OCCURRENCE AND DISTRIBUTION OF GROUNDNUT ROSETTE DISEASE IN WESTERN KENYA

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A proposal submitted in partial fulfillment of the requirements for the degree of Master of Science in Crop Protection of Masinde Muliro University of Science and Technology.

December 2016

DECLARATION

This proposal 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.

Signature..... Date 05-12-2016.....

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SCP/G/04/15.

APPROVAL

The undersigned certify that they have read and hereby recommend for acceptance of Masinde Muliro University of Science and Technology a proposal entitled "*Occurrence and Distribution of groundnut rosette disease in Western Kenya*".

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ABSTRACT

Groundnut (*Arachis hypogaea* L.) is an economically important food legume, grown in Western Kenya. Groundnut rosette disease (GRD) is a major constraint in sub-Saharan Africa, which can cause serious yield losses in an epidemic situation. Rosette is transmitted in nature by the viruliferous aphid vector, *Aphis craccivora* Koch (Insecta: *Homoptera*), in a persistent circulative manner. The disease 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). The GRD viruses occur in three predominant symptom forms; chlorotic rosette, green rosette and mosaic rosette. This study will determine the symptomatology, occurrence, distribution, biological and molecular characterization of groundnut rosette in Western Kenya. Two disease diagnostic surveys, will be conducted during the short and long rain seasons in eight counties; Bungoma, Busia, Homabay, Kakamega, Migori, Nandi, Siaya and Vihiga. The counties represent the Lower Midland (LM) and Upper Midland (UM) agro-ecological zones (AEZs). Symptomatic leaves will be collected from the groundnut farms. Total RNA will be extracted by RNeasy Mini Kit (Qiagen), and sequenced using next generation sequencing technologies (NGS). Disease incidence and severity will be scored on the disease score sheet. The data collected on occurrence, distribution, diversity and characterization, will be compared by analysis of variance (ANOVA), using Statistical Analysis Software (SAS) program (SAS Institute lnc.). Pairwise comparison of means will be done using Least Significance Difference (LSD) at $P \leq 0.05$ confidence level. This research will provide comprehensive knowledge of GRD viruses, rosette symptoms and better agronomic farming technologies, for considerable increased groundnut production.

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LIST OF ABBREVIATIONS AND ACRONYMS

AEZ	- Agro-Ecological Zones.
ANOVA	- Analysis of Variance.
bp	- Base Pair.
dsRNA	- Double Stranded Ribonucleic Acid.
FAO	- Food and Agricultural Organization.
FAOSTAT	- Food and Agricultural Organization Statistics.
GRAV	- Groundnut Rosette Assistor Virus.
GRD	- Groundnut Rosette Disease.
GRV	- Groundnut Rosette Virus.
ICRISAT	- International Crops Research Institute for the Semi-Arid Tropics.
KARI	- Kenya Agricultural Research Institute
LM	- Lower Midland.
NGS	- Next Generation Sequencing.
ORF	- Open Reading Frame.
RT-PCR	- Reverse Transcription Polymerase Chain Reaction.
SADC	- Southern African Development Community.
Sat-RNA	– Satellite Ribonucleic Acid.
SSA	– Sub-Saharan Africa.
ssRNA	- Single Stranded Ribonucleic Acid.

UM - Upper Midland.

CHAPTER ONE

BACKGROUND OF THE STUDY

1.1 Groundnut production and importance

Groundnut (*Arachis hypogaea*) is native to Southern America. It is a key crop for small scale farmers that produce aerial flowers, but fruiting below the soil level. *Arachis hypogaea L.* is often called peanuts, belonging to the family *Fabaceae,* is the only domesticated species in the genus (Usman, 2013). Other local 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 Virginia, Espanola, Malgache groundnut, Kersting groundnut and Roja Tennessee. Groundnut is the fifth most important annual oilseed and food legume crop, grown in diverse environments throughout the semi-arid and sub-tropical regions, in nearly 100 countries, in the six continents of the world (Kumar *et al.,* 2007). The most important groundnut producing countries are Argentina, Chad, China, India, Indonesia, Myanmar, Nigeria, South Africa, Senegal, Sudan, USA, and Vietnam (Kumar *et al*., 2007). Africa accounts for 40% of the global area planted to groundnuts. Only 26% of the highest average were observed in South Africa, and the lowest in East Africa. (ICRISAT, 2012, FAOSTAT, 2013, World Bank, 2015). In Kenya, groundnut (*Arachis hypogaea),* have a local name "njugu karanga". Groundnuts are mainly grown in Western Kenya, by small scale farmers for food and sale. 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, maturing in 120-150 days. The common varieties grown include Red Valencia, Manipita, Makulu Red, Bukene, Asyria Mwitunde, Texas Peanut, Serere 116 (white), Alika and Homabay. Western Kenya has a tropical climate suitable for farming. The present

growers yield in Kenya is 450-700kg/ha. This yield can be improved by adopting good management and agronomic practices.

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%s, 0.42%, 0.50%), and Iron (0.40%, 0.47%, 0.43%) respectively (Ayoola *et al.,* 2012). The possible use of groundnut seeds, is in animal feeds (poultry), complete human diet (balance diet for elderly ones who need little carbohydrate but much protein), and an antidote for children suffering from malnutrition is recommended (Ayoola *et al.,* 2012). The haulms and groundnut cakes are used as hay for feeding livestock, while the groundnut seed is consumed as whole seed, or processed as snack foods. Groundnut is also a source of vitamins like niacin, falacin, riboflavin, and thiamine. As a legume, groundnut improves soil fertility, by fixing nitrogen and thereby increases productivity of the semi-arid cereal cropping systems (Smartt. 1994) and other agro- ecological soils.

