OCCURRENCE, DISTRIBUTION AND MOLECULAR DIVERSITY OF GROUNDNUT ROSETTE ASSISTOR VIRUS CAUSING GROUNDNUT ROSETTE DISEASE IN WESTERN KENYA

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Award of the Degree of Master of Science in Crop Protection of Masinde Muliro University of Science and Technology

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DECLARATION

This thesis is my original work prepared with no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award.

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of Masinde Muliro University of Science and Technology a thesis entitled "*Occurrence, distribution and molecular diversity of groundnut rosette assistor virus causing groundnut rosette disease in western Kenya*".

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DEDICATION

To my parents, Mr Nehemiah Muke, Mrs Truteah Muke and my daughter Sharleen Mabele.

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ABSTRACT

Groundnut (*Arachis hypogaea* Linn) is an important legume in western Kenya, but yields are low and declining. Pests and diseases are ranked high among the yield reducing factors. Groundnut rosette disease (GRD) is the main disease and can cause up to 100% yield loss. Rosette is transmitted mainly by the groundnut aphid, *Aphis craccivora* Koch and to a lesser extent by *Aphis gosypii* Glover and *Myzus persicae* Sulzer. Rosette is caused by two synergistic viruses; groundnut rosette assistor virus (GRAV, genus *Luteovirus*) and groundnut rosette virus (GRV, genus *Umbravirus*) associated with a satellite-ribonucleic acid (sat-RNA). Inadequate current information on the occurrence, distribution and diversity of GRAV causing GRD in western Kenya, is a limiting factor on proper diagnosis and management of GRD which gave the impetus for this study. This study determined the occurrence, distribution and diversity of GRAV on groundnuts in western Kenya. A survey of GRD was conducted in Bungoma and Kakamega Counties during the short rains (October – December 2016) and long rains (May – June 2017). Symptomatic leafy samples were collected in falcon tubes containing RNALater solution, and preserved for laboratory analysis. The data collected on incidence and severity was subjected to analysis of variance and pairwise comparison of means done using Least Significance Difference at $P \leq 0.05$. Screening for resistance to GRAV was done on five popular legume varieties and one solanaceous *Physalis peruviana* Linn. The plants at three leaf-stage were mechanically inoculated with GRD inoculum prepared from leaves of RT-PCR positive samples. The plants were monitored for symptom development in the screenhouse for 8 weeks. Total RNA was extracted from the leaf samples using RNeasy Mini Kit **(**Qiagen**)** according to the manufacturers' protocol. The extracted total RNA was used for double stranded cDNA synthesis using the SuperScript II kit. DNA libraries were prepared and sequenced on the MiSeq platform (Illumina). Quality check on reads was done using FastQC. Trimmed reads were used for de novo assembly and contigs aligned to the viral genomes database using CLC Genomics Workbench 10.1.2. The assembled contigs were subjected to a BLASTn search against the GenBank database. Phylogenetic analyses and comparisons were performed using the MEGA X software. Primers used in RT-PCR were designed using Primer3Plus software from consensus sequences. A total of 144 farms were surveyed. Rosette was observed in all the surveyed areas with chlorotic symptoms being dominant followed by green rosette and mosaic. Mean rosette incidence was higher in Bungoma (66.51%) than Kakamega (60.52%). Short rains had higher mean incidence than the long rains season. Nucleotide sequences of GRAV coat protein (CP) gene revealed 97-99% identity among the western Kenya isolates and those from Ghana, Malawi and Nigeria. All tested plants developed viral symptoms and tested positive for GRAV by RT-PCR. The fact that GRD occurs wherever groundnuts are grown in western Kenya, is of great concern and may be the reason for the observed low yields. Incorporation of GRD resistant genes in the local cultivars/varieties may be the only practical solution.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS AND ACRONYMS

AFLP	- Amplified Fragment Length Polymorphism
BLAST	-Basic Local Alignment Tool
BSA	-Bulked Segregant Analysis
cDNA	-Complementary Deoxyribonucleic Acid
CDS	- Coding Sequences
CP	- Coat Protein
dNTP	- Deoxyribonucleotide Triphosphate
dsRNA	- Double Stranded Ribonucleic Acid
EtBr	- Ethidium Bromide
GPS	- Global Positioning System
GRAV	- Groundnut Rosette Assistor Virus
GRD	- Groundnut Rosette Disease
GRV	- Groundnut Rosette Virus
GTR	- General Time Reversible model
HKY	- Hasegawa-Kishino-Yano model
ICGV	- ICRISAT Groundnut Variety Number
ICRISAT	- International Crops Research Institute for the Semi-Arid Tropics
IPM	- Integrated Pest Management
JC	- Jukes Cantor model

K2	- Kimura 2-parameter model
K26	- Kenya green gram variety
KALRO	- Kenya Agricultural and Livestock Research Organization
KK15	- KALRO-Kakamega local bean variety
MEGAX	-Molecular Evolutionary Genetics Analysis Across Computing Platforms
MLT	- Maximum Likelihood Tree
M-MuLV	- Moleney-Murine Leukemia Reverse Transcriptase enzyme
MOA	- Ministry of Agriculture
NGS	- Next Generation Sequencing
NSAAM	- Nucleotide Sequence Alignment Analysis by MUSCLE
nt	- Nucleotide
ORF	- Open Reading Frame
PDR	- Pathogen Derived Resistance
PBS	- Phosphate Buffered Saline
RPKPMMR	- Reads Per Kilobase Per Million Mapped Reads
RT-PCR	- Reverse Transcription-Polymerase Chain Reaction
SAS	- Statistical Analysis Software
Sat-RNA	- Satellite Ribonucleic Acid
SSA	- sub-Saharan Africa

ssRNA	- Single Stranded Ribonucleic Acid
T92	-Tamura 3-parameter model
TN93	- Tamura-Nei model
UPGMA	-Unweighted Pair Group Method with Arithmetic mean
WB	-Washing Buffer

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Groundnut (*Arachis hypogaea* Linn) is native to southern America. It is a key annual leguminous crop for small scale farmers that is self-pollinated, producing aerial flowers but fruiting below the soil level. Groundnut is often called peanut belonging to the family *Fabaceae*, genus *Arachis* and species *hypogaea,* the only domesticated species in the genus (Usman *et al*., 2013). The other common names include Arachides, Goober peas, Mani, Pinders, Earthnuts, Monkey nuts, Pygmy nuts and Pig nuts. The most cultivated groundnut varieties distributed in the different continents are Espanola (Spanish type), Kersting groundnut, Malgache groundnut, Roja Tennessee, Valencia type and Virginia type (Karanja *et al*., 2009). However, the four major cultivar groups distinguished by branching habit and branch length include the Spanish, Virginia, Valencia and Runner types (Muthoni *et al*., 2010).

The Spanish types are grown in South Africa, Southwestern and Southeastern United States. They are higher yielding and more disease resistant with higher oil content compared to other varieties (Ntare *et al*., 2002). Virginia types are large seeded and grown in Virginia, North Carolina and Tennessee in the United States of America (USA). They are increasing in popularity due to demand for large peanuts for processing. Valencia types are coarse with heavy reddish stems and large foliage grown on a small scale in Mexico. It is the most preferred type for boiled groundnuts because they have the best flavor (Ayoola *et al*., 2012). Runner types are found in Georgia, Alabama, Florida and South Carolina. They have good flavor, better roasting characteristics and higher yields compared to Spanish types. Specific

cultivar groups are preferred for particular uses because of differences in flavour, oil content, size, shape and disease resistance (Bucheyeki *et al*., 2008).

Groundnut is the fifth most important annual oilseed and food legume crop after dry pea (*Pisum sativum*), garden bean (*Phaseolus vulgaris*), Black gram (*Vigna mungo*) and cowpea (*Vigna radiata*) (Asif *et al*., 2013), grown in diverse environments throughout the semi-arid and sub-tropical regions between 40^0N and 40^0S in nearly 100 countries in the five continents of the world (Kumar *et al.,* 2007). The most important groundnut producing countries are Argentina, Chad and China (40%), India (16%), Indonesia (4%), Myanmar and Nigeria (8%), South Africa, Senegal, Sudan, United States of America and Vietnam (6%) (Kumar *et al*., 2007). Africa accounts for 40% of the global area planted to groundnuts. Only 26% of the highest averages were observed in South Africa, and the lowest in East Africa (Herselman *et al*., 2004), probably due to pests, diseases and poor agronomic practices.

In Kenya, groundnut (*Arachis hypogaea* L.) have a local name 'Njugu karanga'. Groundnut is mainly grown in western Kenya by small scale farmers as oilseed, cash crop and animal feed. The two main groundnut types in Kenya are the bunch type, for example Red Valencia maturing within 90-100 days, and the runner type, for example Homabay Red maturing in 120-150 days. The common varieties grown include Alika, Bukene, Homabay Red, Manipintar, Makulu Red, Red Valencia, Texas Peanut and Uganda Red. Western Kenya has a tropical climate suitable for groundnut farming. The present growers' yield in Kenya is 450-700 kg/ha (MOA, 2016a; 2016b). With the high population growth rate in western Kenya and the decreasing smallholder farm sizes (MOA, 2016b), groundnut yield can be improved by breeding resistant varieties and practicing better crop protection agronomic practices (Usman *et al*., 2013; Appiah *et al*., 2017).

Groundnut production is an enterprise of economic and nutritional value for farmers in east Africa (Kidula *et al*., 2010; Okello *et al*., 2010). Groundnut seeds (raw, sun dried and roasted) contain moisture content of 7.405 %, 3.40 %, 1.07 % ; ash content of 1.48 %, 1.38 %, 1.41 %; crude protein of 24.70 %, 21.80 %, 18.40 %, crude fat of 46.10 %, 43.80 %, 40.60 % ; crude fiber of 2.83 %, 2.43 %, 2.41 % ; and carbohydrate of 17.41 %, 27.19 %, 36.11 % respectively. Groundnut mineral ions include; Sodium (0.71 %, 0.69 %, 0.57 %), Phosphorus (0.68 %, 0.65 %, 0.69 %), Potassium (0.47 %, 0.51 %, 0.55 %), Zinc (0.44 %, 0.42 %, 0.50 %), and Iron (0.40 %, 0.47 %, 0.43 %) respectively (Ayoola *et al.*, 2012). Potential use of groundnut seeds is in animal feed (poultry), entire human diet (balance diet for elderly people who need less carbohydrate but a lot of protein), and an antidote for malnourished children (Ayoola *et al*., 2012). The haulms and groundnut cakes are used to feed livestock as grass, while groundnut seed is eaten as whole oilseed, or refined as snack food. Groundnut is also a source of vitamins like niacin, falacin, riboflavin and thiamine. As a legume, groundnut improves soil fertility through biological fixation of free atmospheric nitrogen, and thereby increase productivity of the semi-arid cereal cropping systems (Smartt, 1994) and other agro-ecological soils. The non-food products made from groundnut include soaps, medicines, cosmetics, pharmaceuticals, emulsions for insect control, lubricants and biofuel for diesel engines (Yaranal *et al.*, 2005).

