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Molecular diagnostics of groundnut rosette disease agents in Uganda: Implications on epidemiology and management of groundnut rosette disease

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The objective of this study was to use molecular diagnostic tools to detect the agents of groundnut rosette disease (GRD) to guide in varietal development and disease management. Samples were collected from both GRD infected and healthy plants and sites geo-referenced. RNA extraction, cDNA synthesis, polymerase chain reaction (PCR) amplification, electrophoresis, staining and visualization were performed according to standard procedures. Molecular diagnosis of the samples showed various combinations of the GRD agents, some in isolation and others a combination of two or three agents. This distribution is attributed to dependence on the aphid feeding behaviour and pathogenicity of GRD agents. Chlorotic and green rosette symptoms were observed throughout the sampling sites signifying the presence of satellite RNA (sat-RNA) variants. Some plants showing GRD symptoms tested negative for GRD, whereas some healthy-looking plants tested positive for the GRD complexes pointing to the ineffectiveness of phenotypic screening and the need for a molecular diagnostic tool that detects all three GRD agents both in absence or presence of disease symptoms. The absence of groundnut rosette assistor virus (GRAV) in some symptomatic samples signifies that they are epidemiologically dead end sources since GRV and sat-RNA must be packaged within the GRAV coat protein to be aphid transmissible. Oyado (*Cassia obtusifolia*) tested positive for all the GRD agents making it a potential alternative host. There is an urgent need for validation of the phenotypic screening with molecular tools in efficient diagnosis of the multi-pathogenic GRD in guiding both plant breeding and pathology work.

Key words: *Arachis hypogaea* L., groundnut rosette disease, molecular diagnostics, polymerase chain reaction (PCR).

INTRODUCTION

Groundnut rosette disease (GRD) on groundnut *(Arachis hypogaea* L.) was first documented at the beginning of the 20th century in present-day Tanzania and South Africa (Zimmermann, 1907). Since then, GRD has been

reported in all groundnut-growing regions of sub-Saharan Africa (SSA) and in Madagascar (Storey and Bottomley, 1928; Storey, 1935; Naidu et al., 1998a; Naidu et al., 1999). GRD is exclusively endemic to SSA, causing an estimated annual loss of US$156 million (Waliyar et al., 2007). Although GRD epidemics are sporadic, yield losses approach 100% whenever the disease occurs in epidemic proportions (Naidu et al., 1999; Okello et al., 2014). The disease is caused by synergistic interactions between two viruses and a satellite RNA - groundnut rosette assistor virus (GRAV, family *Luteoviridae*), groundnut rosette virus (GRV, family *Tombusviridae*) and a satellite RNA (sat-RNA) of GRV (Casper et al., 1983; Reddy et al., 1985; Murant, 1989; Waliyar et al., 2007). The sat-RNA plays a crucial role in encapsidation of GRV RNA into GRAV coat protein and is required in aphid transmission (Murant, 1990; Robinson et al., 1999). The sat-RNA is the most essential part for the complex to survive in nature (Waliyar et al., 2007).

GRAV acts as a helper virus (Hull and Adams, 1968) for aphid transmission of GRV (Storey and Bottomley, 1928; Casper et al., 1983; Reddy et al., 1985) and sat-RNA (Murant et al., 1988). All three agents must be present in the host plant for transmission of the disease (Murant et al., 1988; Ansa et al., 1990). Successful transmission occurs in a persistent, circulative manner by the aphid, *Aphis craccivora* Koch (Storey and Bottomley, 1928; Storey and Ryland, 1955; Okusanya and Watson, 1966; Hull and Adam, 1968). Presently, there is no evidence of seed transmission (Anitha et al., 2014). While GRAV infection alone can contribute to yield losses in groundnut (Naidu and Kimmins, 2007), GRD occurs when a groundnut plant is infected with all three components (Murant et al., 1988; Ansa et al., 1990).