1.2 Constraints to groundnut production

Nearly 75% to 80% of the world's groundnut, is grown by resource poor smallholder farmers in developing countries, who routinely obtain yields of 500-800kg/ha, as opposed to the potential yield of >2.5t/ha (Kayondo *et al.,* 2014). In Western Kenya, farmers achieve less than 30-50% of the potential yield with an average output of 600-700 kilograms per hectare (kg/ha) (Kidula *et al*., 2010). Low yields are mainly attributed to viral diseases like GRD,

which is not adequately managed due to high cost and unavailability of insecticides to control the vector insects (Appiah *et al*., 2016). Other constraints include low quality seeds, drought, poor agronomic practices, numerous pests and diseases caused by fungi, viruses, bacteria and nematodes. In Kenya, Kidula *et al*., (2010) noted that, groundnut is grown mainly as an oil crop for the market as a source of income. However, adoption of new varieties is given lower priority due to lack of efficient seed production systems. Viral diseases are the most important. 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,* and two each to *Cucumovirus, Pecluvirus, Soymovirus* and *Umbravirus,* and one each to *Begomovirus, Bromovirus, Carlavirus, Ilarvirus, Luteovirus, Potexvirus, Rhabdovirus* and *Tymovirus.* Of these, 19 were first isolated from groundnut, and the remaining first isolated from other hosts, but they commonly occur on groundnut (Salem *et al*., 2010).

Pests, diseases, weeds, lack of appropriate production technologies, inadequate markets and poor post-harvest handling practices, are among the factors that influence the low production, and profitability of groundnuts in East Africa (Mutegi, 2010; Okello *et al.,* 2010). Improved varieties are developed for better disease resistance, tolerance, higher yields and good market acceptability, which could enhance overall productivity (Ntare *et al*., 2002). 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), are the most economically important, and are responsible for serious yield losses regionally and even globally (Okello *et al.,* 2014). These viruses are also known to naturally infect several other crops, and inflict significant losses in them. Although not present every year, when epidemics of GRD viruses occur, they can result in devastating losses. In 2006, the average groundnut yield recorded in sub-Saharan

Africa was 980kg/ha, considerably less than the world's average of 1690kg/ha (Bucheyeki *et al.,* 2008).

1.3 Statement of the problem

Groundnut Rosette Disease (GRD) is by far the most endemic, destructive virus disease of groundnut in sub-Saharan Africa (SSA) and Madagascar (Wangai *et al.,* 2001). Groundnut rosette disease (GRD) causes significant losses in groundnut production, in Western Kenya and Eastern Uganda (Okello *et al.,* 2010, 2014). Only limited field resistance is available for GRD cultivars that have less than superior agronomic traits (Usman, 2013).

The complex etiology and lack of diagnostic tools, are major constraints in understanding the epidemiology of GRD viruses, and developing appropriate management strategies for the disease. To date, lack of sufficient research surveyed, on the occurrence, distribution and diversity of GRD virus symptom types in Western Kenya (Wangai *et al*., 2001; Kidula *et al*., 2010; Thuo *et al*., 2014), has resulted in continued and increased yield losses amongst groundnut farmers. Research by Okello *et al*. (2014), reports that in Uganda, GRD viruses resulting in green rosette symptoms predominate. This is in contrast with Wangai *et al*. (2001), who reported that chlorotic rosette symptom type, has been the predominant form throughout sub-Saharan Africa.

In Western Kenya, virus variability and symptom types within the virus populations, is not known and therefore, a need to understand virus-plant interactions. This study will determine the occurrence, distribution and diversity of GRD viruses within the study site, to generate an in depth analysis of GRD viruses occurring in the region. There is need to document the whole range of symptom types encountered in Western Kenya, and develop improved rosette disease identification, monitoring and recommend appropriate management and control methods. This will help reduce high cost of insecticides to resource poor persons,

ineffectiveness of pesticides against insect vectors, negative impact of insecticides on the terrestrial, aquatic macroinvertebrates, environment as a whole and resistance to pesticides by the transmitting vectors. Inaccurate molecular and biological characterization of genotype adaptability, on host plants and indicator plants, may lead to poor productivity in environments that interact negatively with specific genotypes of groundnut cultivars (Tillman *et al*., 2009).

1.4 Justification of the study

It is necessary to understand the occurrence and distribution of GRD epidemiology, which is complex, and involves interactions between two distinct viruses, a Sat-RNA, an aphid vector and the host plant, in the unpredictable environments of SSA. The deleterious impact of GRAV and GRV with its sat-RNA, on groundnut host plants in a synergistic manner is not known. Taliansky *et al., (*2000) reported that GRAV or GRV infection alone in groundnut, results in transient mottle symptoms with insignificant impact on the plant growth and yield. These results have however, been contradicted by Naidu and Kimmins (2007) who reported that, GRAV infection alone affects plant growth and contributes to significant yield losses in susceptible groundnut cultivars. Because the disease is endemic to SSA and the offshore islands of Madagascar, it is presumed that there are alternate indicator plants, from which the GRD viruses spread into groundnut with the help of a polyphagous aphid, *Aphis craccivora.* The spread of GRD viruses is complicated, because a single aphid may not always transmit the three agents; GRV, GRAV and sat-RNA.

