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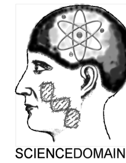

Regeneration Procedure for Three *Arachis hypogaea* L. Botanicals in Uganda through Embryogenesis

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Authors' contributions

This work was carried out in collaboration between all authors. Author DKO designed the study, managed the literature searches and together with authors JA, CM and CMD developed and optimized the protocol and drafted the manuscript. Author TLO performed the statistical analysis and together with author DKO managed the analyses of the study. Authors LBA, PT and SMO read and provided the overall supervision of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: A procedure was developed for embryogenesis from embryo explants derived from mature seeds of freshly harvested Serenut 4T, Serenut 1R and Acholi-white groundnut cultivars representing the three broad groundnut botanical classifications.
Methodology: This study explored the use of mature embryo axes as explants for somatic

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embryogenesis, and determined the factors that affect regeneration of three Ugandan groundnut cultivars. Freshly harvested mature seeds of the three groundnut cultivars were collected and the embryo explants were initiated on 3 media namely; Murashige and Skoog (MS) basal media with varying concentrations of the growth regulator 2,4-Dichlorophenoxy acetic acid (2,4-D); Chu N6 basal medium with vitamins (N6); and Callus Induction Medium (CIM). The shoot formation and elongation medium contained MS basal medium supplemented with indolebutyric acid (IBA) and 6-Benzylamminopurine (BAP) in isolation, and BAP in combination with a-naphthaleneacetic acid (NAA) and indoleacetic acid (IAA). For root induction, elongated shoots were transferred to MS medium supplemented with various combinations of NAA with IBA, BAP and a combination of IBA and Kinetin.

Results and Conclusion: Different concentrations of 2,4-D elicited different callogenesis responses in the cultivars with Acholi white (Valencia botanical) and Serenut 4T (Spanish botanical) giving the optimal response at 5mg/l whereas Serenut 1R (Virginia botanical) showed best response at a concentration of 30 mg/l. N6 and CIM supported callogenesis in Acholi white (AW) and Serenut 4T only. In all cultivars, maximum root production was gained when using MS medium supplemented with NAA- 1 mg/l and IBA -2.0 mg/l. On the other hand, for Serenut 1R and Serenut 4T, BAP 2.5 mg/l; NAA 0.5 mg/l combination yielded higher shoot regeneration percentage whereas for AW BAP 3 mg/l; NAA 0.5 mg/l supported maximum shoot production.This is the first ever report of successful regeneration of the three groundnuts botanicals in Uganda. These results are likely to facilitate genetic transformation of three preferred Ugandan groundnut varieties.

Keywords: Groundnuts; cultivars; callus induction; 2,4-D, N6; callus induction medium; in vitro morphogenesis.

1. INTRODUCTION

Groundnut or peanut (*Arachis hypogaea* L.) is globally an economically important legume crop, whose seeds contain about 43% oil and 25% protein. The crop has a significant nutritional value in tropical and sub-tropical regions of Asia, Africa, and North and South America [1]. It is cultivated in over 100 countries worldwide on over 24 million hectares of land with a total production of 38 million tons [2]. There are several constraints to the production of groundnut that result in reduced yields and great economic losses annually [3-7].

Conventional breeding has contributed towards the improvement of groundnuts to mitigate the effects of the constraints [8,9] but with limitations. Although some of the wild relatives of *A. hypogaea* have been identified as sources of resistance to several diseases and pests [10], the success in transferring these desirable traits to cultivated cultivars has been limited due to reproductive barriers, frequent failures in interspecific crosses, low recovery of hybrids, and linkage with undesirable traits [11-13]. Genetic transformation overcomes these limitations and allows the introduction of agronomically important traits across taxa into established cultivars [3,12,14-19]. This greatly compliments traditional breeding and selection approaches. However, efficient regeneration of totipotent cells is an essential prerequisite of genetic engineering systems, whether the regenerated plant results from a preformed shoot meristem, an undifferentiated callus cell or embryo that is competent to express a morphogenetic ability [20]. In general auxins (e.g. IAA, NAA and IBA) will stimulate regeneration of roots, and cytokinins (e.g. BAP and kinetin) will promote regeneration of shoots or embryos [20]. There is no regeneration procedure in place for groundnuts botanicals in Uganda.

Groundnuts have three botanical groupings which have effects on agronomy, management, hybridization and selection [21-24]. Three groundnut cultivars widely grown in Uganda, Acholi white (Valencia botanical), Serenut 1R (Virginia botanical) and Serenut 4T (Spanish botanical), representing the three Arachis botanicals, were purposely selected for this study.

