Reprint **ISSN 1923-7766 (Web Version)**

International Journal of Experimental Agriculture

(Int. J. Expt. Agric.)

Volume: 10	**Issue: 1**	**January 2020**

Int. J. Expt. Agric. 10(1): 26-37 (January 2020)

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ISSN-1923-7766 (Online)
Int. J. Expt. Agric. 10(1):26-37(January 2020)

IN VITRO CALLUS DEVELOPMENT AND PLANT REGENERATION FROM IMMATURE COTYLEDONS-THE BEST TECHNOLOGY FOR SUSTAINABLE GROUNDNUT (*Arachis hypogaea* L.) PRODUCTION IN THE MODERN ERA

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Accepted for publication on 6 January 2020

ABSTRACT

Debsharma SK, Roy KK, Ghosh SR, Razzak MA, Nath UK, Azad AK (2020) *In vitro* callus development and plant regeneration from immature cotyledons- the best technology for sustainable groundnut (*Arachis hypogaea* L.) production in the modern era. *Int. J. Expt. Agric.* 10(1), 26-37.

Groundnut (*Arachis hypogaea* L.) is an important oilseed and protein-rich crop in the world. Plant regeneration system from explants through the application of the biotechnological approach is the best technology in the modern era. Therefore, callus induction and organogenesis from different groundnut explants were studied on MS medium supplemented with different concentrations and combinations of 2,4-D, BAP, Kn, IBA and NAA hormones. Different types of explants *viz*., cotyledon, epicotyl and hypocotyl have been used by various researchers worldwide to study *in vitro* plant regeneration but in our study, we used immature cotyledon. Four groundnut genotypes such as GC(1)-35-1, GC(1)-4, GC(1)-3-2-1 and Zhingabadam were used as plant materials. The experiment was conducted in the tissue culture laboratory of the Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh-2202 during the periods of 2010 to 2012. Our results demonstrated a wide range of variations in callus induction under different hormonal concentrations and combinations. Among the chemicals, applications of 2,4-D was found as the best hormone for callus induction in alone or in combination with kinetin. The maximum (75%) proliferated callus development was appeared on in combination of MS + 1.5 mg/L 2,4-D + 0.5 mg/L BAP. The highest number of shoot regeneration (61.53%) were observed when MS medium supplemented with 1.5 mg/L BAP + 1.0 mg/L NAA for genotype GC(1)-35-1 while the maximum percentage of roots induction (75%) was observed in combinations of half MS + 0.5 mg/L IBA from previously regenerated shoots. Among the four genotypes, the explants GC(1)-35-1 was successfully established in soil with the highest (75%) rate of survival and it finally yielded with flowering in the earthen pots.

Key words**:** *groundnut, immature cotyledons, in vitro, hormones, plant regeneration*

INTRODUCTION

The oilseed crop *Arachis hypogea* L. (groundnut/peanut) belongs to the family Leguminosae and sub-family Papillionacea. It is one of the most important oil- and protein-producing legume of the world and holds 13th position among other food crops (Varnell and Mc-Cloud, 1975). In Bangladesh, it occupies 3rd place in terms of area of production, however, considering of yield it ranked first among the other oilseed crops *viz.*, mustard, sesame, sunflower and linseed. Groundnut seed contains around 50% oil and 25-30% protein which can be used as a meal for food and feed (Ahmad and Rahim, 2007). Regarding the nutritional aspect, it is better than mustard oil. The chemical composition of essential amino acid such as linoleic acid is higher in groundnut than mustard.

Groundnut is a very important oilseed crop in Bangladesh. It mostly used as edible oil, to craft cake, bakery and biscuit in several food industries. Traditionally it is eaten as fried 'badam' and oil cake is used for cattle feeding. Bangladesh imports groundnut oil from abroad and shelled groundnut on a usual basis. The climatic conditions and soil status of Bangladesh are fairly appropriate for the production of groundnut. It is cultured typically in sandy soils and river beds (Nath and Alam, 2002). So, there is a huge scope for increasing its production. To improve its agronomic traits, conventional breeding is not much suitable in the current era for releasing a high yielding variety. Besides this, sexual breeding techniques are not worthy.Therefore, the regeneration of plants using tissue culture approaches is an important and essential way in biotechnological research and sometimes it also required for the genetic manipulation. According to Scowcroft *et al*. (1987), tissue culture techniques can play an important role for the enhancement of inherent variability giving rise to variations/mutations in a higher rate and might be a narrative source of genetic variability in many crop species. The techniques of plants tissue culture have been developed as a new and powerful tool for crop improvement. High frequency of plants from in vitro cultured tissue is a pre-requisite for successful application of tissue culture technique in crop improvement. But callus differentiation and morphogenesis is difficult because it is considered extremely recalcitrant seed. Now-a-days, plant tissue culture techniques have emerged as a worldwide accepted concept (Haque 2003). Widespread research has been done in recent decades to develop the methods for in vitro plant regeneration and somatic embryogenesis of peanut crop using various explants (cotyledonary nodes, de-embryonated cotyledons) and media combinations in different countries (Tiwari and Tuli, 2008; Radhakrishnan *et al.* 2000; Akasaka *et al.* 2000; Little *et al.* 2000; Ozias-Akins *et al.* 1992). Recently, Tiwari and Tuli (2009), developed a protocol using leaflet explants and obtained a higher rate of