Disease symptoms are largely due to sat-RNA and its variants (Murant and Kumar, 1990). Symptoms occur in two predominant forms, chlorotic and green rosette although other symptomatic forms have been reported (Naidu et al., 1999; Deom et al., 2000). On their own, either GRAV or GRV cause symptomless infection or transient mild mottle symptoms (Naidu et al., 1999; Deom et al., 2000).

All resistant germplasm and breeding lines available so far are susceptible to GRAV (Subrahmanyam et al., 1998; Olorunju et al., 1991). Resistance in these lines is to GRV and thus indirectly against its satellite RNA. Such genotypes do not develop symptoms (Bock et al., 1990). However, resistance to GRV does not amount to immunity and can be overcome under high inoculum pressure or adverse environmental conditions (Bock et al., 1990). In addition, all previous studies done on inheritance of disease resistance were based on visual symptoms and are applicable only to GRV and its sat RNA, but not GRAV (Deom et al., 2000).

Methods that have been investigated to manage GRD include pesticides to reduce vector aphid populations, cropping practices to delay onset and spread both vector and disease, and breeding for virus and vector resistance (Naidu et al., 1999; Deom et al., 2000). Deployment of host resistance is the most cost-effective way to manage epidemics given that groundnuts are produced primarily by subsistent smallholder farmers in SSA. However, breeding of GRD resistant genotypes and their deployment is most effective when supported by efficient pathogen diagnostic systems, even in the absence of symptoms (Anitha et al., 2014).

In Uganda, breakdown of resistance in a widely grown GRD resistant variety -Serenut 4T had been observed (Okello et al., 2010, 2014). Symptom types vary in various regions throughout Uganda (Okello et al., 2014). Yield declines are reported in released varieties and the national groundnut breeding program deploys phenotypic screening methods to identify GRD resistant sources. The objective of this study was to use molecular diagnostic tools to detect the agents of GRD to understand disease epidemiology and guide in varietal development and management of the disease.

METHODOLOGY

Sample collection

Groundnut samples were collected from both GRD infected plants (manifesting chlorotic and green symptoms) and symptomless samples. Also collected were samples from Oyado (*Cassia obtusifolia*), a local leguminous vegetable commonly found in groundnut gardens (Figures 1 to 4). All the sampling sites were tablegeo-referenced (Table 2).

RNA extraction

To extract RNA, 100 mg tissue frozen in liquid nitrogen was macerated in 1 mL of accuZol RNA extraction reagent (Bioneer Inc, Korea) using a chilled pestle and mortar. Then 200 µL of chloroform was added and the mixture shaken for 15 s and incubated on ice for 10 min. The mixture was then centrifuged at 12,000 rpm for 5 min and 400 µL of the aqueous layer was transferred to a fresh 1.5 mL Eppendorf tube.

An equal volume of isopropanol was added, the solution mixed by aspirating 5 times using a pipette, and incubated at -20°C for 10 min. The solution was then centrifuged at 12,000 rpm for 5 min and the aqueous layer was transferred to a new 1.5 mL tube and 1 mL of 80% ethanol was added. The solution was centrifuged at 12,000 rpm for 5 min to pellet the RNA. The RNA was dissolved in 100 µL of DEPC treated water and stored at -80°C.

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Figure 1. Yellow rosette.

Figure 2. Green rosette surrounded by healthy looking plants.

DNase treatment

Prior to DNase treatment, the RNA was quantified using the NanoDrop 2000C spectrophotometer (Thermo Fisher, Wilmington MD) and all samples were standardized to 100 ng/µL. DNAase treatment to remove endogenous DNA was performed by digesting 1 ug of RNA in 20 µL of 1X DNase buffer using 1 µL of 1U/ µL DNase enzyme (Fermentas). The solution was incubated at 37°C for 1 h and the reaction was stopped by adding 1 µL of 25 mM EDTA and incubating at 65°C for 15 min. The solution was centrifuged at 12,000 rpm for 2 min and the supernatant was stored at -80°C.

cDNA synthesis

cDNA was synthesized using the Accupower RT-PCR kit (Bioneer Inc, Korea). A 50 µL reaction consisting of 1 µg DNase treated RNA and 0.4 µm of each of the reverse primers (R) targeting the GRV, GRAV and SAT components of GRD (Table 1) was incubated at 70°C for 5 min. Samples were then transferred to a 200 µL tube containing the Accupower RT-PCR premix and incubated at 42°C for 1 h for cDNA synthesis. RT was inactivated by incubating samples at 94°C for 5 min.