This study will determine the indicator host plants of GRD viruses through simple biological characterization procedures, which can be utilized to integrate applied and basic groundnut research to increase knowledge at molecular and physiological levels. This will unravel the virus-plant interaction systems, whose combinations may give rise to asymptomatic or

possibly latent infection that serves as virus reservoirs in the field, causing serious biosecurity in virus disease control (Salem *et al.,* 2010). This knowledge will be utilized in developing viable strategies that will expand protection methods of groundnut plants against GRD viruses for the benefit of groundnut farmers in Western Kenya and sub-Saharan Africa.

Several methods have been used to investigate and manage GRD viruses. They include pesticides application to reduce vector populations, cropping practices to delay onset and spread of both vector and disease, and cultural practices, but only limited success has been achieved with each of these approaches (Naidu *et al.,* 1999a). The poor documentation of the impact of GRD, in sub-Saharan Africa, is probably due to misdiagnosis, as a result of lack of adequate resources to conduct reliable surveys, and a lack of in depth knowledge of the rosette disease.

The international crops research institute for the semi-arid tropics (ICRISAT), estimates that groundnut rosette disease, causes greater yield loss than any other virus disease, affecting groundnut in the semi-arid tropics of the world (Naidu *et al.*, 1998b). ICRISAT and its partners have made significant contributions towards understanding GRD epidemiology based on molecular diagnostic assays. However, despite much advancement in knowledge on rosette, critical information pertaining to the off-season survival, of the disease agents and aphid vector is lacking. This requires intensive studies on aphid dispersal patterns, off-season survival and long distance migration of aphids, identification of possible biotypes and alternative hosts of GRD viruses.

1.5 General objective

To determine the occurrence, distribution and diversity of groundnut rosette disease (GRD) in Western Kenya.

1.5.1 Specific objectives

1. To determine the occurrence of groundnut rosette disease (GRD) in Western Kenya.

2. To determine the distribution and diversity of groundnut rosette disease (GRD) in Western Kenya.

3. To establish the biological and molecular characteristics, of groundnut rosette isolates on known and new indicator plants.

1.6 Hypothesis

The research objectives will be tested by the following null hypothesis;

H_O1: Groundnut rosette disease occurs in Western Kenya.

H_O2: There exist distinct strains, of groundnut rosette disease viruses in Western Kenya.

H_O3: Isolates of groundnut rosette disease viruses from Western Kenya, express similar symptoms on indicator plants.

CHAPTER TWO

LITERATURE REVIEW

2.1 Occurrence and distribution of groundnut rosette disease (GRD)

Groundnut rosette disease (GRD) was first documented in 1907 from Tanganyika, now called Tanzania (Waliyar *et al.,* 2007). Since then, GRD has been reported in several other sub-Saharan African Countries: Angola, Burkina Faso, Cote d'lvoire, Gambia, Ghana, Kenya, Madagascar, Malawi, Niger, Nigeria, Senegal, South Africa, Swaziland, Uganda and the Democratic Republic of Congo DRC) (Thuo *et al*., 2014; Kidula *et al*., 2010; Wangai *et al.,* 2001). The virus agents of GRD have not been detected elsewhere in the world, except in SSA, despite the fact that groundnut is grown in more than 100 countries around the world, and the vector aphid, *Aphis craccivora,* is found in almost all the groundnut growing regions.

Symptoms similar to GRD, have been reported in some countries of Asia and South America, but diagnostic tests, to unequivocally confirm the presence of the disease, have not been conducted (Reddy, 1991). This disease is considered to be endemic, to groundnut growing countries of SSA, and its offshore islands of Madagascar. Since GRD is limited to SSA, it is likely that groundnut introduced from South America, sometime during the sixteenth century by the Portuguese, was infected by a pathogen endemic to SSA, and is therefore an example of a new encounter phenomenon (Olorunju *et al.,* 2001). The new encounter phenomenon occurs, when a crop has been introduced into a new geographical region, and pests or pathogens that evolved with other host species, attack the newly introduced crop (Deom *et al*., 2000)

Epidemics of GRD viruses in SSA, often reduce groundnut production, and cripples rural economy. Although not present every year, epidemics occur with devastating losses. 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 (Olorunju *et al.,* 2001). In 1995-1996, Eastern Zambia lost 43,000 ha of groundnut to GRD viruses estimated at US$ 5 million. 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 (Taliansky *et al.,* 2000; SADC/ICRISAT., 1996).

Yield losses due to GRD viruses, depends on the growth stage of the plant when infection occurs. If infection occurs before flowering, over 90% loss in pod yield may result. Yield loss is variable if infection occurs between flowering and pod maturing stage, whereas subsequent infections cause negligible effects (Kumar *et al.,* 2007). Although the debilitating impact of GRD epidemics, was documented in a few instances (Herselman *et al*., 2004), the international crops research institute for the semi-arid tropics (ICRISAT), estimates that groundnut rosette disease, causes greater yield loss, than any other virus disease affecting groundnut in the semi-arid tropics of the world (Subrahmanyan *et al.,* 1998).

