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An optimized protocol for high frequency regeneration of selected groundnut (Arachis hypogaea L) varieties from East Africa using cotyledons

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ABSTRACT: An efficient protocol for regenerating high yielding groundnut varieties; ICGV-CG2 and CG2 through direct organogenesis has been optimized using 6-benzylaminopurine and 2,4-dichlorophenoxyacetic acid. Murashige and Skoog medium with B5 vitamins was supplemented with individual treatments of BAP and 2,4-D and with their combinations to induce shoot bud formation. Preliminary results showed no shoot formation on media with the individual plant growth regulators. The highest and lowest regeneration frequencies in CG2 were 93.33% and 36.67% respectively on medium supplemented with 1 mg/L 2,4-D and 4.5 mg/L BAP. In ICGV-CG2 the highest frequency was 90% on media with 1.5 mg/L 2, 4-D and 4.5mg/L BAP with a low of 46.67%. Three (3) to ten (10) shoots were recorded per explant, with a maximum of ten shoots recovered from the best performing plant growth regulator regimes. Shoots elongated on MS with reduced BAP levels (0.75-4.5mg/L), rooted on media with α-naphthalene acetic acid and later acclimatized in the glasshouse on peat moss. Hardening and growth of plants to maturity was achieved in the glasshouse on a sand-soil mixture (1:1 w/w) in pots. In vitro induction of flowers in one of the explants could have been as a result of somaclonal variation. This work has developed an optimized protocol that sets up a platform for improvement of these varieties that are adapted to East Africa with novel genes through genetic transformation
Keywords: Direct organogenesis; genotype dependent; optimized protocol; somaclonal variation; vapor-phase sterilization.
Abbreviations: 2,4-D; 2, 4-dichlorophenoxyacetic acid, BAP; 6-benzylaminopurine, MS; Murashige and Skoog, NAA; α-naphthalene acetic acid, PGR; Plant growth regulator, SEM; Shoot elongation medium, SIM; Shoot induction medium, RIM; Root induction medium.

INTRODUCTION

Groundnut, also known as peanut (Arachis hypogaea L) is an important leguminous food crop (Sharma and Anjaiah, 2000). It is cultivated in over 100 countries worldwide on over 24 million hectares of land with a total production of 38 million tons (FAOSTAT, 2010). Groundnut seed contains about 43-55% oil and 25-28% protein which are important to human and livestock nutrition (Gohari and Niyaki, 2010). Groundnut also contributes to income generation to farmers through trade, provides fodder for livestock and helps improve soil fertility through nitrogen fixation (Giller et al., 2002). The crop is affected by many biotic and abiotic stresses such as soil fertility issues, prevalence of weeds and pests and diseases that cause major production losses in terms of quality and quantity (Pandey et al., 2012). Contamination of groundnut by fungi which produce aflatoxins that cause spoilage in the field or during storage is a major food security concern (Bankole et al., 2006).

Genetic transformation could introduce genes in groundnuts that could provide a solution to these problems (Anuradha et al., 2006). However, as in other crops the lack of an efficient and reproducible regeneration protocol makes improvement via genetic engineering a challenge. Regeneration of groundnuts from various tissues such as leaf discs, cotyledons, cotyledonary nodes, hypocotyls, epicotyls and embryos has been reported (Kim et al., 2003;

Anuradha et al., 2006; Iqbal et al., 2011). However, the frequencies of plants regenerated have been low and this limits rapid concept testing of candidate genes targeted for introduction via genetic transformation. Groundnut regeneration is genotype dependent and therefore optimization of an efficient in vitro regeneration protocol for specific genotypes is imperative to their micro propagation and transformation (Iqbal et al., 2011).

Direct organogenesis using cotyledons as explants is preferred since shoot regeneration occurs at the cut edges and this may allow easy transfer of genes of interest via Agrobacterium-mediated transformation into the tissues. Regeneration protocols for groundnuts adapted to Eastern and Southern Africa have only been reported for a handful of genotypes hence the importance of optimizing a protocol for the other genotypes (Maina et al., 2010). This study therefore aimed at regenerating var. ICGV-CG2 and var. CG2 via direct organogenesis using varying concentrations of BAP and 2,4-D.