Acholi white (AW) is a popular Valencia landrace grown for its early maturity (85 days), off-white seed coat colour and high oil content (51%). It is however low yielding (<1250 kgs/ha), susceptible to leaf spots (LS) and groundnut rosette virus disease (GRD). Genetic improvement for higher yields and resistances to LS and GRD, using conventional approaches to recover the original off-white seed coat has been difficult due to the complex nature of inheritance in its multiline

background. Advanced progenies beyond the sixth filial (F6) generations still have unstable seed coat phenotypes and manifest many transgressive segregation patterns typical of the multi-progenitor genomes. Genetic transformation would help curb the seed coat inheritance problem and aid fast stacking of multiple traits in this popular landrace.

Serenut 1R (ICRISAT line, CG7), a Virginia botanical type, was released in 1998 in Uganda. It is a very high yielding (potential yield of 3000kg/ha) confectionery type variety with a deep attractive red seed coat colour. However, the cultivar is very susceptible to GRD and cannot be grown in fields unless sprayed with insecticides to control aphids (GRD vector) leading to its abandonment by most farmers who could not spray their crop [6,25].

Serenut 4T (ICRISAT line, ICGV 12291), a Spanish botanical type groundnut variety was released in Uganda in 2002 as aphid (GRD vector) resistant, small seeded, tan coloured, early maturing (90 days) cultivar with a shelling turnover of 73% [6]. Recently, breakdown in GRD resistance in Serenut 4T has been observed at high vector populations and disease pressure. Its production is declining rapidly as farmers are abandoning it for other GRD-resistant cultivars.

Numerous protocols for *in vitro* groundnut regeneration have been reported in the last decade. Many of these protocols describe somatic embryogenesis using a great variety of explants such as leaflets [26], immature cotyledons [26-28], axes of immature embryos [28], hypocotyls [14] and epicotyls [29].

This study describes the first regeneration methods for three groundnut cultivars widely grown in Uganda representing the Spanish, Virginia and Valencia botanicals using cotyledonary explants in MS basal media with varying 2, 4-D concentrations, CIM and Chu N6 Media.

2. MATERIALS AND METHODS

2.1 Groundnut Cultivars Used

Freshly harvested mature groundnut seeds representing the three botanical groupings obtained from the Groundnut Improvement Programme of National Semi-Arid Resources Research Institute (NaSARRI), Serere were used in the study (Table 1). The experiment was conducted at the National Agricultural Research Laboratories at Kawanda, Kampala.

2.2 Explant Preparation

The pods were washed 3 times with sterile distilled water and liquid soap to reduce the level of surface contaminating organisms. They were then transferred to a laminar flow hood and the pods were surface sterilized with 5% sodium hypo-chlorite for 10 min, 70% ethanol for 10minutes and rinsed 3 to 5 times with sterile water. The seeds were extracted from the pods and the embryos axes excised by removing the cotyledons aseptically from the sterilized seeds. The radical and plumule portions of embryo axis were excised and used as explant (Fig. 1).

2.3 Regeneration via Organogenesis

Ten plates per media concentration and standards (2, 4-D at 5, 10, 20, 30, 40 and 50 mg/L, N6 and CIM standards) were plated with 5 explants from each groundnut cultivar. The explants were initiated on MS basal medium supplemented with 20 mg ascorbic acid, 30 mg sucrose, 2.4 g gelrite and varying 2,4-D concentrations (5, 10, 20, 30, 40 and 50 mg/L); Chu [14,30,31] N6 medium (100 ml N6 macros salts, 5 ml MS micro salt, 10 ml MS iron, 5ml Gamborg's B5 vitamin, 30 mg Sucrose, 20mg ascorbic acid, 400 mg L-proline, 100 mg L-glutamine, 100 mg casein hydrolysate, 1 mg/l 2,4-D, 1 mg/l NAA, 1 mg/l IAA, 0.25 mg/l kinetin, 2.4 g gelrite) and callus induction medium (CIM) (MS basal salts and MS vitamin, supplemented with 20 mg ascorbic acid, 30 mg Sucrose, 1mg/l 2,4-D, 1 mg/l NAA, 2mg/l biotin, 2.4 g gelrite). The pH of each medium was adjusted to 5.8 before autoclaving for 15 minutes at 121ºC. The cultures were incubated under 16/8 h light/dark cycle at 25±2°C in a growth room. Light was supplied by Philips–lux lamps providing an intensity of 80 µEm-2s-1. Cultures were transferred to fresh medium under laminar flow hood at 7-day intervals and routinely examined for morphological development and *in-vitro* responses. The calli obtained were placed in embryo growth and differentiation medium consisting of MS basal salts and vitamins supplemented with 30 mg/l sucrose and growth regulators singly or in combinations and incubated under the same conditions described above. The calli were then proliferated and differentiated into shoots and roots. The shoot formation and elongation medium contained MS

