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Full Length Research Paper

Improvement of physic nut (*Jatropha curcas* L.) by intraspecific hybridization between ecotypes of Africa and Americana

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***Jatropha curcas* is a plant with several attributes, multiples uses and considerable potentials. To mitigate the effects of climatic change, it is necessary to alleviate fossil power energy and increase biofuel energy. Plant-based fuels are among the best renewable sources, and their use can lead to a better balance of CO_2 and other greenhouse gases responsible for global warming. The aim of this work was to study the progeny of intraspecific cross of *J. curcas* (Equator × Senegal (Dialocoto)) in order to improve the productivity of the cultivated species through intra specific hybridization. The results showed that hybrids obtained from the cross between Equator ecotypes and Senegal ecotype (Dialocoto) showed the best production traits. For the width and length of the leaves, Equator ecotypes and hybrids had almost the same size (19.79 and 17.73 cm for Equator ecotypes and 19.0 and 17.23 cm for hybrids). The physical fruit properties of the hybrid showed dominance in comparison with the best parent due to heterosis effect. The hybrids showed a positive heterosis in fruit length, with significant H (30.17%) and Hb (3.47%) values. For the qualitative data, hybrids had the same leaf and petiole color as the Senegal ecotype, but the same leaf size with the Equator ecotype. The introduction of genetic variability can be performed by intraspecific cross.**

Key words: Benin, ecotype, *Jatropha curcas*, heterosis, hybrids.

INTRODUCTION

Jatropha curcas L. is an oleaginous plant which is not very exacting and can be adapted comfortably to the most climates conditions of tropical and subtropical regions. The better propitious zones to the culture of the plant are those where the annual mean of temperature varies from 18 to 29°C, with optimum values between 26 and 27°C (Trabucco et al., 2010). It has low requirements to soil fertility and can grow under low rainfall conditions (Sarin et al., 2007). It is a plant with several attributes, multiples uses and considerable potentials (Heller, 1996; Opensahw, 2000). *J. curcas* is a plant belongs to the family Euphorbiaceae, native to South America having a great economic, environmental and medical value. In the recent years, it has drawn attention as a source of seed oil that can provide an economically viable substitute for motor fuel (Opensahw, 2000; Adebowale and Adedire,

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2006; Chen et al., 2006). Banerji et al. (1985), Kaushik et al. (2007) and Sunil et al. (2008) mentioned that the seed contains 23 to 42% of oil which can be converted in a biodiesel, renewable energy source alternative to conventional petrodiesel (Mandpe et al., 2005; Ghosh et al., 2007). Biodiesel derived from *J. curcas* seed oil has the desirable physiochemical characteristics; performance was demonstrated to be superior to conventional petro-diesel (Heller, 1996; Opensahw, 2000; Sudheer et al., 2010). It is also a biofuel which allows reduction of the environment pollution by the greenhouse gas emissions. *Jatropha* is highly cross-pollinated and variations among the same species are limited. The majority of the vegetal material used to date comes from a simple selection within the semi-natural populating or local varieties. The inter-plants variations concerning the vigor and yield in seeds are enormous and we can expect that a good systematic selection with hybridization give great genetics improvements for yield in seeds and others significant characteristics. However, it is now well documented that some selections are rare and possess beneficial characteristics such as high yield, high oil content, drought resistance, photoperiod insensitivity, resistance/tolerance to major insect pests and diseases (Mohan et al., 2011). There were also significant differences ($P < 0.05$) in seed size, 100 seeds weight, oil content between accessions and low phorbol ester (PE) content (Ginwal et al., 2004; Ovendo-Medina et al., 2011). An approach to increase the productivity of physic nut (*J. curcas*) is to exploit hybrid vigor of the F1 progeny for possible production of hybrid varieties. Genetic improvement of physic can be done through many options like classical breeding, combining ability, heterosis breeding, mutation breeding, interspecific hybridization and genetic transformation (Divakara et al., 2010). Before using all potentialities of *J. curcas*, genetic improvement is necessary. Selection work and particularly genetic improvement is important to day so as to guarantee a high and stable yield for this culture. Physic nut has been recently domesticated and because of its high oil content, breeders want to select superior genotype for this character and others. The exploitation of heterosis is a common objective in plant breeding (Mayo, 1987). Application of heterosis breeding can boost physic nut oil content, yield, phorbol ester content, number of branches, seed size, size leaves, earlier maturity, reduced plant height, resistance to pests and diseases, drought tolerance, higher ratio of female to male flowers and improved fuel properties (Sujatha, 2006). Literature on *Jatropha curcas* improvement through heterosis are scarce (Divakara et al., 2010). A global effort to evaluate the genetic variability in *J. curcas* was initiated by Montes et al. (2008) using 225 and accessions collected from 30 countries in Asia, Africa and Latin America. Intraspecific diversity analysis by RAPD and AFLP of *J. curcas* collected from different geographical regions of India indicated the existence of low genetic diversity (Sudheer et al., 2009). The same work was done by Ouattara (2013) using 103 accessions. They found low genetic variation in African and Indian accessions and high genetic variation in Guatemalan and other Latin American accessions. That is the reason why Equator Hyderabad ecotype was chosen in the cross. Thus, the aim of this work was to improve the productivity of the cultivated species through intra specific hybridization involving two ecotypes of *J. curcas* from Senegal and Equator, respectively.