shoot regeneration efficiency (80%). Numerous protocols of in vitro groundnut regeneration have been reported by many researchers in the last decade. Based on the above information, the present study has been aimed to regenerate groundnut plant from immature cotyledons of four groundnut cultivars to see their in-vitro regeneration efficiency and approval the process is the best technology for sustainable groundnut (*Arachis hypogaea* L.) production in the modern era.

MATERIALS AND METHODS

Experimental location and plant materials

The experiment was conducted in the tissue culture laboratory of the Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh-2202 and conducted during the periods of 2010 to 2012. The plant materials (immature cotyledons) were collected from the organization Bangladesh Institute of Nuclear Agriculture (BINA), BAU Campus, Mymensingh-2202. The following local cultivars (Table 1) of groundnut (*Arachis hypogaea* L.) were used in the present investigation to see their in-vitro regeneration efficiency.

In the present study following cultural methods were practiced or used depending on specific purposes. These are mentioned below:

a) Immature cotyledon was taken after peg formation and sterilized with 70% alcohol
b) For callus inductions and shoot differentiation
   (i) MS (Murashige and Skoog, 1962) medium
c) For root induction
   (i) MS (Murashige and Skoog, 1962) medium
   (ii) Half-strength of MS (Murashige and Skoog, 1962) medium
d) For watering of the plantlets after their transplantation from culture vessel to the soil
   (soil containing 50% soil + 50% sand)

Table 1. Name of the local groundnut cultivars used in the study

Sl No.	Name of the genotypes	Chromosome number
i.	*Arachish ypogaea* L. var. Zhingabadam	2n=20
ii.	*Arachis hypogaea* L. var. GC(1)-35-1	2n=20
iii.	*Arachis hypogaea* L. var. GC(1)-4	2n=20
iv.	*Arachis hypogaea* L. var. GC(1)-3-2-1	2n=20

Table 2. Constituents of stock solution for MS medium

Constituents	Concentration(mgL^{-1})	Concentration(gL^{-1})
a) Macro nutrients (10x)		
KNO_3	1900.00	19.0
NH_4NO_3	1650.00	16.5
KH_2PO_4	170.00	1.70
$CaC1_2.2H_2O$	440.00	4.40
$MgSO_4.7H_2O$	370.00	3.70
b) Micro nutrients (100x)		
$MnSO_4.4H_2O$	22.30	2.23
H_3BO_3	6.20	0.62
$ZnSO_4.7H_2O$	8.60	0.86
KI	0.83	0.083
$Na_2MoO_4.2H_2O$	0.25	0.025
$CuSO_4.5H_2O$	0.025	0.0025
$CoC1_2.6H_2O$	0.025	0.0025
c) Iron source (10x)		
$FeSO_4.7H_20$	27.80	0.278
Na_2-EDTA	37.30	0.373
d) Organic nutrients (100x)		
Glycine	2.00	200.0
Nicotinic acid	0.50	50.0
Pyridoxine-HCI	0.50	50.0
Thiamine-HC1	0.10	10.0
Myo-inositol	100.0	-

Preparation of stock solutions

The first step for the preparation of MS medium was the preparation of stock solutions. Various constituents (Table 2) of the medium were prepared into stock solutions to accelerate its preparation. Separate stock solutions were prepared and used for macronutrients, irons, vitamins, growth regulators etc. The stock solutions were as-

1. Stock solution A (macro-nutrients)

These macronutrients were made at 10 folds (10x) oftheir final strength and dissolved in 1000 ml of distilled water. Ten times the weight of salts was weighed accurately and dissolved it in 750 ml of distilled water, and the final volume was made up to 1000 ml by further addition of distilled water. This stock solution was filtered and poured into a clean brown bottle and stored in a refrigerator at 4°C for later use.