2.2 Etiology of groundnut rosette disease (GRD)

The etiology of GRD is a complex, involving three agents; Groundnut rosette assistor virus (GRAV), Groundnut rosette umbravirus (GRV) and a Satellite-RNA (Sat-RNA) of GRV (Taliasky *et al.,* 2003). These three components are intricately dependent on each other, and all three play a crucial role in the biology and perpetuation of GRD. Groundnut rosette virus (GRV) needs assistor GRAV for mechanical transmission by the aphid, *Aphis craccivora*. The virus-like nucleic acid molecule, Satellite-RNA, occurs in different forms, and results in at least three types of field symptoms, chlorotic (yellowing), green and mosaic rosette (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 4.6kbp (dsRNA-1) and 1.3kbp (dsRNA-2), a very abundant species

of 900bp (dsRNA-3), and numerous minor species of intermediate mobility. The GRV satellite-RNAs associated with chlorotic and green rosette disease, in different regions of Africa, are 895-903 nucleotides long, and are at least 87% identical. 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. Evidence suggests that, sat-RNA plays a key role in symptom expression (Taliansky *et al.,* 2000).

Groundnut rosette assistor virus (GRAV) is a member of the family *Luteoviridae.* Deom *et al.* (2000) characterized the virus, and identified it as *luteovirus.* GRAV virions are non-enveloped, isometric shaped with 28nm diameter particles of polyhedral symmetry. Their genome is a non-segmented, single molecule of linear positive sense, single-stranded RNA of ca.6900 nucleotides, which encodes for structural and non-structural proteins (Murant *et al.,* 1990). Like other members of the *luteovirus,* GRAV is thought to encode for six Open Reading Frames (ORFs). Only coat protein region of the genome is sequenced (Gene Bank Accession # z 68894 af195502, af195825). Virions are made of single coat protein subunits of size 24.5kDa, and the virus is antigenetically related to *Bean/pea leaf roll virus, Beet western yellow virus* and *Potato leaf roll virus* (Scott *et al.,* 1996). The virus replicates autonomously, in the cytoplasm of the phloem tissue. Groundnut rosette assistor virus (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. Groundnut is the only known natural host of the GRAV. The virus is reported to occur, wherever GRD has been reported. The GRAV on its own, causes symptomless infection or transient mottle, and can cause significant yield loss in susceptible groundnut cultivars (Waliyar *et al.,* 2007).

Groundnut rosette virus (GRV) belongs to the genus *Umbravirus.* On isolation and characterization, the virus has no structural (coat) protein (Taliansky *et al.,* 2003), and thus

forms no conventional virus particles. Taliansky *et al.* (2003) detected, enveloped bullet-shaped structures, in the ultra-thin sections, due to GRV infection as opposed to real virions. The virus genome is a non-segmented, single linear molecule of single-stranded, positive sense RNA of size ca.4019 nucleotides, that encodes for four open reading frames (ORFs) (Taliansky *et al.*, 2003).The genome of GRV isolate when completely sequenced (Gene Bank Accession #z66910), and several partial sequences, are available in the Gene bank. The GRV replicates autonomously, in the cytoplasm of the infected tissues (Taliansky *et al.,* 2003).

Groundnut rosette virus (GRV), on its own causes transient symptoms, but a Sat-RNA associated with GRV, is responsible for rosette disease symptoms (Waliyar *et al.,* 2007). Groundnut rosette virus (GRV) depends on GRAV for encapsidation of its RNA, and transmission by *Aphis craccivora* in a persistent mode (Robinson *et al.,* 1999). The virus is transmitted by grafting and mechanical inoculation but not through seed, pollen or by contact between the plants (Waliyar *et al.,* 2007). Groundnut is the only known natural host, but several experimental hosts in the families *Chenopodiaceae* and *Solanaceae* have been reported (Waliyar *et al.,* 2007). No strains of GRV have been reported, and the virus is restricted to SSA and its offshore islands of Madagascar (Okello *et al.,* 2013).

The Satellite-RNA, 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 (Block *et al.,* 1994). It totally depends on GRV for its replication, encapsidation and movement, both within and between the plants. Sat-RNA is responsible for rosette symptoms, and plays a critical role in helper virus dependent transmission of GRV (Taliansky *et al.,* 1997). Different variants of Sat-RNA have been shown to be responsible for different rosette symptoms, in other indicator plants (Murant & Kumar., 1990). Upto five open reading frames (ORFs) in positive or negative sense are predicted to occur in Sat-RNA, but no protein products have been isolated (Block *et al.,* 1994). It is mechanically transmissible along with

the GRV, and is also transmitted by aphids, in the presence of GRV and GRAV (Waliyar *et al.,* 2007,). The sequences of 10 variants of GRV Sat-RNA have been determined (Block *et al.*, 1994).

All the three agents, GRAV, GRV and the Sat-RNA, are intricately dependent on each other in GRD etiology. The Sat-RNA and its variants are responsible for GRD symptoms (Olorunju *et al.,* 2001). The GRAV and GRV can replicate autonomously, but Sat-RNA totally depends on GRV for replication. The GRAV acts as a helper virus in vector transmission of GRV and Sat-RNA (Robinson *et al.*, 1999). The Sat-RNA plays a crucial role in encapsidation of GRV RNA into GRAV coat protein, and thereby assists in aphid transmission (Robinson *et al.,* 1999). The Sat-RNA is the most essential part for the GRD complex to survive in nature.