Figure 3. Healthy looking leaves among rosetted ones.

Figure 4. Aphids on green mottled Oyado leaves.

PCR amplification, electrophoresis, staining and visualization

PCR reactions (20 µL) contained 100 ng cDNA, 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq DNA polymerase and 0.4 µm of both forward (F) and reverse (R) primers were performed. PCR was programmed for 35 cycles of 20 s at 94°C, 40 s at temperatures described in Table 1, and 1 min at 72°C, preceded by an initial denaturation for 5 min at 95°C and followed by a 7 min extension at 72°C. The PCR products were analyzed on 1.2% agarose gels. Gel images were captured using a Syngene G: BOX gel documentation system (Syngene, Fredrick, MD).

RESULTS

RNA was obtained from leaf samples (Table 2) and subsequently used to synthesize cDNA that was used in PCR assays as a diagnostic tool for the presence of GRD agents.

There was differential amplification of DNA from collected samples. The PCR amplification of cDNA from samples showed various combinations of the GRD agents, some were detected in isolation, or as a combination of two or three agents (Table 2 and Figures 5 to 7). PCR amplicons of 380 bp for GRV, 280 bp for GRAV, and 300 bp for sat-RNA were generated by the RT-PCR (Figure 6).

Table 1. Primer names, sequences, annealing temperatures and source reference of the primers used for detecting components of the groundnut rosette virus.

Primer name	Primer sequence (5'-3')	Specific to	Annealing temperature (°C)	Source references
GRV1 GRV2	F-GGAAGCCGGCGAAAGCTACC R-GGCACCCAGTGAGGCTCGCC	GRV ORF3P and 4P	53	Taliansky et al., 1996; Deom et al., 2000; Anitha et al., 2014
GRAV1 GRAV2	F- ATGAATACGGTCGTGGTTAGG R- TTTGGGTTTTGGACTTGGC	GRAV-CP	55	Murant and Kumar, 1990; Deom et al., 2000; Anitha et al., 2014
Sat- RNA1 Sat- RNA3	F- GAAAAGGTGAGGGGTGTGT R- TAGCTTGATTTCAAGCTCGC	sat-RNA	50	Scott et al., 1996; Naidu et al., 1998b; Deom et al., 2000; Wangai et al., 2001

Most of the samples tested positive for sat-RNA, followed by GRAV and GRV. Ten samples, which had characteristics green and yellow rosette symptoms, tested negative for all GRD agents (Table 2). Five samples were positive for all the GRD agents (GRAV, sat-RNA and GRV), two samples were positive for both GRAV and sat-RNA and only one sample was positive for GRV and sat-RNA (Tables 2 and Figure 7). The groundnut samples, which tested positives for all three GRD agents, were from Busia, Jinja (Nakabango) and Serere (NaSARRI) districts. Nakabango and Serere are known hotspots for the GRD (Okello et al., 2010, 2014).

A symptomless sample (17 h) tested positive for the sat-RNA. One Oyado sample (*Cassia obtusifolia*), a local edible legume, which phenotypically resembles groundnuts and is always present in groundnuts fields tested positive for the three GRD agents.

DISCUSSION

GRD is caused by synergistic interactions between GRAV, GRV and sat-RNA. Symptomatic leaves samples had either chlorotic or green rosette symptoms signifying the existent of the variants of the sat-RNA. Earlier studies by Murant (1989) and Naidu et al. (1998a, 1999), reported that the type of GRD symptom (chlorotic, green and mosaic) was dependent on the sat-RNA and not on GRV or GRAV. All agents of GRD are persistently transmitted by aphids (*Aphis craccivora* Koch) (Storey and Bottomley, 1928; Storey and Ryland, 1955).