Virtually 75% to 80% of the globe's groundnut is sprouted by resource-poor smallholder farmers in third world countries who frequently yield between 500 and 800 kg / ha compared to prospective yields of > 2.5 t / ha (Kayondo *et al*., 2014). In western Kenya, with an average output of 600-700 kg / ha, farmers attain less than 30-50 percent of prospective yield (Kidula *et al*., 2010). Numerous pests and diseases largely caused by fungi, viruses, bacteria, and nematodes are primarily due to low yields. Weeds, drought, poor agronomic practices, inadequate markets and poor post-harvest handling practices also contributes to low yields.

(Okello *et al.,* 2010). Plant virus diseases like GRD are the most important because they are not adequately managed due to lack of enough information on their complex etiology in occurrence, distribution, diversity, management, lack of resistant varieties, high cost and unavailability of insecticides to control the aphid vector (Appiah *et al*., 2016).

About 31 viruses were reported to naturally infect groundnut around the world (Kumar *et al.,* 2007). Nine of them belong to the genus *Potyvirus,* six to *Tospovirus,* two each to *Cucumovirus, Pecluvirus, Soymovirus* and *Umbravirus,* and one each to *Begomovirus, Bromovirus, Carlavirus, Ilarvirus, Luteovirus, Potexvirus, Rhabdovirus* and *Tymovirus.* Out of this, 19 were first isolated from groundnut and the remaining first isolated from other hosts, but they commonly occur on groundnut crops (Usman *et al*., 2013). The most common diseases in groundnut are caused by *Cucumber Mosaic Virus* (CMV), *Groundnut bud necrosis virus* (GBNV), *Groundnut rosette virus* (GRV), *Indian peanut clump virus* (IPCV), *Peanut clump virus* (PCV), *Peanut mottle virus* (PeMoV), *Peanut stripe virus* (PStV), *Tobacco streak virus* (TSV) and *Tomato spotted wilt virus* (TSWV). These viruses which are also known to naturally infect several other crops, inflict significant losses to groundnut (Naidu *et al*., 1998b). Although not present every year, outbreaks of GRD is sporadic and unpredictable in Africa due to inadequacy of rains in most areas resulting in severe devastating yield losses of upto 100 % (Subrahmanyam *et al*., 2001). In 2006, the global average groundnut yield in sub-Saharan Africa was 980 kg / ha, which was significantly lower than the average worldwide of 1 690 kg / ha (Bucheyeki *et al*., 2008).

1.2 Statement of the problem

Groundnuts are nutritious oilseeds that supplement the human diet, animal feeds, source of income and ameliorates soil fertility. Groundnut rosette disease (GRD) is by far the most destructive virus disease of groundnut in Kenya (Wangai *et al.,* 2001; Anitha *et al*., 2014). Rosette disease causes significant yield losses in groundnut producing regions of western

Kenya and neighbouring eastern Uganda (Okello *et al.,* 2010; 2014). Only limited field resistance is available for GRD cultivars which have less than superior agronomic traits (Usman *et al.*, 2013).

To date there is not enough documented information on the occurrence, distribution and diversity of GRAV pathogen in western Kenya. This has resulted in continued yield losses amongst groundnut farmers. The wide variability of the three field rosette symptom types (chlorotic, green and mosaic) occurring in the western Kenya region, have not been documented in a geo-referenced map to help understand the occurrence, distribution and diversity of GRAV in the region.

In western Kenya, GRAV strains within the virus populations are not known and therefore, there is need to understand virus-plant interactions to generate an in-depth analysis on breeding for resistance/tolerance. Inaccurate biological and molecular characterization of genotype adaptability on host plants and indicator plants, may lead to poor pathotyping and productivity in environments that interact negatively with specific genotypes of groundnut cultivars (Tillman & Stalker., 2009). The variability of field isolates of the three causal agents of GRD has been ascertained in Malawi, Nigeria (Deom *et al*., 2000) and Kenya (Wangai *et al*., 2001), but distinct symptom diversity and distribution has not been reported in western Kenya.

1.3 Justification of the study

The demand for groundnuts in western Kenya has gone up to improve livelihoods and nutrition, but its production is not commensurate due to GRD. Taliansky et al., (2000) reported that GRAV infection alone in groundnut, results in transient mottle symptoms with insignificant impact on the plant growth and yield. Nevertheless, Naidu and Kimmins (2007) disputed these findings by stating that GRAV alone influences plant growth and significant

loss of yield in prone groundnut cultivars. Thus, the need for biological characterization of GRAV on plant growth parameters. A survey in the groundnut growing seasons of 1997-1998 in western Kenya by Wangai *et al*., (2001), showed that GRD incidence ranged between 24 - 40%. This is a long time more than a decade, and the dynamics of rosette disease new encounter phenomenon might have changed contributing significantly to the low productivity of the crop, therefore the need for a current study.

In western Kenya, the diversity of GRAV has not been documented because the coat protein sequences obtained by RT-PCR using primers of already characterized viruses are not deposited in the GenBank. Next Generation Sequencing (NGS) can detect all the GRAV causal agent variants in a single run. This will unveil the strains available in western Kenya for molecular characterization and diversity studies. The limitation in the documentation of alternative natural hosts of GRD pathogenicity in western Kenya, is probably due to misdiagnosis as a result of a lack of in depth knowledge of GRAV causal agent. Therefore, there was need to document the occurrence and distribution of alternative hosts of GRD and molecular characteristics of GRAV in western Kenya to facilitate the understanding of GRD complex etiology and recommend appropriate management and control strategies.

1.4 General objective

To determine the distribution of GRD and molecular diversity of groundnut rosette assistor virus (GRAV) in western Kenya.

1.4.1 Specific objectives

i. To determine the occurrence and distribution of groundnut rosette disease (GRD) in western Kenya.
ii. To determine the molecular diversity of groundnut rosette assistor virus (GRAV) in western Kenya.

iii. To screen popular legumes for resistance to groundnut rosette disease (GRD) in western Kenya.

1.5 Hypotheses

H_{O1}: Occurrence of groundnut rosette disease (GRD) is not widely distributed in western Kenya.

H_{O2}: The groundnut rosette assistor virus (GRAV) strains in western Kenya constitute a homogenous population to those found elsewhere.

H_{O3}: Popular legumes grown in western Kenya are susceptible to groundnut rosette disease (GRD).

CHAPTER TWO

LITERATURE REVIEW

2.1 Occurrence and distribution of groundnut rosette disease (GRD)

Groundnut rosette disease (GRD) was first documented in 1907 from present-day Tanzania (Naidu *et al*., 1999a; Waliyar *et al.,* 2007). Since then, GRD has been reported in several other sub-Saharan African (SSA) countries of Angola, Burkina Faso, Cote d'lvoire, Democratic Republic of Congo (DRC), Gambia, Ghana, Kenya, Madagascar, Malawi, Niger, Nigeria, Senegal, South Africa, Swaziland, Uganda and Zambia (Bucheyeki *et al*., 2008). In 1975, GRD affected 0.7 million ha of groundnut in northern Nigeria and caused an estimated yield loss of 0.5 million tonnes valued at US$ 5 million (Deom *et al.,* 2000). In 1995-1996, eastern Zambia lost 43,000 ha of groundnut to GRD pathogens estimated at US$ 5 million (Olorunju *et al*., 2001). In 1994-1995, farmers in central Malawi abandoned the crop by 23% following an unpredictable epidemic, whose annual loss was estimated at US$ 155 million (Appiah *et al.,* 2017; Taliansky *et al.,* 2000). Key market class cultivars, including landraces have succumbed to GRD resulting in yield reduction to as low as 800 kg/ha compared with 3,000 kg/ha reported from on-station plots in Uganda (Okello *et al*., 2017).

GRD-like symptoms have been confirmed in some Asian and South American countries, but there have been no diagnostic tests to corroborate that rosette diseases have been present (Reddy, 1991; Subrahmanyam *et al*., 2001). GRD is confined to SSA and is therefore likely to have been contaminated by GRD causal agents native to SSA, as groundnut imported from South America somewhere during the sixteenth hundred years. The new confrontation arises when a crop has entered a new geographical area, and when the newly launched crop attacks pests or pathogens developed with other host species (Olorunju *et al*, 2001)

2.2 Symptom diversity of groundnut rosette disease (GRD)

Distinct field rosette symptoms of chlorotic, green and mosaic caused by synergistic interaction among groundnut rosette assistor *Luteovirus* (GRAV), groundnut rosette *Umbravirus* (GRV) and its satellite RNA (sat-RNA), makes it three diseases in one from the phenotypic symptom expression. Variability in sat-RNA is mainly responsible for symptom variations (Taliansky & Robinson, 1997). In addition, differences in genotypes, plant stage infection, variable climatic conditions and mixed infections with other viruses, contributes to symptom variability under field conditions (Naidu & Kimmins, 2007; Naidu *et al*., 1999a). In eastern Uganda, GRD pathogens resulting in green rosette symptoms predominate (Okello *et al*., 2014). This is in contrast with (Wangai *et al*., 2001) who reported that chlorotic rosette symptoms of GRD have been the predominant form throughout SSA. This findings are of utmost importance because eastern Uganda and partly neighbouring western Kenya are major groundnut growers in SSA. The dynamics of the GRD pathogen symptomatology needs further research that this study unravels.

RNA viruses exist as "quasispecies" (Roossinck, 1997) in the infected plants and thus the population complexity of GRAV in the field has the potential to be large. The potential permutations among variants of GRAV, GRV and its sat-RNA are able to form viable alternatives with enormous capacity to adapt to diverse and changing econiches. With time, this continuous evolution of GRD causal agents under strong selection pressure can lead to new disease patterns. For instance in Nigeria, a clear shift occurred from green to chlorotic rosette over a period of about 20 years (Appiah *et al*., 2017). The shift could be due to changes in the genome sequences of GRD causal agents or to different vector biotypes and cropping patterns (Usman *et al*., 2013). Rosette disease manifests in two major symptom types; chlorotic (yellow) and green rosette which override the isolated occurrence of mosaic rosette throughout SSA, and sometimes occur in the same field (Okello *et al*., 2014; Mugisa

et al., 2016). Mosaic rosette variant results from mixed infection of the groundnut cultivars by the sat-RNA causing chlorotic and green mottled variant (Scott *et al*., 1996; Waliyar *et al*., 2007). Mosaic rosette is of low incidence but of wide occurrence in Southern and East African region (Kayondo *et al*., 2014)

The major chlorotic and green rosette symptom distribution in SSA countries include Burkina Faso, Ghana, Kenya, Malawi, Mali, Mozambique, Nigeria, Niger Republic, Tanzania, Uganda and Zambia (Subrahmanyam *et al*., 1998) while mosaic rosette was diverse in western Kenya. In chlorotic rosette symptom type, leaves are usually bright yellow with fewer green islands, bushy appearance and stunted in growth with shortened internodes, mottled and twisted reduced leaf lamina that curls upwards. Chlorosis of the lamina of the young leaves upon which the veins form a green network, they progressively become dark green than normal. Green rosette symptom type makes the leaves appear dark green with bushy appearance, shortened internodes, stunted growth and distorted shoots. Mosaic rosette symptom type includes light green to dark green mosaic, distortion of the shoots and bushy appearance with shortened internodes. However, shortening of the internodes and stunting is not pronounced in mosaic rosette as in chlorotic rosette and green rosette. The white-green patches with brilliant yellow blotch mosaic pattern retards elongation of axes of the stem and leaves which appear to progress more slowly than in healthy plants and eventually ceases.