METHODOLOGY

Study area

The study was conducted at Ouedo-Adjagbo (06°29 N and 02°16 E), an administrative district of Abomey-Calavi Township, located in the south of Benin, around 22 km of Cotonou (Figure 1). The soil is clayey with sand. The climate is the sub equatorial with two rainy seasons and two dry seasons. The annual average temperature is 27.5°C, mean altitude is 41.25 m, average annual of rain is 1200 mm and mean humidity 84.47% (ASECNA, 2015).

Materials

The plant material used in this study consisted of two *J. curcas* ecotypes from Equator and Senegal (Dialacoto) and hybrids obtained from the crossing between *J. curcas* Equateur (♀) × *J. curcas* (♂) (Senegal). The crosses were performed in Senegal and the seeds of hybrids and ecotypes (Senegal and Equator) were sent to Benin by Pr. Guy Mergeai of Belgium. After harvesting, seeds were put into envelopes and kept to room temperature until sowing.

Approach

The seeds of the two ecotypes and 18 seeds of F1 hybrids were sown in 22.5 × 12.5 cm plastic bags filled with 2.5 kg of soil at Ouedo-Adjagbo district and were watered daily. It should be noted that one seed was sown per bag. Three months old, healthy seedlings (about 30 cm height and 8 to 10 true leaves) were randomly transplanted together in the experimental field in an interval of 2 × 2 m with their parents. A total of 15 plants for Senegal ecotype, 15 for Equator and 7 for hybrids were grown in the field and water was applied as needed to supplement natural rainfall.

Agronomic evaluation

Seven plants were chosen per ecotype and hybrids for measurement. The following parameters were measured: canopy height (cm) from the ground at the stem base to the top apex, number of branches, stem base diameter (cm), color of the stem, color of the leaf, color of the petiole, leaf length (cm) and leaf width (cm) of the 6^{eme} leaf completely opened from apex, fruit length (cm), fruit width, fruit weight, number of fruits per plant, number of kernel, average weight (g) of seed per fruit, proportion of kernel per seed (kernel ratio) kernel length, kernel width, and kernel weight (Table 1). A total of 16 quantitative and 3 qualitative traits were evaluated.