basal medium supplemented with BAP-2.5 mg/l and NAA-0.5 mg/l. Shoot buds appeared on the explants within three weeks after transferring to shoot formation and elongation medium and increased in numbers resulting in clusters. Subsequently, shoot clusters were sub-cultured on the same medium every three weeks to enhance shoot elongation. After 3–4 cycles, shoots (3–4 cm) derived from the shoot clusters were excised aseptically and rooted on MS medium supplemented with NAA- 1mg/l and IBA 0.5 mg/l.

2.4 Hardening of the Plants

Rooted shoots were observed till the formation of secondary roots (28 days). They were then washed with running tap water to remove the traces of medium from the roots and the plants were potted in sterilized sandy loam soil and kept in a humid chamber in a glass-house with a watering schedule of twice a day till maturity (Fig. 6 G and H).

All biochemicals and media constituents, unless stated otherwise were molecular biology/cell culture grade from Duchefa Biochemie BV - The Netherlands.

2.5 Data Analysis

To determine the effect on treatment (MS media with various concentrations of 2, 4-D; N6 and CIM) and varieties, data were collected on the number of callus formed, % of callus formed, days to callus formation and number of ideal calluses). These dataset were first explored using MS pivot tables then subjected to analysis of variance using Genstat 14th Edition [32]. Ranking of the varieties and concentration was done using the Kruskal-Wallis one-way test [33]. Data was also collected on conditions for shoot formation and rooting and this is presented graphically (Figs. 4 and 5).

3. RESULTS AND DISCUSSION

The present report describes a reproducible and efficient protocol for *in-vitro* propagation of groundnuts. Induction and maturation of the somatic embryos was achieved on three media, namely MS basal medium supplemented with varying 2, 4-D concentrations, CIM and N6 media (Fig. 1). Data were collected on days to callus formation, percentage callus formation, percent of ideal (embryogenic) callus on 2, 4-D concentrations (5, 10, 20, 30, 40,and 50 mg/l), CIM and N6. Data was also collected on conditions for shoot formation and rooting. Exploratory analysis using pivot tables and pivot charts in MS excel on the effect of both cultivars and media (2, 4-D, N6 and CIM) concentrations are shown in Fig. 2.

3.1 Days to Callus Formation

Days to calli formation was faster in N6 and CIM (5 days and 6 days respectively) for AW and S4T, while for MS medium supplemented with different 2,4-D concentrations, it took 7 days to

Table 1. Groundnut cultivars used for regeneration

Cultivar	Botanical	Market status	Seed coat phenotype	Year released
Acholi white	Valencia (multiline)	Landrace	Off-white	1966
Serenut 1R	Virginia	Commercial	Red	1998
Serenut 4T	Spanish	Commercial	Tan	2002

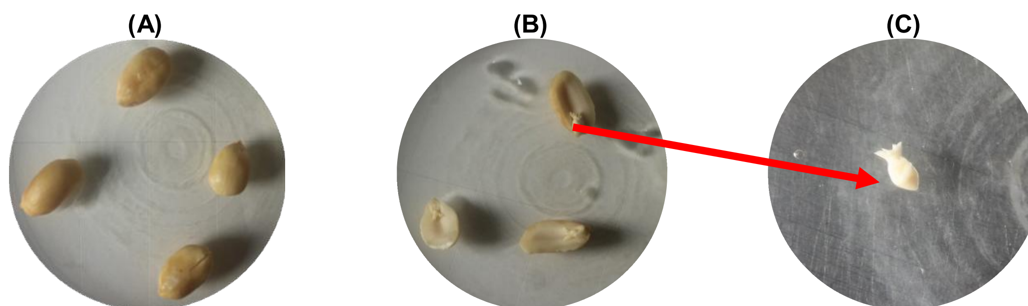

Fig. 1. Stages in the preparation of embryo explants; (A) testa peeled off from pre-soaked surface-sterilized seed, (B) embryonated cotyledon, and (C) embryo axis aseptically excised by removing the cotyledon (arrow) and used as explant.