Infected groundnut leaves may also show symptoms other than the typical chlorotic or green rosette. This suggests wider variability of the visible symptoms of the diseased plants that appear severely stunted with shortened internodes and reduced, twisted leaf size resulting into a bushy appearance (Naidu *et al.*, 1998b). Rosetted plants may flower but few pods and seeds are produced or at times no seeds are formed at all in the pods (Mabele *et al*., 2019a). This strange phenomenon on rosetted groundnut occurring in countries that forms the East African region (Burundi, Democratic Republic of Congo (DRC), Ethiopia, Kenya, Rwanda, South

Sudan, Sudan, Tanzania and Uganda), needs extensive field survey to determine the distribution of major symptom types and screen for resistance/tolerance to establish appropriate crop protection and agronomic management technologies.

2.3 Etiology and genome organization of GRD causal agents

A few researchers have shown interest in GRD studies mainly because of its complex etiology that involves synergism among the three causal agents; Groundnut rosette assistor virus (GRAV), Groundnut rosette virus (GRV) and its associated satellite-RNA (sat-RNA) of GRV (Taliansky & Robinson, 2003). The virus-like nucleic acid molecule of sat-RNA occurs in different variant forms and is responsible for the three distinct field symptom types (Taliansky *et al*., 2000; Kayondo *et al*., 2014). No virus-like particles have been reported for GRV, but infected plants yield infective ssRNA. Infected leaves also contain dsRNA with prominent electrophoretic species of 4.6 kbp (dsRNA-1) and 1.3 kbp (dsRNA-2), a very abundant species of 900 bp (dsRNA-3), and numerous minor species of intermediate mobility. The GRV sat-RNAs associated with chlorotic and green rosette disease in different regions of Africa are 895-903 nucleotides long, and are at least 87 % identical (Deom *et al*., 2000). The sat-RNA contains upto five open reading frames (ORFs) in either positive or negative sense, but the role of any proteins expressed from these ORFs is unknown (Taliansky *et al*., 2000). The intricate interaction between GRAV, GRV and sat-RNA is crucial to the development of rosette disease. GRV is a member of the genus *Umbravirus* with a single-stranded positive-sense RNA genome of 4,019 nt that contains four large open reading frames (ORFs). ORF 2 is a putative RNA-dependent RNA polymerase and is likely expressed as a fusion protein with the product of ORF1 by a –1frameshift mechanism (Taliansky & Robinson, 2003). The 3′ ORFs (3 and 4) are almost completely overlapping. The protein encoded by ORF 3 was shown to be a trans-acting long-distance movement protein that can traffic nonrelated viral RNA systemically, while analysis of the ORF 4

putative amino acid sequence suggests that it may be involved in cell-to-cell movement (Taliansky & Robinson, 2003).

Groundnut rosette assistor virus (GRAV) is a member of the genus *Luteovirus* and family *Luteoviridae* (Deom *et al*., 2000). GRAV virions are non-enveloped, isometric shaped with 28 nm diameter particles of polyhedral symmetry. Their genome is a non-segmented, single molecule of linear positive sense, ssRNA of ca.6900 nucleotides, which encodes for structural and non-structural proteins (Murant & Kumar., 1990). Like other members of the *luteovirus,* GRAV is thought to encode for six ORFs. Only the coat protein (CP) region of GRAV genome is sequenced (Gene Bank Accession # z 68894 af195502, af195825). Virions are made of single CP subunits of size 24.5 kDa, and the virus is antigenetically related to *Bean leaf roll virus (BLRV), Beet western yellows virus (BWYV), Chickpea Luteovirus (CPLV), Pea leaf roll virus (PLRV), Potato leaf roll virus-1(PLRV-1)* and *Tobacco necrotic dwarf Luteovirus (TNDLV)* (Scott *et al*., 1996). GRAV is transmitted by *Aphis craccivora* in a persistent circulative manner and experimentally by grafting but not by mechanical sap inoculation, seed and pollen or by contact between the plants. GRAV acts as a helper virus for aphid transmission of GRV and sat-RNA. Unlike sat-RNA and GRV, GRAV is phloem limited and the virus replicates autonomously in the cytoplasm of the phloem tissue (Murant & Kumar., 1990). Groundnut (*Arachis hypogaea*) is the only known natural host of groundnut rosette assistor virus (GRAV) (Naidu & Kimmins., 2007). The *Luteovirus* is reported to occur wherever GRD has been reported and on its own, it causes symptomless infection or transient mottle and can cause significant yield loss in susceptible groundnut cultivars by reducing total dry mass of the plant and seed weight (Naidu & Kimmins., 2007)

GRAV, GRV and sat-RNA are dependent on each other synergistically for survival, and all the three causal agents play a crucial role in the biology and perpetuation of rosette disease. GRV RNA and sat-RNA are packaged in the CP gene of GRAV to form virus particles that

can be transmitted by aphids. The sat-RNA depends entirely on GRV for its replication while GRV depends on sat-RNA for its packaging and encapsidation into the GRAV coat protein and subsequent aphid transmission. However, GRV replicates autonomously (Taliansky *et al*., 2000; Taliansky & Robinson, 2003). GRAV and GRV contribute little to disease symptoms in groundnut apart from yield loss because the sat-RNA variants induce symptom development in rosetted groundnut (Taliansky *et al*., 2000). The persistent nature of GRD in the field is through the ability of GRV and its sat-RNA to utilize the coat protein of GRAV epidemiologically during the process of aphid transmission although the causal agents get separated (Taliansky *et al*., 2000).

Groundnut rosette virus (GRV) belongs to the genus *Umbravirus* and family *Tombusviridae* (Waliyar *et al*., 2007). On isolation and characterization, the *Umbravirus* has no structural coat protein (Taliansky & Robinson, 2003) and thus forms no conventional virus particles. Taliansky and Robinson (2003) detected enveloped bullet-shaped structures in the ultra-thin sections due to GRV infection as opposed to real virions. The *Umbravirus* genome is a non-segmented, single linear molecule of single-stranded, positive sense RNA of size ca.4019 nucleotides which encodes four ORFs (Taliansky *et al*., 2000). The genome of GRV isolate when completely sequenced (GenBank Accession #z66910), has several partial sequences available in the GenBank. The GRV replicates autonomously in the cytoplasm of the infected tissues (Waliyar *et al*., 2007).

The satellite-RNA which forms the subviral RNAs of GRV, belongs to the sub-group-2 (small linear) satellite-RNAs. It is a single-stranded linear non-segmented RNA of 895 to 903 nucleotides (Blok *et al*., 1994). It totally depends on GRV for its replication, encapsidation and movement both within and between the plants. The sat-RNA variants are responsible for rosette symptoms and plays a critical role in GRAV helper virus dependent transmission of GRV (Olorunju *et al*., 2001). Different variants of sat-RNA have been shown to be

responsible for the typical rosette symptom types in other indicator plants which acts as alternative hosts for GRD inoculum (Murant & Kumar, 1990; Mukoye & Mabele., 2019). The sat-RNA has upto five ORFs in positive or negative sense but no protein products have been isolated (Blok *et al*., 1994). It is mechanically transmissible along with GRV, and is also transmitted by aphids, in the presence of GRV and GRAV (Waliyar *et al*., 2007; Alegbejo & Abo., 2002). The sequences of 10 variants of GRV sat-RNA have been determined (Blok *et al*., 1994; Taliansky *et al*., 2000).

2.4 Structure of GRAV *Luteovirus* particle

The GRAV *Luteovirus* has a VPg bound at 5^1 end. There is no Poly (A) tail or tRNA-like structure at the 3^1 end (Deom *et al*., 2006) (Fig. 1).

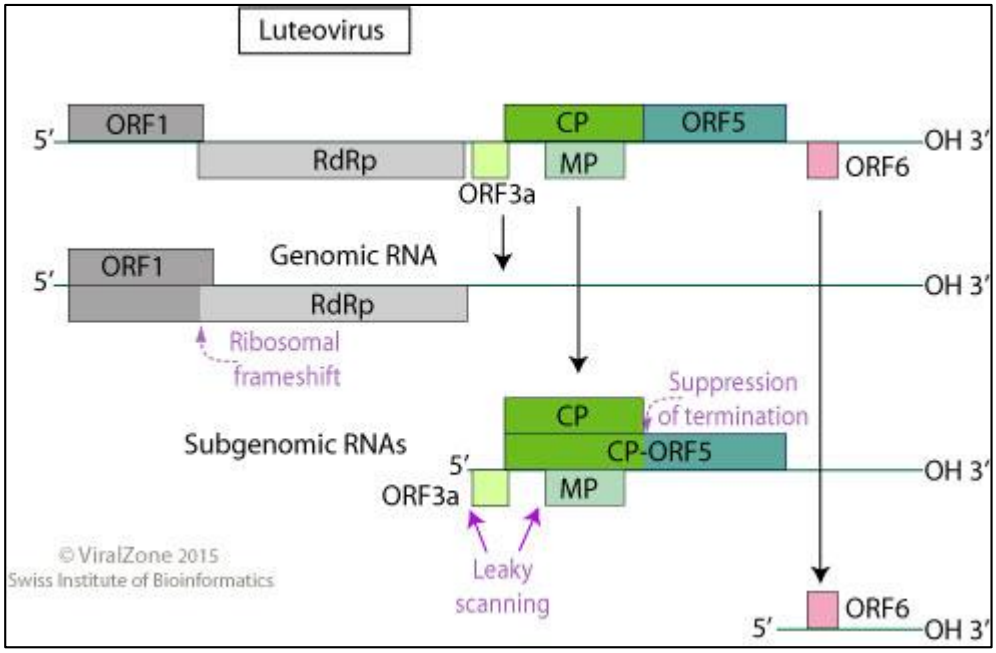


Figure 1: GRAV *Luteovirus* particle.

Source: Viral zone 2015. Swiss Institute of Bioinformatics.

2.5 Epiphytology of groundnut rosette disease (GRD)

GRD is efficiently transmitted by the polyphagous groundnut aphid *Aphis craccivora* Koch in a persistent manner, and inefficiently by *Aphis gosypii* Glover and *Myzus persicae* Sulzer

(Todd *et al*., 1993) because the latter two vectors are not significant in the ecology of GRD perpetuation. There is no evidence available for seed transmission of GRD pathogens (Anitha *et al*., 2014). The groundnut aphid maintains itself successfully throughout the dry and wet seasons because it is anholocyclic, parthenogenetic and ovoviviparous reproducing almost everywhere throughout the year on some host crops with preference to groundnuts which are not drought stressed (Alegbejo & Abo, 2002). GRD epidemiology is a complex involving synergistic interaction between and among the aphid vector, GRAV, GRV and its sat-RNA, the host plant and environment (Naidu *et al*., 1998; Okello *et al*., 2017).

