Groundnut Rosette Disease and its Management

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List of Acronyms, Abbreviations and Symbols

A	Absorbance
ALP	Alkaline phosphatase
DNA	Deoxyribonucleic acid
DNTP	Deoxy nucleotide triphosphate (dATP, dGTP, dCTP, dTTP)
DsRNA	Double stranded RNA
ELISA	Enzyme-linked immunosorbent assay
et al.	And others
G	Gravitational force
GRD	Groundnut rosette disease
GRAV	Groundnut rosette assistor virus
GRV	*Groundnut rosette virus*
ha	Hectare
H	Hour
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IgG	Immuno-γ-globulin
Kb	Kilo bases
KDa	Kilo Daltons
Nts	Nucleotides
M	Molarity
M	Milli
Mabs	Monoclonal antibodies
Ml	Milliliter
ORF	Open reading frame
Nm	Nanometers
PCR	Polymerase chain reaction
PLRV	*Potato leaf roll virus*
PH	Hydrogen ion concentration
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SADC	Southern African Development Community
SatRNA	Satellite RNA
SsRNA	Single stranded RNA
SSA	Sub-Saharan Africa
TAS-ELISA	Triple antibody sandwich-ELISA
V	Volume
Var.	Variety
W	Weight
%	Percent
µ	Micro
$	United States Dollar

1. Introduction

Groundnut (Peanut, *Arachis hypogaea* L.), an important food and cash crop in sub-Saharan Africa (SSA), is predominantly grown by small-land holding farmers under rainfed conditions (see Box 1). Groundnut rosette disease (GRD), first reported in 1907 from Tanganyika (presently Tanzania), is the most devastating disease of groundnut in SSA (Zimmermann 1907; Reddy 1991) (Figs. 1 & 2). The disease is endemic in groundnut growing areas of SSA, including its offshore islands such as Madagascar. GRD is limited to groundnut crop and the African continent (Fig. 3). There is no evidence of GRD occurrence anywhere outside Africa. Earlier reports on its occurrence based on rosette-like symptoms in groundnut in India, Java and Australia were later confirmed as caused by other viruses (such as *Indian peanut clump virus*).

Figures 1 and 2: Rosette disease affected groundnut in farmer fields in Malawi.

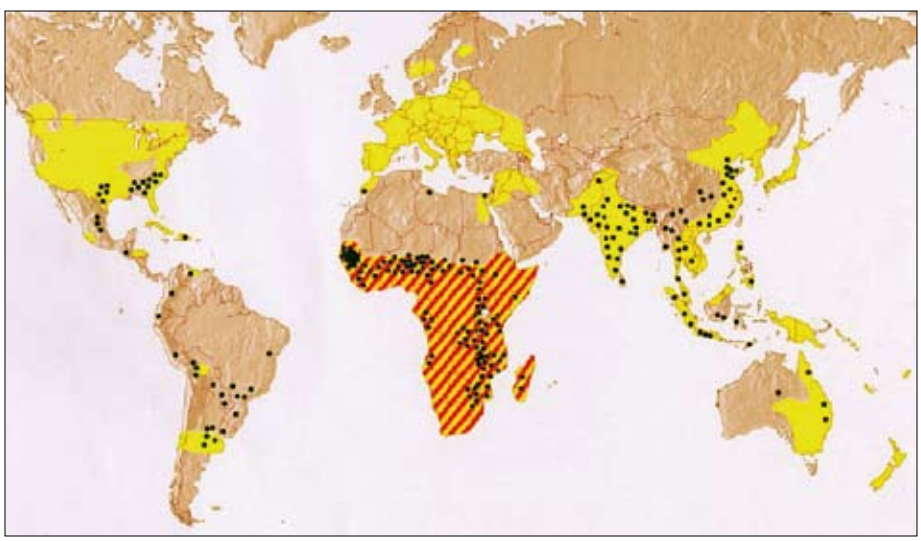


Figure 3: Distribution map of groundnut producing regions in the world (black dots), groundnut rosette disease (area indicated with crossed lines) and distribution of Aphis craccivora Koch (yellow region) (Source: Naidu et al. 1999a)

Box 1: About groundnut

Groundnut or peanut (*Arachis hypogaea* L; allo-tetraploid; 2n=40; x =10) is a major oilseed legume crop grown on 24.4 million ha with a total annual production of 35.4 million tons (FAOSTAT, 2005). It is the world's 13th most important food crop, 6th most important source of edible oil and 3rd most important source of vegetable protein.

The crop, native to South America, is currently grown under a wide range of environmental conditions between 40°S and 40°N latitude in over 100 countries in Asia, Africa, Australia and Americas. The largest producers of groundnut are China and India, followed by SSA countries and Central and South America.

Developing countries account for 97% of the global groundnut area and 94% of the global production. The production of groundnut is concentrated in Asia and Africa (56% and 40% of the global area and 68% and 25% of the total global production, respectively, as of 2006). It is a valuable food and cash crop cultivated by millions of smallholding farmers in the SSA and South and Southeast Asia. It is cultivated as sole crop, intercrop or mixed crop. Groundnut seeds contain high quality edible oil (44-52%), easily digestible protein (26-28%) and carbohydrates (20%), besides vitamins (E, K, B1 and B3), minerals and dietary fiber. Haulms (leaves and stalk) are utilized as fodder and the cakes, formed after the oil extraction, are a high protein animal feed. Groundnut shells are used as fuel, as filler in feed industry and in making cardboards. Being a legume with root nodules, it enriches the soil by fixing atmospheric nitrogen, thereby contributing to soil fertility. It generates employment on the farm and in marketing, transportation and processing, and cash income from sale of pods in local markets.

Being a low input rain-fed crop with characteristics that provide economic returns from each and every part of the plant, groundnut cultivation has a direct bearing on the overall economic and financial well-being, and on the nutritional status of subsistence farmers particularly in the SSA and Asia.

GRD usually occurs in small proportions every growing season, but its severity increases in groundnut crops sown late in the season. When epidemics do occur, groundnut production is significantly reduced and the disease has the potential to cripple rural economies in SSA (Naidu et al. 1999a). An epidemic in northern Nigeria in 1975 destroyed approximately 0.7 million ha of groundnut, with an estimated loss of US$250 million (Yayock et al. 1976). Similarly, an epidemic in 1995 in eastern Zambia affected approximately 43,000 ha causing an estimated loss of US$4.89 million. In the following year in the central region of Malawi, groundnut production was reduced by 23% (SADC/ICRISAT Groundnut Project, 1996). As per the estimates of ICRISAT, GRD causes an annual yield loss of US$156 million in SSA.

GRD is a virus disease, transmitted by an aphid, *Aphis craccivora* Koch (Insecta: Homoptera) (Storey and Bottomley 1928; Storey and Ryland 1955, 1957; Hull and Adams 1968). Three causal agents are involved in GRD etiology: *Groundnut rosette assistor virus* (GRAV), *Groundnut rosette virus* (GRV) and a Satellite-RNA (SatRNA) (Reddy et al. 1985a,b; Murant *et al*. 1988; Taliansky et al. 2000). The complex association of the three agents in causing GRD makes it a unique and fascinating virus disease whose origin and perpetuation in nature, in spite of significant advance in our knowledge, still remain a mystery.