2.3 Symptoms and host range of groundnut rosette disease (GRD)

Both chlorotic and green rosette symptoms occur throughout the SSA, and sometimes occur in the same field (Mugisa *et al*., 2016). A less common third symptom variant, called mosaic rosette, resulting from mixed infection of the groundnut cultivars by the sat-RNA causing chlorotic and green mottled variant, has been reported from East Africa (Scott *et al.,* 1996; Waliyar *et al.,* 2007). 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 that results in a bushy appearance (Naidu *et al.,* 1998b). In chlorotic rosette, leaves are usually bright yellow with a few green islands and leaf lamina is curled. In the green rosette, leaves appear dark green, with light green to dark green mosaic (Naidu *et al.,* 1999a). Variability in Sat-RNA is mainly responsible for symptom variations (Taliansky *et al*., 2007). 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 *et al.,* 2007), also resulting into biotic and abiotic constraints of groundnut production in Western Kenya. In the neighbouring Eastern Uganda, GRD viruses 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 and Western Kenya. This finding is of utmost importance because, Eastern Uganda and partly neighbouring Western Kenya is a major groundnut grower in SSA. This study suggests that, it would be worthwhile to perform more-detailed surveys in groundnut growing areas in Western Kenya, and other regions to establish the predominant rosette symptoms. Furthermore, recommend for adoption of new certified seed varieties and better management and control farming technologies. The dynamics of the GRD virus symptomatology, and the vector behaviour, needs further research (Okello *et al.,* 2014). Jinja and the surrounding areas of Eastern Uganda, for instance, recorded the highest severity of GRD (Okello *et al.,* 2014). This further affirms that the region is a GRD virus hotspot (Okello *et al.,* 2010). Rosette symptoms include yellowing, mottling, stunting, mosaic, shortened internodes, bushy appearance and distortion of the shoots.

RNA viruses exist as "quasispecies" (Roossinck, 1997) in the infected plants, and thus the population complexity of GRAV, GRV and Sat RNA in the field, has the potential to be large. The potential permutations among variants of the three agents, are able to form viable alternatives, and their capacity to adapt to diverse and changing econiches, are thus enormous. With time, this continuous "evolution" of GRD viruses, under strong selection pressure, can lead to new disease patterns (Okello *et al.,* 2014).

In Nigeria, a clear shift occurred from green to chlorotic rosette, over a period of about 20 years. The shift could be due to changes, in the genome sequences of GRD agents, or to different vector biotypes and cropping patterns (Okello *et al.,* 2014). Routine documentation of the predominant GRD symptom types, is therefore necessary, to enhance research efforts,

which are geared towards development of novel strategies, to support crop protection measures currently in use, for effective management and control of GRD in Western Kenya. Since groundnuts are important, and widely grown in Sudan, DR Congo, Tanzania, Rwanda and Burundi, it would be interesting to determine the distribution of GRD virus symptom types, in these countries that forms the East African region (Okello *et al.,* 2014), and recommend appropriate agronomic control technologies.

2.4 Epidemiology of groundnut rosette disease (GRD)

Groundnut and some of its wild relatives are the only natural hosts of GRAV, GRV and Sat-RNA. GRD epidemiology is complex, involving synergistic interaction between and among GRAV, GRV and a Sat-RNA, the aphid vector, the host plant and environment (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* (L.) Taub, S. *mucronata* Wild, S. *sundaica* Taub, *Trifolium incarnatum* L., T. *Pratense* L., *Caspella bursa-Pastoris* (L.) Medicus, *Gomphrena globosa* L., *Montia Perfoliata* L. and *Spinacia Oleracea* L. (Ayoola *et al*., 2012). All these plants showed symptomless infections, and virus replication that was confirmed by diagnostic assay. 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; C. *amaranticolor, Glycene max, Phaseolus vulgaris, Nicotiana benthamiana* and N. *Clevelandii* are systematic hosts of GRV (Waliyar *et al.,* 2007). Apart from groundnut, experimental hosts of both GRAV, and GRV and Sat-RNA are *Gomphrena globosa, Stylosanthes gracilis,* S. *mucronata,* S. *Sundaica, Spinacia oleracea, Trifolium incarnatum* and T. *repens* (Murant *et al*., 1990).

Aphis craccivora, commonly known as the cowpea aphid or groundnut aphid, is the principal vector involved in the transmission of all the GRD viruses, in a persistent circulative manner. The GRV and Sat-RNA must be packaged within the GRAV coat protein, to be aphid transmissible. Studies have shown that all the GRAV particles, whether they contain GRAV-RNA or GRV-RNA and Sat-RNA, are acquired by the aphid vector, from phloem sap in 4hr and 8hr acquisition access feeding, for chlorotic and green rosette respectively. *Aphis craccivora* does not always transmit, all the three GRD viruses together (Naidu *et al.*, 1998a). During short inoculation feeding (test probe or stylet pathway phase), *Aphis craccivora* probe groundnut leaves, without reaching the phloem, transmitting only GRV and Sat-RNA, which multiply in 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.,* 1996b). *Aphis craccivora* 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 agents together is high. The vector aphid, A. *craccivora,* fails to acquire or transmit GRV, and Sat-RNA from diseased plants lacking GRAV, and 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. Reports of groundnut crop damage by GRD, underscores the need for further epidemiological studies and appropriate control and management strategies, that reduces the inoculum sources for viral diseases, to prevent resistant and tolerant varieties from succumbing to GRD at high inoculum pressure (Appiah *et al*., 2016).