2. Stock solution B (micro-nutrients)

These micronutrients were made at 100 folds (100x) of their final strength and dissolved in 1000 ml of distilled water (DW) with received salts. The stock solution was filtered, labelled and stored in a refrigerator at 4°C for after-wards use.

3. Stock solution C (iron source)

This solution was prepared at 10 folds (10x) and the final strength of $FeSO_4.7H_2O$ and Na_2-EDTA dissolved in 100 ml of distilled water and chelated by heating on a hot plate stirrer and then the volume was made up to 1000 ml by further addition of distilled water. Finally, the stock solution was filtered and stored in a refrigerator at 4°C for later use.

4. Stock solution D (vitamins)

Each of the desired ingredients except myo-inositol were taken at 100 folds (100x) of their final strength in a measuring cylinder and dissolved in 400 ml ofdistilled water. The solution was dispensed into 10 ml aliquots and stored at -20°C. Myoinositol has been used directly during the time of media preparation.

5. Hormonal stock solutions

Different hormonal supplements were used in the present studies which are mentioned below-

- Auxins: Indole-3 acetic acid (IAA)
- Indole butyric acid (IBA)
- 2, 4 dichlorophenoxyacitic acid (2,4-D)
- α-napthalene acetic acid (NAA)
- Cytokinins: 6-benzyl amino purine (BAP)
- 6-furfuryl amino purine (kinetin, Kn)

For preparingthe stock solution of any of these hormones, 10 mg of hormone was taken on a clean watch glass and then dissolved it in 1 ml of particular solvent. The mixture was washed off with distilled water and collected in a 100 ml measuring cylinder and made the volume up to 100 ml by adding distilled water. The solution was then poured into a clean plastic container and stored it at O°C temperature for further use.

One litre of MS medium was prepared by following the below-stated steps:

- Firstly, different constituents *viz.* 100 ml of macronutrients, 10 ml of micronutrients, 100 ml of irons and 10 ml of vitamins were taken from each of these stock solutions into a 2-litre Erlenmeyer flask on a hot plate stirrer.
- Then add 500 ml of distilled water in the flask to dissolve all the ingredients.
- Add 100 mg of Myo-inositol directly to the solution and dissolved vigorously.
- Add 30 gmof sucrose (3%) to this solution and agitatedgently to dissolve completely.
- Different concentrations of hormonal supplements were added to the solution either in a single or in combinations as required and mixed well.
- The p^H of the medium was adjusted to 5.8 with a digital p^H meter with the help of 0.1 N NaOH or 1 N HCl when ever was necessary.
- After adjusting the p^H, 8 gmof Difco-brand Bacto Agar was added to solidify the medium. The mixture was then gently heated with continuous stirring till complete dilution of agar.
- The required volume of hot medium was dispensed into culture vessels or conical flasks. After dispensing the medium, the culture vessels were plugged with cork and non-absorbent cotton bung and then marked each vessel with different codes with the help of a glass marker to indicate specific hormonal combinations.

Sterilization of the medium

The culture vessels were then autoclaved at 15-lbs/sq. inch pressure at 121°C for 20 minutes. After autoclaving, the medium was cooled before going to use. The inoculation of cotyledon into media was carried out in a laminar air flow cabinet by maintaining aseptic condition. The cabinet was switched on for half an hour before use and cleaned inside it with 90% ethyl alcohol to reduce the chances of contamination. Equipments such as scalpels, forceps, needle etc. were sterilized by an alcoholic dip and flaming method inside the inoculation chamber. Other requirements like Petridishes, distilled water and glassware were sterilized by steam autoclave method.

Culture methods for cotyledon regeneration

The soft part of immature cotyledons was used as explants. The attempts have been taken for the induction of embryogenesis and organogenesis using different explants in MS medium supplemented with different hormones. Immature cotyledons were cut off from the small seedlings and inserted into a culture tube. Four cotyledon segments of 2 to 6 mm long were inserted in each culture tube. Sub-culture was done when the calli attained a size of about 20-25 mm in diameter. These calli were removed aseptically from tubes on a sterilized Petridish and were cut into 4 to 5 mm pieces with the help of a sharp sterile scalpel, the pieces were again cultured on freshly prepared medium containing different hormonal supplements for shoot induction from the cells. The sub-culturing media used in the present investigation were MS containing different combinations and concentrations of 2,4-D, NAA, IAA, IBA, Kn, and BAP. After shoot initiation, higher light intensity was supplied for shoot elongation. In some cases, repeated sub-cultures were needed to shoot differentiation. The plantlets transplanted to small pots which containing of garden soil, sands and cow dung in the ratio of 1:2:1. Immediately after transplantation, the plants along with pots were covered with a moist polythene bag to prevent desiccation. To reduce sudden shock, the pots were kept in a controlled environment in the growth chamber. After 2-3 days of incubation, the polythene bags were gradually perforated to expose the plants to the natural environment. The polythene bags were completely removed after 7 to 10 days of staying in the chamber. The plantlets at this stage were placed in the natural environment for 3-10 hrs daily. Finally, after 15-20 days of cultivation, they were transferred to the field condition.