2. Causal Agents

GRAV: GRAV is a member of the family Luteoviridae. It was first recognized as a component of groundnut rosette disease by Hull and Adams (1968). Casper et al. (1983) and Reddy et al. (1985a) characterized the virus and identified it as a luteovirus. GRAV virions are non-enveloped, isometric shaped with 28 nm diameter particles of polyhedral symmetry (Fig 4). The genome is a non-segmented, single molecule of linear positive-sense, single-stranded RNA of c. 6900 nucleotides that encodes for structural and non-structural proteins (Murant et al. 1989). Like other members of the luteovirus, GRAV is thought to encode for six Open Reading Frames (ORFs). Only coat protein region of the genome was sequenced (Gene

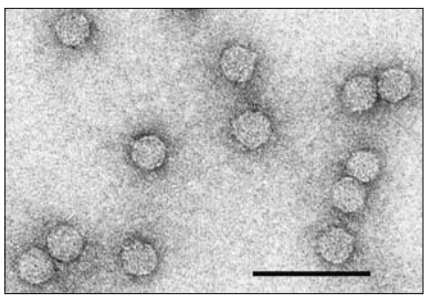


Figure 4: Negatively stained electron micrograph of GRAV particles. Bar = 100 nm (Reproduced with permission from Dr DJ Robinson, Scottish Crop Research Institute)

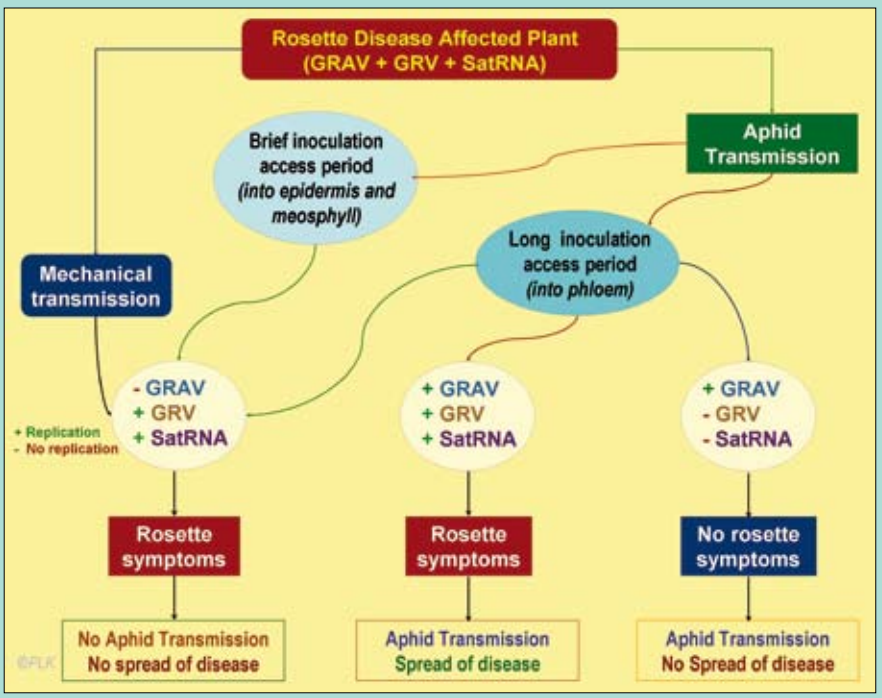
Bank Accession# z68894, af195502, af195825; Murant et al. 1989, Scott et al. 1996). Virions are made of single coat protein subunits of size 24.5 kDa, and the virus is antigenically related to Bean/Pea leaf roll virus, Beet western yellows virus and Potato leaf roll virus (Scott et al. 1996). The virus replicates autonomously in the cytoplasm of phloem tissue. GRAV is transmitted by A. craccivora in a persistent manner, and experimentally by grafting, but not by mechanical sap inoculation, seed, 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 virus on its own causes symptomless infection or transient mottle, and can cause significant yield loss in susceptible groundnut cultivars (Naidu et al. 2007). There are no reports on occurrence of strains of GRAV.

GRV: GRV belongs to the genus Umbravirus. It was first isolated and characterized by Reddy et al. (1985b). The virus has no structural (coat) protein (Taliansky et al. 2003), and thus no conventional virus particles of GRV are formed. Enveloped bullet-shaped structures detected in the ultra-thin sections of infected cells were shown to be cytopathological structures due to GRV infection, as opposed to real virions (Taliansky et al. 2003). The virus genome is a non-segmented, single liner molecule of single-stranded, positive sense RNA of size c. 4019 nucleotides that encodes for four ORFs (Taliansky et al. 1996). The genome of an isolate was completely sequenced (Gene Bank Accession# z66910) and several partial sequences are available in the Gene Bank. The virus replicates autonomously in the cytoplasm of the infected tissues (Taliansky et al. 2003). GRV on its own causes transient symptoms, but a SatRNA associated with GRV is responsible for rosette disease symptoms. GRV depends on GRAV for encapsidation of its RNA and transmission by A. craccivora in a persistent mode (see Box 2) (Robinson et al. 1999). The virus is transmitted by grafting and mechanical inoculation, but not through seed, pollen or by contact between the plants. Groundnut is the only known natural host, but several experimental hosts in the families Chenopodiaceae and Solanaceae have been reported (Murant et al. 1998). No strains of GRV have been reported. The virus is restricted to SSA and its offshore islands.

SatRNA: The SatRNA (subviral RNAs) of GRV belongs to the Subgroup-2 (small linear) satellite RNAs. It is a single-stranded, linear, non-segmented RNA of 895 to 903 nucleotides (Murant et al. 1988; Block et al. 1994; Taliansky et al. 2000). It totally depends on GRV for its replication, encapsidation and movement, both within and between the plants. SatRNA is responsible for rosette symptoms and plays a critical role in helper virus

Box 2: Synergistic interaction among the GRD agents for vector transmission

The three agents, GRAV, GRV and SatRNA, synergistically interact with each other for survival and spread. As shown in the diagram below, aphids fail to transmit GRD in the absence of GRAV, and plants lacking GRV and SatRNA do not show rosette symptoms. GRAV replicates autonomously in plants and is transmitted by an aphid, *Aphis craccivora*. GRAV alone causes no obvious symptoms. The SatRNA depends entirely on GRV for its replication. GRV replicates autonomously, but it must be associated with its SatRNA for its packaging in the GRAV coat protein and subsequent transmission by the aphid vector. Through the ability to utilize the coat protein of GRAV, GRV-SatRNA gain epidemiologically by acquiring a persistent relationship with the aphid vector for survival and spread. During the process of aphid transmission, the three agents can get separated (see diagram below). Plants that lack GRAV become dead-end sources.



dependent transmission of GRV as discussed in the following section (Murant et al. 1988; Robinson et al. 1999). Different variants of SatRNA have been shown to be responsible for different rosette symptoms, such as green rosette and chlorotic rosette (Murant and Kumar, 1990; Taliansky et al. 1997). Up to five ORFs in positive or negative sense are predicted to occur in SatRNA, 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 presence of GRV and GRAV. The sequences of 10 variants of GRV SatRNA have been determined (Block et al. 1994).

