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# Distribution, Molecular Detection and Host Range of Groundnut Rosette Assistor Virus in Western Kenya

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**Abstract:** Groundnut (*Arachis hypogaea* L.) is an important legume in western Kenya, but yields are low and declining due to pests and diseases. Groundnut rosette disease (GRD) is the main disease causing upto 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. The disease is caused by synergistic interaction among groundnut rosette assistor virus (GRAV, genus *Luteovirus*), groundnut rosette virus (GRV, genus *Umbravirus*) and its associated satellite RNA (sat-RNA). The GRAV plays a crucial role in packaging the other two agents for vector transmission, and therefore a key factor in the spread of GRD. Limited information was available on distribution and host range of rosette disease in western Kenya. This study determined the distribution, molecular detection and host range of GRAV in western Kenya. A survey was conducted in Bungoma and Kakamega Counties during the short and long rains of 2016-2017. Symptomatic leafy samples were collected in RNAlater® stabilization solution and analyzed by RT-PCR. Host range studies were conducted at the Kenya Agricultural, Livestock and Research Organization (KALRO)-Kakamega. Five popular legumes of beans (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogaea* L.), green gram (*Vigna radiata*), soybean (*Glycine max*) and one solanaceous golden berry (*Physalis peruviana* L.) were planted in a 4×6 factorial design. The plants at three leaf-stage, were mechanically inoculated with GRD inoculum prepared from 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. A two-step RT-PCR was done using primers designed targeting GRAV CP gene. Rosette incidence and severity was significantly higher during the short rains than the long rains. All tested plants from the screen house developed typical GRD symptoms, and were found positive by RT-PCR. It is concluded that GRD is the major virus disease of groundnuts and infects most legumes grown in western Kenya. Breeding for resistance to both the groundnut aphid and GRAV coat protein (CP) gene, might be the only practical solution.

**Keywords:** *Arachis hypogaea*, *Groundnut Rosette Assistor Virus*, Groundnut Rosette Disease, Host Range, Two-step RT-PCR

## 1. Introduction

Leguminous groundnut (*Arachis hypogaea* L.) crop is grown for food, oilseed, cash crop, animal feed and amelioration of soil fertility through $N_2$ fixation in western Kenya. The major groundnut producing countries include in

Asia (China, India, Indonesia, Myanmar and Vietnam), Africa (Burkina Faso, Chad, Democratic Republic of Congo, Ghana, Kenya, Malawi, Mali, Mozambique, Nigeria, Senegal, South Africa, Sudan, Uganda, Zambia and Zimbabwe), United States of America and South America (Argentina, Brazil and Mexico). Africa contributes about 24.4% of the world production, and yields per hectare in Eastern and South Central Africa averages 1,604 kg/ha, which is consistently below 3,393 kg/ha and 3,801 kg/ha recorded in China and the United States of America respectively [1]. The western Kenya farmers achieve less than 30-50% of the potential yield with an average output of 600-700 kg/ha. The present farmers' yield in Kenya is 450-700 kg/ha below the expected yield of 3000-4000 kg/ha obtained in on-station experimental fields [2]. Low yields are mainly attributed to numerous pests and diseases. Among other virus diseases, groundnut rosette disease (GRD) causes significant yield losses of upto 100%.

Rosette is a unique and fascinating virus disease whose origin and perpetuation in nature still remains inconclusive, in spite of substantial advance in knowledge since 1907 when it was first documented in Tanzania [3]. 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 and Uganda [4]. 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 [5]. 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 [6]. Key market class cultivars including local 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 [7]. Adoption of new varieties and specific cultivar genotypes is constrained by the low priority given due to inadequate outreach field days to educate the groundnut farmers on GRD or "witches bloom" as commonly referred to in western Kenya, identification, management, efficient seed production systems and pest/disease pressure tolerance/resistance awareness.