Data collection

To investigate the effect of different treatments following data were collected on the below-mentioned parameters:

a) Percent callus induction

The percentage of callus induction was collected, based on the number of explants placed and the total number of callus induced.

$$\text{Callus induction (\%)} = \frac{\text{No. of explants induced calli}}{\text{No. of explants inoculated}} \times 100$$

b) Percent plant regeneration

The percentage of plant regeneration was calculated based on the number calli transferred to a regeneration medium and the number of calli produced plantlets.

$$\text{Callus regeneration (\%)} = \frac{\text{No. of calli with plantlets}}{\text{No. of inoculated calli}} \times 100$$

c) Percent plant establishment

The percentage of established plants was calculated based on the number of plantlets placed in the pot and the number of plants finally established or survived.

$$\text{Plant established (\%)} = \frac{\text{No. of established plantlets}}{\text{Total no. of plantlets}} \times 100$$

RESULTS

The experiment was conducted with four selected genotypes *viz*., GC(1)-35-1, Zhingabadam, GC(1)-4 and GC(1)-3-2-1 of groundnut. The efficiency of callus induction and their maintenance, organogenesis and plant regeneration of the tested cultivars were observed.

Callus induction

(a) Response of 2,4-D on callus induction

Immature cotyledon of four selected groundnut genotypes was used for callus induction. In all kinds of explants, three different concentrations (1.0, 1.5 and 2.0 mg/L) of 2,4-D were used in MS medium and callus induction was recorded in Table 3.

Table 3. Effect of different concentrations of auxin (2,4-D) in MS medium for callus induction in groundnut genotypes

Explants			Immature Cotyledon		
Genotypes	Supplements (mg/L) 2,4-D	No. of explants inoculated	No. of explants showing callus induction	Callus induction (%)	Days to callus induction
GC(1)-35-1	1.0	12	8	66.67	7-8
	1.5	12	6	50.00	7-8
	2.0	12	5	41.67	6-8
Zhingabadam	1.0	12	7	58.33	6-7
	1.5	12	6	50.00	6-7
	2.0	12	4	33.33	4-6
GC(1)-4	1.0	12	6	50.00	5-6
	1.5	12	3	25.00	5-6
	2.0	12	7	58.33	6-7
GC(1)-3-2-1	1.0	12	6	50.00	6-7
	1.5	12	4	33.33	5-6
	2.0	12	5	41.67	6-7

Immature cotyledon culture

Immature cotyledons of groundnut genotypes were cultured in MS medium which supplemented with 2,4-D at different concentration of 1.0, 1.5 and 2.0 mg/L. Callus induction frequency was found highest (66.67%) at 1 mg/L concentration of 2,4-D in genotype GC(1)-35-1 followed by Zhingabadam (58.33%). The concentration of 1.5 mg/L and 2.0 mg/L showed the lowest frequency (33.33%) of callus induction in genotypes GC(1)-3-2-1 and Zhingabadam respectively. The initiation of callus from immature cotyledon of GC(1)-35-1 with MS medium + 1.0 mg/L of 2,4-D showed with the Fig. 1a.

(b) Response of BAP on callus induction

Three different concentration of BAP (1.0, 1.5 and 2.0 mg/L) were used with MS medium and callus induction frequency was observed on immature cotyledon in groundnut genotypes (Table 4).

Immature cotyledon culture

Immature cotyledons of groundnut genotypes were cultured in MS medium which supplemented with different concentration of 1.0, 1.5 and 2.0 mg/LBPA solutions. The highest (66.67%) ratio of callus induction was observed when cultured on MS medium with the supplement of 1.5 mg/L and 1.0 mg/L BAP solutions in genotypes GC(1)-35-1 and GC(1)-4 respectively while the lowest (33.33%) frequency of callus induction was exhibited with the concentration of 2.0 mg/L BAP by GC(1)-3-2-1 cultivar. A clear image of callus initiation from immature cotyledon of GC(1)-4 with MS medium + 1.5 mg/L of BPA hormones expressed with Fig. 1b.