Interaction between and among GRAV, GRV and SatRNA: All the three agents are intricately dependent on each other in GRD etiology, which is crucial in the biology and perpetuation of the disease (Table 1) (Taliansky et al. 1997; Naidu et al. 1999a). The SatRNA and its variants are responsible for GRD symptoms (Murant et al. 1988; Murant and Kumar 1990; Taliansky et al. 1997), whereas GRAV or GRV alone causes symptomless infection or transient mild mottle. GRAV and GRV can replicate autonomously, but SatRNA totally depends on GRV for replication. GRAV acts as a helper virus in vector transmission of GRV and SatRNA (Robinson et al. 1999). SatRNA plays a crucial role in encapsidation of GRV RNA into GRAV coat protein and thereby assists in aphid transmission (Murant 1990; Robinson et al. 1999). The SatRNA is the most essential part for the complex to survive in nature.

Table 1. Properties of GRD agents

Agent	Replication	Transmission		Symptoms
		Mechanical	Aphid	
GRAV	Autonomous	No	Yes	Symtomless to mild mottle
GRV	Autonomous	Yes	Yes, requires GRAV & SatRNA	Symptomless to mild mottle
SatRNA	Requires GRV	Yes, requires GRV	Yes, requires GRAV & GRV	Rosette symptoms

3. Distribution

GRD has been reported in Angola, Burkina Faso, Côte d'Ivoire, Gambia, Ghana, Kenya, Madagascar, Malawi, Niger, Nigeria, Senegal, South Africa, Sudan, Swaziland, Tanzania, Uganda, and Zaire (Gibbons 1977; Naidu

et al. 1999a). The agents of GRD have not been detected elsewhere in the world, despite the fact that groundnut is grown in more than 100 countries around the world and *A. craccivora* is found in almost all these groundnut growing regions (Fig. 3).

4. Symptoms and Effects on Yield

GRD occurs as two symptom variants, chlorotic rosette and green rosette, with considerable variation within each type (Murant 1989; Naidu et al. 1998b; 1999a). Both forms of the disease cause plants to be severely stunted, with shortened internodes and reduced leaf size, resulting in a bushy appearance of plants (Figs. 5, 6 & 7). In chlorotic rosette, leaves are usually bright yellow with a few green islands and leaf

Figure 5: Leaf symptoms of chlorotic rosette affected groundnut plants

Figure 6: Chlorotic rosette symptoms on groundnut

lamina is curled (Fig. 7). In the green rosette, leaves appear dark green, with light green to dark green mosaic (Fig. 7). Chlorotic rosette occurs throughout the SSA, whereas green rosette has been reported from Angola, Kenya,

Figure 7: Experimentally infected groundnut with three symptom variants of groundnut rosette disease. From left to right: chlorotic rosette, green rosette and mosaic mottle rosette. (Reproduced with permission from Dr DJ Robinson, Scottish Crop Research Institute)

Malawi, Swaziland, Uganda and West Africa (Naidu et al. 1999a). A less common symptom variant, mosaic rosette (Fig. 7), due to mixed infection of the plants by the SatRNA causing chlorotic variant and mottle variant, was reported from East Africa (Storey and Ryland 1957). Variability in SatRNA is mainly responsible for symptom variations (Murant and Kumar 1990; Taliansky et al. 1997). In addition, differences in genotypes, plant stage at infection, variable climatic conditions and mixed infections with other viruses also contribute to symptom variability under field conditions (Naidu et al. 1998b; 2007).

Yield losses due to GRD depend on the growth stage at which infection occurs (Olorunju et al. 1991). Infection due to chlorotic or green rosette disease occurring in young plants (prior to flowering) will result in 100% yield loss. In contrast, plants infected during later growth stages (between flowering and pod setting) may show symptoms only in some branches or parts of branches and yield loss depends on severity of infection. Infection after pod setting/ maturation causes negligible effects on pod yield. An average annual yield loss due to GRD is estimated to be between 5 and 30% in non-epidemic years and epidemics often result in 100% yield loss.

The deleterious impact of GRAV or GRV on host plant together with SatRNA in a synergistic manner is not known. Ansa et al. (1990) have reported that stunting is more severe in diseased groundnut plants containing all the three agents than in diseased groundnut plants containing only GRV and SatRNA. Some reports have suggested that GRAV or GRV infection alone in groundnut results in transient mottle symptoms with insignificant impact on the plant growth and yield (Taliansky et al. 2000). These results have, however, been contradicted by more recent studies that demonstrated that GRAV infection alone affects plant growth and contributes to significant yield losses in susceptible groundnut cultivars (Naidu et al. 2007).

5. Host Range

Groundnut and some of its wild relatives are the only natural hosts of GRAV, GRV and SatRNA (Okusanya and Watson 1966). Under experimental conditions using viruliferous *A. craccivora*, 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. (Adams 1967; Hull and

Adams 1968; Okusanya and Watson 1966; Murant 1989). All these plants showed symptomless infection and virus replication in these plants was confirmed by diagnostic assay. Exception is C. *bursa-pastoris*, which was reported to show chlorotic symptoms.

By artificial mechanical sap inoculations, experimental hosts of GRV and SatRNA were identified in several species in Leguminosae, Chenopodiaceae and Solanaceae (Okusanya and Watson 1966, Adams 1967, Hull and Adams 1968, Dubern 1980, Reddy et al. 1985a,b, Murant et al. 1998). *Chenopodium amaranticolor* and C. *murale* are local lesion hosts; C. *amaranticolor*, *Glycene max*, *Phaseolus vulgaris*, *Nicotiana benthamiana* and *N. clevelandii* are systemic hosts of GRV.

Apart from groundnut, experimental hosts of both GRAV, and GRV and SatRNA are *Gomphrena globosa*, *Stylosanthes gracilis*, *S. mucronata*, *S. sundaica*, *Spinacia oleracea*, *Trifolium incarnatum* and *T. repens* (Murant 1989; Murant et al. 1998).

