc]	5.6 [ab]	5.67	4.67
F1C9	6.08 [c]	20.27[bc]	6.033[a]	14.67	4.00
F3C1	3.96 [c]	26.67[abc]	4.667 [ab]	18.33	5.00
F4C11	3.5 [c]	32.84[ab]	3.667 [abc]	21.67	4.67
F3C3	2.36 [c]	29.85[abc]	3.8 [abc]	36.33	4.00
F3C5	0.64 [c]	34.49[a]	3.633 [abc]	2.33	4.33

Mean values within a column followed by the same letter are not significantly different at $p \leq 0.05$ by LSD.

4.1.4. Phenotypic correlation between disease severity score, beta carotene, dry matter . Iron and Zinc.

The 13 sweet potato clones and two checks (clone Kenspot 4 and 5) were used to determine the phenotypic correlation between virus challenge responses and the root content of β carotene levels, dry matter, iron and zinc (Table 6). Pearsons correlation analysis revealed negative correlation (r= -.443**) between dry matter and beta-carotene content. Root dry

matter and iron content were also negatively correlated (r=-.296*). There was no correlation between average virus score and the root attributes assayed in this study

Table 6: Phenotypic correlation coefficients between virus response and root content of beta carotene, dry matter and micronutrients in sweet potato clones.

	ß-Carotene	Dry weight	Iron in ppm	Zinc in ppm	Average virus score
ß-Carotene		-.443**	-.090	-.071	-.017
Dry weight			-.296*	.122	-.059
Iron in ppm				-.031	-.263
Zinc in ppm					-.073

**. Correlation is significant at p=0.01 level.

*. Correlation is significant at p=0.05 level.

4.1.5 Determination of putative resistance traits by characterizing OFSP clones using SSR markers linked to virus resistance.

Genomic DNA was successfully isolated from 17 sweet potato genotypes (listed in Table 7) that included thirteen test OFSP clones, two checks namely Kenspot 4 and Kenspot 5 and two varieties known to be virus susceptible (cultivars Ejumula and Carrot C) for screening with ten SSR markers that are linked to virus resistance. The isolated total genomic DNA was intact and of high molecular weight (Plate 7). The genomic DNA was therefore suitable for PCR analysis using SSR primers.

Table 7. Seventeen genotypes that were evaluated for putative resistance markers using SSRs.

Sample Number	Family/ Clone	Test Attribute(s)
1	F1/C2	Test clone
2	F1/C7	Test clone
3	F1/C9	Test clone
4	F2/C13	Test clone
5	F2/C9	Test clone
6	F3/C1	Test clone
7	F3/C13	Test clone
8	F3/C3	Test clone
9	F3/C5	Test clone
10	F3/C9	Test clone
11	F4/C15	Test clone
12	F4/C9	Test clone
13	F4/C11	Test clone
14	Kenspot 4	Medium resistance to virus diseases
15	Kenspot 5	Medium resistance to virus diseases
16	Ejumula	Virus susceptible variety
17	Carrot C	Virus susceptible variety

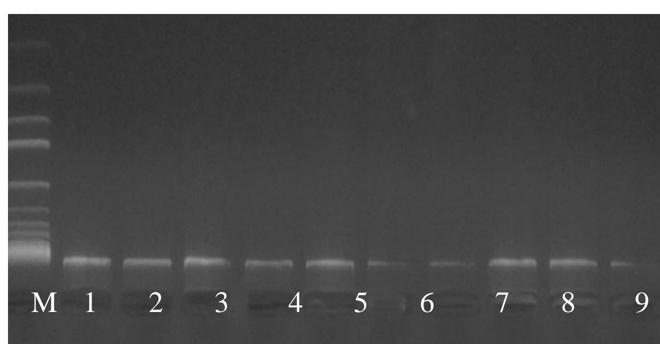

Plate 7: Genomic DNA isolated from sweet potato genotypes samples 1-9 listed in Table 7 and resolved in 1 % agarose gel after digesting with RNase A. M is a 100bp DNA ladder.

SSR-PCR assays of the 17 sweet potato clones revealed positive amplicons. The genotypes were scored for the presence (1) or absence (0) of specific fragment (alleles) in a binary

matrix. Only those fragments (alleles) with medium or high intensity were taken into account in the subsequent analysis. Fragments with the same mobility on the gel but with different intensities were not distinguished from each other when genotypes were being compared. The

SSR microsatellites used in this study revealed different levels of polymorphism across test genotypes. Different profiles were obtained when clones from Family 1 (F1C2, F1C7 and F1C9) were screened against markers IB-R03, IB- CIP, IB-316 and IBJ 522 (Plate 7). The screening for putative resistance traits in the 17 genotypes using different SSR markers revealed varying alleles among test samples. Reliability of SSR microsatellites in putatively identifying resistance and/or susceptibility traits was revealed by absence of some loci in the two susceptible genotypes which were available in the other test clones using marker IB-316 (Plate8). General polymorphism was identified among test clones when marker JB 1809 was used to screen the clones (Plate 10). Some clones had notable absence of some alleles such as F2C9, F3C13, F3C3 and F3C9. The clones are predominantly from Family 3. Clone F2C9 could be related to Family 3 closely. The clones were however characterized by low carotene content.

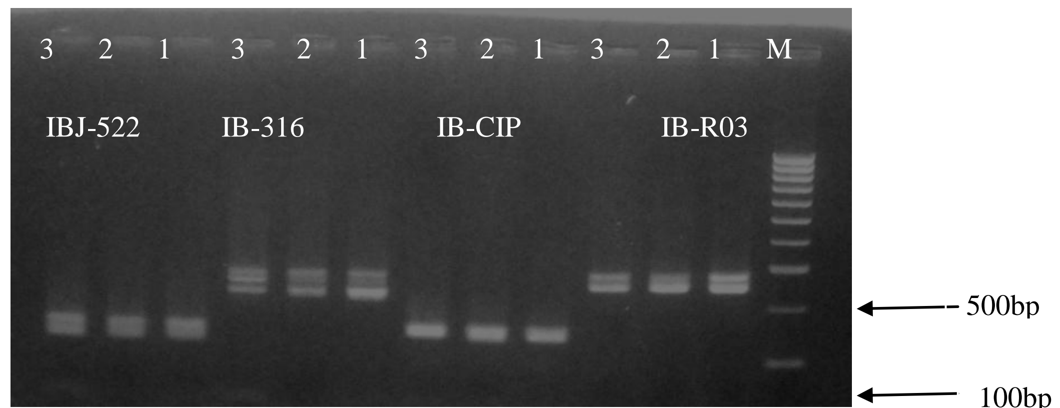

Plate 8: 3% Agarose gel profiles of clones F1C2 (lane 1), F1C7 (lane 2) and F1C9 (lane 3) screened with markers IB-R03, IB- CIP, IB-316 and IBJ 522. Lane M is a 100bp DNA ladder. Arrow shows polymorphic SSR Marker IB-CIP 316.