The samples showed various combinations of the GRD agents, some in isolation, combination and with all three agents present (Table 2). Twenty leave samples assayed showed various combinations of the GRD agents. This distribution is attributed to aphids feeding behavior. Naidu et al. (1999) and Deom et al. (2000) noted a single aphid vector acquires GRAV, GRV, and sat-RNA. However, it does not always transmit the three disease agents together to a host plant: GRAV or GRV plus sat-RNA can be transmitted separately. However, for the disease to perpetuate in nature, all three agents must be transmitted by the aphid vector to a plant.

The most detectable GRD agent was sat-RNA, with fourteen of twenty six samples testing positive for the satellite. One sample tested positive for only GRV + sat-RNA and two tested for GRAV and sat-RNA (Figure 7). This situation could be due to differences in inoculation feeding behavior of the vector. During short inoculation feeding (test probes), vector aphids probing groundnut leaves without reaching the phloem can transmit GRV and sat-RNA, which multiply in the mesophyll cells. Whereas GRAV, which is phloem limited in infected plants, either does not replicate in mesophyll cells or fails to move from cell to cell (Naidu et al., 1999). Therefore, the success of transmitting all three agents together is high when the inoculation feeding period is longer or when the number of aphids per plant is increased (Misari et al., 1988). Vector aphids fail to acquire or transmit GRV and sat-RNA from diseased plants lacking GRAV and such plants become dead-end sources for the disease. However, if such plants receive GRAV later due to vector feeding, the plants again might serve as source of inoculum (Deom et al., 2000).

A sat-RNA positive sample (17 h) did not show characteristic GRD symptoms. It tested negative for both GRV and GRAV. The RT-PCR assay used in this study is more sensitive for sat-RNA than for GRV (they are usually found together in nature). This could also be a result of recent inoculation by the aphid and the symptom development is still at infancy stage or the host plant is resisting the low levels of both viruses. Deom et al. (2000) noted that initial symptom appearance is greatly delayed in resistant varieties suggesting a restriction of viral replication and/or movements. This further calls for incorporation of molecular diagnostics in GRD screening and GRD resistant varietal development.

Some of the disease plants sampled had normal leaves and branches without any symptom of the GRD (Figure 1 and sample 17 h). This tends to show restriction of virus movement within the plants. Deom et al. (2000) also reported restrictions of virus movements within the plants

Table 2. Groundnut samples ID, varieties, locations, GRD symptoms and agents.

Sample	Variety	GRD symptoms	District	Region	GPS Coordinates	GRV	sat-RNA	GRAV
76g	Local	Green	Wakiso	Central	N00°23’23.562” E032°39’30.383”	-	+	-
79g	Local	Green	Wakiso	Central	N00°23’23.562” E032°39’30.383”	-	+	-
5y	Local	Yellow	Jinja	Eastern	N00°31’21.57” E033°13’00.000”	+	+	-
2g	Local	Green	Jinja	Eastern	N00°31’21.57” E033°13’00.000”	-	+	-
28g	Improved	Green	Serere	Eastern	N01°32’53.446” E033°23’31.427”	-	+	+
30a	Improved	Yellow mottled	Serere	Eastern	N01°32’53.446” E033°23’31.427”	+	+	+
41a	Oyado leguminous vegetable	Green mottled	Lira	Northern	N02°17’57.766” E032°54’45.709”	+	+	+
3y	Local	Yellow	Jinja	Eastern	N00°31’21.57” E033°13’00.000”	+	+	+
17h	Local	None (Healthy leaves)	Busia	Eastern	N00°28’53.475” E034°05’45.975”	-	+	-
27y	Improved	Yellow	Serere	Eastern	N01°32’53.446” E033°23’31.427”	+	+	+
20y	Improved	Yellow	Busia	Eastern	N00°28’53.475” E034°05’45.975”	-	+	+
25y	Improved	Yellow	Serere	Eastern	N01°32’53.446” E033°23’31.427”	-	-	-
15y	Improved	Yellow	Busia	Eastern	N00°28’53.475” E034°05’45.975”	+	+	+
14g	Improved	Green	Bugiri	Eastern	N00°35’58.245” E033°42’15.521”	-	+	-
48a	Oyado leguminous vegetable	Green mottled	Lira	Northern	N02°17’57.766” E032°54’45.709”	-	-	-
31g	Improved	Green	Serere	Eastern	N01°32’53.446” E033°23’31.427”	-	-	-
52a	Improved	Green	Arua	North Western	N03°04’48.092” E030°56’46.847”	-	+	-
36y	Improved	Yellow	Serere	Eastern	N01°32’53.446” E033°23’31.427”	-	-	-
37y	Improved	Yellow	Serere	Eastern	N01°32’53.446” E033°23’31.427”	-	-	-
33g	Improved	Green	Serere	Eastern	N01°32’53.446” E033°23’31.427”	-	-	-
34g	Improved	Green	Serere	Eastern	N01°32’53.446” E033°23’31.427”	-	-	-
40g	Local	Green	Serere	Eastern	N01°32’53.446” E033°23’31.427”	-	-	-
46g	Improved	Green	Lira	Northern	N02°17’57.766” E032°54’45.709”	-	-	-
50g	Improved	Green	Lira	Northern	N02°17’57.766” E032°54’45.709”	-	-	-