Table 4. Effect of different concentrations of Cytokinin (BAP) in MS medium for callus induction in groundnut genotypes

Explants			Immature Cotyledon		
Genotypes	Supplements (mg/L) BAP	No. of explants inoculated	No. of explants showing callus induction	Callus induction (%)	Days to callus induction
GC(1)-35-1	1.0	12	6	50.00	8-9
	1.5	12	8	66.67	7-8
	2.0	12	7	58.33	10-11
Zhingabadam	1.0	12	7	58.33	8-9
	1.5	12	5	41.67	8-9
	2.0	12	6	50.00	10-12
GC(1)-4	1.0	12	8	66.67	7-8
	1.5	12	8	65.67	7-9
	2.0	12	5	41.67	9-10
GC(1)-3-2-1	1.0	12	6	50.00	7-9
	1.5	12	7	58.33	8-9
	2.0	12	4	33.33	9-11

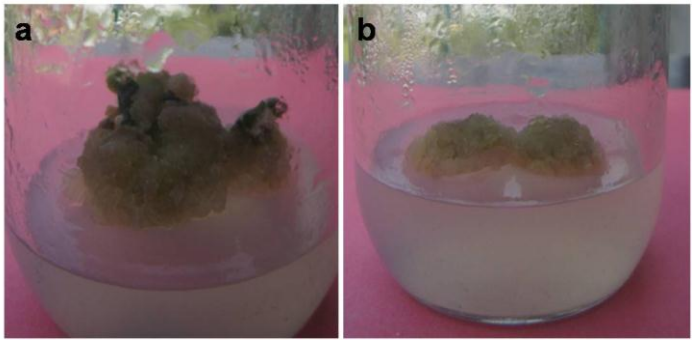

Fig. 1. Callus induction from immature cotyledons of groundnut genotypes; (a) initiation of callus from immature cotyledon of GC(1)-35-1 with MS medium + 1.0 mg/L of 2,4-D, and (b) callus initiation from immature cotyledon of GC(1)-4 with MS medium + 1.5 mg/L of BPA hormones

(c) Response of groundnut genotypes with combined/single concentrations of of 2,4-D and BAP on callus induction

Different concentrations of 2,4-D (1.0, 1.5 and 2.0 mg/L) and a fixed concentration of BAP (0.5 mg/L) were used for callus induction on different explants in groundnut genotypes (Table 5).

Table 5. Effect of different concentrations of auxin (2,4-D) and a fixed concentration of cytokinin (BAP) for callus induction in groundnut genotypes

Genotypes	Explants			Immature Cotyledon		
	Supplements (mg/L)		No. of explants inoculated	No. of explants showing callus induction	Callus induction (%)	Days to callus induction
	2,4-D	BAP				
GC(1)-35-1	1.0		12	6	50.00	8-9
	1.5	0.5	12	8	66.67	9-11
	2.0		12	7	58.33	6-7
Zhingabadam	1.0		12	7	58.33	8-9
	1.5	0.5	12	5	41.67	7-9
	2.0		12	6	50.00	8-9
GC(1)-4	1.0		12	8	66.67	9-11
	1.5	0.5	12	8	66.67	6-7
	2.0		12	5	41.67	8-10
GC(1)-3-2-1	1.0		12	6	50.00	8-9
	1.5	0.5	12	7	58.33	6-7
	2.0		12	4	33.33	8-10

Immature cotyledon culture

Among the genotypes, GC(1)-35-1 and GC(1)-4 showed the highest (66.67%) frequency of callus induction in MS + 1.5 mg/L of 2,4-D + 0.5 gm/L BAP followed by Zhingabadam (Fig. 2a) and GC(1)-3-2-1. The genotype GC(1)-3-2-1 showed minimum (33.33%) callus induction in MS + 2.0 mg/L 2,4-D + 0.5 mg/L BAP solution.

d) The response of different concentrations of 2,4-D and a fixed concentration of kinetin (kn) on callus induction in groundnut genotypes

Callus induction ability was also observed by different concentration of 2,4-D (1.0, 1.5 and 2.0 mg/L) and a fixed concentration (0.5 mg/L) of kinetin (kn) in different explants of groundnut genotypes (Table 6).