6. Transmission and Virus-Vector Interactions

Aphis craccivora (Fig. 8; see Box 3), commonly known as the cowpea aphid is the principal vector involved in the transmission of all the GRD agents in a persistent and circulative manner (Storey and Bottomley 1928, Storey and Ryland, 1955, Watson and Okusanya 1967, Hull and Adams 1968). GRV and SatRNA 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 SatRNA are acquired by

Figure 8: Aphis craccivora Koch, the vector of groundnut rosette disease. A colony of nymphs (light brown colour) and adults (black colour). (Figure source: www.forestryimages.org. Reproduced with permission from Prof. K Douce, University of Georgia, Tifton)

Box 3: About the aphid vector

Aphis craccivora (Aphididae: Homoptera: Arthropoda), first described by Koch in 1854, is an economically important pest on groundnut and cowpea in SSA. It is worldwide in distribution, abundant in the tropics and in the Mediterranean (see Fig. 3). It causes crop losses by direct feeding damage and indirectly as the vector of plant viruses. Besides GRD agents, *A. craccivora* vectors about 30 different plant viruses. It is polyphagous, occurring on nearly 142 plant species, mainly in the Leguminosae. Several non-crop plant hosts serve as reservoirs of *A. craccivora* and associated viruses throughout the year in the fields. However, no alternative host to GRD agents has been found.

Adult Aphis craccivora (alatae) (Reproduced from ©CABI, Wallingford, UK, 2005)

Only females have been recorded in the tropics (anholocyclic), which reproduce parthenogenetically throughout the year. It is ovoviviparous, with females retaining eggs inside their bodies, giving birth to small larvae. These undergo four nymphal stages before developing into adults. Adult apterae (wingless) measures around 1.4 to 2.2 mm and alatae (winged) about 1.4 to 2.1 mm. Viviparous females have a shiny black or dark brown body with a prominent cauda and brown to yellow legs. Under favorable conditions, the aphid is capable of rapid population development, increasing infestation by 5 to 8 fold in each generation. The rate of reproduction and development of various morphological forms is largely dependent on climatic factors, especially temperature, and the nutritional status of the host plant.

The aphid infestation on groundnut can be controlled by undertaking: (i) early sowings in the rainy season to allow plants to mature before build up of aphid populations to high levels; (ii) dense planting, which deter landing of aphids; (iii) insecticidal sprays between germination and 40th day; and (iv) cultivation of groundnut genotypes resistant to aphids. Sanitary measures such as removal of GRD-affected plant material, and any volunteer plants or weeds that can harbor *A. craccivora*, reduces the insect harboring sources in the fields. Integrated Pest Management (IPM) combining all these options, can be most effective in reducing the aphid populations on groundnut.

the aphid vector from phloem sap in 4 h and 8 h acquisition access feeding for chlorotic and green rosette, respectively (Misari et al. 1988). Then, there is a latent period of 26 h 40 min and 38 h 40 min for chlorotic and green rosette, respectively, and the inoculation access feeding period of 10 min for both forms (Misari et al. 1988). Once acquired, aphid can transmit virus particles for up to two weeks and beyond. All stages of the aphid can acquire and transmit the disease agents. Transmission rates of 26-31% have been reported with one and two aphids per plant, and 49% with five aphids per plant (Misari et al. 1988).

Aphid vector does not always transmit all the three agents together (Naidu et al. 1999b). Under natural conditions, some GRD-affected plants (GRV and SatRNA positive) were found to be free from GRAV, and GRAV was detected in some non-symptomatic plants (no GRV and SatRNA) (Naidu et al. 1999b). This situation was due to difference in inoculation feeding behavior of the vector leading to transmission of (i) all the three agents together, (ii) only GRAV or (iii) GRV and SatRNA, as demonstrated by the electrical penetration graph (EPG) studies of aphid stylet activities (Naidu et al. 1999b). This showed that during short inoculation feeding (test probe or stylet pathway phase) vector aphids probe groundnut leaves without reaching the phloem, transmitting only GRV and SatRNA, which multiply in the epidermal and mesophyll cells. Even if GRAV particles are deposited in the mesophyll cells, they cannot replicate, as they can replicate only in the phloem cells (Naidu et al. 1999b). However, vector aphids can transmit GRAV, and GRV-SatRNA when the stylets penetrate sieve elements (salivation phase) of the phloem cells. Therefore, the success of transmitting all the three agents together is high when inoculation feeding period is longer or increasing the number of aphids per plant (Misari et al. 1988). Vector aphids fail to acquire or transmit GRV and SatRNA from diseased plants lacking GRAV and such plants become dead-end sources (see Box 2; Fig. 12). However, if such plants receive GRAV later due to vector feeding, the plants again serve as source of inoculum.

7. Protocols for Purification of GRD Agents

Purification of GRAV: Luteoviruses, such as GRAV are phloem-limited, occur in low concentrations and purification of such viruses is often difficult. The procedure given below was developed by Murant (1989). In general, any luteovirus purification protocol can be applied for the purification of GRAV particles from infected groundnut plants.

Harvest entire shoots of infected groundnut plants and grind each 100 g tissue with liquid nitrogen in a mortar. Homogenize the powder in a

blender with 400 ml 60 mM phosphate buffer, pH 8.0, containing 10 mM disodium ethylenediamine-tetraacetate (EDTA) and 0.5% (v/v) of monothioglycerol. Stir the extract for 2 h at 20°C in the presence of 5% (v/v) cellulase enzyme. Filter the extract through muslin cloth and to this add equal volumes of a 1:1 mixture of *n*-butanol and chloroform. Separate the emulsion by centrifugation for 10 min at 13,000 *g*. Mix the supernatant fluid with polyethylene glycol (PEG; M. Wt. 6000) at 8% (w/v) and NaCl at 0.2 M, and stir for 1.5 h at 4°C. Centrifuge the preparation for 20 min at 13,000 *g*. Resuspend the pellets in 40 ml of 6 mM phosphate buffer (PB), pH 7.2 and stir overnight at 4°C. Centrifuge the resuspension for 10 min at 13,000 *g*, and add Triton X-100 to the supernatant to 1% (v/v). Layer each 18 ml of the preparation over a 7 ml of 20% (w/v) sucrose solution in PB containing 8% PEG 6000 and 0.2 M NaCl in Beckman 50.2 Ti rotor tubes, and centrifuge for 2 h at 50,000 revolutions/min in a Beckman 50.2 Ti rotor. Resuspend the pellets in 16 ml PB for 1 h at 4°C. Remove the insoluble material by centrifugation for 10 min at 13,000 *g*. Layer the preparation on 10-40% linear sucrose density gradients prepared in PB and centrifuge for 3 h at 110,000 *g*. Fractionate the gradient into four 2.5 ml fractions. Fraction 2 usually contains maximum concentration of virus. Pool the fractions containing the virus particles and centrifuge for 1 h at 250,000 *g*, and resuspend the pellets in 0.5 ml PB for further use.

GRAV particle preparations contain a single component with a sedimentation coefficient ($s_{20,w}$) of 115 S in sucrose gradients and buoyant density in cesium sulphate gradient is 1.34 g/cc. Yields of virus particles are *c.* 0.5-1.0 mg/kg plant material (Murant 1989).