Since *Aphis craccivora* commonly known as the cowpea aphid or groundnut aphid is the principal vector involved in the transmission of all the GRD pathogens in a persistent circulative manner, studies have shown that all the GRAV particles, whether they contain GRAV-RNA, GRV-RNA or sat-RNA, are acquired by the aphid vector from phloem sap in 4 hr and 8 hr acquisition access feeding for chlorotic and green rosette respectively (Waliyar *et al*., 2007). The groundnut aphid does not always transmit all the GRD pathogens together (Naidu *et al.,* 1998a). During short inoculation feeding (test probe or stylet pathway phase), the vector probes groundnut leaves without reaching the phloem, hence transmitting only GRV and sat-RNA that multiply within the epidermal and mesophyll cells. Even if GRAV particles are deposited in the mesophyll cells, they cannot replicate because they can only replicate in the phloem cells (Naidu *et al.,* 1999b). The groundnut aphid vector can transmit GRAV and GRV-sat-RNA when the stylets penetrate sieve elements (Salivation phase) of the phloem cells. When the inoculation feeding period is longer or the number of aphids per plant is increased, the success of transmitting all the three causal agents together is high. The aphid vector can fail to acquire or transmit GRV and its sat-RNA from diseased plants lacking GRAV. Such plants become dead-end sources of heavy inoculum for volunteer indicator plants. However, if such plants receive GRAV later due to A. *craccivora* feeding, the plants

again serve as source of inoculum (Olorunju *et al*., 2001; Anitha *et al*., 2014). Reports of groundnut crop damage by GRD underscores the need for further epidemiological studies and appropriate control and management strategies unveiled to reduce the inoculum source for rosette disease. This will prevent resistant and tolerant varieties from succumbing to GRD at high inoculum pressure (Appiah *et al*., 2016).

2.6 Host range of groundnut rosette disease (GRD) pathogens

Groundnut and some of its wild relatives are the only natural hosts of GRAV, GRV and sat-RNA. Under experimental conditions using viruliferous *Aphis craccivora* vector, GRAV has been transmitted to *Pisum sativum* L., *Stylosanthes gracilis* Taub, S. *hamata* Taub, S. *mucronata* Wild, S. *sundaica* Taub, *Trifolium incarnatum* L., T. *Pratense* L., *Caspella bursa-pastoris* L., *Gomphrena globosa* L., *Montia Perfoliata* L. and *Spinacia Oleracea* L. (Olorunju *et al*., 1992; Ntare *et al*., 2002). All these plants showed symptomless infections and virus replication that was confirmed by diagnostic bioassays. Exception is C. *bursa-pastoris* which was reported to show chlorotic symptoms (Waliyar *et al*., 2007). By artificial mechanical sap inoculation, experimental hosts of GRV and sat-RNA in the West, East and Southern Africa were identified in several species in *leguminosae, chenopodiaceae* and *solanaceae*. *Chenopodium amaranticolor* and C. *murale* are local lesion hosts while C. *amaranticolor, Glycene max, Phaseolus vulgaris, Nicotiana benthamiana* and N. *clevelandii* are systemic hosts of GRV (Naidu *et al*., 1999b; Waliyar *et al.,* 2007). There is not enough information on screening for GRAV and this study screened for resistance and tolerance by indicator plants that acts as source of inoculum during the on-season and off-season periods.

Apart from groundnut crop, experimental hosts of both GRAV, GRV and sat-RNA are *Gomphrena globosa, Stylosanthes gracilis,* S. *mucronata,* S. *sundaica, Spinacia oleracea, Trifolium incarnatum* and T. *repens* (Murant & Kumar., 1990). In western Kenya, A. *craccivora* appears in groundnut farms early in the rainy season on beans (*Phaseolus*

vulgaris), cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogaea L.*), green gram (*Vigna radiata*), soybean (*Glycine max*) and wild host plants like golden berry (*Physalis peruviana* L.), which acts as source of inoculum for GRD causal agents that this study attempted to determine through pathotyping screening of phenotypic symptoms and molecular analysis.

2.7 Screening of indicator plants

Groundnuts and some of its wild relatives are the only natural hosts of GRAV, GRV and sat-RNA (Naidu *et al*., 1998). Under experimental conditions using viruliferous *Aphis craccivora* vector, GRAV has been transmitted to *Pisum sativum* L., *Stylosanthes gracilis* Taub, *S. hamata* Taub, S. *mucronata* Wild, S. *sundaica* Taub, *Trifolium incarnatum* L., T. *Pratense* L., *Caspella bursa-Pastoris* L., *Gomphrena globosa* L., *Montia Perfoliata* L. and *Spinacia Oleracea* L. (Waliyar *et al.*, 2007; Olorunju *et al*., 2001; Amoah *et al*., 2015). All these plants showed symptomless infections, and virus replication that was confirmed by molecular diagnostic assays. Exception is *C. bursa-pastoris* which was reported to show chlorotic symptoms (Waliyar *et al*., 2007). By artificial mechanical sap inoculations, experimental hosts of GRV and Sat-RNA in the West, East and Southern Africa, were identified in several species in *leguminosae, chenopodiaceae* and *solanaceae*. *Chenopodium amaranticolor* and C. *murale* are local lesion hosts while *C. amaranticolor, Glycene max, Phaseolus vulgaris*, *Nicotiana benthamiana* and *N. Clevelandii* are systemic hosts of GRV (Waliyar *et al.*, 2007). Apart from groundnuts, experimental hosts of both GRAV, GRV and sat-RNA are *Gomphrena globosa, Stylosanthes gracilis, S. mucronata, S. Sundaica, Spinacia oleracea, Trifolium incarnatum* and *T. repens* (Murant & Kumar., 1990).

Research by Murant and Kumar (1990) showed that mechanical sap inoculation of GRD inoculum on experimental hosts, *Chenopodium amaranticolor* and C. *murale* indicated the presence of GRAV while infected indicator plants showed rosette symptoms about four days after inoculation. Plants infected with GRAV showed significant reduction in seed weight.

This findings indicate that GRAV infection without GRV and its sat-RNA affects plant growth contributing to yield loss (Naidu & Kimmins, 2007). Research by Waliyar *et al* (2007) observed that all GRD resistant cultivars and germplasm lines contain resistance to GRV and sat-RNA only but not to GRAV. This complexity in selective resistance poses a challenge to breeders screening the groundnut lines with the aim of trying to develop durable resistance (Amoah *et al*., 2015) to GRD pathogens indigenous to Africa, because they have not been recorded elsewhere. However, Reddy (1991) reported that they were introduced to Africa by the Portuguese from South America in the 16th Century.

2.8 Management of groundnut rosette disease (GRD)

Most strategies aimed at GRD management are largely directed at preventing virus infection, by eradicating the source of inoculum to prevent the virus from reaching the crop, minimizing the spread of the disease by controlling the transmitting aphid vector, utilizing virus-free planting material and incorporating host-plant resistance to GRD causal agents (Naidu *et al*., 1998b; Mabele *at al*., 2018a). Various methods are available for protecting groundnut against rosette disease. These include the rogueing of volunteer groundnut plants that serve as inoculum source, cultural practices that can interfere with vector movement through integrated pest management (IPM), use of insecticides to control vector aphids and use of rosette disease resistant cultivars (Reddy *et al*., 1985; Olorunju *et al*., 1991). However, these approaches are seldom feasible for the subsistence farming systems of SSA (Appiah *et al*., 2017). Studies have shown that resistance to the aphid vector is controlled by a single recessive gene, which is mapped on linkage Group-1 at a distance of 3.9 cm from a marker originating from a susceptible parent (ICGV-SM 93541) (van der Merwe & Subrahmanyam, 1997; Herselman *et al.*, 2004). Identification of this DNA marker offers a scope to develop a simple DNA-marker based method for screening aphid resistance, which may accelerate breeding progress (Usman *et al*., 2013). The reliance of GRV on its sat-RNA for

encapsidation and aphid transmission explains why sat-RNA free-isolates of GRV have not been found in nature (Appiah *et al*., 2017).

Biotechnological attempts have been made to exploit pathogen-derived resistance (GRAV replicase and CP genes, movement protein genes and sat-RNA derived sequences) to groundnut rosette disease in developing broad based agronomically superior groundnut cultivars (Taliansky *et al.,* 1996). Pathogen-derived resistance (PDR) (Deom *et al*., 2006) provides a good strategy for controlling GRD through the generation of transgenic groundnut. This could potentially be achieved by introducing GRAV or GRV genomic sequences or genes, or sat-RNA–derived sequences that down regulate GRV replication, into suitable groundnut cultivars. However, the success of PDR-, RNA- or protein-mediated resistance (Deom *et al*., 2006), is highly influenced by the degree of sequence homology between the sequence of the transgene and the challenging virus (Taliansky *et al.,* 1996; Usman *et al*., 2013). An earlier effort to develop PDR, the degree of genetic variability within the three GRD agents from two diverse groundnut-growing regions of SSA (Malawi and Nigeria) was examined by Deom *et al* (2000) with the hypothesis that this type of resistance could be introduced by deducing the most conserved region in the GRD causal agents from different regions of SSA. The CP of GRAV is suggested to be the most probable candidate for developing PDR for GRD across SSA, because it appeared to be the most highly conserved between the two distinct regions of Malawi and Nigeria (Deom *et al*., 2000).

Several wild *Arachis hypogaea* species have been screened and found resistant to GRAV (Subrahmanyam *et al*., 2001). The wild groundnut accessions belong to *Arachis diogoi,* A. *hoehnei*, A. *kretschmeri*, A. *cardenasii*, A. *villosa*, A. *pintoi*, A. *kuhlmanui*, and A. *stenosperma*. Some accessions in A. *appressipla,* A. *diogoi,* A. *stenosperma,* A. *decora,* A. *triseminata,* A. *kretschmeri,* A. *kuhlamannui* and A. *pintoi* have been found to be resistant to all the three causal agents of GRD (GRAV, GRV and its sat-RNA). This GRD resistance

immunity is based on resistance to initial infection, restriction of virus movement, restricted production of sat-RNA which induces rosette symptoms and this trait can be transferred to cultivated groundnut through biotechnological plant breeding approaches to reduce disease incidence (Karanja *et al*., 2009).

Resistance in groundnut landraces against chlorotic and green rosette is effectively governed by two independent recessive genes (Olorunju *et al*., 1992; 2001). Over 12,600 groundnut germplasm lines have been screened and identified to be field resistant to GRD (Subrahmanyam *et al*., 1998). These germplasm lines have shown resistance to GRAV but not absolute as a small proportion of plants or a few branches show rosette symptoms (Subrahmanyam *et al*., 1998). This plants act as source of inoculum for the vector leading to spread and survival of the disease. Only limited field resistance is available for either causal agent in popular groundnut cultivars and landraces which have less than superior agronomic traits (Olorunju *et al*., 2001; Usman *et al*., 2013; Appiah *et al*., 2017). This phenomenon needs further evaluation of the germplasm in popular groundnut genotypes.