The distinct phenotypic symptom expression of chlorotic, green and mosaic rosette caused by synergism among groundnut rosette assistor *luteovirus* (GRAV), groundnut rosette *umbravirus* (GRV) and its associated satellite RNA (sat-RNA), makes it three diseases in one. The pathogens of GRD have not been detected elsewhere in the world except in SSA [8-9], despite the fact that groundnut crop is grown in more than five continents around the world. In addition, the efficient polyphagous groundnut aphid *Aphis craccivora* Koch and the inefficient *Aphis gosypii* Glover and *Myzus persicae* Sulzer, are also found in almost all the groundnut growing regions of the world [10-12]. Limited information was available on the host range and distribution of rosette isolates in western Kenya. Management of GRD is dependent on resistant/tolerant cultivars and cultural measures that include rogueing of volunteer diseased groundnut crop and weeds, intercropping with other legumes and cereals, early planting and crop rotation. This study determined the distribution, host range and validated seed transmission studies. To date, there is no work on the host range studies of indicator plants for GRD causal agents in western Kenya, which this study accurately determines through phenotypic screening, pathotyping biological characterization and molecular diagnostic assays.

# 2. Materials and Methods

### *2.1. Field Survey*

Two disease diagnostic surveys to determine GRD occurrence and distribution were conducted in major groundnut growing areas of Bungoma and Kakamega Counties of western Kenya. Symptomatic leafy samples were collected from farmers' fields and placed into falcon tubes containing RNAlater® stabilization solution, stored in a cool box and taken to the laboratory for molecular analysis. Groundnut fields were sampled during the short rains season (September to November) of 2016 and long rains season (April to June) of 2017. Sampling was done in Bungoma County (Bumula, Bungoma Central, Bungoma East, Bungoma South, Bungoma West and Mount Elgon) and Kakamega County (Kakamega Central, Kakamega East, Kakamega North, Kakamega South and Mumias). Purposive sampling of groundnut farms was done by stopping at regular pre-determined intervals along motorable roads that traverses each sampling area. The survey was conducted by walking through groundnut fields, and visually inspecting groundnut crops for symptomatic leaves. Depending on the farm size, quadrats were estimated, disease incidence and severity was scored on the disease diagnostic score sheet for each quadrat through random sampling. Disease incidence was calculated according to [10] as the proportion of plants showing GRD symptoms. Disease severity was scored using a severity scale of 0 – 3, where: 0 = No disease, 1 = Mild, 2 = Moderate and 3 = Severe. Geographical position system (GPS) coordinates were recorded for each sampled field to produce a geo-referenced map of GRD occurrence and distribution in western Kenya. Data on incidence and severity was recorded and subjected to analysis of variance (ANOVA) using Statistical Analysis Software (SAS) program version 9.3 [13]. Pairwise comparison of means was done using the Least Significance Difference (LSD) at $P \leq 0.05$. Correlation analysis was also done to establish the relationship between symptomatic variables.

### *2.2. Host Range Studies*

A total of seventy two indicator plants of five popular legumes of beans (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogaea L.*), green gram (*Vigna radiata*), soybean (*Glycine max*) and one common

plant/weed of solanaceous golden berry (*Physalis peruviana* L.), were screened at the Kenya Agricultural, Livestock and Research Organization (KALRO)-Kakamega in the screen house. The experiment was arranged in a 4×6 factorial design with three replications in three plot sizes of 4 m by 2 m, with 60 cm spacing between the rows, 30 cm between the plants, and 1 m spacing between the three plots. The 30 cm diameter by 30 cm height pots were filled with solarized soil and planted with 4 seeds of each legume and *P. peruviana*. The seeds were watered daily in the morning and evening throughout the growing period. At two leaf stage, the plants were thinned to two to ensure a good crop stand. At three leaf stage, they were inoculated with GRD inoculum prepared from RT-PCR positive symptomatic leafy samples obtained from the field survey. The GRD inoculum was prepared by macerating the mixed distinct chlorotic, green and mosaic rosette symptomatic leaves with a mortar and pestle, in a chilled sterilized 0.01M cold phosphate buffer ($K_2HPO_4$ +$KH_2PO_4$), PH 7.0 containing 0.2% Sodium sulphite ($Na_2SO_3$) and 0.01M Mercaptoethanol (1:6 [w/v]) tissue:buffer. Inoculation at three leaf stage was mechanically done using the rub method on Carborundum dusted leaves. The plants were observed weekly for 8 weeks for symptom development. Molecular analysis was done by RT-PCR to verify the presence of GRD causal agents. Seeds from less severe rosette diseased groundnut crops, were obtained from Bungoma and Kakamega Counties, and planted in caged pots then observed for 8 weeks for GRD symptom development. The screened symptomatic leafy samples were collected from each indicator plant based on visual symptoms in falcon tubes containing RNAlater® stabilization solution, stored in a cool box and taken to the laboratory for molecular analysis.