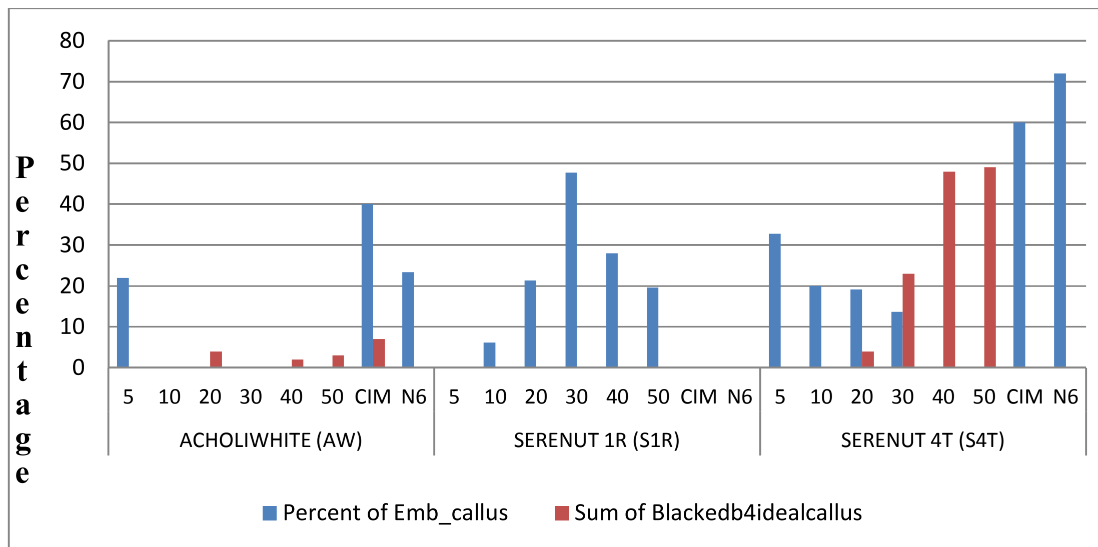

Fig. 2. Effect of groundnut cultivars used, media, and media concentrations on callus formation

callus formation for all the cultivars. Days to ideal calli (Fig. 6A) formation for S4T is 42 days on all media. For AW it takes 42 and 56 days on CIM and N6 media respectively to reach ideal calli formation whereas 2, 4-D concentrations above 5mg/l resulted in blackening. There were no calli formed for S1R in both N6 and CIM media (Table 2, Fig. 2) while lower concentrations of 2,4-D (less than 20 mg/l) increased day to ideal calli formation to 49 days as compared to 42 days under higher concentrations.

Some callus formed black mass (blackened) which did not result into ideal/embryonic callus (Red bars). Ideal calli formed per cultivar are represented by blue bars.

There were insignificant variations in the number of callus formed, % of callus formed, and days to callus formationamong the cultivars and botanicals. The analysis of variance for percentage ideal callus formation revealed that there is a significant variation in the media compositions, varietal performances and an interaction between concentrations and varieties. The three groundnut cultivars responded differently to growth media (Tables 2 and 3). This agrees with earlier reports that groundnut organogenesis have a strong genotypic influence and requires specific protocols for each botanical type [34-39].

The ranking of the varieties and medium type and 2, 4-D concentration using the Kruskal-Wallis one-way analysis of variance is shown below:

The highly significant chi-square probability (<0.001) shows that the observed differences in the dataset are not by chance. With 2,4-D media, Acholi white and Serenut 4T gave the best performance for ideal callus (Fig. 6A) formation at 5 mg/l. Serenut 1R on the other hand had the highest rank for ideal callus formation at 30mg/l of 2,4-D. Media N6 and CIM favoured only Acholi white and Serenut 4T cultivars for ideal callus formation and the subsequent embryogenesis. This could be attributed to the media composition of the N6 and CIM which have lower (1 mg/l) 2, 4-D concentration.

Different concentrations of 2, 4-D elicited different responses in the cultivars to callogenesis (Tables 2, 4). Acholi white and Serenut 4T gave the best response at 5mg/l whereas Serenut 1R showed best response at a concentration of 30 mg/l. This is in agreement with earlier studies [37] on organogenesis potential among four market types groundnuts from different countries and it was found that different concentrations of growth hormone were required for efficient response of different cultivars. Earlier studies on *in-vitro* organogenesis in groundnut also showed strong influence of culture conditions [5,40-43] on the response of explants. Embryogenesis is induced by compounds such as 2, 4-D and other compounds of auxinic effect such as picloram.