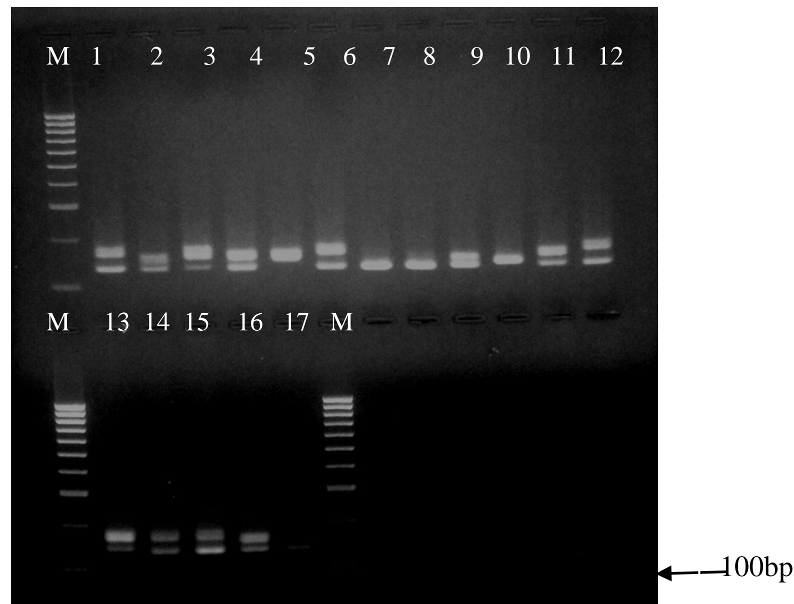

Plate 9**:** PCR products resolved in 3% agarose gel from 17genotypes (Table 8) showing polymorphic alleles using marker JB-1809. Lane M is a 100 bp DNA ladder. Some alleles present I sample six are missing in samples 5,7, 8 and 10 and susceptible genotypes (Lane 16 and 17).

4.1.6 Major allele frequency, allele number, genetic diversity and polymorphism information content in the 17 genotypes.

Power Marker Version 3.0 was used to give summary statistics output. Six simple sequence repeat markers (1B-316, IBJ-324,IBJ-522,IB 1809,IB-R03 and IB-CIP) for 6 loci were used to characterize 17 sweet potato genotypes. A total of 18 alleles were detected. The number of alleles per locus ranged from 6 to 2 for markers IBJ-324, IB-1809, IB R03 and IB-CIP with an average of 3.0 alleles per locus. The highest major or abundant alleles was observed in marker IBJ-324 (0.94) whereas marker IB-316 showed the lowest value abundant alleles at 0.2941.Major allele frequency mean for the six markers was 0.696. Genetic diversity among the six markers was highest in marker IB-316 at 0.78 while the least diversity was recorded in marker IBJ-324. Mean genetic diversity of the markers was 0.41. The polymorphic information content (PIC) ranged from the lowest, 0.10for marker IB-324 and IB-CIP highest value of 0.74 for marker IB-316. Average polymorphic information content was 0.36. (Table7).

Table 8: Summary statistics for major allele frequency, allele number, genetic diversity and polymorphism information content in the 17 genotypes.

Marker	Major allele frequency	Sample size	Number of observations.	Allele numbers	Availability	Gene diversity	PIC
1B-316	0.29	17.0	17.0	6.0	1.0	0.78	0.74
IBJ-324	0.94	17.0	17.0	2.0	1.0	0.11	0.10
IBJ-522	0.64	17.0	17.0	3.0	1.0	0.49	0.41
IB 1809	0.70	17.0	17.0	3.0	1.0	0.45	0.41
IB-R03	0.70	17.0	17.0	2.0	1.00	0.41	0.32
IB-CIP	0.88	17.0	17.0	2.0	1.00	0.20	0.18
Mean	0.69	17.0	17.0	3.0	1.00	0.41	0.36

4.1.7 SSR markers allele counts, frequencies and band patterns for Binary Data

The allele counts ranged from 1 to 16. Highest allele count was recorded in marker IBJ- 324 while lowest counts was found in some alleles of markers IB-316, IB-324 and IBJ-322. Allele frequencies ranged from 0.05 in an allele in marker IB -316 and the highest (0.94) was recorded in marker IBJ-324. Lowest allele frequencies were observed in allele 374 and 206 bp, respectively, both at locus IBCIP-13 (Table 8). Test clones revealed highest numbers of bands and bands frequency 5 % followed by Kenspot 4 and Kenspot 5. Virus susceptible genotypes showed the least number of bands and bands frequency. Similarly, Number of private bands was highest in the test clones followed by Kenspot 4 and Kenspot 5. Ejumula and Carrot C did not reveal any private bands (Appendix 5).