Immature cotyledon culture

The groundnut genotype GC(1)-35-1 exhibited the highest (75%) percentage of callus induction ability in combination of 1.5 mg/L 2,4-D + 0.5 mg/L kinetin chemicals while the other genotypes Zhingabadam and GC(1)-3-2-1 showed the lowest (33.33%) callus induction ability in combination of 1.5 mg/L 2,4-D + 0.5 mg/L kinetin (Fig. 2b).

Table 6. Effect of different concentrations of auxin (2,4-D) and a fixed concentration of kinetin(kn) in MS medium for callus induction in groundnut genotypes

Genotypes	Explants			Immature Cotyledon		
	Supplements (mg/L)		No. of explants inoculated	No. of explants showing callus induction	Callus induction (%)	Days to callus induction
	2,4-D	Kn				
GC(1)-35-1	1.0		12	8	66.67	8-9
	1.5	0.5	12	9	75.00	7-9
	2.0		12	5	41.67	8-10
Zhingabadam	1.0		12	7	58.33	5-7
	1.5	0.5	12	4	33.33	8-9
	2.0		12	6	50.00	9-11
GC(1)-4	1.0		12	6	50.00	7-9
	1.5	0.5	12	8	66.67	7-8
	2.0		12	5	41.67	5-7
GC(1)-3-2-1	1.0		12	7	58.33	8-10
	1.5	0.5	12	4	33.33	8-9
	2.0		12	6	50.00	7-9

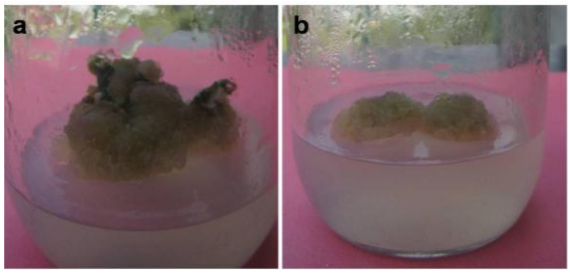

Fig. 2. Callus induction from immature cotyledons of groundnut genotypes; (a) callus initiation from immature cotyledon of Zhingabadam in MS + 1.0 mg/L 2,4-D + 0.5 mg/L BPA, and (b) callus initiation from immature cotyledon of GC(1)-3-2-1 in MS + 1.0 mg/L 2,4-D + 0.5 mg/L Kn

Maintenance of callus culture

In the present study two different concentrations (1.0 and 1.5 mg/L) of 2,4-D in the combination of 0.5 mg/L BAP were used with MS medium for maintenance of raised callus (Table 7). Among the genotypes, GC(1)-35-1 showed the maximum callus proliferation (84.62%) in combination of MS + 1.0 mg/L 2,4-D + 0.5 mg/L BAP (Fig. 3b). The second highest (76.92%) percentage of callus proliferation was observed in the genotypes Zhingabadam and GC(1)-3-2-1 (Fig. 3a) in combination of MS medium + 1.0 mg/L 2,4-D + 0.50 mg/L BAP while the lowest proliferation intensity was observed by GC(1)-3-2-1 in 1.5 mg/L 2,4-D + 0.50 mg/L BAP. Combination of 1.0 mg/L 2,4-D + 0.50 mg/L BAP, most of the proliferating callus showed slightly greenish colour while in another combination (1.0 mg/L 2,4-D + 0.5 mg/L BAP), callus colour was observed with whitish to creamy-white or brownish. From the above study, it was found that at higher concentrations of auxin and lower concentrations of cytokinin gave the best results for callus maintenance.

Table 7. The response of different concentration combinations of 2,4-D and BAP in MS medium for callus maintenance derived from different explants of groundnut

Genotypes	Supplements (mg/L)		No. of tubes to which callus inoculated	No. of tubes showing callus proliferation	Callus proliferation (%)	Days to Sub-culture
	2,4-D	BAP				
GC(1)-35-1	1.0	0.5	13	11	84.62	15
	1.5	0.5	13	9	69.23	15
Zhingabadam	1.0	0.5	13	10	76.92	15
	1.5	0.5	13	9	69.23	15
GC(1)-4	1.0	0.5	13	8	61.54	15
	1.5	0.5	13	9	69.23	15
GC(1)-3-2-1	1.0	0.5	13	10	76.92	15
	1.5	0.5	13	7	53.84	15