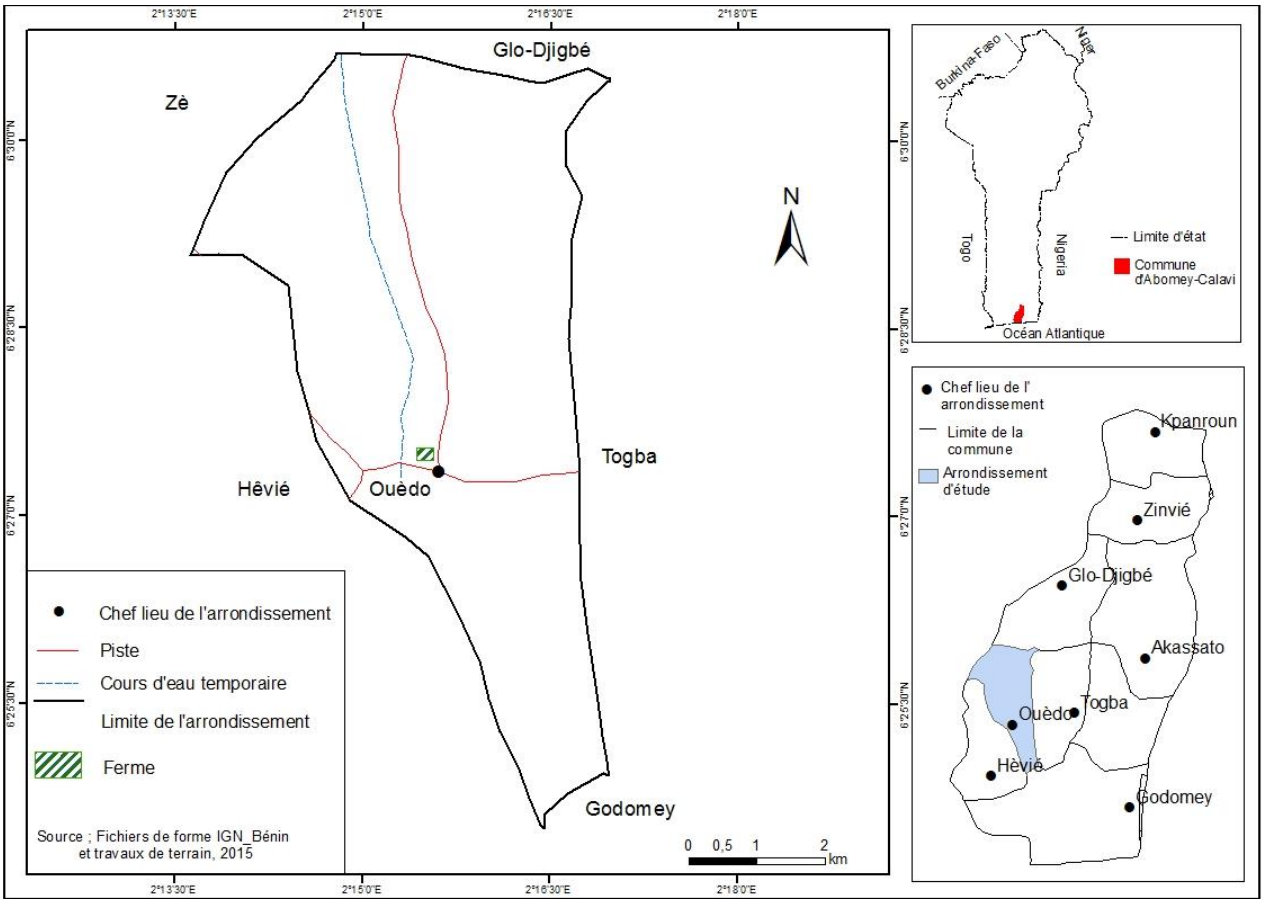

Figure 1. Map of Benin showing Abomey-Calavi Township and the study area at Ouedo.

Table 1. List of quantitative variables.

Designation	Abbreviations	Unity
Production	Prod	Number of fruits
height	Heig	cm
Number of branches	Npbr	-
Collar Circumference	Ccir	cm
Leaf width	Lwid	cm
Leaf length	Llen	cm
Fruit width	Fwid	mm
Fruit length	Flen	mm
Fruit weigth	Fwei	g
Seed width	Swid	mm
Seed length	Slen	mm
Number of kernel	Nker	-
Kernel weigth	Kwei	g
Kernel ratio	Krat	%
Kernel width	Kwid	mm
Kernel length	Klen	mm
Leaf color	Lcol	-
Petiole color	Pcol	-
Stem color	Scol	-

Table 2. Germination percentage of *J. curcas* from Senegal, Equator and hybrids.

Parameter	Number of seeds sowed	Harvesting year	Date of sowin	Date of counting	Number of seeds germinated	% of germination
Equator	40	2013	25/05/14	10/06/14	33	82.5
Senegal (Dialacoto)	40	2013	25/05/14	10/06/14	15	37.5
Hybrids (Equator (♀) × Senegal (♂)	18	2013	25/05/14	10/06/14	9	50

Statistical analysis

The collected data were encoded and entered into Excel 2007 software. Genstat 9 was used to compute the classical parameters such as mean, standard deviation, coefficient of variation, minimum and maximum. It was also used for the analysis of variance (ANOVA). For fruit weight, kernel weight, kernel length which conditions of normality and equality of variances were not satisfied, those variables are transformed. For other variables (kernel ratio, kernel width, kernel number, fruit length and fruit width), the test of Kruskal-wallis (a non-parametric alternative test analysis of variance) was used to separate means at P = 0.05. A simple description for frequency or proportion of different categories of each categorical variable was performed for qualitative data.

The values of mid-parent heterosis (H) and better-parent heterosis (heterobeltiosis, Hb) were calculated according to Equations 2 and 3, respectively (Tar et al., 2011).

$$\%H = (F1 - MP) \times 100/MP \quad (1)$$

$$\%Hb = (F1 - BP) \times 100/BP \quad (2)$$

where MP (mid-parent value) = (P1 + P2) /2; P1and P2 are the mean values of parents 1 and 2, respectively; F1 is the mean value of hybrid progeny; BP is the mean value of the better parent (showing the more desirable value of that trait) in the cross. The data from different characters were analyzed and statistically tested according to Soehendi and Srinives (2005), in this study, the significance of H and Hb in each character was determined by t-tests using Equations 3 and 4, respectively:

$$t\text{-test for } H = (F1 - MP)/ SH \quad (3)$$

$$t\text{-test for } Hb = (F1 - BP)/ SHb \quad (4)$$

where SH and SHb are the standard error of estimates of H and Hb, respectively.

RESULTS

Germination rate

Table 2 shows that the lowest germination % was recorded with Senegal ecotype while the highest germination rate was obtained with Equator ecotypes. Before transplanting the plants, two seedlings of hybrids were died.

Results of quantitative data

Table 3 shows the mean, standard deviation, coefficient of variation, minimum and maximum values of phenotypic characteristics for hybrids and ecotypes. The fruit production varies from 0 to 33 for ecotypes and hybrids. Senegal ecotype had the highest production and the lowest production was recorded in Equator ecotype (Table 3). For plant height, Equator ecotype had the best height and the Senegal ecotype had the lowest height. Hybrids' height was between the two parents and had the low coefficient of variation (CV= 6.30). Equator ecotypes and hybrids had almost the same leaf width and length (19.79 and 17.73 cm for Equator ecotypes and 19.0 and 17.23 cm for hybrids). There was no significant difference between hybrids and Equator parent for the leaf size (Table 4). Hybrids had the best fruit weight and the average weight of hybrids was better than the Senegal ecotype which was the best parent.