Table 9: SSR markers allele counts and frequencies

Marker	Allele	Count	Frequency
1B-316	155/0/0/0/0/0	4	0.23
1B-316	155/0/0/155/0/0	1	0.05
1B-316	155/0/165/0/0/0	2	0.11
1B-316	155/160/0/0/0/0	5	0.29
1B-316	155/160/165/0/0/0	4	0.23
1B-316	160/0/0/0/0/0	1	0.05
IBJ-324	150/0/0	16	0.94
IBJ-324	150/155/0	1	0.05
IBJ-522	220/0/0	5	0.29
IBJ-522	220/240/0	1	0.05
IBJ-522	220/240/270	11	0.64
IB 1809	0/250/0	2	0.11
IB 1809	230/0/0	3	0.17
IB 1809	230/250/0	12	0.70
IB-R03	302/0/0	5	0.29
IB-R03	302/312/0	12	0.70
IB-CIP	155/0/0	15	0.88
IB-CIP	155/160/0	2	0.11

4.1.8 Phylogenetic Analysis.

The resultant dendogram constructed using Dissimilarity Analysis Representation for Windows (DARwin version 6.0.010) revealed three clusters. Cluster 1, 2 and 3 represented 53% 29.4% and 17.6% of the 17 genotypes analyzed. Test clones clustered independently from the checks except Kenspot 5 and virus susceptible varieties. Carrot C and Ejumula were clustered so closely revealing their SPVD susceptibility trait. Kenspot 4 however was placed in the same cluster. Summarized data on tested attributes among clones in clusters indicated that most clones in cluster 1 had high beta carotene contents with an average of 25.59ppm compared to clones in cluster 2 which had an average of 6.73ppm, however 3 out of 9 clones had an average virus score of above 5 and 5.67. Cluster 2 had 4 out of five clones with virus score average below 5. Cluster 2 had higher dry matter content with an average of 27.26gm compared to 25.9 gm in cluster 1. Similarly, cluster 2 had higher iron content averaging 4.80ppm against 3.96ppm in cluster 1. Differences in zinc content was not significant since cluster 1 had an average of 17.73ppm while cluster 2 averaged at 17.33ppm. Cluster 1 revealed a higher average virus score of 4.70 while cluster 2 had an average of 4.60.

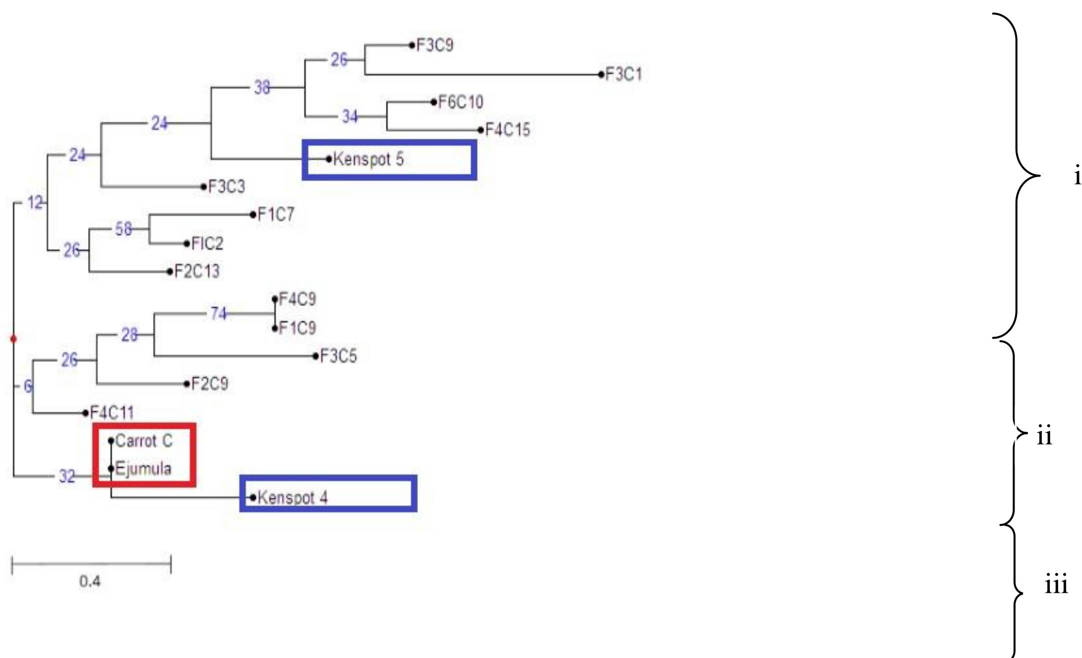

Figure 1. Neighbor joining tree showing 17 sweet potato genotypes grouping in 3 distinct clusters. Varieties in highlighted in blue represent checks (Kenspot 4 and Kenspot 5) used in the while red represents virus susceptible varieties (Ejumula and Carrot C)

Generally, most clones had acceptable dry matter, iron content and medium severity score among clusters (Table 10).

Table 10: Root beta carotene iron and zin data in OFSP based on genetic clusters derived from the dendogram in Figue 3.

Cluster	Family/clone	Carotene in ppm	Dry matter in gm	Iron in ppm	Zinc in ppm	Average virus score
Cluster 1	F3C9	17.33	19.67	4.33	35.00	5.67
	F3C1	4.00	26.33	4.67	18.33	5.00
	F6C10	10.00	29.33	1.67	4.67	5.67
	F4C15	47.33	24.67	4.67	3.33	4.00
	Kenspot 5	19.67	28.67	3.33	24.67	4.67
	F3C3	2.33	30.00	4.00	36.33	4.00
	F1C7	52.00	23.67	4.00	19.67	4.67
	F1C2	7.00	30.00	5.67	6.00	4.00
	F2C13	34.00	18.00	3.33	8.00	4.33
	Mean	**21.51**	**25.59**	**3.96**	**17.33**	**4.66**
Cluster 2	F4C9	17.00	28.67	5.00	44.33	5.33
	F1C9	6.00	20.33	6.00	14.67	4.00
	F3C5	0.33	34.33	3.67	2.33	4.33
	F2C9	6.67	21.33	5.67	5.67	4.67
	F4C11	3.67	32.67	3.67	21.67	4.67
	Mean	**6.73**	**27.46**	**4.80**	**17.73**	**4.6**
Cluster 3	Carrot C	-	-	-	-	-
	* Ejumula	-	-	-	-	-
	* Kenspot 4	30.67	24.67	3.67	47.67	4.67

* Genotypes that were used as virus susceptible varieties in SSR analysis.