Table 2. Time taken to calli and ideal calli formation for the three groundnut cultivars in the media tested

Data collected	Treatments																							
	2,4-D concentrations (mg/l)																		N6			CIM		
	5			10			20			30			40			50								
	AW	**S4**	**S1R**	**AW**	**S4**	**S1R**	**AW**	**S4**	**S1R**	**AW**	**S4**	**S1R**	**AW**	**S4**	**S1R**	**AW**	**S4**	**S1R**	**AW**	**S4**	**S1R**	**AW**	**S4**	**S1R**
Days to callus formation	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	5	5	0	6	6	0
Days to ideal callus formation	42	42	49	All blackened	42	49	All blackened	42	42	All blackened	42	42	All blackened	42	42	All blackened	42	42	56	42	0	42	42	0

Table 3. Analysis of variance for percentage ideal callus formation

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Concentration	5	3371.1	674.2	3.96	0.002
Variety	2	7964.4	3982.2	23.41	<.001
Concentration. variety	10	18462.2	1846.2	10.85	<.001
Residual	162	27560.0	170.1		
Total	179	57357.8			

N6 and CIM having lower 2, 4-D concentrations (1 mg/l) did not support callogenesis in S1R, but instead the explants grew straight into plants with shoots and roots (Fig. 3). This is consistent since already at 5mg/l there was no callogenesis in S1R and this can be attributed to endogenous hormonal interaction. The high positive response of cultivars Acholi white and S4T on N6 and CIM (Fig. 2) having low 2, 4-D concentrations suggests that even lower concentration of 2,4-D could be ideal for callogenesis in these cultivars.

The percentage of ideal callus proliferating into whole plants ranged between 0 – 72% (Fig. 2). This percentage is however higher than the 2% regeneration success rate from AKWA variety [44]. Overall, for 2, 4-D the percentage of ideal callus formed were highest in S1R (48%); N6 was highest in S4T (72%) and for CIM was highest in S4T (60%).

3.2 Shoots and Roots Regenerations

Regeneration of shoot buds (Fig. 6B) from ideal callus were achieved through the use of MS medium supplemented with IBA, BAP and a combination of BAP and IAA (Fig. 4). In the present study, BAP and IBA alone were able to induce proliferation of shoot buds in all the three cultivars. This finding agrees earlier report [45] that BAP alone was able to induce shoot proliferation. The combination of BAP 2.5 mg/l; NA 0.5 mg/l NA 0.5 mg/l yielded over 50% shoot regeneration among all the cultivars. For AW however, BAP 2.5 mg/l; NA 0.5 mg/l had the highest (80%) shoot regeneration. BAP at concentrations higher than 2.5 mg/l seems to suppress shoot production in S1R and S4T. For AW BAP 2.5 mg/l; NA 0.5 mg/l and BAP 3 mg/l; NA 0.5 mg/ yielded over 50% shoot regeneration. It is only in AW cultivar where BAP -3 mg/l alone yielded less than 50% shoot regeneration (Fig. 4). With exception of Acholi white cultivar, lower concentrations of BAP were proved to be effective in inducing multiple shooting whereas higher concentrations proved to be inhibitory. Higher concentration of BAP has been reported to inhibit shoot regeneration [45]. The observed variation in shooting potential of all the cultivars under the influence of BAP might be due to their genotypic differences.

The well-formed shoots (Fig. 6C) were transferred to shoot induction media where they continued to elongate and often formed clusters of multiple shoots (Fig. 6D). Healthy and elongated shoots of approximately 4 cm in length were excised and rooted (Fig. 6E) using a combination of NAA, IBA and Kinetin (Fig. 5). MS medium supplemented with NAA- 1 mg/l and IBA -2.0 mg/l supported maximum root production in all the three groundnut cultivars with AW and S1R having 100% rooting gains. Serenut 4T recorded 70% rooting (Fig 5). Overall rooting percentage was higher in S1R (Virginia botanical cultivar) than in the AW (Valencia) and S4T (Spanish). Virginia-type groundnuts shoots have a natural capacity to form adventitious roots in medium even without plant growth regulators [46].