2.5 Biotypes and diversity of groundnut rosette disease (GRD) viruses

The existence of biotypes of A. *craccivora,* that differ in host-plant specificity, and transmission efficiency of GRD viruses, has important implications on the epidemiology of

GRD complex (Waliyar *et al.,* 2007). Since none of the causal agents is seed-borne, primary infection of crops depend on the survival of infected plants (Virus sources) and vectors (aphids) (Naidu *et al.,* 1998b). Possible source from which rosette could spread, are infected groundnut plants surviving between cropping seasons (Waliyar *et al.,* 2007). In regions where there are no sources of infection, initial infection may depend on the influx of viruliferous aphids, from other parts of Africa on prevailing wind currents (Olorunju *et al*., 2001). The vector *Aphis craccivora* is polyphagous and can survive on as many as 142 plant species in addition to groundnut. One or more of these 142 plant species, could be a source of the rosette complex (Naidu *et al.,* 1998b). GRV and Sat-RNA must be packaged within the GRAV coat protein to be aphid transmissible. Research efforts have failed to identify any alternative natural hosts of the GRD viruses (Waliyar *et al.,* 2007).

Groundnut rosette disease (GRD) is a polycyclic disease, because each infected plant, serves as a source for initiating subsequent disease spread in the field. Winged aphids are responsible for primary spread of the disease. Secondary spread from the initial foci, of rosette disease within the fields, also occur by way of migration of aphid vector, but largely apterae and nymphs (Naidu *et al.,* 1998b). Primary infection at early stages of the crop growth, provides a good opportunity for repeating cycles of infection to occur, before crops mature, and vector populations decline. The nature and pattern of disease spread, is influenced by plant age, cultivar type, crop density, time of infection, transmission efficiency of aphids, proximity to the source of infection, and climatic conditions (Waliyar *et al.,* 2007).

2.6 Rosette disease management

Various methods are available for protecting groundnut against rosette disease. They include; the removal of volunteer groundnut plants that serves as inoculum source, cultural

practices that can interfere with vector movement, use of insecticides to control aphids and use of rosette disease resistant cultivars (Naidu *et al.,* 1998b, 1999a).

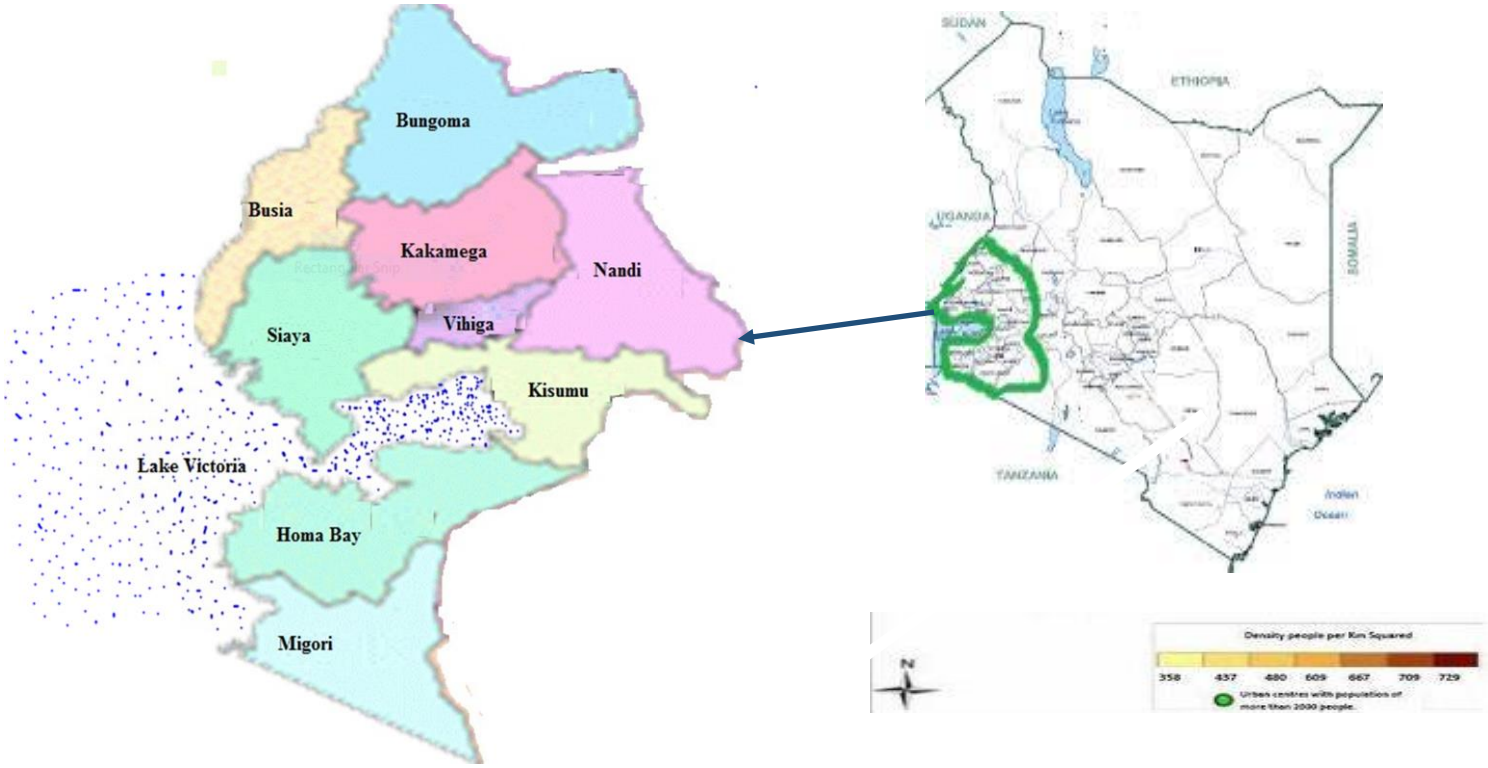
Studies have shown that resistance to the aphid vector, is controlled by a single recessive gene (van der Merwe & Subrahmanyan., 1997), which is mapped on linkage Group-1, at a distance of 3.9 cm from a marker, originating from a susceptible parent (ICGV-SM 93541) (Herselman *et al.,* 2004). Identification of this DNA marker, offers a scope to develop a simple DNA-marker based method, for screening aphid resistance, which may accelerate breeding progress.