Correlation between variables

Significant correlation was observed between some variables (Table 5). The production (number of fruits per plant) was positively correlated with fruit width, fruit length, fruit weight, number of kernels, kernel weight, kernel ratio, width and length of kernel. On the other hand, the width and length of the leaves were negatively correlated with the number of fruits. Plant height had a positive correlation with the collar circumference, leaf dimensions and negatively with the number of branches. The collar circumference had a positive correlation with some variables: fruit length, fruit width, fruit weight, number of kernel weight, kernel ratio, kernel length and width. An increase in the collar circumference affects positively the fruit physical proprieties. It has been noticed that the fruit width correlated with the fruit length, fruit weight, kernel weight, the kernel ration kernel width and length. These different positive correlations can improve crossing results.

Heterosis of agronomic characters

The two ecotypes used in this study, demonstrated a wide diversity with interesting characters which can be transferred into hybrids plants. These interesting characters which were transferred into hybrids were

Table 3. Quantitative parameters' values of ecotypes and hybrids.

Ecotypes and hybrids	Variables	Total plant	Mean	Standard deviation	CV (%)	Minimum	Maximum
Equator (E)	Prod	7	7.29	8.48	116.39	0.00	19.00
	Tall	7	142.64	19.27	13.51	120.00	171.00
	Nbra	7	2.43	1.81	74.64	1.00	6.00
	Ccir	7	15.26	1.05	6.90	14.10	17.00
	Lwid	7	19.79	3.92	19.83	11.30	23.40
	Llen	7	17.73	1.03	5.83	15,80	18.60
	Fwid	7	11.67	10.98	94.09	0.00	21.87
	Flen	7	15.70	14.69	93.58	0.00	28.35
	Fwei	7	1.74	1.65	94.98	0.00	3.38
	Swid	7	11.66	10.97	94.09	0.00	21.87
	Slen	7	15.70	14.70	93.58	0.00	28.35
	Nker	7	1.50	1.44	95.65	0.00	2.90
	Kwei	7	1.23	1.21	98.48	0.00	2.54
	Krat	7	39.88	37.91	95.06	0.00	76.09
	Kwid	7	6.60	6.18	93.61	0.00	11.84
	Klen	7	11.04	10.33	93.56	0.00	19.61
Senegal Dialacoto (DS)	Prod	7	17.14	8.93	52.11	4.00	33.00
	Tall	7	119.64	15.80	13.21	88.00	138.50
	Nbra	7	5.43	2.51	46.18	3.00	10.00
	Ccir	7	15.51	1.44	9.25	13.40	17.40
	Lwid	7	11.61	2.36	20.35	8.60	14.80
	Llen	7	11.53	1.60	13.89	8.70	13.20
	Fwid	7	20.72	0.62	3.00	20.06	21.90
	Flen	7	26.63	1.26	4.73	24.51	27.97
	Fwei	7	2.87	0.29	10.07	2.54	3.40
	Swid	7	20.87	0.56	2.70	20.07	21.90
	Slen	7	27.03	0.79	2.93	25.78	27.97
	Nker	7	2.61	0.33	12.61	1.93	3.00
	Kwei	7	1.98	0.31	15.76	1.53	2.58
	Krat	7	69.10	8.87	12.84	80.87	77.19
	Kwid	7	11.01	1.28	11.60	8.15	11.76
	Klen	7	18.62	0.20	1.06	18.34	18.84
Equator × Senegal (ES)	Prod	7	13.00	10.49	80.68	1.00	32.00
	Tall	7	135.16	8.52	6.30	126.10	149.00
	Nbra	7	3.29	2.21	67.40	0.00	5.00
	Ccir	7	15.20	0.70	4.59	14.30	16.50
	Lwid	7	19.00	4.33	22.81	11.70	23.70
	Llen	7	17.23	2.34	13.55	14.10	20.90
	Fwid	7	21.05	1.09	5.20	19.25	22.09
	Flen	7	27.55	0.94	3.41	25.98	28.69
	Fwei	7	3.11	0.36	11.68	2.51	3.44
	Swid	7	20.95	1.11	5.27	19.26	22.09
	Slen	7	27.55	0,94	3.41	25.98	28.69
	Nker	7	2.81	0.19	6.68	2.54	3.00
	Kwei	7	2.27	0.32	14.27	1.72	2.58
	Krat	7	72.70	2.89	3.98	68.53	76.58
	Kwid	7	11.52	0.23	1.97	11.29	11.95
	Klen	7	19.10	0.36	1.91	18.64	19.61

Table 4. Segregating mean of agronomic characters of hybrids and their parents.