MATERIALS AND METHODS

Plant material and explant preparation

Mature groundnut seeds var. ICGV-CG2 and var. CG2 used in regeneration were obtained from Dr. Santie de Villiers of ICRISAT. MS salts (Murashige and Skoog, 1962) with B5 vitamins (Gamborg et al., 1968) were used in all experiments with SIM supplemented with different concentrations of BAP (1.5-9 mg/L) and 2,4-D (1, 1.5 and 2 mg/L). Seeds were first washed with tap water for 20 minutes to remove adherent particles and excess water blotted off before vapor sterilization using a local bleach (Jik) containing 3.85% m/v sodium hypochlorite (NaOCl) with 3 ml concentrated hydrochloric acid (HCl) in a dessicator for 5 hours according to Clough and Bent, (1999). The seeds were washed 3 times and then soaked in sterile distilled water for 2 hours before use. The embryo was surgically excised off following the removal of the seed coat, and the cotyledons dissected vertically into halves to obtain the explants. Explants were cultured on SIM in such a way that the cut edges were in contact with the medium.

Organogenesis

The SIM comprised 4.4 g/L MS salts with B5 vitamins (Duchefa), 30 g/L sucrose, pH adjusted to 5.8 before adding 8 g/L plant agar prior to autoclaving at 121ºC for 20 minutes. The medium was supplemented with appropriate combinations of filter sterilized BAP and 2,4-D before dispensing into sterile petri plates. Ten (10) explants were cultured per petri plate, sealed with parafilm and incubated at 27±1ºC in the growth room under 16/8 hour photoperiod from fluorescent lamps (100 mEs^{-1} m^{-2}). SEM comprised MS salts with B5 vitamins, 30 g/L sucrose, 8 g/L plant agar and reduced levels of BAP (0.75-4.5 mg/L). Shoot buds were transferred onto the SEM after 14 days for 2 to 3 passages of 21 days each. Elongated shoots were rooted on RIM containing 4.4 g/L MS salts with B5 vitamins, 30 g/L sucrose, 8 g/L plant agar at pH 5.8 supplemented with NAA (0.5-1.5mg/L). Rooting plants were maintained on the RIM for 28 days. Plants with well developed roots were transplanted into plastic pots containing autoclaved peat moss and maintained at 25ºC in a glasshouse and later transferred to bigger pots with sand-soil (1:1) mixture and hardened for 10 days with irrigation using tap water.

Data analysis

Three (3) replicates were used for each treatment with ten (10) explants cultured per petri plate. Analysis of the data was done according to Tuky's test using SAS version 9.1.3.

RESULTS

When cotyledons from mature groundnut seeds were inoculated on MS medium with B5 vitamins containing different combinations of BAP and 2,4-D, they showed direct shoot bud formation within 14 days. Within 3-7 days of culture initiation, the explants considerably enlarged in size and turned green (Figure 1A). This was later followed by multiple shoot differentiation at the proximal cut end of the explants in all treatments although the frequency varied across the PGR combinations.

Among the various BAP and 2,4-D concentrations tested, the highest shoot induction frequency was obtained following culture of explants on MS medium supplemented with 4.5 mg/L BAP and 1 mg/L 2,4-D and 4.5 mg/L BAP in combination with 1.5 mg/L 2, 4-D for CG2 and ICGV-CG2 respectively (Figure 2). A preliminary finding in this study had earlier shown no shoot bud formation on media supplemented with either of the PGRs separately. A maximum of 93.33% and 90% of explants in var. CG2 and ICGV-CG2 respectively showed direct shoot bud formation. The lowest frequencies were 36.67% and 46.7% for CG2 and ICGV-CG2 respectively.

The average number of shoots recovered from each explant ranged from 3 to 10 although more shoots could be obtained if the explants were transferred and maintained on SEM for two or more extra sub-cultures. Further, the number of shoots per explant was highest on PGR regimes that produced the best frequencies of regeneration with both varieties having a maximum of 10 shoots recovered per explant (Table 1).

Interestingly, however, was the presence of flowers in vitro from one of the explants in var. CG2 on SEM (Figure 1F).

Successful induction of roots in the elongated shoots was achieved using MS with B5 vitamins supplemented with varying concentrations of NAA. Root buds were first observed appearing as a cluster 14 days after culture on the RIM. Most shoots (except in media without NAA) developed roots which elongated and were fully developed within 25 days. Among the treatments tested, 1mg/L NAA produced the highest frequency of root induction in both varieties (Table 2). Rooted plants were hardened in the glasshouse on peat moss and later allowed to develop to maturity in the greenhouse on a sand-soil (1:1) mixture. The shoots were healthy, appeared phenotypically normal and later produced many pods with viable seeds (Figure 1H).