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). Only limited field resistance is available for either virus, in popular groundnut cultivars and landraces, which have less than superior agronomic traits. This phenomenon needs further evaluation of the germplasm in popular groundnut genotypes.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study site map



Source: 24kenyan.com/wp-content/uploads/2013/08/E93Kenyan-counties.jpg

Figure 1: Study site map.

3.2 Rosette disease diagnostic survey

This study is based on symptomatology, biological and molecular data of GRD viruses in Western Kenya. Two disease surveillance surveys, to determine GRD virus occurrence and distribution, will be conducted in all the major groundnut growing areas in the region. Leafy symptomatic samples will be collected from the farmers' fields and taken to the laboratory for molecular characterization. Total RNA will be extracted and sequenced using the next generation sequence technology (NGS). Groundnut fields will be sampled during the short rain season (October to December) of 2016 and long rain season (March to May) of 2017. The following agro-ecological zones (AEZs) will be covered: Lower Midland; LM1 (Butula, Rongo and Teso South), LM2 (Bumula, Bungoma East, Bungoma South, Bungoma West and Busia), LM3 (Gem, Rarieda, Siaya and Teso North), LM4 (Bondo and Suba). Upper Midland; UM1 (Emuhaya, Nandi Central, Nandi South, Sabatia and Vihiga) and UM2 (Nandi North). Sampling of groundnut farms will be done by stopping at regular predetermined intervals, of 3-8 KM along motorable roads that traverses each sampling area. The survey will be conducted, by walking through groundnut fields, and visually inspecting groundnut crops for symptomatic leaves. Depending on the farm size, quadrats of $10m^2$ will be estimated, disease incidence and severity will be scored, for each quadrat through random sampling. A disease diagnostic score sheet, will be used to record GRD virus incidence and severity in each farm. Disease incidence will be calculated according to Reddy, (1991), as the percentage of plants showing GRD virus symptoms, to the total number of plants observed in the field as shown in the following equation:

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

GRD virus incidence will be 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 virus severity will be scored using a severity scale of 0 – 3, where: 0 = No disease, 1 = Mild, 2 = Moderate and 3 = Severe. The symptomatic leaves collected in falcon tubes containing RNAlter solution and put in a cool box, will be taken to the laboratory for total RNA extraction using RNeasy Mini Kit (Qiagen) and sequenced using the next generation sequencing technologies (NGS) (Eichmeier *et al*., 2016). The Geographical Positioning Remote System (entrex venture HC GARMIN^{TM}), will be used to record the latitude, longitude and altitude of the sampled regions.

3.3 Indicator plants and mechanical inoculation of GRD virus symptoms

Biological characterization experiments will be carried out in a screenhouse, through mechanical inoculation of the germplasm with GRD virus isolates. Low concentrations of the rosette disease agents in host plants, makes it essential to develop a reliable and sensitive method for their detection (Usman, 2013; Salem *et al*., 2010). Groundnuts (*Arachis hypogaea),* common beans (*Phaseolus vulgaris),* cowpea (*Vigna unquiculata),* soyabean (*Glycine max),* and peas (*Pisum sativum* L.*),* which are leguminous indicator plants (Mugisa *et al*., 2016), will be planted in pots and mechanically inoculated with GRD viruses from symptomatic leaf samples. Popular groundnut landrace cultivars; Red Valencia, Serere 116 (white) and Homabay, will be intercropped alternately with indicator plants in one kilogram pot capacity in a screenhouse at Masinde Muliro University of Science and Technology Research Farm, in Kakamega County. Three seeds per variety will be planted in each pot. After germination, the seedlings will be thinned to remain with one seedling per pot.

The GRD virus symptomatic leaf samples from the survey, will be 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 (K_2HP0_4 + KH_2P0_4), P^H 7.0, containing 0.2% Sodium Sulfite and 0.01M Mercaptoethanol (1: 6 [w/v] tissue: buffer), will be added to the ground tissue, mixed and transferred to a falcon tube, and allowed to stand for 5 minutes in

ice, for debris to settle at the bottom of the tube. The sap will be kept on ice, until inoculation is completed. A separate inoculum will be made, by crushing GRD virus symptomatic leaf samples in distilled water. At 3 leaf stage, the plants will be inoculated with GRD virus inoculum. The test plants will be dusted with Carborundum to act as an abrasive. The inoculum drops, will be applied gently on the leaf surfaces, using saturated cotton wool swab. After inoculation, the excess inoculums on the groundnut leaves will be gently sprayed, and washed with sterilized distilled water. This will be carried out to forestall confusing residue, because of the GRD virus infected sap extract left on the leaf. Hands will be washed with detergent, before proceeding to the next inoculation, to prevent contamination.