Table 4. Kruskal-Wallis one-way analysis of variance for ideal callus formation

Sample	Size	Mean rank
Group AW_5	10	127.45
Group AW_10	10	69.00
Group AW_20	10	69.00
Group AW_30	10	69.00
Group AW_40	10	69.00
Group AW_50	10	69.00
Group AW_CIM	10	185.90
Group AW_N6	10	145.35
Group S1R_10	10	95.85
Group S1R_20	10	148.00
Group S1R_30	10	195.90
Group S1R_40	10	168.05
Group S1R_5	10	69.00
Group S1R_50	10	132.70
Group S1R_CIM	10	69.00
Group S1R_N6	10	69.00
Group S4T_10	10	136.40
Group S4T_20	10	133.80
Group S4T_30	10	117.45
Group S4T_40	10	69.00
Group S4T_5	10	166.90
Group S4T_50	10	69.00
Group S4_CIM	10	220.50
Group S4_N6	10	227.75

Degrees of freedom = 23; Chi-square probability <0.001; Key: AW=Acholi white; S1R= Serenut 1 Red, S4T= Serenut 4 Tan

Fig. 3. S1R growth on N6 media

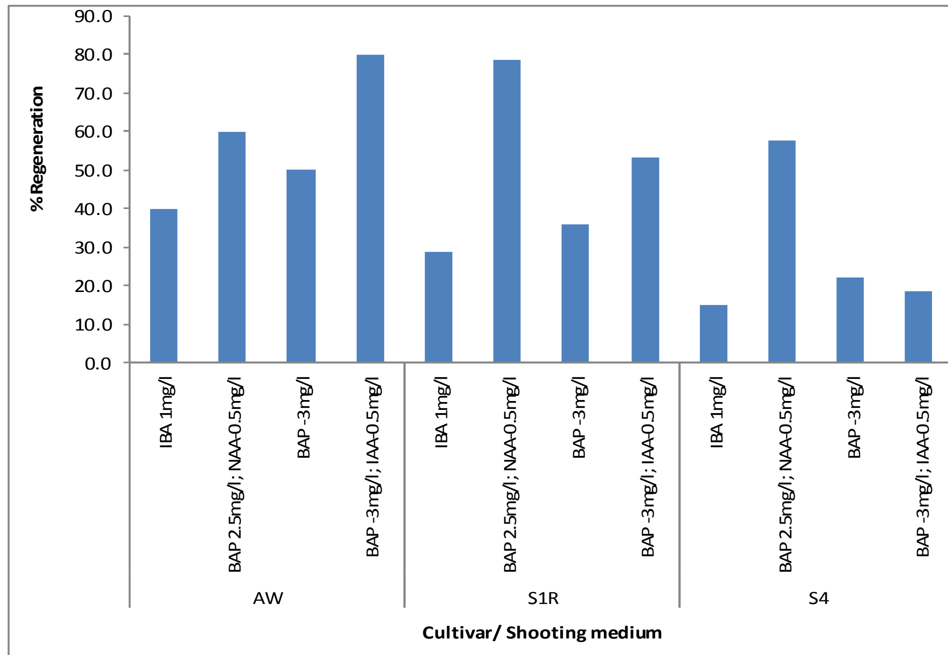

Fig. 4. Response of the cultivars to shooting media

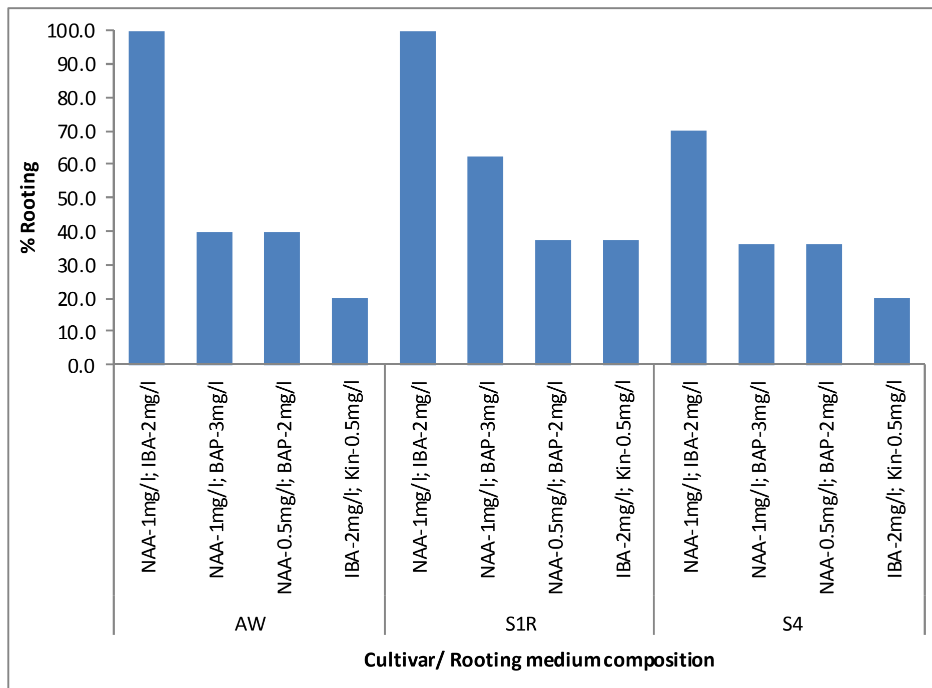

Fig. 5. Response of the cultivars to rooting media

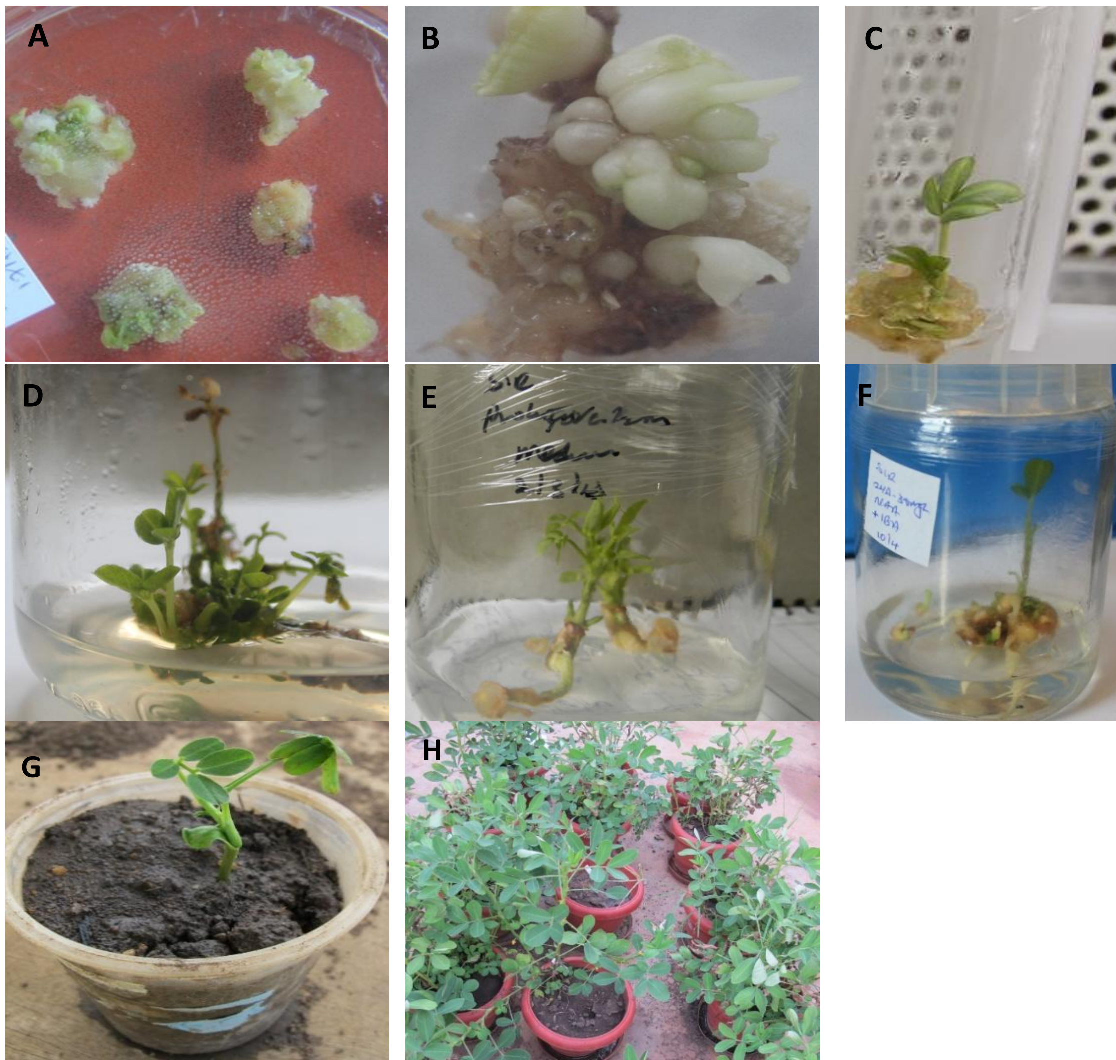

Fig. 6. Multiple shoots and root developmental steps from embryo explants
(A) Embyogenic callus formed after incubating the embryo explant (B) Leaf shoot primordia growing from ideal callus (C) Fully formed leaflets growing on MS medium supplemented with BAP-3 mg/l and NAA-0.5 mg/l (D) Shoot cluster proliferated and maintained on MS medium supplemented with BAP-3mg/l and NAA-0.5 mg/l. (E) Elongated shoot on rooted on MS medium supplemented with NAA- 1mg/l and IBA -0.5 mg/l. (F) Rooted shoot on MS medium containing 4.95 mM NAA. (G) Tissue culture plant acclimatizing in a plastic pot. (H) Well adapted and matured plants in the glasshouse

The mature embryo axes explants responded well and gave satisfactory somatic embryogenesis (Fig. 5). The somatic embryos could be sub-cultured several times, and in turn proliferated to form clusters of secondary embryos upon transfer to fresh medium.