4.1.8 Principal Component Analysis (PCA) based on genetic distances of the 17 sweet potato genotypes

Two major clusters were obtained in the PCA plot. The clustering pattern was similar to the neighbor joining tree since almost all clones in cluster 1 of the tree clustered together of while clones in cluster 2 of the tree grouped closely and distant from the virus susceptible varieties (Ejumula and Carrot C). Four principle components were revealed in the plot based on Eigen values obtained with the first and second coordinates accounting for a total of 69 %. (Appendix 3).

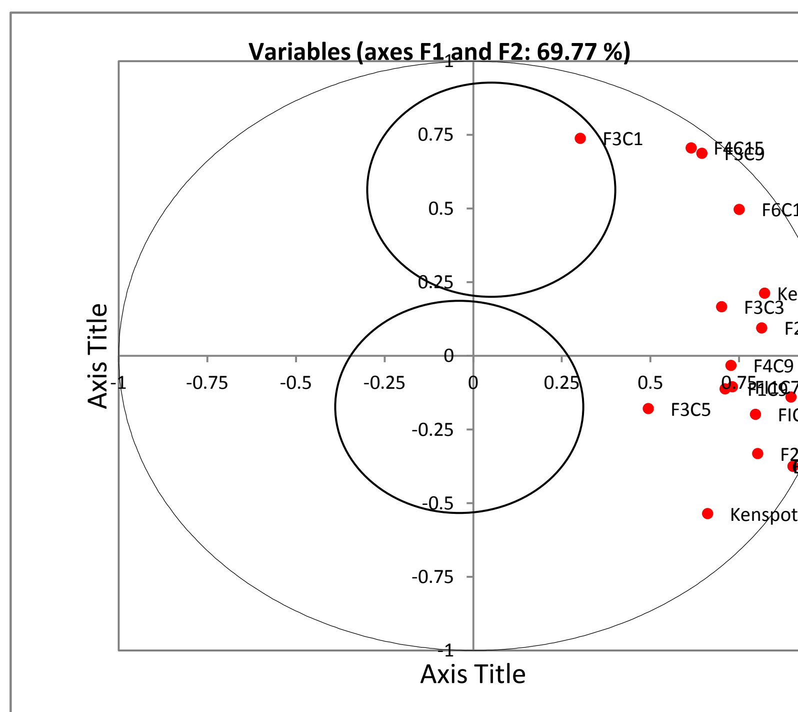

Figure 3. Principle component analysis for 17 genotypes revealing two major clusters.

4.1.9 Determination of genetic diversity and Analysis of Molecular Variance (AMOVA) in OFSPs clones

Genetic distance matrix revealed that the most identical genotypes were Carrot C and Ejumula (GD= 0), F1C2 and F1C7 (GD= 1), F1C2 and Ejumula (GD=1), F1C9 and F3C5 (GD = 2), F2C13 and F1C2 (GD=1), F2C13 and F4C11 (GD=2), F2C9 and F4C11 (GD= 2), F2C9 and F4C9 (GD= 2) and F2C9 and Kenspot 4 (GD=2) among others. The most remotely related genotypes were F1C9 and F3C1 (GD=7) F3C1 and Kenspot 4 (GD=7), F3C1 and Ejumula (GD=6), F3C1 and Carrot C (GD=6), F1C7 and F3C1 (GD=6) and F4C15 and F2C9

(GD=6). The similarity matrix showed that the genotypes such as Ejumula and Carrot C usually characterized by low virus resistance are not genetically similar to test clones such as F3C1. AMOVA data revealed 91% variance within the population accounting for 1.61 estimated variation. Molecular variation among population was 9% representing 0.15% of estimated variation (Appendix 4).

A reduced Unbiased distance matrix revealed a distance of 0.05 when Checks 2 were compared to Checks 1. This was the least distance among assayed populations. Checks 2 and test clones revealed a distance of 0.065. The highest distance of 0.068 when test clones were compared to checks 2.

Table 11: Nei reduced unbiased genetic distance matrix between test clones, Checks 1 (Kenspot 4 and Kenspot 5) and Checks 2 Ejumula and Carrot C.

Test clones	Checks1	Check 2	
0.00			Test clones
0.06	0.00		Checks 1
0.06	0.05	0.00	Checks 2

Genetic identity matrix revealed close relationship (0.96) when test clones were compared to Checks 1. Lower identity matrix value (0.93) was obtained between test clones and checks 2. The closest similarity value (1.00) was obtained between both checks.

Table 12: Relationship among test clones, Checks 1 (Kenspot 4 and Kenspot 5) and Checks 2 (Ejumula and Carrot C) based on Nei unbiased genetic identity matrix.

Test clones	Check 1	Check 2	
1.00			Test clones
0.96	1.00		Check 1
0.93	1.00	1.00	Check 2

DISCUSSIONS

4.1 Screening OFSP clones for multiple virus infections reactions, dry matter, Iron and Zinc levels

Serology of virus particles and coat proteins has been widely used for the identification of plant viruses. Enzyme Linked Immunosorbent Assay (ELISA) has consequently become the ideal test for routine plant viruses' detection due its ease, adaptability, sensitivity, and accuracy (Valverde *et al*., 2007). Even though ELISA has been used for sweet potato virus detection, there are limitations to use of this technique in sweet potato viruses. The low titre and irregular distribution of viruses in sweet potato have been identified as some detection constraints (Esbenshade and Moyer 1982). In this study, NCM ELISA detection recorded 75 %, 62.5% and 96% of SPFMV, SPMMV and SPCSV of positive samples respectively while RT- PCR revealed that 94, 90 and 88 percent respectively of test samples were positive for the SPCSV, SPFMV and SPMMV respectively two tests however concur in high detections of SPFMV among test samples. Among the samples tested in Table 5, 6 and 10 positive detections were found in SPMMV and SPFMV respectively. Single detections of CMV, SPCSV, SPCFV were found in the samples. SPFMV occurs in all sweet potato areas the crop is grown and is reported to be the most widespread in Eastern Africa region (Tairo *et al*., 2004). SPMMV virus is second in prevalence in the East Africa region, however, the virulent SPCSV is less prevalent in Kenya. SPCSV has been reported as the second most prevalent virus and occurs in single and mixed infections. Very severe symptoms are observed in dual or mixed infections with SPCSV (Nyaboga *et al*., 2008). Only four viruses have been detected among sweet potato in Kenya These include the Sweet potato feathery mottle virus (SPFMV), the crinivirus Sweet potato chlorotic stunt virus (SPCSV), the ipomovirus Sweet potato mild mottle virus (SPMMV) and Sweet potato chlorotic fleck virus (SPCFV). Single dual and multiple infections occur in the field with the SPFMV and SPCSV being the most common dual infection (Ateka *et al*., 2004a,b). Viruses C-6, SPCaLV, SPLV and SPMSV were not detected in the test samples. These viruses have not been reported in the East African region. Analysis and scoring of NCM ELISA data is hampered by different purple intensities which may lead to false positives scoring or the reverse.