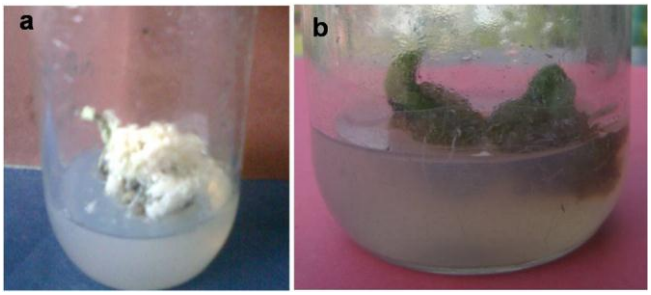

Fig. 3. Maintenance of callus cultures with different hormones; (a) calli of GC(1)-3-2-1 in MS medium + 1.0 mg/L 2,4-D + 0.5 mg/L BPA, and (b) calli of GC(1)-35-1 in MS medium + 1.0 mg/L 2,4-D + 0.5 mg/L BPA solutions

Organogenesis

After maintenance of calli, the unorganized calli were transferred to new combination media in order to inducing shoots as well as roots. Usually 13 tubes were inoculated for each genotype, where each genotype contained callus from immature cotyledon (Table 8). Among the genotypes, GC(1)-35-1 and GC(1)-3-2-1 were found as the best for shoot regeneration (61.53%) when MS medium supplemented with 1.0 mg/L BAP + 0.5 IAA mg/L for GC(1)-3-2-1 (Fig. 4a) and 1.0 mg/L BAP + 1.0 mg/L IAA, 0.5 mg/L BAP + 0.5 mg/L 2,4D and 1.5 mg/L BAP + 1.0 mg/L NAA for GC(1)-35-1 (Fig. 4b). The lowest frequency of shoot regeneration (15.38%) was observed in MS + 1.0 mg/L BAP + 1.5 mg/L IAA by Zhingabadam and GC(1)-4, and in MS + 1.0 mg/L BAP + 0.5 mg/L IAA for GC(1)-4 while no shoot regeneration was observed in 1.0 mg/L BAP + 1.0 mg/L IAA by GC(1)-4. During the present study very few genotypes were showed with root regeneration. The genotypes GC(1)-35-1 and GC(1)-3-2-1 were found with the highest (23.07) root regeneration in combinations of 1.0 mg/L BAP + 1.5 mg/L IAA.

Table 8. The response of different combinations of BAP, IAA and 2,4-D in MS medium on the shoot and root initiation from different explants derived calli of groundnut

Supplements (mg/L)	Genotypes	No. of tubes in whichcallus inoculated	No. of tubes in which shoot initiated	No. of tubes in which root initiated	Shoot regeneration (%)	Root regeneration (%)	Days to regeneration	
							Root	Shoot
1.0 BAP + 0.5 IAA	GC(1)-35-1	13	4	2	30.76	15.38	6-7	8-10
	Zhingabadam	13	3	-	23.07	-	-	7-10
	GC(1)-4	13	2	-	15.38	-	-	7-9
	GC(1)-3-2-1	13	8	1	61.53	7.69	5-7	7-8
1.0 BAP + 1.0 IAA	GC(1)-35-1	13	8	-	61.53	-	-	8-10
	Zhingabadam	13	3	-	23.07		-	7-8
	GC(1)-4	13	-	-	-		-	-
	GC(1)-3-2-1	13	4	-	30.76	-	-	7-9
1.0 BAP + 1.5 IAA	GC(1)-35-1	13	6	3	46.15	23.07	7-8	8-10
	Zhingabadam	13	2	-	15.38	-	-	8-9
	GC(1)-4	13	2	-	15.38	-	-	8-9
	GC(1)-3-2-1	13	4	3	30.76	23.07	7-8	7-1
0.5 BAP + 0.5 2,4-D	GC(1)-35-1	13	8	2	61.53	15.38	9-12	10-12
	Zhingabadam	13	4	2	30.76	15.38	8-9	8-10
	GC(1)-4	13	3	1	23.07	7.69	7-9	7-9
	GC(1)-3-2-1	13	5	1	38.46	7.69	8-9	8-9
1.5 BAP + 1.0 NAA	GC(1)-35-1	13	8	2	61.53	15.38	8-9	9-10
	Zhingabadam	13	7	2	53.84	15.38	6-7	6-7
	GC(1)-4	13	5	-	38.46	-	-	7-9
	GC(1)-3-2-1	13	5	2	38.46	15.38	8-9	9-10
1.0 BAP + 1.5 NAA	GC(1)-35-1	13	7	-	53.84	-	-	7-10
	Zhingabadam	13	6	-	46.15	-	-	7-8
	GC(1)-4	13	-	-	23.07	-	-	6-9
	GC(1)-3-2-1	13	4	-	30.76	-	-	8-9