However, the somatic embryos that were sub-cultured only once gave the best regeneration of plantlets in subsequent tests (Okello, unpublished). The *in vitro* regeneration varied considerably among the groundnut botanicals. It has been reported that *in vitro* regeneration competence varied considerably among various species of a genus or among cultivars within the same species [36]. Such variable response of different varieties in culture might be due to their differential genomic constitution and different levels of endogenous plant growth regulators (PGRs) within the explants [37]. Lastly, the effect

of plant growth regulators not only depends on the concentration applied but also on its interaction with the endogenous growth regulators [47].

Well-developed rooted plants (Fig. 6F) were taken out from jars and transferred into plastic pots (Fig. 6G and H) for hardening in the glasshouse. The regenerated plants showed normal growth, survival and fruit set in glasshouse.

4. CONCLUSION AND RECOMMENDATIONS

Crop improvement by conventional breeding in groundnuts is not as rapid as envisaged to meet the demands of increasing population, especially in seed quality improvement and developing virus and insect-resistant cultivars. Tools of genetic engineering such as pathogen derived resistance can be exploited as an additional method for introduction of valuable traits into established cultivars. However, efficient regeneration of highly totipotent cells is an essential prerequisite of genetic engineering systems. This study optimized regeneration protocol for above three groundnut cultivars in Uganda representing the Spanish, Virginia and Valencia botanicals.

In this study we successfully regenerated complete plantlets from the explants of groundnut via embryogenesis. Groundnut cultivars showed significant divergence for their *in-vitro* response to ideal callus induction and the subsequent regeneration suggesting that genetic factors are primordial in the determination of *in-vitro* tissue culture response