Though NCM- ELISA has widely been used, analysis and scoring of NCM ELISA data is hampered by different purple intensities which may lead to false positives scoring or the reverse. This study shows that some samples deemed to be positive on ELISA may be negative on RT-PCR and vice versa, For instance, the detection of SPCSV on a section of test

samples revealed that some samples with weak signal on NCM ELISA were indeed positive in RT-PCR (Plate 4). This could be attributed to limitations experienced in NCM-ELISA. Through research, it has been demonstrated that RT- PCR has a higher detection capacity. When applied to serially diluted RNA samples, RT-PCR has been demonstrated to result in amplification from On the other hand other research findings indicate that RT- PCR has detection capacity was tested by serially diluting RNA samples. RNA resulted in amplification from undiluted to $1:10^6$ dilution that while beyond this no band was observed (Abarshi *et al.*, 2010., Kathurima *et al* ., 2011).This findings demonstrate the superiority of PCR assays. This implies that NCM ELISA is an ideal tool for the screening of these viruses on large sample numbers then validate samples that were priory tested showed a poor signal on NCM membranes due to low virus titre among other factors. Indeed, molecular diagnostics of viruses, such as PCR-based, have been described as sensitive than protein-based techniques (Souto., 2003) and therefore NCM-ELISA could be used as a complimentary diagnostic test.
Determination of beta carotene, dry matter and iron revealed significant differences among clones. However, no significant differences were found in zinc content. Field experiments conducted by Courtney, 2007 and Gruneberg *et al*, 2009 showed similar variations in dry matter and micronutrients among test materials. Generally, carotene, dry matter and micronutrients values were lower as compared to those previously reported (Tumwegamire *et al.*, 2011a).

Analysis of dry matter and micronutrients through regression analysis did not reveal significant effect of the virus scores on dry matter, iron, zinc and β carotene this is attributed to the fact that virus tolerance formed the strongest basis for selection of the clones and their subsequent analysis. Analysis of OFSP varieties (N=15 genotypes) using two way ANOVA and separation of means using LSD revealed that in clones, most traits can only selected and improved autonomously.

4.2. Determination of correlation between disease severity score, dry matter, iron and zinc

Correlation analysis of dry matter and micronutrients through regression analysis did not reveal significant effect of the virus scores on dry matter, iron, zinc and beta carotene.

This is attributed to the fact that virus tolerance formed the strongest basis for selection of the clones and their subsequent analysis. Analysis of OFSP varieties (N=15 genotypes) using two

way ANOVA and separation of means using LSD revealed that in clones, most traits can only be selected and improved autonomously.

Negative correlation (r= -.443**) between β-carotene and dry matter has been reported previously (Andrade *et al*., 2009). Therefore, the findings in this study agrees with earlier findings. Among sweet potato genotypes, there are large differences in storage root accumulation levels of protein, starch, sucrose, total sugars, β-carotene, calcium and magnesium. Interestingly, these differences are less prominent for iron and zinc concentrations. Occurrence of negative genetic correlations largely leads to unwanted and unpredicted genetic responses to selection which can result in whole breeding populations being rendered useless for variety development. Such a critical negative genetic correlation exists in sweet potato genotypes. For instance, there is strong negative genetic correlation between β-carotene and storage root dry matter content. Marker studies have established that quantitative trait loci (QTLs) for β-carotene and storage root dry matter content may be found in the same linkage groups and infrequently are closely associated with the same genetic marker. This phenomenon is referred to as pleiotropy, meaning that one gene determining two different traits. It has been observed that the inheritance of β-carotene is controlled by additive gene effects Ma 2009). The additive gene effects were identified in the inheritance of dry matter and β-carotene content (Chiona., 2009). Sweet potato breeders in Sub-Saharan Africa are aware that high dry matter and β-carotene-rich materials are hard to find as a result of the negative genetic correlation between these two traits (Andrade *et al*., 2009). Iron content in this study are similar with the findings by Bradbury and Holloway (1988); however zinc content in test clones was higher in 40% of the tested genotypes as compared to the average they reported. Negative correlation (r=-.296*) between dry matter and iron is the first to be reported in sweet potato genotypes. This could be attributed to genetic instability for the dry matter and iron traits among the clones since they are progenies of open pollinated populations. Broad-sense heritability estimates for iron and zinc 0.74 and 0.82 respectively are promising since iron and zinc content can be improved using traditional mass-selection techniques (Singleton., 2008). Dry matter heritability estimates of 0.92 is encouraging since consumers in third world countries prefer sweet potatoes with high dry matter content. Since dry matter has a high heritability estimate, iron and zinc can be improved and still produce a sweet potato that is high in dry matter (Courtney., 2007).

Moderate genetic variation for protein and minerals such as iron and zinc is found among sweet potato genotypes. The absence of correlation in most traits in this study indicates that each trait can be selected and improved independently (Acquaah., 2007).

4.3. Determination of putative resistance traits by characterizing OFSP clones using SSR markers

Availability of putative resistance traits for characterizing OFSP clones using SSR markers is key in marker assisted breeding and selection of genotypes with sources of resistance to SPVD. In recent times, SSR markers have been used to characterize sweet potato genotypes for SPVD resistance and high dry matter content in the germplasm collection in Kenya (Karuri *et al*., 2009). This study used 10 SSR markers (Table 6) of the markers showed considerable levels of polymorphism. Seventeen genotypes were screened using Marker JB-1809. This present study used 10 SSR markers which revealed considerable levels of polymorphism. Seventeen genotypes were screened using Marker IB-316 which revealed that certain SSR alleles were absent in all the susceptible genotypes and not the moderately tolerant genotypes clearly demonstrating the potential to identify putative SSR markers that maybe linked to tolerance / resistance to SPVD. However, to validate the linkage of such markers to the resistance/tolerance trait, a large number of genotypes that segregate for the SPVD trait need to be screened.