Parameter	Equator	Senegal (Dialacoto)	Hybrids	CV (%)	Probability
Prod	7.29 ± 8.48[a]	17.14±8.93[a]	13.00±10.49[a]	74.86	0.169[ns]
Tall	142.64 ± 19.27[a]	119.64±15.8[b]	135.16±8.52[ab]	11.48	0.0326*
Nbra	2.43 ± 1.81[b]	5.43±2.51[a]	3.29±2.21[ab]	59.14	0.0534[ns]
Ccir	15.26 ± 1.05[a]	15.51±1.44[a]	15.20±0.70[a]	7.21	0.853[ns]
Lwid	19.79 ± 3.92[a]	11.61±2.36[b]	19.00±4.33[a]	21.67	0.000854***
Llen	17.73 ± 1.03[a]	11.53±1.60[b]	17.23±2.34[a]	11.23	0.00003***
Fwid	11.67 ±10.98[b]	20.72±0.62[a]	21.05±1.09[a]	35.81	0.0202*
Flen	15.70 ± 14.69[b]	26.63±1.26[a]	27.55±0.94[a]	36.63	0.0324*
Fwei	1.74 ± 1.65[b]	2.87±0.29[a]	3.11±0.36[a]	38.50	0.0413*
Swid	11.66 ±10.97[b]	20.87±0.56[a]	20.95±1.11[a]	35.76	0.0198*
Slen	15.70 ±14.70[b]	27.03±0.79[a]	27.55±0.94[a]	36.34	0.0292*
Nker	1.50 ± 1.44[b]	2.61±0.33[a]	2.81±0.19[a]	37.19	0.0226*
Kwei	1.23 ± 1.21[b]	1.98±0.31[ab]	2.27±0.32[a]	40.89	0.0484*
Krat	39.88 ±37.91[b]	69.10±8.87[a]	72.70±2.89[a]	37.22	0.0266*
Kwid	6.60 ± 6.18[ab]	11.01±1.28[a]	11.52±0.23[a]	37.54	0.0404*
Klen	11.04 ± 10.33[ab]	18.62±0.20[a]	19.10±0.36[a]	36.73	0.0362*

*Significant at P < 0.05; **Significant at P < 0.01; ***Significant at P < 0.001; ns: no significant. On the same row, means with the same letters are not significantly different.

Table 5. Correlation between quantitative variables.

Prod	1.00														
Tall	0.19	1.00													
Nbra	0.34	-0.29	1.00												
Ccir	0.46	0.48	0.47	1.00											
Lwid	-0.15	0.46	-0.46	-0.15	1.00										
Llen	-0.28	0.40	-0.52	-0.13	0.84	1.00									
Fwid	0.53	0.03	0.37	0.36	-0.36	-0.26	1.00								
Flen	0.53	0.08	0.30	0.36	-0.35	-0.23	0.99	1.00							
Fwei	0.52	0.07	0.36	0.39	-0.29	-0.17	0.98	0.96	1.00						
Swid	0.54	0.02	0.37	0.36	-0.36	-0.26	1.00	0.99	0.98	1.00					
Slen	0.53	0.06	0.32	0.36	-0.36	-0.24	0.99	1.00	0.96	0.99	1.00				
Nker	0.51	0.06	0.35	0.32	-0.28	-0.19	0.98	0.97	0.97	0.98	0.96	1.00			
Kwei	0.49	0.10	0.35	0.33	-0.19	-0.11	0.95	0.92	0.97	0.95	0.92	0.98	1.00		
Krat	0.53	0.06	0.35	0.30	-0.28	-0.21	0.98	0.97	0.95	0.98	0.97	0.99	0.96	1.00	
Kwid	0.51	0.06	0.32	0.33	-0.33	-0.20	0.98	0.99	0.95	0.98	0.98	0.98	0.94	0.99	1.00
Klen	0.52	0.05	0.33	0.35	-0.35	-0.22	0.99	0.99	0.97	0.99	0.99	0.97	0.92	0.97	0.98	1.00

revealed through the highest values of phenotypic traits recorded in the hybrids. These gains can be observed through heterosis. Significant values of H and Hb were detected in the cross (Table 6). The F1 hybrids had more length and width in fruit than their two parents. Hybrids had the highest kernel (length, width, weight) (19.10 mm, 11.52 mm and 2.27 g, respectively) than the two parents (18.62 mm, 11.01 mm and 1.98 g for Senegal parent and 11.04 mm, 6.60 m and 1.23 g for Equator) and significant heterosis values were observed (Table 6). The crosses between Equator and Senegal showed a positive heterosis in fruit length, with significant H (30.17%) and Hb (3.47%) values. Overall, it has been observed that most of the H (mid-parent) values were significant except for production, height, collar circumference and leaf width. On the other hand, Hb (heterobeltiosis) values were significant for kernel, fruit length and height.

Results of qualitative characters

From qualitative point of view, leaves were palmatilobed

Table 6. Heterosis of quantitatives agronomic characters.

Variable	%H	%Hb
Prod	6.43^{ns}	-24.17^{ns}
Tall	3.06^{ns}	12.97**
Nbra	-16.36*	-39.47*
Ccir	-1.21^{ns}	-2.03^{ns}
Lwid	21.02^{ns}	-3.97^{ns}
Llen	17.77*	-2.82^{ns}
Fwid	30.00***	1.59^{ns}
Flen	30.17***	3.47*
Fwei	34.94***	8.35^{ns}
Swid	28.80***	0.38^{ns}
Slen	28.93***	1.91^{ns}
Nker	36.76***	7.78*
Kwei	41.50**	14.81^{ns}
Krat	33.43***	5.22*
Kwid	30.89***	4.70***
Klen	28.75***	2.55*

*Significant at P < 0.05; **Significant at P < 0.01; * **Significant at P < 0.001; ns: non-significant.

regardless of the ecotype considered with a green-pale color for Senegal but dark for Equator. The petiole color of Equator ecotype was brown, while it was green-pale with a tiny brown line for Senegal. Hybrids had the same type of petiole like Senegal petiole. The stem of all hybrids were green-pale like Senegal parent. Taking into account the leaf size, we observed that these characters of Equator were dominant. Hybrid plants had the same color of leaf and petiole like Senegal ecotype (Figure 2).