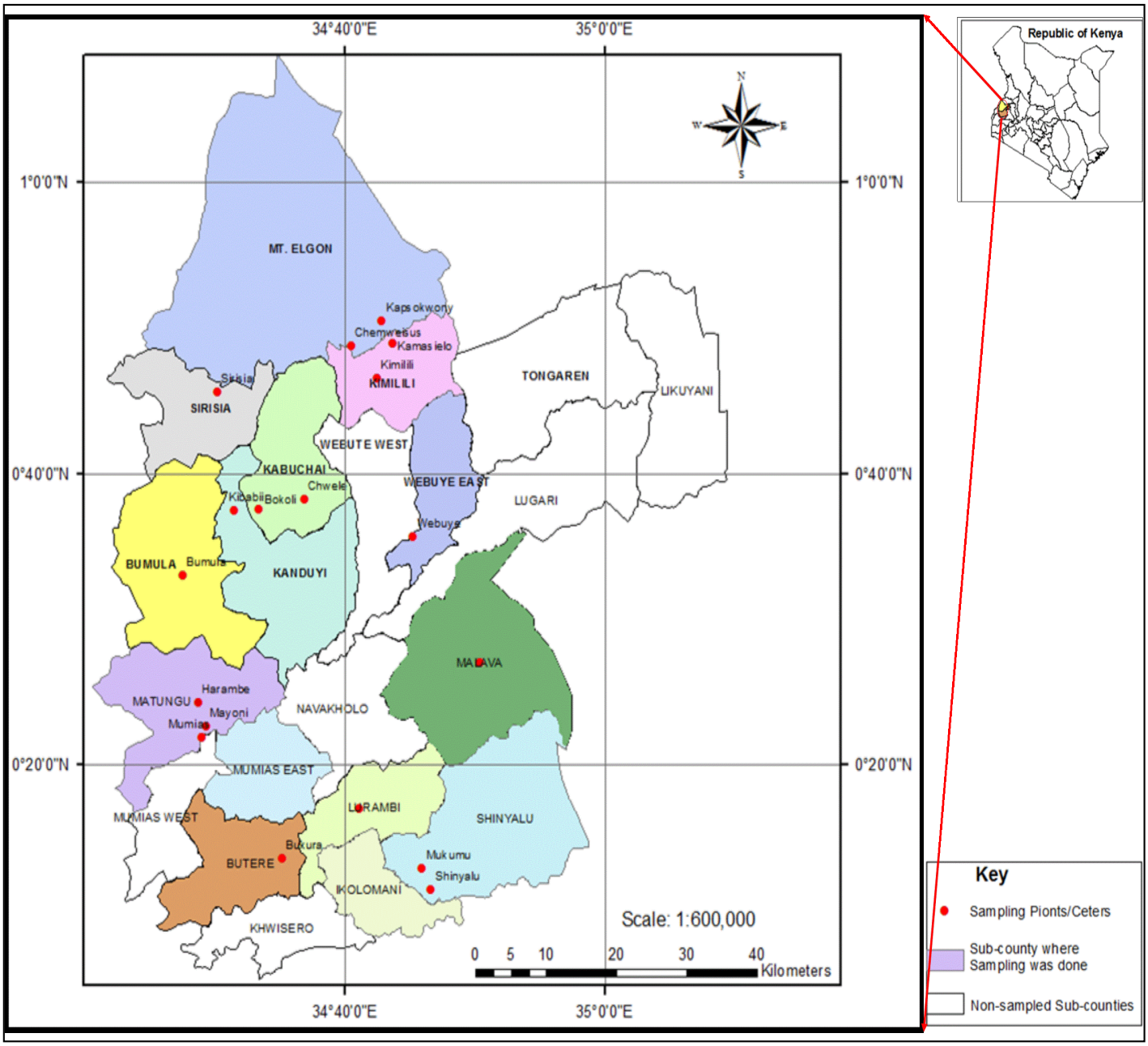
Adoption of new varieties and specific cultivar genotypes is constrained by the low priority given due to lack of efficient seed production systems and pest/disease pressure tolerance/resistance. In Kenya, Kidula *et al*., (2010) noted that groundnut is grown mainly as an oilseed crop for the market as a source of income. However, adoption of new varieties is constrained by the low priority given due to inadequate information and unavailability of improved varieties developed for better disease resistance, tolerance, higher yields and good market acceptability (Ntare *et al*., 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study area

The GRD field survey and symptomatic leaf sampling was conducted in the main groundnut



growing areas of Bungoma and Kakamega Counties (Fig. 2).

Figure 2: Map of western Kenya showing location of the counties surveyed.

3.2 Survey of GRD in western Kenya

Two disease surveys to determine GRD occurrence and distribution were conducted in all the major groundnut growing areas of Bungoma and Kakamega counties. Leafy symptomatic samples were collected from 144 farmers' fields and placed into falcon tubes containing RNAlater solution, and kept in a cool box till use. Groundnut fields were sampled during the short rains season (September to December) of 2016 and long rains season (April to July) of 2017 when the groundnut crop was at flowering stage. In Bungoma County, the sub-counties where sampling was done include Bungoma Central, Bungoma East, Bungoma South, Bungoma West and Mount Elgon. In Kakamega County, sampling was conducted in Kakamega Central, Kakamega East, Kakamega North and Kakamega South. Purposive sampling of groundnut farms was done by stopping at regular predetermined intervals along motorable roads that traversed each study area. The survey was conducted by walking through groundnut fields, and visually inspecting groundnut crops for symptomatic leaves. Depending on the farm size, quadrats of $10m^2$ were estimated with disease incidence and severity scored on the disease diagnostic score sheet for each quadrat through random sampling. Disease incidence was calculated according to Reddy (1991) as the percentage of plants showing GRD symptoms to the total number of plants observed in the field as shown in the following equation:

$$\text{Disease incidence} = \frac{\text{Number of GRD symptomatic plants}}{\text{Total number of groundnut plants sampled}} \times 100\%$$

GRD incidence was scored using a rating scale according to Reddy (1991) where: low incidence = 1-20%; moderate incidence = 21-49% and high incidence = 50-100%. The GRD severity was scored using a severity scale of 0 – 3, where: 0 = No disease, 1 = Mild, 2 =

Moderate and 3 = Severe. The Geographical Positioning Remote System (entrex venture HC GARMIN^{TM}) was used to record the latitude, longitude and altitude of the sampled farms.

3.3 Total RNA extraction, Next generation sequencing (NGS) and sequence analysis

Total RNA was extracted from the symptomatic leafy samples using RNeasy Mini Kit **(**Qiagen**)** according to the manufacturers' protocol. The extracted total RNA was quantified with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and column-purified with the DNA Clean & Concentrator TM-5 – DNA kit (Zymo Research, Irvine, USA). The purified total RNA was used for double stranded cDNA synthesis using the SuperScript II (Thermo Fisher Scientific, Waltham, USA) kit. The samples were then processed with the transposon-based chemistry library preparation kit (Nextera XT, Illumina) following manufacturer's instructions. The fragment sizes structure of the DNA libraries was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The indexed denatured DNA libraries were sequenced (200-bp paired-end sequencing) on the Illumina MiSeq platform.

Reads quality check was done using FastQC (version 0.11.5). Reads were then trimmed to remove poor quality sequences. Trimmed reads (Haas *et al*., 2013) were used for de novo assembly and contigs aligned to the viral genomes database (ftp://ftp. ncbi.nih.gov/genomes/Viruses/all.fna.tar.gz/, downloaded on October 2017) using CLC Genomics Workbench 10.1.2. The assembled contigs were subjected to a BLASTn search against the GenBank database (Altschul *et al*., 1990). GRAV CP gene sequences used for comparison and phylogenetic analyses were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). Phylogenetic analyses and comparisons were performed using the MEGA X (Kumar *et al*., 2018) and DnaSP v.5 (Librado & Rozas, 2009) programs.

3.3.1 Designing GRAV primers

Designing oligonucleotide primers is a crucial step for successful molecular biology experiments that require the use of Real Time-Polymerase Chain Reaction (RT-PCR), which involves cycles of denaturation, annealing and extension. All of these steps are temperature sensitive and the common choice of temperatures is 94°C, 60°C and 70°C respectively.

A new version of Primer3Plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Untergasser *et al*., 2007) was used to design the GRAV primers used in this study. The software which has an option of directly uploading a file with the target sequence for primer design, has many different input parameters that you control and consistently picks good primers.

Upon opening Primer3Plus webpage (http://fokker.wi.mit.edu/primer3/input.htm), the sequences of GRAV_1 and GRAV_18 were uploaded in the organization of Primer3Plus software web interface (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi and http:// sourceforge.net/projects/primer3/files/primer3-web/) that communicates with primer3_core using the boulder IO format, as described in the software program. The product size range of 20 – 35 bp was entered with no optimum for the Left (forward) and Right (reverse) primers into the sequence box. Primer Tm of between 55 - 65^0C was chosen with the optimum being 60^0C and a maximum Tm difference between the primers of 5^0C. The product Tm was left blank. A primer % GC content between 40 - 60% with the optimum being 50% was chosen and the rest of the setting was left at default, except GC clamp which was set at 1, 2 and 3 for the program to pick primers with 1, 2, or 3 G's or C's at the 3' end to work great. The primers that Primer3Plus chose were checked by use of Integrated DNA technologies website Oligo Analyzer (http://www.idtdna.com/SciTools/SciTools.aspx?cat=DesignAnalyze) in the primer3_core main program that uses libprimer3 library (Table 1).

Table 1: Oligonucleotide primers designed and used for the amplification of GRAV

Oligo Name	Primer sequence (5' - > 3')	Specific to	Reference
GRAV F	GCAATGGACGAGCTAACAGG	GRAV CP	This study
GRAV R	ACTTGATGGTGAACCGGAAG	GRAV CP	

A description of primers from this study from the consensus reads of GRAV_1 and GRAV_18 samples, gave specific primers that had good primer specificity, high annealing efficiency, appropriate melting temperature, proper GC content and prevention of primer hairpins or primer dimers.

3.4 Screening popular legumes for resistance to GRAV

Groundnuts (*Arachis hypogaea),* common beans (*Phaseolus vulgaris),* cowpea (*Vigna unquiculata),* soybean (*Glycine max),* green gram (*Vigna radiata)* and golden berry *(Physalis peruviana),* were planted 3 seeds per indicator plant per pot and replicated three times in a 6x2 factorial design. The 6x2 factors included the five popular legume plants with one solanaceous golden berry and the two factors comprised the inoculated and non-inoculated control indicator plants.

After germination, the seedlings were thinned to remain with two plants per pot for a healthy crop stand. The plants were mechanically inoculated with GRD pathogens at 3 leaf stage as follows: The GRD symptomatic leafy samples from the survey, were ground using a sterilized pestle and mortar, with the aid of dust powdered Carborundum 320 grit. Freshly prepared ice-cold 0.01M Potassium Phosphate buffer, pH 7.0, containing 0.2% Sodium Sulfite and 0.01M Mercaptoethanol (1: 6 [w/v] tissue: buffer), was added to the ground

tissue, mixed and transferred to a falcon tube. The mixture was allowed to stand for 5 minutes on ice for debris to settle until use. The test plant leaves were dusted with Carborundum. The inoculum was applied gently on the leaf surfaces, using saturated cotton wool swab. After inoculation, the excess inoculum on the leaves was gently washed with sterilized distilled water. The plants were observed on weekly basis for any viral symptoms until after flowering.