Sample 17 h: was from healthy leaves within chlorotic rosette plant. Samples 48a and 41a are local leguminous vegetable (Oyado) common in groundnut gardens nationwide.

as a resistant mechanism.

CONCLUSION AND RECOMMENDATIONS

RT-PCR assay was able to detect sat-RNA 14 times in the samples whereas GRV was only detected six times. Sat-RNA can only be present together with GRV, since GRV is required for sat-RNA replication. Since sat-RNA has been detected more often than GRV, it can be deduce that the RT-PCR assay for sat-RNA is more

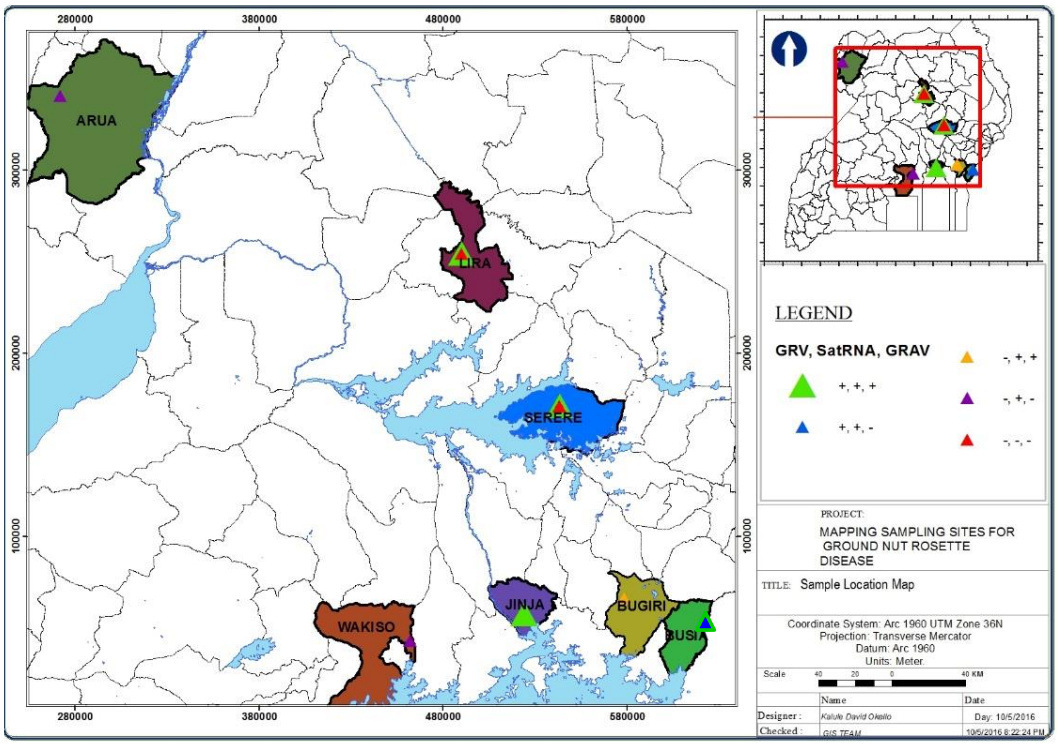

Figure 5. The sampling points and GRD complex distribution.