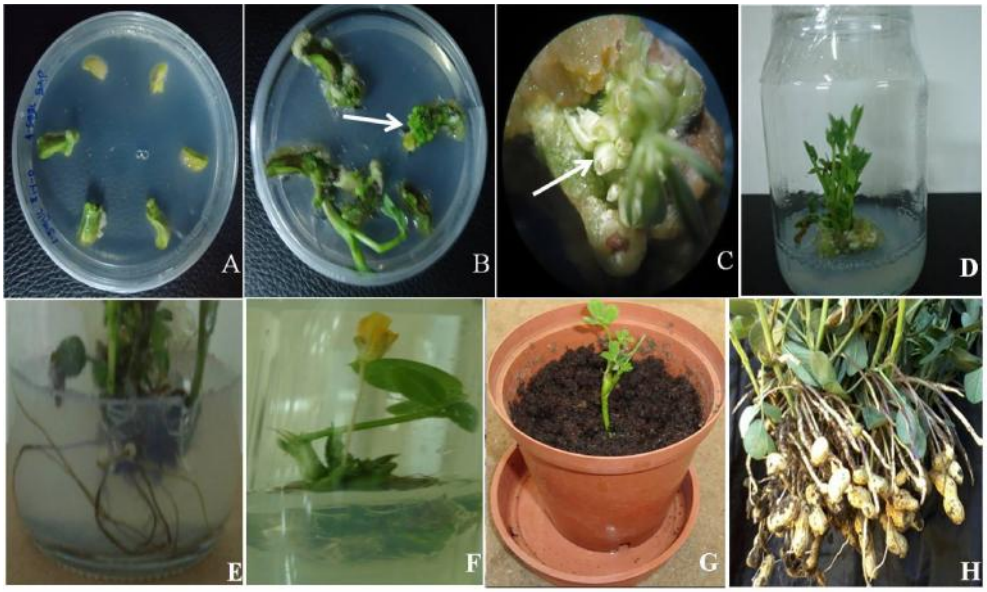

Figure. 1 Developmental stages of groundnuts through direct organogenesis; (A) Swelling and greening of explants 7 days after culture on SIM (B) Differentiation of shoot buds at the proximal cut end 14 days after culture (C) Shoot induction as seen under the microscope. Arrows indicate multiple shoots (D) Developed shoots on SEM (E) Elongated roots on media supplemented with NAA (F) In vitro induction of flowers on SEM observed in var. CG2 (G) Acclimatization of developed plants using a plastic bag (H) Mature groundnut with fully developed pods.

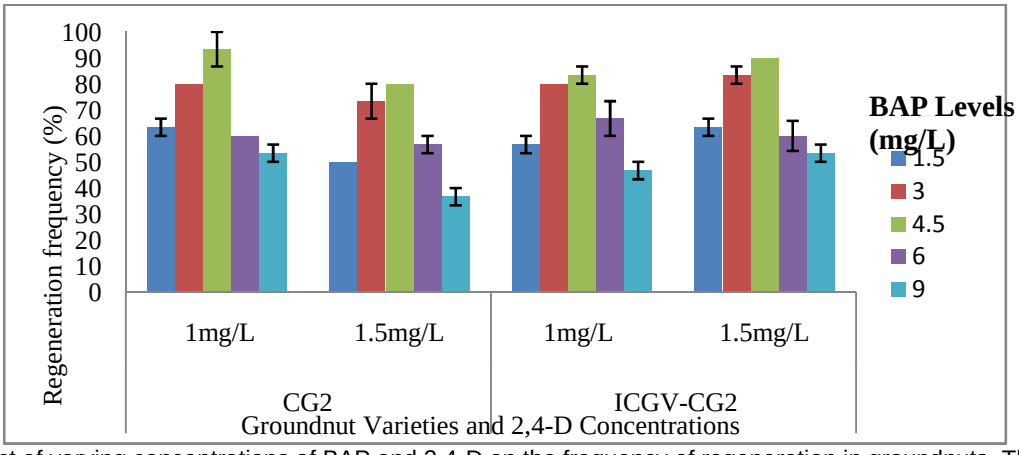

Figure 2. Effect of varying concentrations of BAP and 2,4-D on the frequency of regeneration in groundnuts. The number of shoots was recorded 3 weeks after transfer to SEM. Data presented is the mean of 3 replicates of 10 explants each according to Tuky's test at ($p \leq 0.05$).

Table 1. Effect of 2, 4-D and BAP on the average number of shoots/explant in groundnuts.

Concentration of BAP (mg/L)	No of explants cultured	Average no. of shoots per explant			
		1mg/L 2, 4-D		1.5mg/L 2, 4-D	
		CG2	ICGV-CG2	CG2	ICGV-CG2
1.5	30	1.33±0.3^{b¶}	1.33±0.3^{a}	1.33±0.3^{a}	1.33±0.3^{b}
3	30	2.33±0.3^{ab}	2.67±0.3^{a}	1.67±0.3^{a}	2.67±0.3^{ab}
4.5	30	3.33±0.3^{a}	3.00±0.6^{a}	2.33±0.3^{a}	3.33±0.3^{a}
6	30	1.67±0.7^{ab}	2.33±0.7^{a}	1.33±0.7^{a}	1.67±0.3^{b}
9	30	1.33±0.3^{b}	1.00±0.6^{a}	1.00±0.0^{a}	1.33±0.3^{b}

Shoots were counted after the second subculture (5th) week on SEM. ¶: Means followed by the same letter in each column are not significantly different according to Tuky's test (p≤0.05).