Leafy samples were then collected and tested for GRAV by RT-PCR. Groundnut field samples with chlorotic, mosaic and green rosette were included in the analysis. Total RNA was extracted as described in section 3.3. The primers used were designed using Primer3Plus software with consensus sequences from this study and those from the GenBank as described in section 3.3.1. The RT-PCR was done essentially as described by Naidu *et al*., (1998) with some modifications. Two step RT-PCR was done using One Taqman master mix. Two µl of RNA was initially used in cDNA synthesis which was run at 42°C for 1 hr followed by denaturation step of 5 min at 94°C. The cDNA synthesis reaction was composed of target virus reverse primer (200 ng), MMLV RT, MMLV buffer, dNTPS, DTTS, RNA (2µl) and water. Five µl of cDNA was then used in the amplification step. The amplification mixture was composed of One Taqman master mix, forward and reverse primers, cDNA and water. Amplifications were carried out in an Eppendorf Cycler using the following temperature regime: a denaturation phase at 94°C for 2 min followed by 35 cycles of amplification at 55^0C each for 1 min and an extension of 2 min at 72^0C. Ten µl of PCR products were analyzed by 1.2 % agarose gel electrophoresis in 0.5 µl TBE buffer, stained with Ethidium bromide (EtBr) and finally visualized under UV light.

3.5 Data analysis

Data on incidence and severity was recorded and analyzed by Statistical Analysis Software (SAS) program version 9.3 (SAS Institute, 2013). Pairwise comparison of means was done

using the Least Significance Difference at $P \leq 0.05$ confidence level. Sequence data was analyzed using MEGA X software (Kumar *et al*., 2018).

CHAPTER FOUR

RESULTS

4.1 Survey of GRD Distribution

A total of 301 samples from 144 farms were collected in Bungoma (151 samples) and Kakamega (150 samples) Counties. Groundnut rosette disease was observed in the two Counties surveyed. The disease expressed varied symptoms across the Counties. The incidence and severity of GRD varied between the two Counties.

4.1.1 GRD field symptoms

Rosette infected plants were dwarf with increased tillering, although some were tall but expressed GRD associated symptoms. The main symptoms observed in order of abundance, starting from the most prevalent and virulent were chlorotic rosette, green rosette and severe mosaic rosette (Plate 1).

Plate 1: Major GRD symptoms observed in the field:

a: Chlorotic rosette with leaf curling upwards, could be a sign of co-infection with other viruses. **b**: Green rosette with stuntedness and very dark green leaves **c**: Mosaic rosette with mixed chlorotic lesions. **d**: Healthy groundnut crop

Harvesting of mature rosetted plants, very few or no pods developed on the affected plants as observed in the farmers' fields (Plate 2).

Plate 2: Pod formation on rosetted and healthy plants

a: Rosetted groundnut plant with no viable pod with seeds. **b**: Healthy groundnut plant.

4.1.2 GRD incidence and severity

Majority of groundnut farms recorded incidence of 30-70% chlorotic rosette and green rosette and 30-40% mosaic rosette symptom types. During the short and long rain seasons, GRD incidence was significantly different ($p<0.05$) with short rains season recording higher incidence than the long rains season. Bungoma County recorded 66.51% mean incidence of rosette disease during the short rains season, higher than in the long rains season where it reduced to 30.89%. Similarly, Kakamega County recorded a higher mean incidence in the

short rains season of 47.73% compared to 43.47% in the long rains season. The mean severity in Bungoma County during the short rains season was higher than in the long rains season. Kakamega County also recorded high severity during the short rains season than the long rains season (Table 2).

Table 2: Visual mean rosette incidence and severity scores

County	Season	N	Mean incidence (%)	Mean severity
Bungoma	Short rain	47	66.51	2.21
	Long rain	45	30.89	1.49
Kakamega	Short rain	22	47.73	2.14
	Long rain	30	43.47	1.53
Total	Short rain	69	60.52	2.19
	Long rain	75	35.92	1.51

The incidence of GRD seemed to increase with increase in severity. Where severity was high, incidence was high and predicted to increase significantly (Fig. 3). The line of best fit depicts the relationship of an increasing trend in disease incidence with increased severity (Fig. 3).

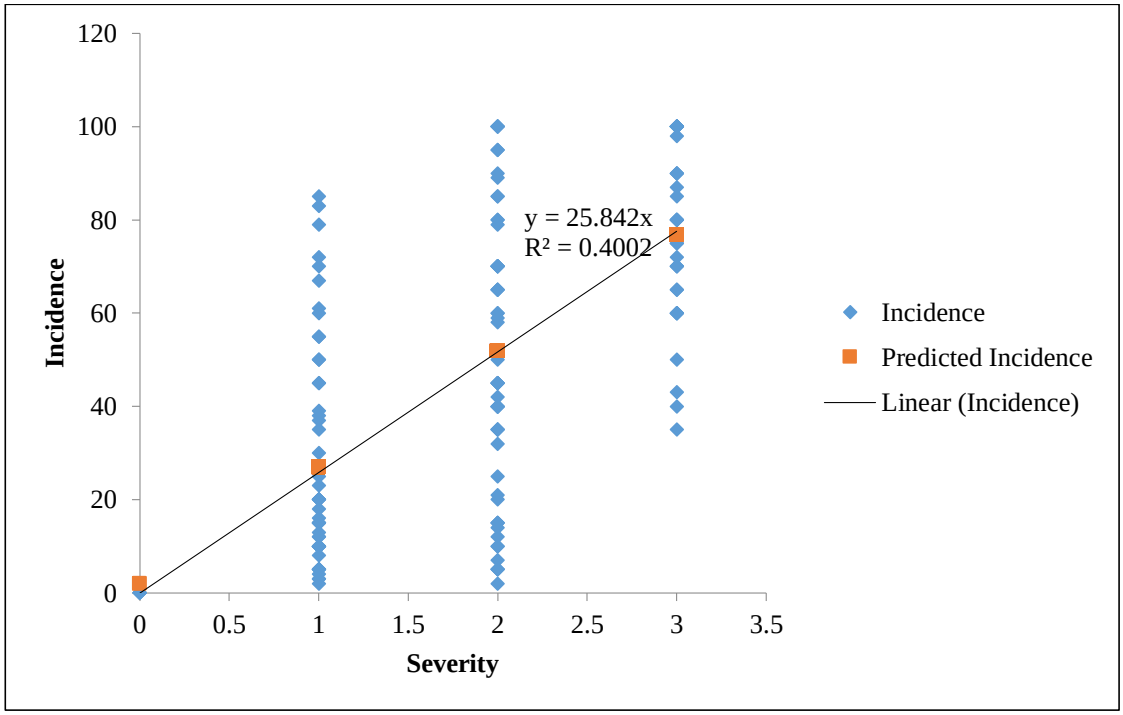


Figure 3: Line graph showing the regression relationship between GRD incidence and severity.

4.1.3 Groundnut varieties planted by farmers

The most popular groundnut varieties were Homabay Red, Local cultivar, Red Valencia, Spanish ICGV-SM 99568, Uganda Red and Virginia CG7. The local cultivars, Red Valencia and Uganda Red were mostly grown by farmers in Bungoma County. In Kakamega County, farmers mainly planted Homabay Red, Red Valencia, Spanish ICGV-SM 99568, Uganda Red and Virginia CG7.

4.2 Diversity of GRAV

Two GRAV coat protein (CP) gene sequences were assembled (600 nt). The two were compared with GRAV CP gene sequences from Malawi, Nigeria and Ghana available in the GenBank. The BLASTn comparison revealed 97-99% identity with the western Kenya isolates. The two isolates clustered together with M16GCP and M8GCP from Malawi in phylogenetic tree. The two GRAV CP gene sequences from western Kenya clustered together

and had 97 – 99% identity with those from Malawi, Ghana and Nigeria implying that there was no much diversity difference among the western Kenya GRAV CP gene isolates. The Kenyan isolates exhibited closest identities with Malawian isolates than Nigerian and Ghanaian isolates. This findings concur with Wangai *et al*., (2001) and Appiah *et al.,* (2017) who observed closer identity between sequences from the same geographical region as compared to those from separate geographical regions. In the study, Wangai *et al*., (2001) found out that Kenyan isolates of GRAV CP gene shared 98% nucleotide identity with Malawian isolates as compared to 96-97% with those from Nigeria. Appiah *et al.,* (2017) found out that Ghanaian GRAV CP gene sequence isolates had 98-99% nucleotide identity as compared to 97-99% with Malawian isolates. Such differences due to geographical distances could be as a result of differences in environmental conditions that bring about variations in the evolutionary biology of the GRD viruses. In general all western Kenya isolates exhibited closest identity and grouped together with Malawian isolates M16GCP and M8GCP than the rest of Malawian, Nigerian and Ghanaian isolates (Fig. 4; Appendix II).

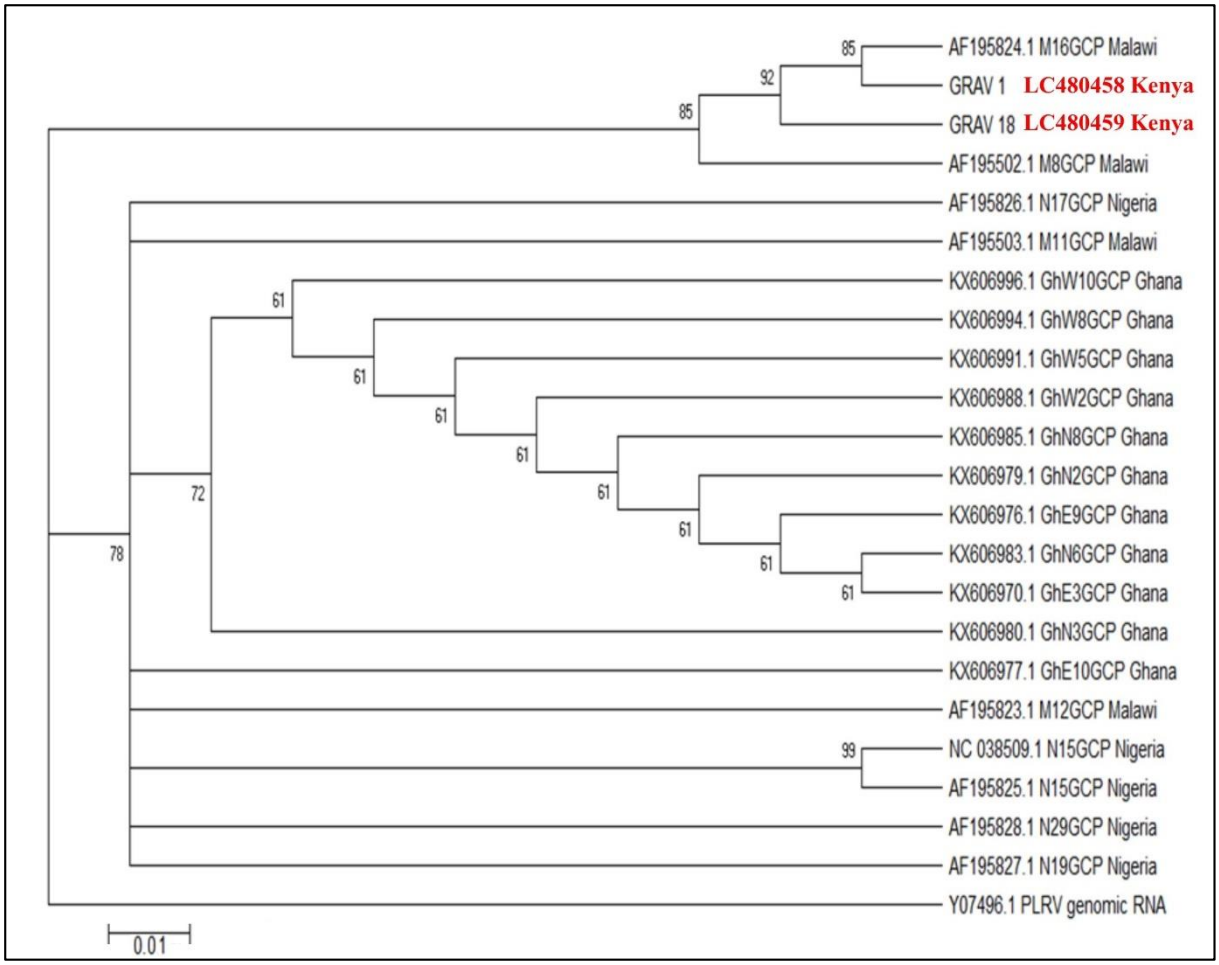


Figure 4: Molecular phylogenetic analysis by Maximum Likelihood method of western Kenya isolates of GRAV with 0.01 genetic distance changes.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei., 1993). *Potato leaf roll virus* (Y07496.1 PLRV) was used as an out-group that gave a better rooting stability than the other possible *Luteoviruses* of *Bean leaf roll virus* (BLRV), *Beet western yellows virus* (BWYV), *Tobacco necrotic dwarf luteovirus* (TNDLV) and *Chickpea luteovirus* (CPLV). The sequences for the western Kenya isolates of GRAV_1 from Bungoma County and GRAV_18 from Kakamega County were deposited in the GenBank with accession numbers LC480458 and LC480459 respectively.