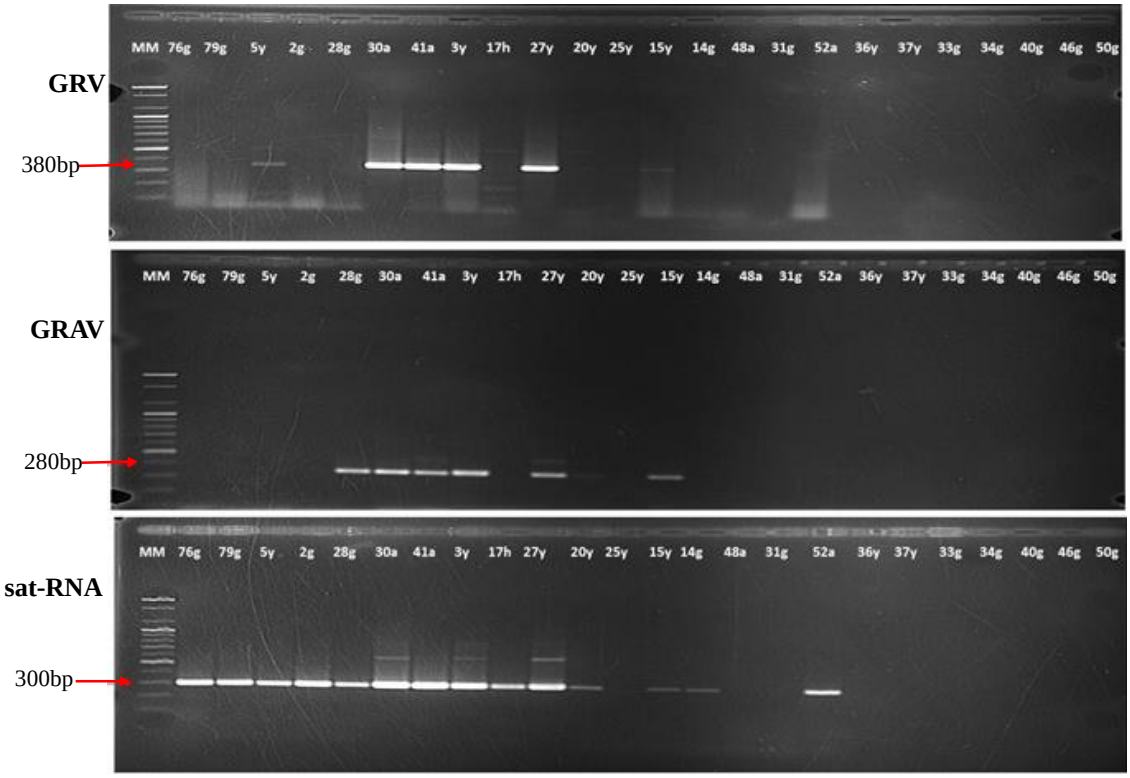

Figure 6. PCR product of GRAV, Sat-RNA and GRV. Lane MM shows a 100-bp DNA ladder (Fermentas). Samples 30a, 15y, 41a, 3y and 27y and 20y amplified for all three complexes (GRAV, sat-RNA and GRV); Samples 5y amplified for both GRV and sat-RNA; Sample 28g and 20y amplified for both GRAV and sat-RNA.