Table 2: Effect of NAA on the frequency of root induction and on the number of roots per shoot in groundnuts.

NAA (mg/L)	Rooting frequency (%)		Average roots/shoot
	CG2	ICGV-CG2	
0.5	63.3±3.3^{b¶}	66.7±6.7^{b}	4.7±0.6^{bc}
1.0	93.3±8.2^{a}	96.7±3.3^{a}	10.1±0.3^{a}
1.5	76.7±3.3^{ab}	83.3±3.3^{ab}	5.8±0.3^{b}

Data presented is the mean of 3 replicates of 10 explants each. ¶: Means followed by the same letter in each column are not significantly different according to Tuky's test at (p≤0.05).

DISCUSSION

A high frequency regeneration protocol for ICGV-CG2 and CG2 groundnut varieties has been optimized using cotyledons derived from mature seeds. Maina et al., (2010) were first to report regeneration of groundnuts adapted to eastern and southern Africa but only a handful of these varieties were reported. Various explants have been used in the past to regenerate different groundnuts via organogenesis (Palanivel et al., 2001; Palanivel and Jayabalan, 2002; Tiwari and Tuli, 2008; Bhatnagar, 2010; Srinivasan et al., 2010). Our work has successfully added to this body of knowledge using these groundnut varieties adapted to east Africa.

The rapid shoot bud formation from the explants (2-3 weeks) and the high frequencies shown by our results are ideal for any regeneration process. Just like in this study, Iqbal et al, (2011) regenerated groundnuts from Pakistan using a combination of BAP and other PGRs and obtained a frequency of 87%. In the present study, we found out that groundnut regeneration via direct organogenesis increased with an increase in BAP (in combination with 1 and 1.5mg/L 2,4-D) in both varieties up to 4.5 mg/L, then decreased with further increase in the cytokinin (Figure 2). This was shown by the percentage mean numbers of explants shooting and the average number of shoots per explant which also increased with increase in BAP. The average number of shoots per explant was also highest in treatments that had the highest percentage of shoot bud regeneration. Higher levels of 2,4-D however led to callus formation with low regeneration frequencies via direct organogenesis.

The effect of cytokinins in shoot bud formation has been extensively reported in regeneration of groundnuts and other plant species (Palanivel and Jayabalan, 2000; Kakani et al., 2009; Verma, 2009). We observed that combining an auxin and a cytokinin at an appropriate concentration is essential for induction of shoots which was consistent with other studies. Our data on shoot bud formation from the two groundnut varieties varied across the treatments. These results demonstrate that groundnut genotypes as well as the concentrations of PGRs play an important role in regeneration. Shoot elongation was achieved using lower levels of BAP for 2-3 passages indicating the importance of the cytokinin in shoot elongation.

Results from this study also indicate that addition of NAA to RIM allowed differentiation and elongation of roots which is consistent with other reported studies (Verma 2009; Iqbal et al., 2011). The highest frequency of rooting was achieved on medium with 1mg/L NAA where 93.3% and 96.7% of shoots in CG2 and ICGV-CG2 respectively formed within the first two weeks and this concentration was significantly different from the others (Table 2). The average number of roots per shoot was highest in the media with highest root induction frequency

indicating the importance of 1 mg/L NAA in root development. Presence of flowers in vitro in CG2 was an interesting phenomenon which could have been due to somaclonal variation due to the use of the PGRs. We also reported vapor-phase sterilization for the first time in groundnuts. This method of effectively sterilizing plant material for in vitro cultures has been reported in other plants including Arabidopsis thaliana (Clough and Bent, 1999). It proved very effective and should therefore be used in Arachis.

CONCLUSION

Cotyledons from mature groundnut seeds show a high potential for regeneration via direct organogenesis using the appropriate combination of BAP (4.5 mg/L) and 2,4-D. This optimized protocol is therefore recommended for regenerating ICGV-CG2 and CG2 groundnut varieties adapted to east Africa since it is efficient and reproducible. The protocol, if adopted has a potential for integration in genetic improvement programs of these two varieties through genetic engineering with novel genes. The use of cotyledons in regeneration will also have an advantage over other explants during transformation since cells that are susceptible to Agrobacterium infection are easily accessible.

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