3.4 Determination of genome sequence of the GRD viruses

Plant viruses contain RNA and DNA genomes, with majority having RNA genomes. Detection of viral RNA and DNA genomes in infected plant material by next generation sequencing (NGS) (Kreuze *et al*., 2009), is possible through the extraction and sequencing of total RNA and DNA (Eichmeier *et al*., 2016). NGS has the ability to sequence whole genomes of known and unknown viruses and the ability to detect multiple viruses from a mixed infection, thus providing a very sensitive diagnostic method for the rapid and routine detection of viruses. NGS being non-specific, it can be used to detect all known viruses present in a host irrespective of their pathogenicity. RNA viruses from infected groundnut crops will be extracted from the sampled symptomatic leaves, and sequenced by NGS. Rosette viral RNAs will then be detected following bioinformatics analysis, an approach involving sequencing and subsequent discarding of significant amounts of host RNA sequence data relative to viral RNA. A larger number of sequence reads are required to ensure the detection of low titre viruses.

3.5 Total RNA extraction

Total RNA will be extracted from semi-purified virions using RNeasy Mini Kit **(**Qiagen**)** according to the manufacturers' protocol. Before starting, 10µl Mercaptoethanol will be added to Buffer RLT or RLC. 44ml ethanol will then be added to concentrate Buffer RPE. 100mg ca. plant material (3cm x 3cm) will be grinded in a sample bag in liquid N_2 and 450-1000 µl RLT or RLC added and grinded again. 450 µl of this lysate will be transferred to a QIAshredder and centrifuged 2' at full speed. The flow-through will be transferred to a new tube without disturbing the cell-debris pellet. 0.5 volume (225 µl) abs. ETOH will be added and mixed by pipetting. The sample including any precipitate that may have formed (650 µl) will be transferred to an RNeasy spin column**,** centrifuged 15s at 10000 rpm and the flow-through discarded. 700 µl buffer RWE will be added and centrifuged 15 s at 10000 rpm to wash the spin column membrane. The flow-through will be discarded and the collection tube reused.

500 µl buffer RPE will be added and centrifuged 15s at 10000 rpm and the flow-through discarded. 500 µl buffer RPE will be added again and centrifuged 2 min at 10000 rpm, the flow-through then discarded. The spin column will be placed in a new 1.5 ml tube and 50 µl RNase free water directly added to the membrane then centrifuged 1 min at 10000 rpm. Final eluate contains purified RNA. RNA isolated from host plants will be used in molecular characterization analysis. RNA will be quantified and applied in RNA Stable (Biomatrica). RNA samples will be sent to Macrogen, Inc, for library construction and next generation sequencing.

3.6 Next generation sequencing

After extraction, libraries will be constructed using Illumina's TruSeq Stranded mRNA Kit: http://www.illumina.com/products/truseq_stranded_mrna_library_prep_kit.html mRNA sample preparation kit. Illumina HiSeq 2000 will then be used for sequencing. Sequence

reads will be analyzed using an in-house, customized version of the PathoScope 2.0 bioinformatics pipeline (Hong *et al*., 2014). Next generation sequence reads will be mapped to a custom database of plant virus sequences, and the proportion of reads mapped to each virus quantified. BLAST analysis of sequence data will be used for analysis by Geneious software. The sequence reads will be assembled to produce the genomewide sequence of each virus, or RT-PCR (Anitha *et al*., 2014) will be used and clones produced to determine the sequence of the lacking parts. A phylogenetical analysis will be done and a phylogenetic tree will be constructed to infer relatedness of these viruses. The sequences will be compared to those from elsewhere in the GenBank database.

3.7 Data analysis

The collected data on GRD virus incidence and severity, will be subjected to analysis of variance (ANOVA), using Statistical Analysis System (SAS) program version 9.3.1 software (SAS Institute, 2013). Pairwise comparisons of means will be made using Least Significance Differences (LSD) for multiple-means comparison method at $P \leq 0.05$ confidence level.

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APPENDICES

Appendix I: Budget

No	Apparatus	Cost in KSHs
1	24 Agarose gel tanks @1,000 KShs	24,000.00
2	6 Packets sterile gloves @1,000 KShs	6,000.00
3	200 Crushing bags @100 KShs	4,000.00
4	3 RNeasy Mini Kits (Qiagen) @40,000KShs	120,000.00
5	4 Nucleotide triphosphates @5,500KShs	22,000.00
6	3 Cooler boxes @8,000KShs	24,000.00
	Sub total	**200,000.00**
	Reagents	
1	400g Sodium citrate @1,000.00KShs	4,000.00
2	500g Glacial acetic acid @1,000KShs	5,000.00
3	2l Bromophenol blue dye @5,000KShs	10,000.00
4	200g Ethidium bromide @100KShs	20,000.00
5	5l Absolute ethanol @1,000KShs	5,000.00
6	500mls RNAlter solution @100KShs	50,000.00
7	6kg Groundnut Seeds @200KShs	1,200.00
8	3kg Diammonium fertilizer @100KShs	300.00
9	121 Oligonucleotide primers @500KShs	60,500.00
	Sub Total	**156,000.00**
	LITERATURE DOCUMENTATION	
1	Journals, conference and seminars	10,000.00
2	Thesis binding and inscription	6,000.00
	Sub Total	**16,000.00**
1	Car hire for local travel survey	80,000.00
2	Accommodation	48,000.00
	Sub Total	**128,000.00**
	Grand Total	**500,000.00**

Appendix 2: Work plan

Period / Activity	2016					2017								
	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	April	May	June	July	Aug	Sept
Proposal writing														
Short rain survey.														
Screenhouse experiment														
Long rain survey														
Thesis Writing														

Appendix 3: 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).