Purification of GRV and SatRNA: They do not form conventional virus particles in the infected cells as GRV lacks coat protein. Therefore, purification of GRV relates to purification of its nucleic acid (RNA). However, purification of single stranded RNA (ssRNA) of GRV from total nucleic acid preparations is extremely difficult due to several co-migrating host RNA species. GRV genomic RNA occurs abundantly in the form of double stranded RNA (dsRNA) in the infected cells (Breyel et al. 1988). Reddy et al. (1985b) developed a procedure to isolate GRV dsRNAs that gives three characteristic bands in 10% polyacrylamide gels (Fig. 9). Two of these species, dsRNA-1 (4.0 kbp) and dsRNA-2 (1.3 kbp), correspond to the genomic and a sub-genomic RNA of GRV, respectively. The dsRNA-3 (0.9 kbp) is a double-stranded form of the satellite RNA. Any procedure for isolation of dsRNAs from plant material is applicable for the isolation of GRV dsRNA. The method developed by Murant et al. (1988) is given below.

Grind fresh leaf tissue (10 g) to a fine powder in liquid nitrogen with a pestle and mortar. Stir the powder for 30 min at room temperature with 20 ml 50 mM Tris-HCl, 0.1 M NaCl, 1 mM disodium EDTA, pH 7.0 (TSE buffer), 3.0 ml 10% SDS, 40 mg bentonite and 30 ml of 9:1 (v/v) water-saturated phenol:m-cresol, containing 0.1% 8-hydroxy-quinoline. To the aqueous phase add ethanol to 20% (v/v) and apply to columns of Whatman CF-11 cellulose as described by Dodds and Bar-Joseph (1983), and elute the dsRNA fraction with ethanol-free TSE buffer. Recover nucleic acid from the eluate by ethanol precipitation. Resuspend the sample in 50 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl2, pH 7.5 (TNM buffer), and treat with 10 μg/ml RNase free DNase I for 30 min at 30°C to remove any contaminating DNA. Adjust the preparation to 0.3 M NaCl and treat with 10 ng/ml RNase A for 1 h at 30°C to remove contaminating ssRNA. Then, adjust the preparation to 2% SDS and re-extract with phenol and precipitate dsRNA with ethanol and resuspend the pellet in TNM buffer. Analyze the preparation in 10% non-denaturing polyacrylamide gel in tris-borate EDTA buffer, pH8.3, and visualize RNA either by staining with ethidium bromide or silver (Kumar and Waliyar 2007).

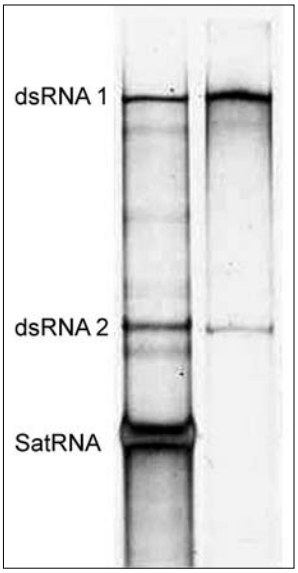


Figure 9: Characteristic dsRNA pattern of a preparation from GRV and SatRNA (left) infected groundnut, and plants infected with GRV alone (right) (Reproduced with permission from Dr DJ Robinson, Scottish Crop Research Institute)

Antiserum production: GRAV-specific polyclonal antibodies have been produced in a rabbit and used for the virus detection in enzyme-linked immunosorbent assay (ELISA). Rabbit polyclonal antiserum produced to an isolate of Potato leaf roll virus-1 (PLRV-1) at the Scottish Crop Research Institute (Tamada and Harrison 1980) and to the Chickpea luteovirus (CPLV) isolate-9 at ICRISAT (Reddy and Kumar 2004), that cross reacts with GRAV coat protein, have also been used for the detection of GRAV in ELISA. A panel of 10 mice monoclonal antibodies (MAbs) to PLRV-1 (Massalski and Harrison 1987), and CPLV (clones 2B2 and IF2) that cross reacts with GRAV are being used as secondary antibody in a triple antibody sandwich (TAS)-ELISA method developed by Massalski and Harrison (1987), and Rajeswari et al. (1987) to detect GRAV in groundnut samples.

A polyclonal antiserum has been raised against the GRV 28K movement protein (the product of ORF-4) by injecting a rabbit with a fusion protein expressed in *Escherichia coli* (Ryabov et al. 1998). The antiserum was shown to react with movement protein transiently expressed in tobacco from a *Potato virus* X (PVX)-based vector (Ryabov et al. 1998), but not with extracts from GRV-infected groundnut or other susceptible plants (PL Kumar and DJ Robinson, unpublished data). This antiserum is mainly used for localization of GRV ORF4 product in the infected cells using transmission electron microscope.

8. Disease Diagnosis

Various diagnostic techniques based on biological, serological (protein-based) and genomic properties (nucleic acid) of the GRD agents have been developed (Table 2).

Table 2. Methods for the detection of GRD agents

Method	GRAV	GRV	SatRNA
Inoculation to indicator plants (biological assay)	No	Yes	Yes (*requires GRV*)
ELISA (serological assay)	Yes	No	No
RT-PCR or nucleic acid byhridization	Yes	Yes	Yes

GRD can be diagnosed in the field based on the characteristic symptoms on groundnut. Mechanical inoculation on to C. *amaranticolor* indicates the presence of GRV (infected plants show minute necrotic lesions on inoculated leaves about four days after inoculation) (Murant et al. 1998). Serological and nucleic acid-based diagnostic methods can be used for the detection of GRAV, but only nucleic-acid based methods can be used for the detection of GRV and SatRNA. TAS-ELISA has been developed for detection of GRAV (Rajeswari et al. 1987) and dot-blot hybridization and reverse transcription-polymerase chain reaction (RT-PCR) to detect all the three GRD agents in plants and aphids (Blok et al. 1995; Naidu et al. 1998a).

TAS-ELISA for the detection of GRAV: A procedure described by Rajeswari et al. (1987) is given below for the detection of GRAV by TAS-ELISA. For standard ELISA procedure and preparations of buffers, refer to Kumar and Waliyar (2007). Coat wells of polystyrine ELISA plates

with 100 μl of first antibody (polyclonal antibodies of GRAV or CPLV or PLRV) diluted in carbonate coating buffer, pH 9.6. Incubate the plates at 37°C for 1 h or 4°C overnight. Wash the plates with phosphate buffered saline containing 0.05% (v/v) tween (PBS-T), pH 7.4. Grind 100-200 mg of plant material (leaf or stems) in 1:10 (w/v) PBS-T containing 0.2% ovalbumin and 2% (w/v) PVP (40,000 MW) (PBS-TPO) and add 100 μl of the extract into the antibody coated wells and incubate at 37°C for 1 h. Then wash wells with PBS-T and add appropriately diluted mice monoclonal antibody (clone# PLRV-SCR 6 or CPLV-CB2F2 or CPLV-IF2) in PBS-TPO and add into the wells of ELISA plate and incubate at 37°C for 1 h. Then wash plates with PBS-T and add alkalinephosphatase enzyme-labelled anti-mice antibody (commercially available) and incubate at 37°C for 1 h. Wash plates with PBS-T and add 0.5 mg/ml p-nitrophenyl phosphate substrate made in 10% (v/v) diethanolamine buffer, pH 9.8, and measure the colorimetric reaction by measuring absorbance values at A405nm in an ELISA plate reader. Absorbance values of infected samples will be at least three times or more than the readings of healthy controls.