4.3 Screening for resistance/tolerance against GRD associated viruses

The screened plants expressed typical symptoms of GRD; stunted growth, dwarfism with bushy appearance, dark green, yellowing with chlorosis lesions, necrosis, mixed mosaic, reduced leaf area with twisted and distorted leaves curling downwards and upwards (Plate 3; Table 3).

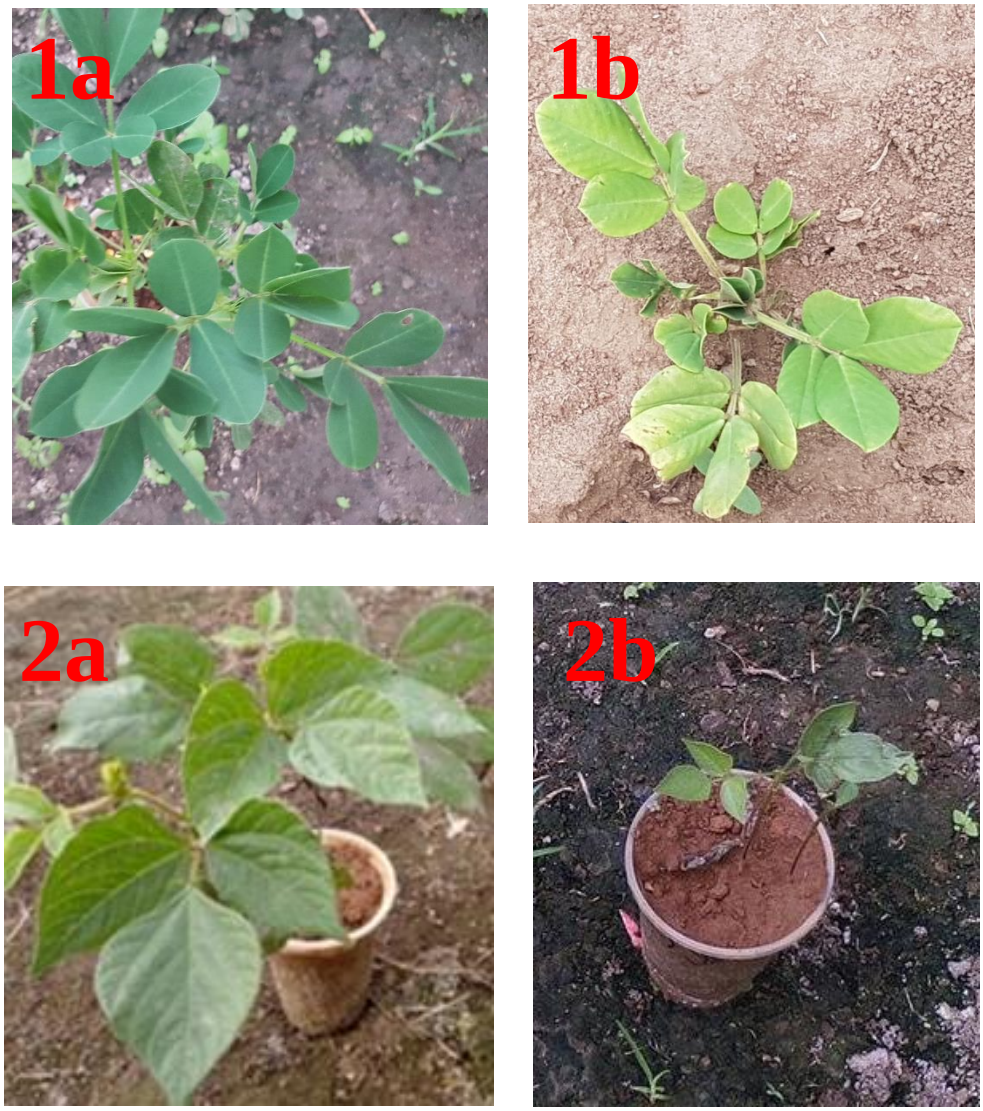


Plate 3: Some symptoms observed upon inoculation of GRD causal agents:
1a: Healthy groundnut, **1b**: Rosetted groundnut; **2a**: Healthy green gram, **2b**: Stunting, leaf deformation and curling in green gram.

Table 3: Greenhouse test crop symptoms and RT-PCR test results

Test plant	Local Symptoms*	Systemic symptoms*	GRAV
Cowpea	N	SS, CS	+
Groundnuts	N	SS, CS, VC	+
Soybean	N	SS, CS, BN	+
Common beans	N	SS, DC, CS	+
Green grams	N	SS, D, CS	+
Golden berry	N	DC, CB	+

*__Key__: **N** – necrosis, **SS**-shiny leaf surface, **CS**-chlorotic spots, **VC**-veinal chlorosis, **DC**-downward leaf curling, **CB**-chlorotic blotches, **BN**-back necrosis, **D**-dwarfing.

4.4 Screening for validation of seed transmission of GRD

The less severe rosetted groundnuts produced one seed per pod in the whole crop. A total of 7 rosetted seeds were obtained during the entire survey in Bungoma County and screened in caged pots to validate seed transmission. Rosetted seeds were found not expressing neither chlorotic, green nor mosaic rosette symptoms (plate 4), validating that GRD is not transmitted by infected rosette seeds. These asymptomatic leaves also tested negative for GRD causal agents on molecular analysis hence confirming that GRD is not transmitted by seed (plate 4).

Plate 4: Healthy groundnut crop observed upon planting rosetted seeds: Both 1a and 1b plates showing healthy groundnut crop in a caged screenhouse.

4.5 RT-PCR detection of GRAV

Total RNA eluted typically ranged between 30 – 55 ng/µl. Gel quantification of the eluted total RNA is shown in Plate 5; Table 4.

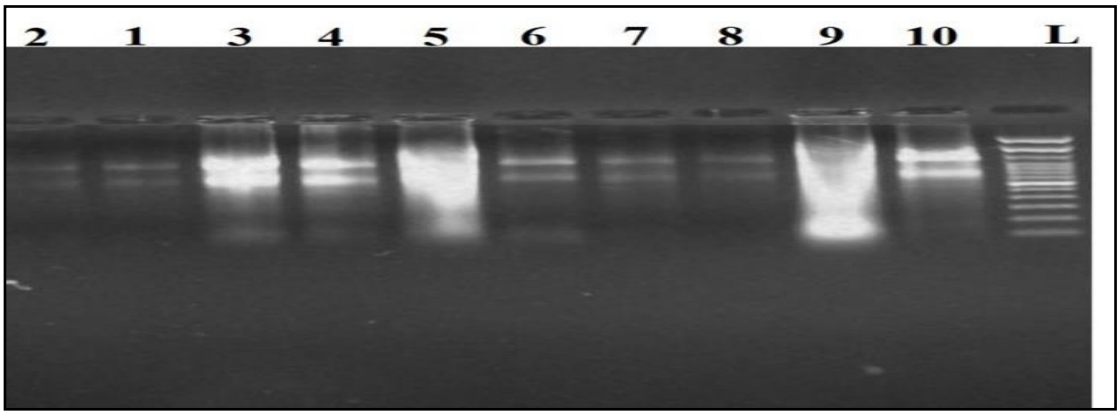
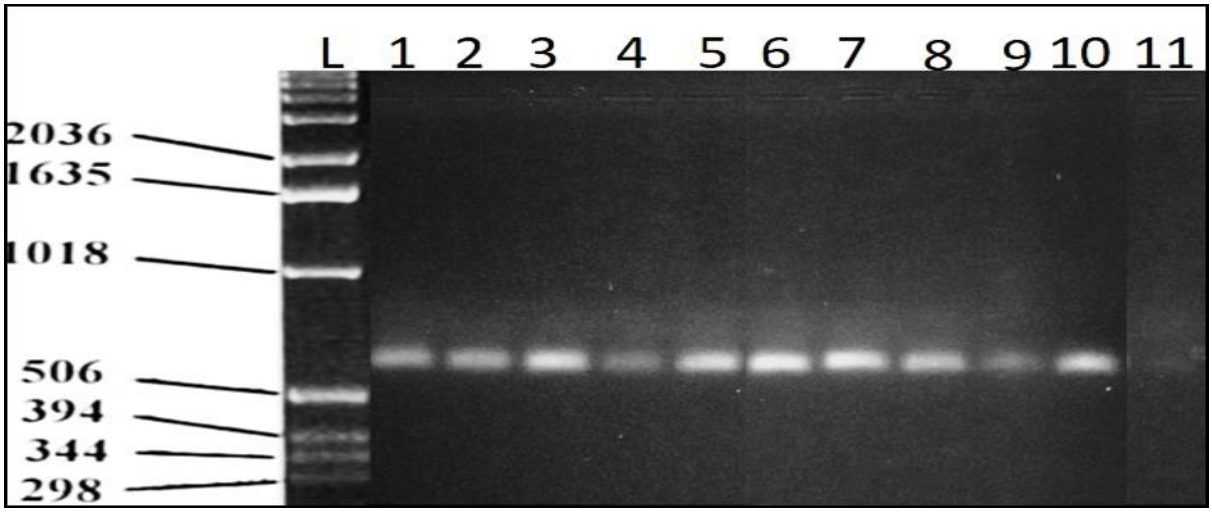


Plate 5: Gel quantification of total RNA. Lanes: 2- green house soybean, 1- green house beans, 3- field green rosette groundnut-a, 4- green house golden berry, 5- field chlorotic rosette groundnut, 6- green house groundnut, 7- green house cowpea, 8- field green rosette groundnut-b, 9- field mosaic rosette groundnut, 10- green house green gram, L – 100 bp DNA ladder.

Table 4: Quantities of GRAV total RNA eluted.