DISCUSSION

The used ecotypes in this work were obtained from two different regions of the world. The germination of the two ecotype seeds showed great variation probably due to their origin. According to Sharma (2007), ecotype had great effect on the germination power of the seed and the germination time. Kobilke (1989), when compared the germination power of the seeds aged from 1 to 24 months, Kobilke observed a germination power inferior to 50% for the seeds of which the time of storage exceeded 15 months. For Moncaleano-Escandon et al. (2013), storage duration, combined with temperature, had strong effects on the germination of *Jatropha* seeds and the germinability decreased as age increased. These two factors certainly affected our seeds and this is why the germination % was low. According to Hartmann et al. (1990), genetic and environmental factors determine germination rate, speed of germination, and vigor of seed and seedling. In this work, the coefficients of variation differed for quantitative data. Similar results were found by Sunil et al. (2008) and Mishra (2009), who selected better plants and accessions of *J. curcas* from India. Correlation coefficients revealed interesting relationship with the seed characters as well as the growth studied parameters. The correlation matrix showed a good correlation between seed weight, seed width; therefore, seed weight can be considered as important trait for early selection from seed sources. Similar results had been reported by Kaura et al. (1998). The number of branches contributed to higher number of flowers which in turn contributed to higher number of female flowers which finally culminated in higher yield. In this study, at the younger stage, the number of branches of hybrids was higher than that of Equator parent. This is vital in the genetic improvement. Morphological characteristics (plant height, collar height and thickness, number of primary branches, petiole length, number of fruits per cluster, pedicel length and seed yield) were also correlated with the oil content of the seed. The two ecotypes plants exhibited some characteristics due to their provenance. The assessment of parameter variability in the study is in close approximation with the findings of genetic parameters in *J. curcas* (Das et al., 2010; Singh et al., 2013; Nath et al., 2014). This variability relative to the ecotype origin was also expressed through the studied qualitative and quantitative variables. For Heller (1996), the key for success of any genetic improvement program lies in the availability of genetic variability for desired traits. The gains from tree breeding programs depend on the type and extent of genetic variability.

Some provenances may differ relatively from others if cultivated at different sites, which is due to genotype ×

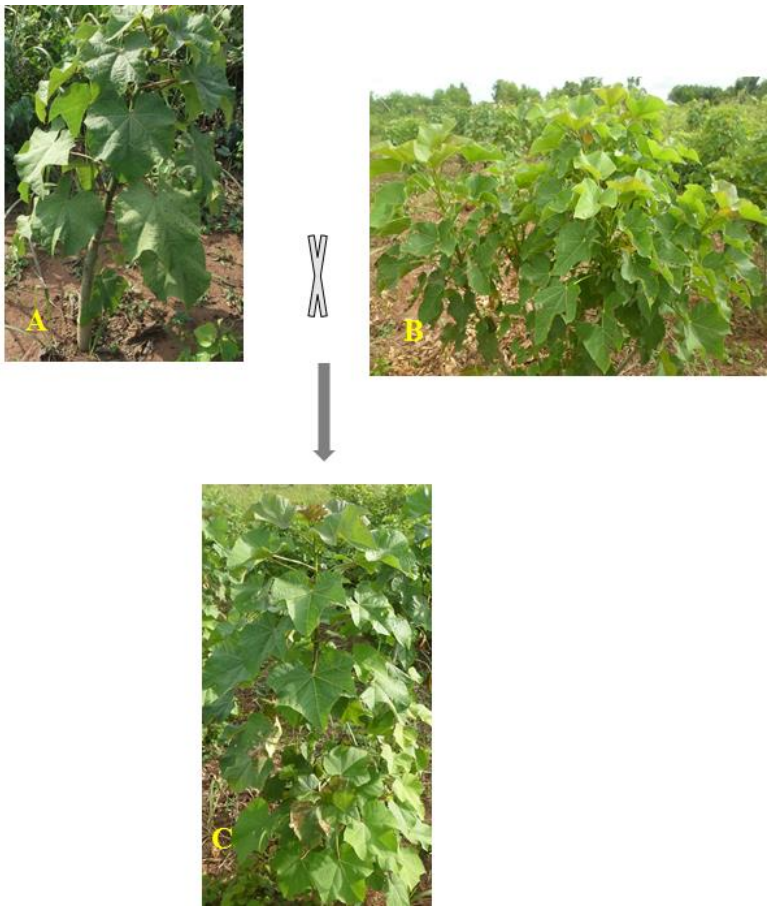

Figure 2. Plants of *J. curcas* studied: A: Equator, B: Dialakoto, Senegal C: Hybrid.