Neighbor joining tree generated using DARwin revealed 3 clusters with Carrot C and Ejumula varieties clustering in the same group. These two varieties are known to have low resistance to SPVD. In the same cluster, Kenspot 4 fell in the group revealing that the genotype has low levels of SPVD resistance. The clustering of Ejumula, Carrot C and Kenspot 4 in the same group exhibits the ability of SSR markers to discriminate virus tolerant sweet potato genotypes from the susceptible ones.

On the contrary, the test clones did not group with the susceptible genotypes. This implies that they possess the putative sweet potato viruses' resistance genes. This findings corroborate findings by earlier workers that microsatellites are useful in identification of resistant genotypes. Analysis of the 57 Tanzanian genotypes against SPVD infection revealed that a high proportion (43.8%) of genotypes has promising resistance against SPVD (Gwandu *et al*., 2012).

Presence of genetic differences among the SPVD resistant genotypes revealed by their clustering into discrete groups suggests the occurrence of different sources of resistance to SPVD clones. Neighbor joining tree generated with markers 1B-316,IBJ-324,IBJ-522,IB 1809,IB-R03 and IB-CIP revealed two groups of SPVD resistant genotypes. Ideally, the relationship between genotypes in the cluster groups could not be attributed to their resistance to SPVD but other attributes such as dry matter or micronutrients. This findings are similar with Karuri *et al.*, 2009 where Kenyan sweet potato genotypes with SPVD resistance clustered in to three independent clusters.

Data on tested attributes among clones in indicated that cluster 1 had high beta carotene contents with an average of 25.59 compared to clones in cluster 2 which had an average of 6.73.Cluster 2 had higher dry matter content with an average of 27.26 compared to 25.59 in cluster. Cluster 2 had higher iron content averaging 4.80 as 3.96 compared to in Cluster 1. Some clones (F2C9, F3C13, F3C3 and F3C9) screened using marker JB-1809 did not reveal some alleles that were present in other samples this was attributed genetic relatedness among them. These clones were characterized by low carotene content. Three clones originated from Family 3 implicating that the family is characterized by low carotene content. This trend in clustering is indicative of the power of SSR markers to be used in selection of OFSPs with the desired root beta carotene, iron and zinc. This trend in clustering is indicative of the power of SSR markers to be used in selection of OFSPs with the desired content of root beta carotene, iron and other attributes. Efforts have been made to develop a genetic linkage map of sweet potato and quantitative trait loci (QTL) analysis has already been attempted in the species (Cervantes-Flores., 2006)Previously, development of a genetic linkage map and QTL analysis in sweet potato molecular markers have been achieved. Subsequently, markers have been used in identification of quantitative trait loci for dry-matter, starch, and β-carotene content in sweet potato germplasm (Cervantes-Flores., 2011).

4..4. Determination of molecular diversity of OFSP clones with putative resistance traits based on SSR markers

Principal component analysis (PCA) based on genetic distances of the 17 sweet potato genotypes provides information about relatedness between the OFSPS genotypes. Key use would be to incorporate information obtained in conventional breeding or marker assisted selection breeding. Results obtained on PCA in this study backed by the result of the neighbor joining tree clustering clearly indicate that SSR markers are proficient in detecting

genetic relationships in sweet potato genotypes with response traits (resistant or susceptible) to SPVD and other attributes such as micronutrients. This is so because even with one principle component, virus susceptible varieties grouped closely as in the neighbor joining tree. Test clones did not group close to Ejumula and Carrot C as demonstrated in the tree. Absence of other principal components could be attributed to similarity in a trait such as micronutrient content or dry matter. Genetic variability among three populations (test clones, Checks 1 and Checks 2) using Unbiased Nei genetic distance revealed that Checks 1 were compared to Checks 5 were more closely related. Checks 2 and test clones revealed a distance of 0.065.

The highest distance of 0.068 when test clones were compared to checks 2 this implies that the test clones have an attribute that is clearly absent in checks 1 and checks 2. The most plausible attribute is resistance to SPVD. Overall, genetic distance obtained in this study is low since the test clones which form majority of the samples were derived from only a few open pollinated varieties raised from different families of OFSPs. Higher Nei unbiased genetic distance have been reported in sweet potato (Ngailo *et al*., 2015). In the present study, Carrot C and Ejumula were the most closely related genotypes as earlier confirmed in the neighbor joining tree and the PCA. In the present study, Carrot C and Ejumula were the most closely related genotypes as earlier confirmed in the neighbor joining tree and the PCA. Jaccard's coefficients obtained by Karuri *et al*., (2009), who similarly employed simple sequence repeats to analyze sweet potato cultivars. SSR based Jaccard's coefficient ranged from 0.5 to 1, with an average of 0.752, accounting for 50% variation among the 89 genotypes. In this study, Carrot C and Ejumula were the most closely related genotypes as earlier confirmed in the neighbor joining tree, PCA. This could be attributed to their viruses' response traits. The least similarity among the genotypes was between F1C9 and F3C1. This distant relationship arose from the clones coming from different families. Clone F3C1 was uniquely different from Ejumula, and Carrot C. This was revealed in the tree and PCA plot. Distinctiveness of this clone against the virus susceptible genotypes is largely attributed to virus tolerance/ resistance. However, the clone was characterized by low carotene content. The mean genetic similarity coefficient of 0.41 obtained in this study is low, implying occurrence of large diversity among the studied genotypes. Comparably, Zhang *et al*. (2000) reported a higher similarity coefficient (0.588) among sweet potato accessions from South America which is also known to be a centre of diversity. Observed similarity coefficient in OFSPs slightly lower. Higher mean similarity coefficient values of 0.64, 0.69, and 0.79 have

been reported by Hwang et al., (2002), Tseng *et al*., (2002) and Abdelhameed *et al*., (2008) respectively

One of the factors contributing to the high degree of variation between the clones found in this study may be related to the mating system of sweet potato, a cross pollinating and hexaploid species (Ozias-Akins and Jarret., 1994). Microsatellite or simple sequence repeat (SSR) markers demonstrate high levels of polymorphism. Currently, several SSR markers have been developed for sweet potato genotyping (Hu *et al.*, 2004).