Sample ID	Total RNA (ng/µl)
2	38.7
1	45.5
3	49.6
4	51.8
5	31.9
6	54.2
7	42.6
8	48.7
9	30.0
10	55.0



All ten samples tested positive for GRAV by RT-PCR (Plate 6)

Plate 6: Amplification of GRAV - CP gene for green house and field samples. Expected band size was 597 bp. Lanes: L- 1 kb ladder, 1- green house beans, 2- green house soybean, 3- field green rosette groundnut-a, 4- green house golden berry, 5- field chlorotic rosette groundnut, 6- green house groundnut, 7- green house cowpea, 8- field green rosette groundnut-b, 9- field mosaic rosette groundnut, 10- green house green gram, 11- negative control (water).

CHAPTER FIVE

DISCUSSION

5.1 Occurrence and distribution of GRD

Groundnut rosette disease (GRD) was widely distributed in the groundnut growing areas of western Kenya due to its high inoculum in the fields from aphid vectors, alternative hosts and volunteer indicator plants that forms dead-end sources of the heavy inoculum. There was no significant difference in mean GRD incidence between Bungoma and Kakamega counties ($p=0.502$). However, during the long rains and short rains seasons, disease incidence was significantly different ($p<0.05$) with short rains season recording higher incidence (60.52%) than the long rains season (35.92%).

Bungoma County recorded 66.51% mean incidence of rosette disease during the short rains season, higher than in the long rains season where it reduced to 30.89%. Similarly, Kakamega County recorded higher mean incidence in the short rains season of 47.73 % as compared to 43.47% in the long rains season. This is attributed to prolonged heavy rains in the long rains seasons than in the short rains. Prolonged heavy rains wash off the aphids from the crop, reducing their build-up and contact hours for inoculum transmission. This concurs with the findings by Mugisa *et al*., (2016) that periods of long rains negatively affected GRD progression as aphid vector pressure was low. Were *et al*., (2013) reported a positive correlation between potato disease incidence and aphid numbers. This further supports the implication that GRD incidence variations between the seasons contributed to by differences in groundnut aphid vector pressure. Incidence increased with increase in severity. This suggests that GRD infections that occur at early stages of crop growth leads to intensification of GRAV as the plant grows and build-up of inoculum for vectors to spread to nearby plants.

Naidu *et al*., (1998a) denotes that GRD is a polycyclic disease whereby diseased plants from previous cropping season serves as inoculum sources for initiating subsequent disease spread.

In western Kenya, the common practice is that groundnuts are grown in two cropping seasons (long rains and short rains) and due to limitation in land size to practice shift cultivation, the same piece of land is continuously used to grow the same or related host crops in the subsequent cropping season. Therefore, GRD infected groundnuts and alternative hosts of any of the GRD causal agents remaining from the long rains season, serve as immediate sources of the GRD pathogens beginning the disease cycle at early stages of crop growth in the short rains cropping season. Such primary infections that occur at early stages of plant growth enhance repeated cycles of infections thus increasing the severity of the disease in the groundnut fields (Waliyar *et al*., 2007).

The three major GRD symptoms (chlorotic, green and mosaic rosette) were observed in the two surveyed Counties. Chlorotic rosette was the most prevalent followed by green rosette and least was mosaic. This supports the findings of Wangai *et al*., (2001) who reported chlorotic rosette to be the most prevalent GRD symptom type in sub-Saharan Africa (SSA). The high prevalence and virulence of chlorotic rosette symptom type could also be attributed to its higher transmission efficiency compared to green rosette (Misari *et al.*, 1988a). The mosaic rosette symptom type has not been previously reported to occur in Kenya but this study recorded it in the surveyed Counties of western Kenya. Dual infection by symptom inducing GRV associated sat-RNA variants, especially the chlorotic and green rosette and/or the mild ones, are likely to induce the mosaic symptoms (Naidu *et al*., 1998a). It is therefore possible that some of these variants occur in western Kenya in mixed infections, thus causing the varied symptoms observed, especially the mosaic rosette type. The nature and pattern of GRD spread is influenced by proximity to the source of infection/inoculum and climatic

conditions. Plants that show rosette disease symptoms but lack GRAV are not important in disease spread because the CP gene of GRAV is needed for encapsidation and transmission of the GRV and its sat-RNA. However, if such plants receive GRAV later due to the groundnut aphid feeding, the plants again serve as source of inoculum. Therefore, it is necessary to characterize the sat-RNA associated with GRV to understand the dynamics in the GRD symptom types observed in western Kenya. Apart from the typical rosette symptoms, other symptoms including severe leaf curling both downwards and upwards, bunching, woodiness and severe reduced leaf size were observed. This suggests that there is wider variability in expression of GRD and could be due to more severe variants of associated viruses or other causal agents.

5.2 Molecular diversity of GRAV

The two GRAV CP gene sequences from western Kenya clustered together and had 97 – 99% identity with those from Malawi, Ghana and Nigeria implying that there was little diversity among the western Kenya GRAV CP gene isolates. The Kenyan isolates exhibited closest identities with Malawian isolates than Nigerian and Ghanaian isolates. This findings concur with Wangai *et al*., (2001) and Appiah *et al.,* (2017) who observed closer identity between sequences from the same geographical region as compared to those from separate geographical regions. In the study, Wangai *et al*., (2001) found out that Kenyan isolates of GRAV CP gene shared 98% nucleotide identity with Malawian isolates as compared to 96-97% with those from Nigeria. Appiah *et al.,* (2017) found out that Ghanaian GRAV CP gene sequence isolates had 98-99% nucleotide identity as compared to 97-99% with Malawian isolates. Such differences due to geographical distances could be as a result of genomic differences in environmental conditions that bring about variations in the evolutionary biology of the viruses that makes them unique to their specific regions. In general all GRAV CP gene sequences both in this study and those in the GenBank shared 97-100% nucleotide

identity. This implies that GRAV CP gene is highly conserved across the wide geographical regions in SSA where GRD occurs. It is therefore a suitable candidate for development of pathogen-derived resistance (PDR) (Deom *et al*., 2000; Appiah *et al*., 2017) through genetic engineering involved in generation of transgenic groundnut cultivars resistant/tolerant to GRD that can be used in western Kenya and across SSA where groundnuts are grown.

5.3 Screening for resistance against GRD causal agents

The screened plants expressed distinct viral symptoms of stunted growth, dwarfism with shortened internodes, thickened stems with bushy appearance, yellowing with chlorosis lesions, mixed mosaic, reduced leaf area with twisted and distorted leaves curling downwards and upwards. GRAV was also detected by RT-PCR in all the screened plants. This is an indication that the major legumes grown in western Kenya can serve as alternative hosts of GRAV. Experimental hosts have been identified in several species in *leguminosae, chenopodiaceae* and *solanaceae* where *Glycene max*, *Phaseolus vulgaris* are among the systemic hosts of GRD agents (Waliyar *et al.,* 2007). These can therefore serve as sources of inoculum when the main natural host is planted adjacent to or intercropped with such infected alternative hosts. This study has achieved the objective of determining that *Leguminosae* and solanaceous *Physalis peruviana* plants are susceptible potential alternate hosts to GRD pathogens, both in the extensive field survey and screenhouse experimental findings, which is in agreement with Okello *et al* (2017) who identified the leguminous weed vegetable "Oyado" (*Cassia obtusifolia*) as a potential alternative host that tested positive for all the GRD causal agents through RT-PCR.

CHAPTER SIX

CONCLUSION, RECOMMENDATIONS AND SUGGESTIONS FOR FURTHER RESEARCH

6.1 Conclusion

This study has concluded that:

- GRD is a major virus disease of groundnuts in western Kenya because it occurs wherever groundnuts are grown and may be the reason for the observed low yields. There is seasonal variations in the incidence and severity of GRD. Short rains season had high incidence than the long rains season. Chlorotic rosette is the dominant GRD symptom type in western Kenya. Mosaic rosette symptom type occurs in western Kenya but sparsely distributed.
- All major legumes in western Kenya are susceptible to GRD while the solanaceous golden berry (*Physalis peruviana*) is a potential alternative host of GRD. Rosette disease is not transmitted through seed.
- There is little diversity in the GRAV CP gene isolates of western Kenya which clustered together at 97 – 99% identity and are highly conserved in their CP genome. The designed primers detected the GRAV and the use of NGS is essential in discovery of new plant viruses and characterization of those that are poorly characterized.

6.2 Recommendations

This study recommends that:

- Crop rotation of groundnuts with non-hosts of GRD crops be adopted as a cultural measure to break the polycyclic nature of rosette disease. Volunteer leguminous crops from previous cropping season be rogued before planting new crop, to reduce the chances of acting as immediate initial sources of GRD inoculum. *Leguminosae* family plants and solanaceous golden berry are susceptible to GRD pathogens, and should be rogued immediately from groundnut farms because they act as sources of GRD inoculum.

- GRD resistant/tolerant genes should be incorporated into the local cultivars/varieties of groundnuts as the only practical solution because GRAV is highly conserved at the CP genomic level which is vital in GRD epiphytology and etiology and can be exploited through pathogen-derived resistance (PDR) breeding.
- There is need for a study to characterize the GRD symptom inducing agent (sat-RNA) to help in understanding the dynamics in the symptom diversity observed in western Kenya. Farmer sensitization on GRD symptom identification and management is urgently required.

6.3 Suggestions for further research

This study suggests further research to be done on the gaps that were not captured including:

- More research to be done to determine the exact mode of GRD transmission by the aphid vector biotypes and etiology, because mosaic rosette symptom type was reported occurring for the first time in western Kenya in isolation and sparsely distributed. This suggests that there is wider variability in new encounter scenarios of virus evolution that could be due to more severe variants of sat-RNA or other causal agents that needs to be unravelled.
- There is need to sequence the RT-PCR results of the few seeds obtained from rosetted plants to authenticate absence of GRD causal agents since GRD is not transmitted through seed. Establish a reliable groundnut seed production and certification system in western Kenya. It is also important to ascertain whether GRAV and GRD associated viruses are soil borne. A study on severely pronounced chlorotic/yellowing symptoms on anthills and termitarium needs an in-depth extensive study to establish GRD inoculum in the soil.
- There is need for urgent measures to manage GRD in western Kenya, possibly through extensive research on other solanaceous and leguminous weeds acting as alternative hosts of GRD inoculum.

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APPENDICES

Appendix 1: Disease diagnostic score sheet

SURVEY DISEASE SCORE SHEET

CROP…….VARIETY……..…

Farmer's name……County……..…

District……Division……

Location……Sub-Location…….….

Village…….Date……..

GPS readings;

Altitude (Metres)……..

Longitude (East or West)……..

Latitude (North or South)…….AEZ…….……

	Disease name…….			
Groundnut variety	No. of plants affected per $10m^2$ quadrat	Part affected (root, stem, leaves, pods)	Distribution (whole field, spots)	Severity 0-3
1				
2				
3				
4				
5				

*Severity: 0= No disease; 1=Mild; = Moderate; 3=Severe.

Number of plants affected per $10m^2$: select the area most affected, 10 steps square quadrat, count infected and total plants, (e.g. $^{20}/_{50}$ indicates 20 plants infected out of 50 plants in the 10x10 steps square quadrat).

Appendix II: Clustal alignment of western Kenya GRAV CP with Malawian, Ghanaian and Nigerian sequences.