RT-PCR for the detection of GRAV, GRV and SatRNA: A procedure described in Naidu et al. (1998a) is given below for the detection of the three GRD agents by RT-PCR using the oligonucleotide primer pairs listed in Table 3.

Table 3. Oligonucleotide primer pairs for the RT-PCR detection of GRD agents

Virus	Primer name	Size (bases)	Sequence (5' to 3')	Size of the amplified product (base pairs)
GRAV	GRAV-1	20	TTTGGGGTTTTGGACTTGGC	597
	GRAV-2	21	ATGAATACGGTCGTGGTTAGG	
GRV	GRV-1	20	GGCACCCAGTGAGGCTCGCC	863
	GRV-2	20	GGAAGCCGGCGAAAGCTACC	
SatRNA	SatRNA-1	20	AAGTGCTGAGGAACCAGCAC	400
	SatRNA-2	20	GGTTTCAATAGGAGAGTTGC	

Isolate total RNA from 100-200 mg of leaf material or aphids (1 to 5) using Rneasy® Plant Mini Kit, (Qiagen) as per the manufacturer protocol. Alternatively, any procedure for isolation of total RNA from plant material can be used (see Naidu et al., 1998a).

RT-PCR: The reaction composition and incubation conditions are given in Table 4. These are useful for the amplification of any of the three agents using appropriate primer pair and RNA sample.

Table 4. RT-PCR reaction mixture and PCR conditions

First strand reaction mixture: Mix the following in a microfuge tube to a total reaction volume of 20 µl:

Component	Volume
Total RNA of plant or aphid	1-4 µg
Primer 1	200 ng
5x RT enzyme buffer	4 (supplied along with the enzyme)
25 mM $MgCl_2$	2 µl
10 mM dNTPs	1 µl
0.1 M DTT	2 µl
Reverse transcriptase	200 U
Sterile distilled water	to 20 µl

Incubate the tubes at 42°C for 1 h. Then terminate the reaction by heating at 70°C for 15 min to inactivate the reverse transcriptase. Proceed to second strand reaction, as shown below.

Second strand reaction mixture and PCR: Mix the following in a microfuge tube to a total reaction volume of 50 µl:

Component	Volume
First strand reaction	3 µl
Primer 1	200 ng
Primer 2	200 ng
10x PCR buffer	5 (usually supplied along with the enzyme)
25 mM $MgCl_2$	3 µl
10 mM dNTP mix	1 µl
Taq polymerase	0.5 U
Sterile distilled water	to 50 µl

Place the tubes in an automated thermal cycler for amplification by denaturation at 94°C for 5 min, followed by 35 cycles of amplification by denaturation at 94°C for 1 min, 55°C for 1 min for primer annealing and 72°C for 2 min for primer extension, and a final extension at 72°C for 10 min. Analyze the amplified products in 1.2% agarose gel electrophoresis as per the procedure described in Kumar and Waliyar (2007)

9. Epidemiology of GRD

The epidemiology of GRD is complex, involving interactions between and among two viruses and a SatRNA, the vector, and the host plant and environment. 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. 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 (Bunting 1950, Adams 1967). The vector *A. 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 (Adams 1967, Eastop 1981, Naidu et al. 1998b). Efforts thus far have failed to identify any alternative natural hosts of the GRD agents.

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 disease within the fields also occurs by way of the movement of aphid vector, but largely apterae and nymphs (Naidu et al. 1998b). In general, 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, crop density, time of infection, transmission efficiency of aphids, proximity to the source of infection and climatic conditions.

10. Disease Control

Various methods are available for protecting groundnut against rosette disease (Table 5). These include the removal of volunteer groundnut plants that serve 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).

Chemical control: Long acquisition access feeding period required by the vector provides an opportunity to control aphids with chemical sprays before they can spread the disease. Various insecticides have been used to control A. craccivora to minimize or prevent spread of rosette disease in field trials (Soyer 1939, Evans 1954, Davies 1975a,b). Timing of spray,

Table 5. Options for the management of GRD

Destroy virus sources	Remove GRD and aphid sources (infected plants after harvest, volunteer plants or weeds that can harbor vector aphids)
Cultural practices	Sow early in the rainy season to take advantage of low-aphid population.
	Remove and destroy early infected plants and fill the gap.
	Sow fast-growing cereals such as maize, pearl millet and sorghum, as inter and border cropping, which interfere with the vector aphid movement
Dense planting	Maintain optimum plant population in the field, which covers ground thereby discourage landing of vector aphids on the crop.
Chemical treatment	Seed treatment with imidaclorprid and followed by regular systemic insecticide spray in the early stages of the crop growth (from emergence to 40th day) will control vector aphids, and consequent protection against GRD.
Cultivate resistant varieties	Cultivate GRD resistant groundnut varieties. (Some resistant cultivars available for cultivation in SSA are: ICG 12991, ICGV-SM 99568, ICGV 93437, ICGV-IS 96894, ICGV-SM 99541, ICGV-SM 01513, ICGV-SM 01514, ICGV-SM 01708, ICGV-SM 01731 and ICGV-SM 03701)

dosage and type of insecticide utilized is critical for controlling aphids. However, insecticides are an unviable option in SSA due to high costs and scarcity, thus seldom preferred by the farmers. Furthermore, insecticide applications pose detrimental effects on health and environment and their usage is being discouraged.

Control through cropping practices: Information on the control of GRD by cultural practices has been obtained in different parts of SSA (Guillemin 1952; Jameson and Thomas 1954; Sauger et al. 1954a,b,c; Smartt 1961; Booker 1963; A'Brook 1964, 1968; Davies 1972; Farrell 1976a,b; Chiyembekeza et al. 1997; Naidu et al. 1998b,1999a). Early sowing in the season to take advantage of low aphid populations, and maintaining good plant density without any gaps (aphids prefer widely spaced plantings for landing) have been shown to reduce rosette disease incidence. However, early sowings may not be effective in areas where groundnut is grown continuously, as this allows perpetuation of virus and vector. Rouging of voluntary sources and early-infected plants prevent the

primary and secondary spread of the disease. Intercropping with cereals such as maize, sorghum, finger millet, beans and cowpea were shown to affect aphid colonization, movement and behavior within crops, thereby GRD incidence (Farrell 1976c, Alegbejo 1997). However, control by cultural practices by smallholder farmers is difficult under subsistence farming conditions due to farmers' pre-occupation with other revenue generating practices, unpredictable climate, small-land holdings and farmers' reluctance to adopt improved cultural practices.

Host plant resistance: Varieties resistant to GRD provide the most economical and practical solution to control GRD in the field, and thus, substantial efforts have been made to identify durable GRD resistant sources.