### *2.3. Molecular Detection of GRAV*

The symptomatic leafy samples collected from the field survey and screenhouse with chlorotic, green and mosaic rosette were tested for GRAV by RT-PCR. Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturers' protocol. Primers used were designed using Primer 3Plus software [14] with consensus sequences from this study and those from the GenBank (Table 1). The RT-PCR was done essentially as described by [15] 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°C each for 1 min and an extension of 2 min at 72°C. 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.

***Table 1.*** *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 | |

# 3. Results

### *3.1. Distribution of GRD*

Rosette symptoms were observed in all farms sampled. A total of 301 symptomatic leafy samples were collected from 144 farmers' fields. Some individual farms recorded up to 100% chlorotic and green rosette symptom incidence respectively and up to 40% mosaic rosette incidence. The mean GRD incidence in most farms was 35.92% in the long rains season and 60.52% during the short rains season. Rosette incidence was significantly different between the rain seasons ($p<0.001$) with the short rains season recording higher incidence than the long rains season. Bungoma County recorded 66.51% mean rosette incidence 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. The mean severity was moderate during the long rains (1.51) and severe in the short rains season (2.19). The mean severity in Bungoma County during the short rains season was significantly higher than in the long rains season. Kakamega County also recorded high severity index during the short rains season than the long rains season (Table 2).

***Table 2.*** *Mean GRD incidence and severity.*

| County | Season | N | Incidence (%) | | Severity | |
|---|---|---|---|---|---|---|
| | | | Mean* | Std. Error of Mean | Mean* | Std. Error of Mean |
| Bungoma | Long rain | 45 | 30.89[a] | 4.534 | 1.49[a] | .104 |
| | Short rain | 47 | 66.51[b] | 4.295 | 2.21[b] | .118 |
| Kakamega | Long rain | 30 | 43.47[a] | 5.283 | 1.53[a] | .124 |
| | Short rain | 22 | 47.73[b] | 6.143 | 2.14[b] | .190 |
| Overall | Long rain | 75 | 35.92[a] | 3.496 | 1.51[a] | .079 |
| | Short rain | 69 | 60.52[b] | 3.653 | 2.19[b] | .100 |

### *3.2. Host Range*

The screened indicator plants expressed typical GRD symptoms of stunted growth, shortened internodes, thickened stems, dwarfism, yellowing with chlorosis lesions, mixed mosaic, reduced leaf area with twisted and distorted leaves curling downwards and upwards (Figures 1-6; Table 3).

***Figure 1.*** *Groundnut rosetted seeds.*

***Figure 2.*** *Arachis hypogaea.*

***Figure 3.*** *Physalis peruviana.*

***Figure 4.*** *Phaseolus vulgaris.*

***Figure 5.*** *Glycine max.*

***Figure 6.*** *Vigna unguiculata.*

Figures 1-6: Screened indicator plants showing typical GRD symptoms.

***Table 3.*** *Screen house 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 gram | 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.

### *3.3. RT-PCR Detection of GRAV*

The total RNA eluted typically ranged between 30 – 55 ng/µl on Gel quantification. All the ten samples tested positive for GRAV by RT-PCR (Figure 7).