The markers have been used effectively in determining the genetic relationship between cultivars or varieties derived from hybrid or polycrossing breeding programs (Hwang *et al.*, 2002) and for analyzing the genetic diversity of East African sweet potato landraces (Gichuru *et al.*, 2006).The polymorphic information content (PIC), refers to the measure of the usefulness of each microsatellite in distinguishing one individual genotype from another (Weir, 1996). Despite the differences between individual marker loci, the microsatellite markers used in this study were generally informative in unraveling the genetic relationships of the 17 sweet potato genotypes. The presence of easily scorable alleles renders the system ideal for genotype identification and diversity analysis using SSRs.

When the sweet potato germplasm assayed in this study was categorized into populations based on their sources and response to SPVD (n=3; OFSP test clones, SPVD susceptible controls, and SPVD tolerant controls), analysis of molecular variance (AMOVA) revealed that 91% of the total variation was partitioned into the within population component and only 9% was partitioned among populations. In their study carried out in Tanzania using SSRs, Abdelhameed *et al*., (2008) reported that 91.2% of the total reported genetic variability was partitioned into within populations while only 3.8% was among regions. This is not unexpected because sweet potato is out crossing. New plants or new genotypes, arising from seeds resulting from cross pollinations, will therefore be diverse due to gene segregation and assortment (Veasey *et al*., 2008). Based on the results obtained from this study, collection strategies for purposes of genetic conservation of the species should focus more on intense sampling within only a few populations ensuring the ecological amplitude of the target populations is adequately covered.

CHAPTER FIVE.

CONCLUSIONS AND RECOMMENDATIONS

This current study concludes the following

Screening of orange fleshed sweet potato clones for presence disease resistance and micronutrients is key to identification of candidates for sweet potato breeding. Use of NCM ELISA and RT-PCR should be applied to complement each other in detection of sweet potato viruses.

Information on possible correlation to virus responses on beta carotene levels, dry matter content and micronutrients levels in sweet potato progenies with medium levels of virus infection has been documented.

Kenspot 4. clustering with SPVD susceptible varieties in the dendogram implies to that the levels of SPVD resistance in the genotype are low. It is therefore imperative that combine field disease score data and SSR analysis to confirm the former.

The present study findings reveal the discriminatory ability of SSR markers into discrete cluster of SPVD susceptible varieties (Carrot C and Ejumula) is a promising finding since future SSR analysis on SPVD tolerance can be successful through use of this two varieties or other susceptible ones such as Resisto and a set of markers used in this study.

This study recommends the following.

1. Screening of orange fleshed sweet potato clones for presence disease resistance and micronutrients should be carried out in sweet potato breeding programmes.
2. Root dry matter and iron content were negatively correlated (r=-.296*). Further studies should be conducted to confirm this finding.
3. Further evaluation of SSR markers is proposed in order to identify more markers resistance to SPVD.
4. Diverse populations of OFSPs should be evaluated in order to establish their genetic diversity which is useful for crop improvement..

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APPENDICES

Appendix 1.Buffers recipes for DNA extraction

CTAB extraction buffer

1.0 M Tris HCl pH 8.0

5 M NaCl

2% CTAB

0.1% β-mercaptoethanol

Chloroform Isoamyl Alcohol 24:1

TE buffer

10 mM Tris HCl pH 8.0

1 mM EDTA

Appendix 2.Buffers for NCM-ELISA

(i) TBS pH 7.5

- 0.02 M Tris base

- 0.5 M NaCl

(ii) T-TBS

- TBS- 0.05, Tween-20

(iii) Substrate buffer pH 9.5 (0.5 l)

-Tris base 6.05 g (0.1 M)

- NaCl 2.92 g (0.1 M)

- $MgCl_2.6H_2O$ 0.51 g (0.005 M)

(iv) Substrate solution for NCM-ELISA

(a) NBT stock solution

- NBT 40 mg

- N, N-dimethylformamide (70%) 1.2 ml

- Mix well and store at 4° C protected from light

(b) BCIP stock solution

- BCIP 20 mg

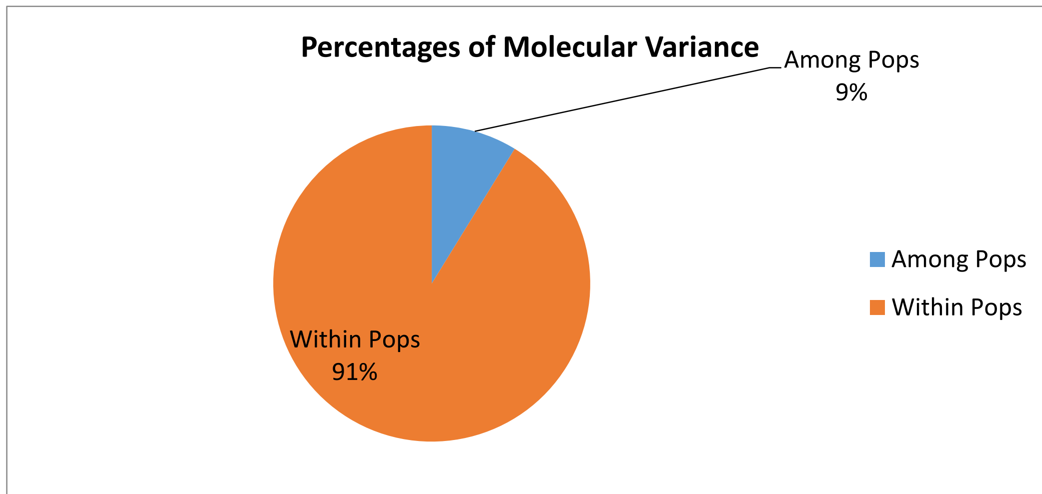
- N, N-dimethylformamide (70%) 1.2 ml

- Mix well and store at 4° C protected from light after preparation.