Resistance to GRD was first found in groundnut germplasm originating from Burkina Faso and Côte d'Ivoire (Sauger and Catharinet 1954a,b,c; de Berchoux 1960). Subsequent efforts in breeding for host plant resistance and evaluation of groundnut germplasm collection held in ICRISAT genebank have contributed to the development of several groundnut genotypes and identification of germplasm lines with acceptable levels of field resistance to rosette disease (Nutman et al. 1964; Olorunju et al. 1991, 2001; van der Merwe and Subrahmanyam 1997; Subrahmanyam et al. 1998, 2001). Evaluation of 12,500 lines from ICRISAT's genebank collection of groundnut germplasm has resulted in the identification of 150 resistant sources, of which 130 are long duration Virginia types and 20 are short duration Spanish types (Subrahmanyam et al. 1998m Olorunju et al. 2001) (Fig. 10). Evaluation of 116 wild *Arachis* accessions

Figure 10: Rosette resistance screening nursery at ICRISAT, Malawi. Scientists demonstrating resistant and susceptible varieties.

representing 28 species identified 25 accessions resistant to rosette disease (Subrahmanyam et al. 2001) (Table 6). Recently, 2,301 germplasm lines were evaluated in Samaru in Nigeria and 65 new sources of resistance to rosette were identified, 55 of which are long duration Virginia types and 10 are short duration Spanish types (Ntare and Olorunju 2001). It is not known whether these resistant sources carry the same or different kinds of resistance genes.

Table 6. Rosette resistance in wild *Arachis* species (Subrahmanyam et al. 2001)

Species	ICG No.
A. appressipila	8127, 8945, 14860
A. decora	14946
A. diogoi	4983
A. hoehnei	8190, 13232
A. kretschmeri	8191, 8216, 11558, 13224
A. kuhlmannii	13225, 14862, 14875
A. pintoi	13222, 14855, 14856, 14888,
A. stenosperma	13171, 13173, 13187, 13210, 14872
A. villosa	13168

Generally, resistance to rosette disease in a genotype was assessed by lack of symptom expression and therefore such resistance was largely against GRV and SatRNA (the two components responsible for rosette symptoms) (Bock et al. 1990, Subrahmanyam et al. 1998, Olorunju et al. 2001). Yield reduction in genotypes that are resistant to GRV and SatRNA was reported, which presumably could be due to their susceptibility to GRAV (Subrahmanyam et al. 1998, Olorunju et al. 2001). This, indeed, was confirmed in a recent study that separated GRAV from GRV and SatRNA, and studied its effect on the agronomic performance of four groundnut genotypes and demonstrated that GRAV infection alone can significantly reduce groundnut seed yield (Naidu et al. 2007).

Resistance to rosette disease was earlier predominantly available in late-maturing cultivars derived from groundnut germplasm collected from the border region between Côte d'Ivoire and Burkina Faso (Sauger and Catharinet 1954, de Berchoux 1958). This resistance, which does not amount to absolute immunity, was shown to be controlled by two independent recessive genes and is effective against both chlorotic and green forms of rosette (Nigam and Bock 1990, Olorunju et al. 1992). This resistance is directed against GRV (and therefore also to SatRNA) and is not effective against GRAV (Bock et al. 1990). Recently, this

form of resistance has been transformed into early-maturing cultivars that are useful for cultivation in regions that are often characterized by short length of growing periods (Fig. 11). Some of these are: ICG 12991 released in four Eastern and Southern Africa (ESA) States; ICGV-SM 99568 released as 'Chitala' in Malawi; ICGV 93437 released as 'Nyanda' in Zimbabwe; and ICGV-IS 96894 released as 'Samnut 23' in Nigeria. Other promising elite short duration Spanish lines, like ICGV-SM 99541, ICGV-SM 01513 and ICGV-SM 01514; and medium duration Virginia lines, like ICGV-SM 01708, ICGV-SM 01731, ICGV-SM 03701 are currently under advanced testing in several countries in ESA. There are also a number of early maturing rosette resistant lines available in West Africa (Ntare et al. 2002). A list of rosette resistant varieties released in the ESA and WCA (West and Central Africa) region is presented in Table 7.

Resistance to rosette disease was also identified in the wild *Arachis* species, several of which seems to be immune to both GRAV and GRV and SatRNA (Murant et al. 1991, Subrahmanyam et al. 2001). A high degree of resistance to rosette was found in a hybrid derivative from an interspecific cross of *A. hypogaea* x *A. chacoense* (Moss et al. 1993), indicating the usefulness of GRD resistance in inter-specific breeding programs. Resistance in groundnut to *Aphis craccivora* was also identified (ICG 12991) (Padgham et al. 1990, Naidu et al. 1999c), which was shown to be susceptible to all the GRD agents (Minja et al. 1999). However, recently we found that under instances of high disease pressure, resistance

Figure 11: Short duration rosette disease resistant groundnut in the farmers' fields.

Table 7. Rosette resistant groundnut varieties released in ESA and WCA and their pedigrees

Variety Name	Type	Pedigree	Source of Resistance	Released in
ICGV-SM 90704	Virginia	RG1 × Mani Pintar	RG1	Malawi, Mozambique, Zambia, Uganda
ICG 12991	Spanish	Landrace	Landrace	Malawi, Mozambique, Uganda, Zambia
ICGV-SM 99568	Spanish	ICGV 93437 × ICGV-SM 93561	RMP 40	Malawi
ICGV 93437	Spanish	ICGV 86063 × ICGV 86065	Unknown (complimentary gene action?)	Zimbabwe
SAMNUT 23 (ICGV–IS 96894)	Spanish	(ICGV-SM 85048 × RG 1) F_2-P_4-B_1-B1-B_1-B1-B1_B1	RG1	Nigeria
SAMNUT 21 (UGA 2)	Virginia	RMP 12 × ICGS 56 (E)	RMP 12	Nigeria
SAMNUT 22 (M572.80I)	Virginia	RMP 91 × (4753.70 × 3520.71)	RMP 91	Nigeria

in ICG 12991 often succumbs to GRD (ES Monyo et al., unpublished). Studies have showed that resistance to the aphid vector is controlled by a single recessive gene (van der Merwe 2001), which was mapped on linkage Group-1, at 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 offered a scope to develop a simple DNA-marker based method for screening aphid resistance, which may accelerate breeding progress.

A possibility for the future to augment resistance to GRD is the deployment of transgenic forms of resistance using genes derived from virus itself (pathogen-derived resistance) (Deom 1999). At the Scottish Crop Research Institute, UK, resistance to GRV was detected in plants transformed with constructs derived from a mild variant of the satellite RNA in *Nicotiana*

benthamiana (Taliansky et al. 1998). However, this strategy has not been tested for protection against rosette disease in groundnut. At ICRISAT, groundnut plants (cv. JL 24) have been transformed with constructs derived from GRAV coat protein (KK Sharma et al., unpublished). The transgenic events are at T3 generation and they are yet to be evaluated for GRAV resistance.