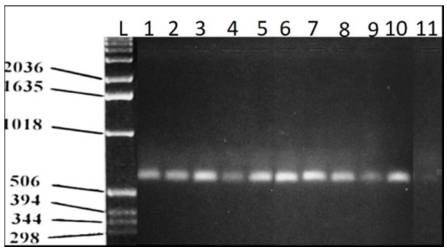


***Figure 7.*** *Amplification for GRAV - CP gene for green house and field samples. Expected band size was 597 bp. Lanes: L- 1 kb ladder; 1-green house beans; 2-green house soybean; 3-field green rosette groundnut-a; 4-green house golden berry; 5-field chlorotic rosette groundnut; 6-green house groundnut;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

# 4. Discussion

Rosette disease was widespread in the groundnut growing areas of western Kenya. During the 2016 to 2017 survey period, GRD incidence in most individual farms of western Kenya recorded up to 100% total crop failure. These findings are in agreement with [1] who estimated yield losses of 60-100% in the groundnut growing areas of western Kenya and the neighbouring eastern Uganda. Study findings by [4] reported that GRD incidence in Kenya ranges from 24 to 40% in western and 30 to 60% in Rift Valley provinces. Rosette incidence and severity was high during the short rains than the long rains. This is attributed to the long rains being heavy and consistent after planting, washing off the vector aphids from the crop, reducing their build-up and contact hours for inoculum transmission, before much damage is done to the groundnut crop [2]. High rosette incidence in the short rains season is attributed to massive build-up, of the vector aphid colonies on the crop to very high densities, leading to further dispersal and secondary spread of the GRD viruses [1].

Research indicated that mosaic rosette is of low incidence, but of wide occurrence in eastern and southern Africa [7]. Different variants of the naturally occurring isolates of sat-RNA nucleic particle, are responsible for the distinct chlorotic and green forms of rosette symptoms [16], while mosaic rosette is phenotypically induced by a mixture of both chlorotic and green sat-RNA variants [8]. Although the sat-RNA is mainly responsible for GRD symptom diversity, groundnut genotypes possessing resistance to rosette disease are highly resistant but not immune to GRV and its sat-RNA, but are fully susceptible to GRAV which intensifies GRV sat-RNA induced symptoms that elevate yield losses. Only limited field resistance is available for GRD cultivars, which have less than superior agronomic traits [9].

This study has shown that legumes and solanaceous *P. peruviana* plants are susceptible and are potential alternate hosts to GRD causal agents. Studies by [7] 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. Research by [8] reported a variant of the satellite RNA of groundnut rosette virus that induced brilliant yellow blotch mosaic symptoms in *Nicotiana benthamiana*. In all forms of rosette disease, early infection causes severe pod loss because rosetted plants may flower but few pods and seeds are produced. Rosette disease causes yield reduction possibly due to impairness of plant performance through limitation of photosynthetic production, thereby retarding growth and interruption of the supply of assimilates to pod and seed development.

Detection of GRAV by RT-PCR in all the screened germplasm samples and selected field isolates that tested positive, infer that the western Kenya legumes and solanaceous golden berry (P. *peruviana*) act as alternative hosts for GRD pathogens. The RNA viruses exist as quasispecies in the infected plants, this makes the population complexity of GRAV in the infected plants have the potential to be large, with potential permutations among the complex synergistic interaction of GRD causal agents. This virus evolution and mutations, may result into new disease patterns and symptom diversity [5] of GRD, making it three (chlorotic, green and mosaic) diseases in one in SSA. The healthy phenotypic expression of the screened seeds of rosetted groundnut crop, is an indicator that GRD is not seed transmissible

# 5. Conclusion

In conclusion, this study confirmed 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 variation in GRD incidence and severity, the short rains season had high incidence than the long rains season. Chlorotic and green rosette symptoms are dominant, overriding the isolated occurrence of mosaic rosette symptom type in the region. All major legumes in western Kenya are susceptible to GRD. The solanaceous golden berry (*Physalis peruviana*) is a potential alternative host of GRD. Rosette disease is not transmitted by seed.

# 6. Recommendations

This study recommends that 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 *Solanaceae* golden berry are susceptible to GRD pathogens, hence 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 and farmer sensitization on rosette disease. 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. Extensive studies on rosetted seeds to unravel the exact mode of GRD transmission and complex etiology.

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## Declaration of Interest

The authors declared that no competing interests exist.

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