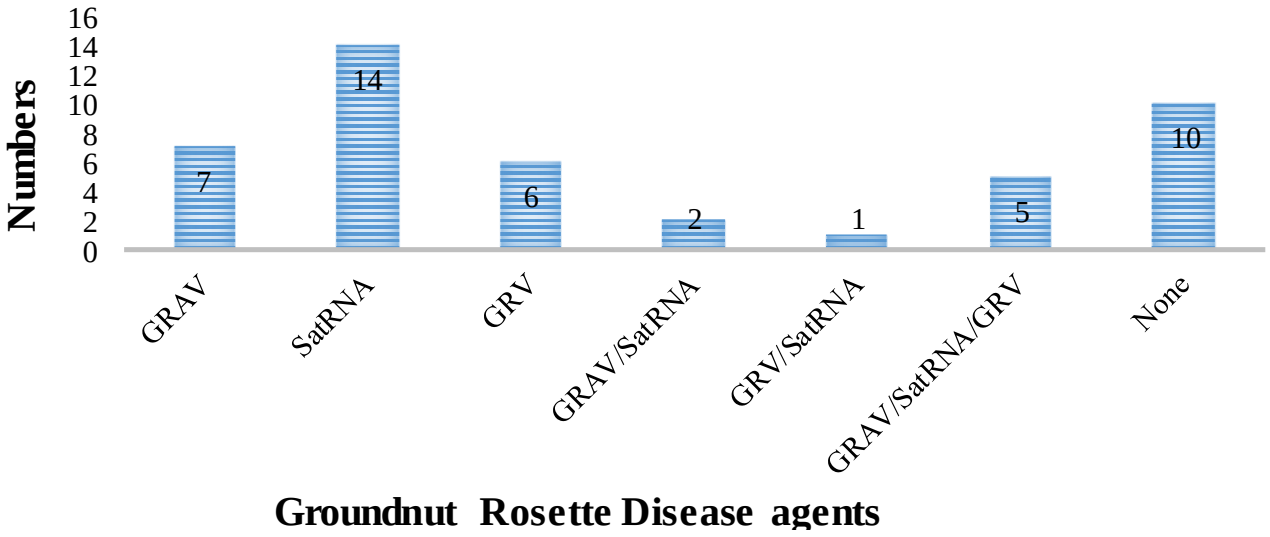

Figure 7. The distribution of GRD agent in the samples.

sensitive than the RT-PCR assay for GRV. There is therefore, need to multiplex for sat-RNA and GRAV to show that a plant has all the components.

In all GRD resistant cultivars and germ lines that have been analyzed, resistance conferred against GRV and not GRAV. However, resistance to GRV does not amount to immunity and can be overcome under high inoculum pressure or adverse environmental conditions (Bock, 1990; Olorunju et al., 1991). Yield reduction in such genotypes which appear resistant to GRV and sat-RNA was reported, which presumably could be due to their susceptibility to GRAV (Olorunju et al., 1991). Additional resistance strategies such as RNA-induced resistance in transgenic groundnut plants against GRAV.

In all conventional GRD resistance breeding programmes, resistance is assessed by lack of symptom expression and therefore resistance is largely due to GRV and sat-RNA, which are responsible for symptoms.

In this study, the inefficiency of the phenotypic screening was demonstrated in detection of three viral agents that cause GRD, the most devastating disease of groundnuts in SSA. Phenotypic screening needs to be supported by molecular analysis to detect all three GRD agents. Breeding of GRD resistant genotypes and their deployment is most effective when supported by efficient pathogen diagnostic systems, even in the absence of symptoms. The simplex RT-PCR assay method used in this study was able to detect all the GRD agents in both the symptomatic and asymptomatic samples in isolated runs thereby increasing our knowledge of the GRD pathogens and their interactions in pathogenesis and epidemiology. Deployment of multiplex PCR in the future for simultaneous detection of all three viral pathogens or just sat-RNA and GRAV in one PCR reaction mixture will provide a cheap, rapid assay thereby expanding the scope for GRD resistance screening. Transmission study on Oyado (*Cassia obtusifolia)* for GRD is needed to helpvalidate this plant as an alternative host.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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