Appendix 3. Principal Component Analysis of 17 genotypes.

	F1	F2	F3	F4	F5	F6	F7	F8	F9
Eigen value	9.222	2.639	1.519	1.140	0.816	0.736	0.575	0.248	0.105
Variability (%)	54.249	15.523	8.934	6.708	4.798	4.327	3.381	1.459	0.620
Cumulative %	54.249	69.772	78.706	85.415	90.213	94.540	97.921	99.380	100.000

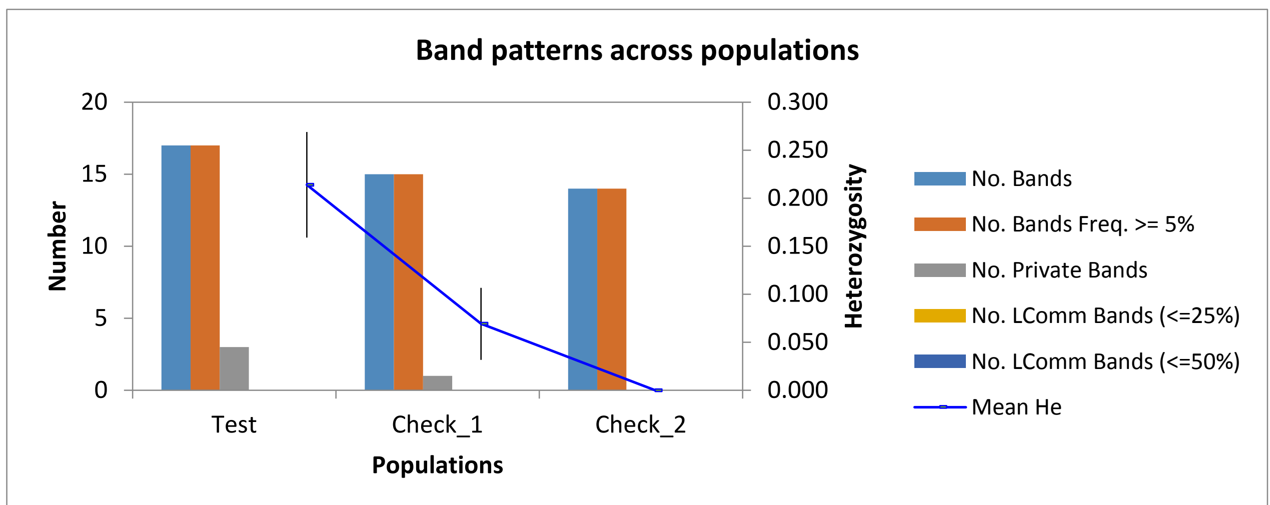
Appendix 4. Analysis of Molecular Variance (AMOVA).



Summary AMOVA Table

Source	df	SS	MS	Est. Var.	%
Among Pops	2	4.247	2.123	0.155	9%
Within Pops	14	22.577	1.613	1.613	91%
Total	16	26.824		1.768	100%

Appendix 5. Band Patterns for Binary Data.



Appendix 6. List of Publications

1. **S.M. Kiarie,** L.S. Karanja, M. Obonyo and F.N. Wachira (2016). Biochemical relationships involving multiple virus infections, dry matter and selected nutritional attributes in orange fleshed sweet potato. Acta Hortic. 1118. ISHS 2016. DOI 10.17660/Acta Horticulturae. Acta Hortic.
2. **S.M. Kiarie,** L.S. Karanja, M. Obonyo and F.N. Wachira (2016). Application of SSR Markers in Determination of Putative Resistance to SPVD and Genetic Diversity among Orange Fleshed Sweet Potato. Journal of Advances in Biology & Biotechnology 9(2): 1-10.

Appendix 7. List of SSR makers sequences

	Name of locus	Primer pairs 5' to 3'	Annealing temperature.	Size range
1	IB-R03	F:GTAGAGTTGAAGAGCGAGCA R:CCATAGACCCATTGATGAAG	58	302-312
2	IB-S07	F:GCTTGCTTGTGGTTCGAT R:CAAGTGAAGTGATGGCGTTT	60	193-211
3	IB-R12	FGATCGAGGAGAAGCTCCACA R:GCCGGCAAATTAAGTCCATC	60	356-395
4	IB242	F:GCGGAACGGACGAGAAAA R:ATGGCAGAGTGAAAATGGAACA	58	136-155
5	IB297	F:GCAATTTCACACACAAACACG R:CCCTTCTTCCACCACTTTCA	58	150-182
6	IB316	F:CAAACGCACAACGCTGTC R: CGCGTCCCGCTTATTTAAC	58	151-167
7	IBCIP-1	F:CCCACCCTTCATTCCATTACT R:GAACAACAACAAAAGGTAGAGCAG	63	155-167
8	IB324	F:TTTGGCATGGGCCTGTATT R:GTTCTTCTGCACTGCCTGATTC	56	140-280
9	IBJ 522a	F:ACCCGCATAGACACTCACCT R:TGACCGAAGTGTATCTAGTGG	57	110-190
10	JB 1809	FCTTCTCTTGCTCGCCTGTTC R:GATAGTCGGAGGCATCTCCA	60	225-298

Appendix 8 Analysis of variance for iron

Variate: Feppm					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	10.3343	5.1671	6.93	0.003
Family	15	50.9725	3.3982	4.56	<.001
Residual	30	22.3743	0.7458		
Total	47	83.6811			

Appendix 9. Analysis of variance for zinc

Variate: Znppm					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	276.6	138.3	0.3	0.744
Family	15	10410.7	694	1.5	0.169
Residual	30	13920.7	464		
Total	47	24608.1			

Appendix 10. Analysis of variance for virus score

Variate: Average virus score					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	0.4018	0.2009	0.24	0.785
Family	15	15.4058	1.0271	1.25	0.294
Residual	30	24.7234	0.8241		
Total	47	40.5309			

Appendix 11. Analysis of variance for dry weight

Variate: DRYWT					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	60.34	30.17	1.74	0.193
Family	15	1015.31	67.69	3.9	<.001
Residual	30	520.93	17.36		
Total	47	1596.57			