11. Screening for GRD resistance

Groundnut genotypes grown in pots under greenhouse conditions or genotypes sown in fields can be evaluated for resistance to all the three GRD agents by using viruliferous aphids and grafting (Olorunju et al. 1992, Naidu et al. 1999a). By mechanical sap inoculation, genotypes can be evaluated for resistance to only GRV and SatRNA. Genotypes can be evaluated for resistance to only GRAV by inoculating test plants with vector aphids fed on GRAV infected groundnut plants or by grafting using scions from GRAV-infected groundnut plants (Olorunju et al. 1992, Naidu et al. 2007). Diagnostic assays such as TAS-ELISA or RT-PCR can be used to confirm the presence or lack of GRD agents during genotype evaluation.

Two methods are being used for routine evaluation of GRD resistance in groundnut genotypes. The rating scale used in both methods primarily accounts for resistance to GRV-SatRNA. One method uses 1-5 disease rating score to evaluate GRD resistance (Table 8) (Pande et al. 1997, Olorunju et al. 2001). The other method, which is widely used is based

Table 8. Evaluation of groundnut genotypes based on 1 to 5 disease rating score

Score	Genotype reaction	Inference
1	No visible symptoms on the foliage	Highly resistant
2	Rosette symptoms on 1-20% foliage, but no obvious stunting	Resistant
3	Rosette symptoms on 21-50% foliage and stunting	Moderately resistant
4	Severe rosette symptoms on 51-70% foliage and stunting	Susceptible
5	Severe symptoms on 71-100% foliage, stunted or dead plants	Highly susceptible

on percent disease incidence (PDI) measured when the crop is at early pod filling stage (Table 9). The total number of plants in each row and the plants showing rosette symptoms (chlorosis with severe stunting) are counted once at 80 days and again at 100 days after germination. PDI in each row and the mean percentage incidence for each plot over the two counts are then computed to assess the genotype resistance to GRD (Table 9). The scheme of screening groundnut genotypes for GRD resistance and interpretation of genotype response is depicted in Figure 12.

Table 9. Evaluation of groundnut genotypes based on percent disease incidence (PDI)

PDI	Inference
Less than 10%	Highly resistant
11-30%	Resistant
31-50%	Moderately resistant
More than 50%	Susceptible

12. Conclusions and Future Directions

Among the plant virus diseases, groundnut rosette disease is of special interest because of its complex etiology. Information on molecular characterization of agents involved in GRD has provided a better understanding of the disease and contributed to the development of diagnostic tools necessary for developing host plant resistance and understand disease epidemiology. Information known so far suggests that all the three agents are vital for survival of the disease complex, and all the agents alone (GRAV and GRV + SatRNA) or in combination causes significant crop yield loss. 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 movement of aphids, identification of possible biotypes, and identification of alternative host(s) of GRD causal agents.

There is also a need for developing a forecasting model that can be used to prevent occurrence of future GRD epidemics. This requires data on risk factors such as aphid population dynamics, inoculum source and its abundance, cropping patterns and weather parameters that favor vectors in order to maximize the effectiveness of various GRD management strategies.

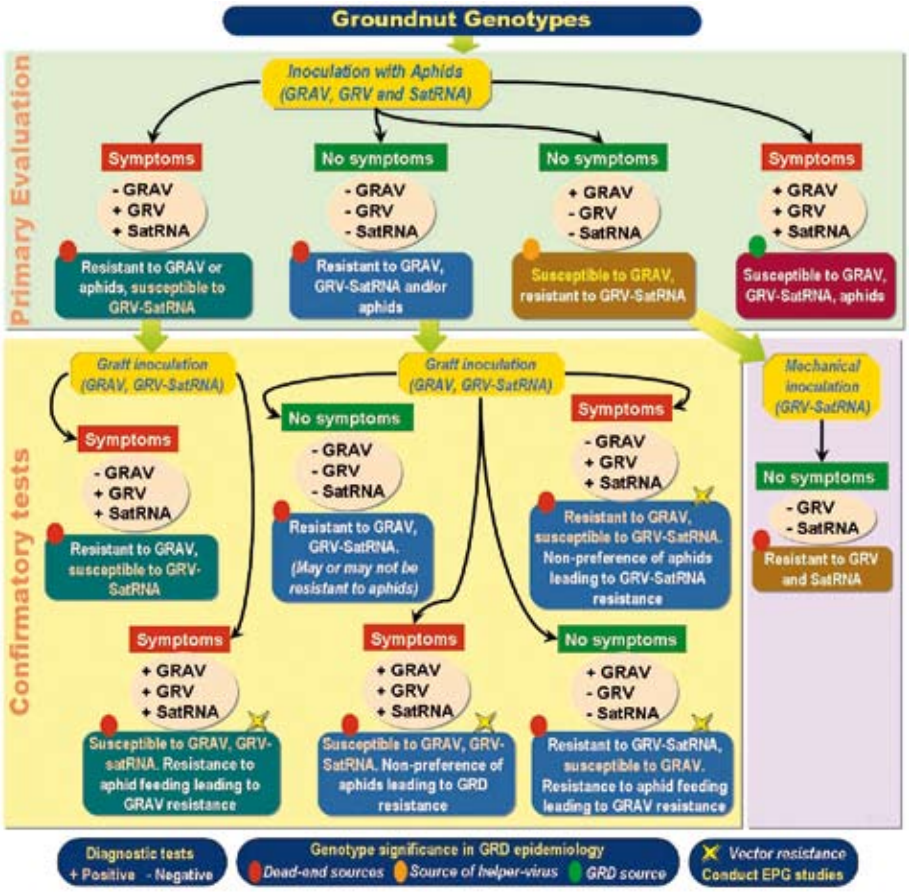


Figure 12: Evaluation of groundnut genotypes for GRD resistance by inoculation with vector aphids, grafting and sap, and interpretation of genotype resistance based on symptom phenotype and detection of the causal agents by TAS-ELISA or RT-PCR.

Although several resistant sources have been identified, they all apparently contain same genes conferring resistance to GRV, and this can be a risky proposition in the long term as there have been reports of the partial breakdown of GRV resistance under heavy disease pressure. Rosette resistant varieties developed in the past did not find wide acceptability among the farming communities in SSA as they were mostly late maturing and lacked preferred pod and seed characteristics. Although some of the preferred traits are now available in late maturing germplasm, the most urgent requirement for SSA is the short duration rosette resistant varieties with high yield and preferred pod and seed characteristics. Further, the

varieties should be suitable for late planting in the cropping season. In addition to GRV resistance, it is also important to have alternative breeding strategies utilizing GRAV (wild *Arachis* species that were shown to be immune to infection) and aphid-resistant genotypes. This could prove a useful approach, especially because this blocks the perpetuation of GRD agents in the fields. It is equally important to insure the seed availability of the new resistant varieties to smallholder farmers on a regular basis for effective management of rosette disease under field conditions.

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