environment interaction. The two ecotypes from Equator and Senegal demonstrated different traits which were advantageous for this cross. The evaluation of hybrids' performance can be done through the analysis of the heterosis values determined by the value of mid-parent and the value of the better parent. It is demonstrated by some authors (Koutroubas et al., 1999; Sunil et al., 2008; Tar et al., 2011) that when physic nut was younger than one year, canopy height, canopy diameter, stem base diameter and number of primary branches were considered important agronomic characters affecting yield per plant. The obtained hybrids from ecotype of Senegal and Equator and used in this work showed similar characters at their younger stage. Thus, the obtained hybrids demonstrated better improvement than their mid-parent for the measured quantitative characters (%H > 0) except the number of branches. Our results are consistent with those obtained by Tar et al. (2011) who worked on the hybrids obtained from the crosses between six toxic parental accessions with a non-toxic accession. It was observed that the physical fruit proprieties showed an improvement of 28% when compared with the mid-parent and 2% more than the best parent. In the present study, moderate genetic gain values obtained for seed characters indicate that improvement could be done with these characters. These hybrids constitute a superior germplasms which stem cutting and their utilization as cultivars can increase the yield in seeds and oil of *Jatropha*.

Conlusion

Conclusively, the present study provided some information about the hybrids from the cross between Senegal and Equator. Thus, the obtained F1 individuals have a great genetic value in comparison with the mid-parent for all quantitative variables studied except the number of branches and the stem collar. These plants possessed also significant great genetic values compared to the best parent for the quantitative variables measured except the production, the number of branches, the stem collar and leaves dimensions. The performances of hybrids were the fruit quality inherited from their parent. The character like leaf size of Equator ecotype is dominant and was transferred to the hybrid, while the

petiole and leaf color of Senegal ecotype were dominant and transferred also to the hybrids. The Equator ecotype expressed a great variability with all quantitative variables. The coefficient of variation was higher for phenotype (quantitative data), indicating a predominant role of the environment. In general, it appears that the environment has a preponderant role in the morphological variation of the two used ecotypes. It was noticed that the introduction of genetic variability can be performed by intraspecific crossing. Classical breeding is suitable for selecting traits, such as seed yield, seed size, number of branches and oil yield, but also for developing planting material adapted to local environmental conditions.

CONFLICT OF INTERESTS

The authors have not declared ant conflict of interests.

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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

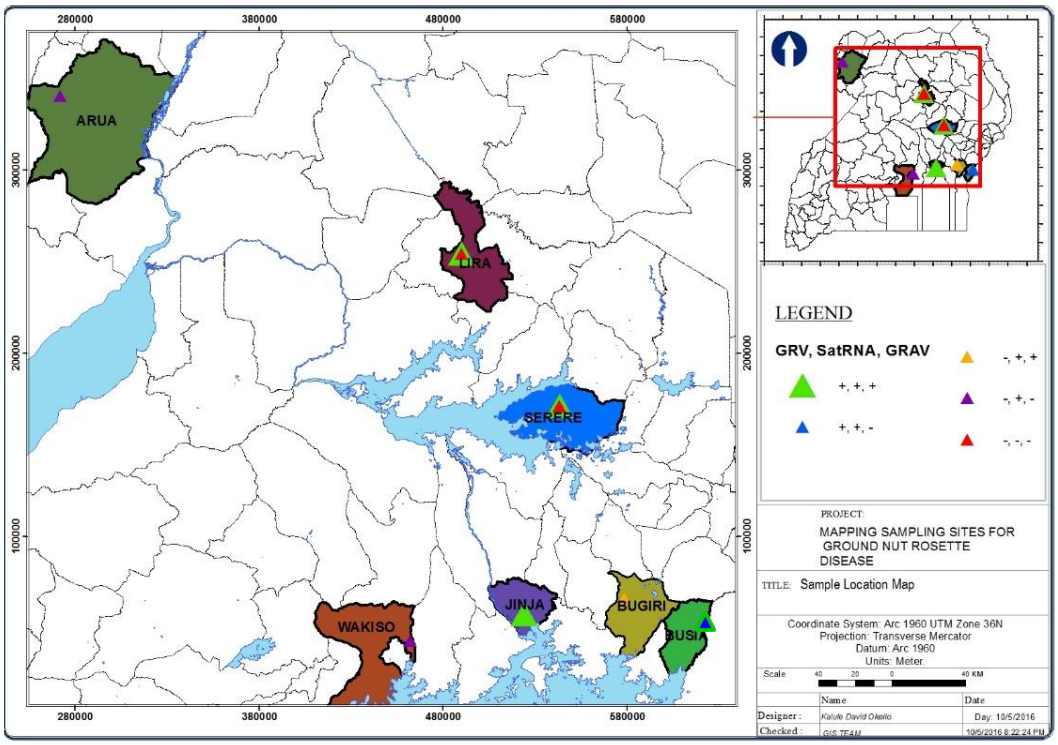

Figure 5. The sampling points and GRD complex distribution.