level [36]. Different concentration of 2, 4-D elicited different responses in the cultivars to callogenesis with Acholi white (Valencia botanical) and Serenut 4T (Spanish botanical) giving the best response at 5mg/l whereas Serenut 1R (Virginia botanical) showed best response at a concentration of 30 mg/l. Given the availability of the three MS media therefore we can deduce that in the presence of 2, 4-D alone, Serenut 1R at a concentration of 30 mg/l and both Serenut 4T and Acholi white at 10mg/l (or even lower) are suitable for ideal callus formation. Media N6 and CIM only support callogenesis and regeneration of AW and S4T cultivars.

When all conditions are optimal for the cultivars' regeneration, peak ideal callus formation takes 42 days from initiation in three media tested. Maximum root production was gained when using MS medium supplemented with NAA- 1 mg/l and IBA -2.0 mg/l. On the other hand, for Serenut 1R and Serenut 4T, BAP 2.5 mg/l; NAA 0.5 mg/l combination yielded higher shoot regeneration percentage whereas for Acholi white BAP 3 mg/l; NAA 0.5mg/l supported maximum shoot production.

The usages of mature dry seeds as explant source guarantee year-round availability of explants for continuous research thereby accelerating the progress of groundnut breeding programmes.

The tissue culture protocol described in the current study promoted regeneration from embryos of all genotypes tested, although the frequencies varied in terms of ideal callus production, root regeneration and number of shoots per explant. This protocol could be improved further to increase the percentage of the regeneration with the potential of enhancing exogenous gene transfer through genetic engineering in the near future. This paper therefore reports the first micro-propagation procedure for groundnuts of the three main botanical classifications in Uganda.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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