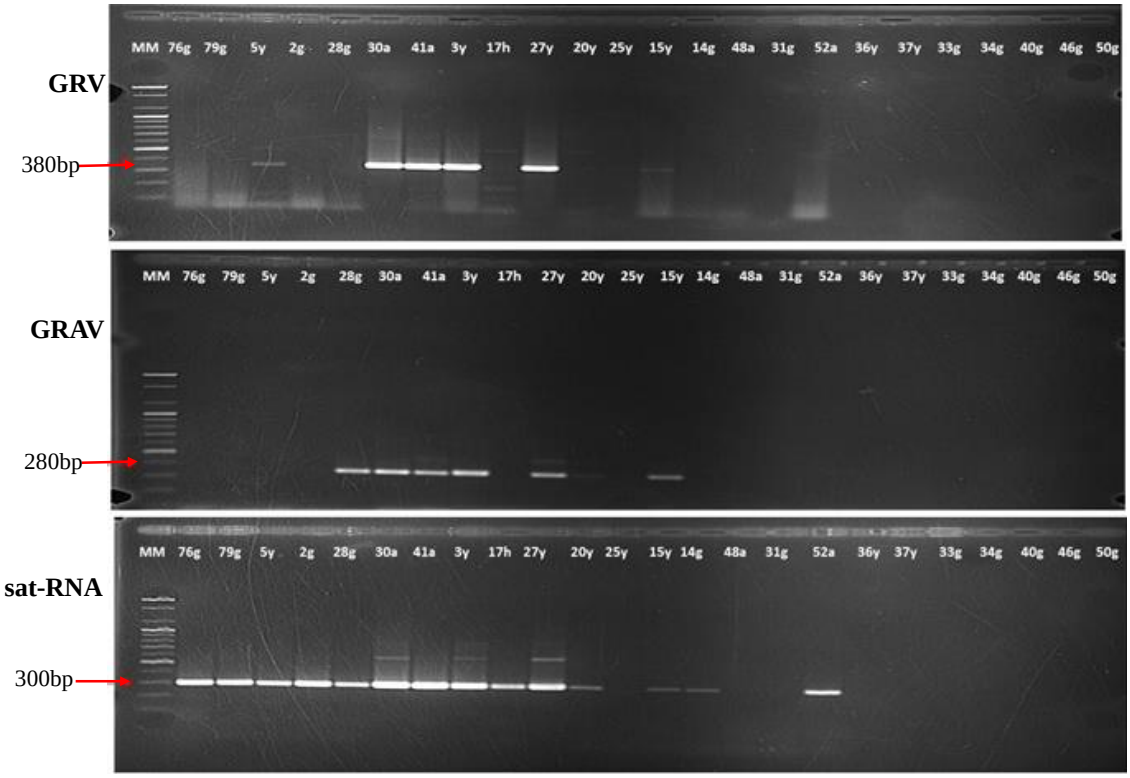

Figure 6. PCR product of GRAV, Sat-RNA and GRV. Lane MM shows a 100-bp DNA ladder (Fermentas). Samples 30a, 15y, 41a, 3y and 27y and 20y amplified for all three complexes (GRAV, sat-RNA and GRV); Samples 5y amplified for both GRV and sat-RNA; Sample 28g and 20y amplified for both GRAV and sat-RNA.

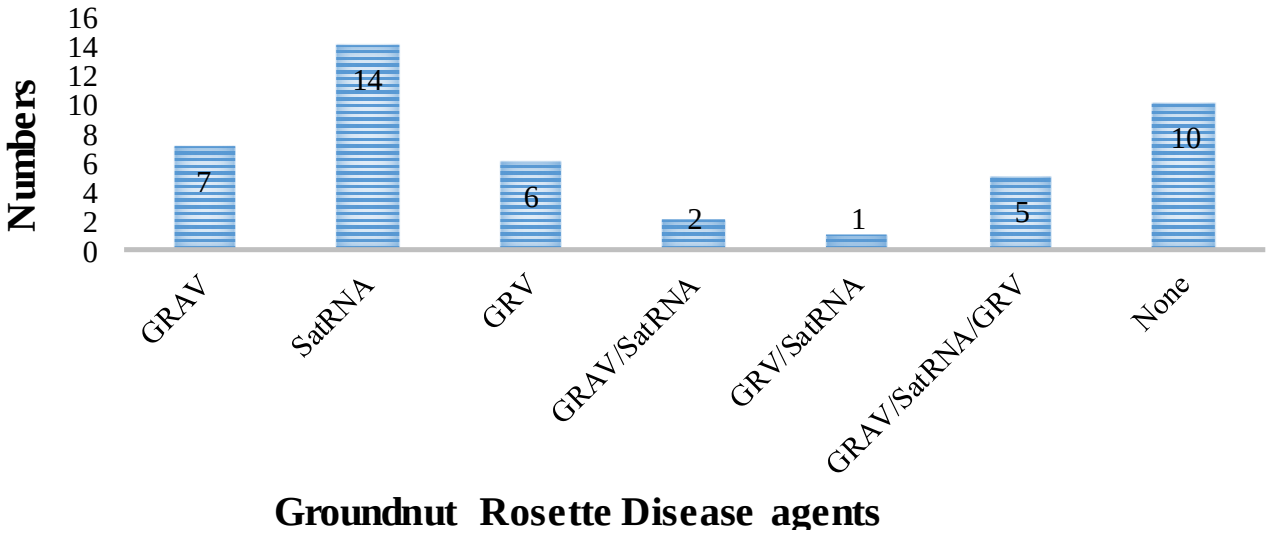

Figure 7. The distribution of GRD agent in the samples.

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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