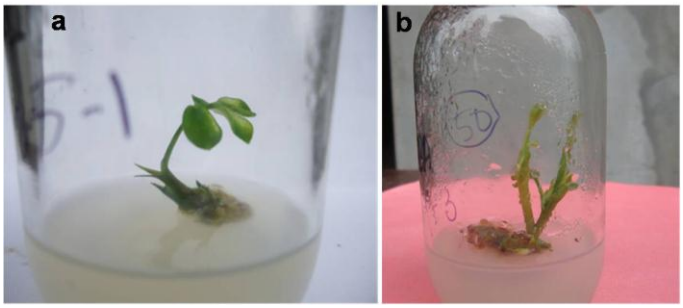

Fig. 4. Shoot regeneration from immature cotyledons; (a) from immature cotyledon of GC(1)-3-2-1 in MS medium + 0.5 mg/L BPA + 0.5 mg/L of 2,4-D, and (b) from immature cotyledon of GC(1)-35-1 in MS + 1.5 mg/L BPA + 1.0 mg/L of NAA

Regeneration of root

Root initiation is an essential step for shoot establishment in the field. Twelve (12) regenerated shoots were transferred against each genotype and were taken from immature cotyledon. The best root induction was found in GC(1)-35-1 when half MS was supplemented with 0.5 mg/L IBA (Fig. 5b) followed by Zhingabadam (0.5 and 1.0 mg/L IBA) (Fig. 5a) and GC(1)-4 (1.0 mg/L IBA). While the lowest root induction ability was observed by GC(1)-3-2-1 in $^{1}/_{2}$ MS + 1.0 mg/L IBA treatment (Table 9).

Table 9. The response of two concentrations of IBA in half strength of MS medium for root initiation from regenerated shoots of groundnut

Supplements (mg/L)	Materials	No. of shoot sub-cultured	No. of shoot from which root induced	Root induction (%)	Days to root induction
0.5 IBA	GC(1)-35-1	12	9	75.00	7-10
	Zhingabadam	12	6	50.00	1-9
	GC(1)-4	12	7	58.33	8-10
	GC(1)-3-2-1	12	5	41.67	9-11
1.0 IBA	GC(1)-35-1	12	7	58.33	7-10
	Zhingabadam	12	8	66.67	8-9
	GC(1)-4	12	5	41.67	8-10
	GC(1)-3-2-1	12	4	33.33	8-11

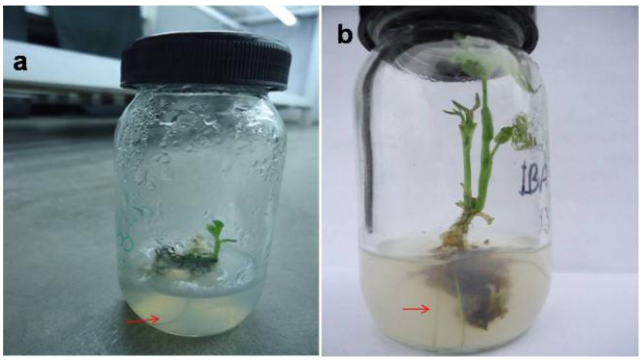

Fig. 5. Root regeneration from immature cotyledons; (a) from regenerated shoot of Zhingabadam in $^{1}/_{2}$ MS medium + 1.0mg/L of IBA, and (b) from regenerated shoot of GC(1)-35-1 $^{1}/_{2}$ MS medium + 0.5 mg/L of IBA

Transplantations and establishment of plantlets

The plantlets with sufficient root systems were transplanted into a small pot which covered with polythene paper for hardening (Fig. 6a). The plantlets were nourished with Hoagland's and subsequently, new leaves were starting to initiate. After 15 days of hardening, the plantlets were transferred into larger pots under natural environment (Fig. 6b) by nourishing with ordinary tap water and the highest (75%) establishment rate was found for genotype GC(1)-35-1 in pot culture (Fig. 6c-d) and subsequently, the genotypes showed healthy plant types with good flowering pattern (Table 10).

Table 10. The survival rate of regenerated plantlet from different explants of groundnut genotypes

Items	Genotypes	No. of plantlets transplanted	No. of plants survived	Survival rate (%)
In pot	GC(1)-35-1	8	6	75.00
	Zhingabadam	8	5	62.50
	GC(1)-4	8	4	50.00
	GC(1)-3-2-1	8	4	50.00
In soil	GC(1)-35-1	6	4	66.67
	Zhingabadam	4	2	50.00
	GC(1)-4	3	0	00.00
	GC(1)-3-2-1	3	1	33.33

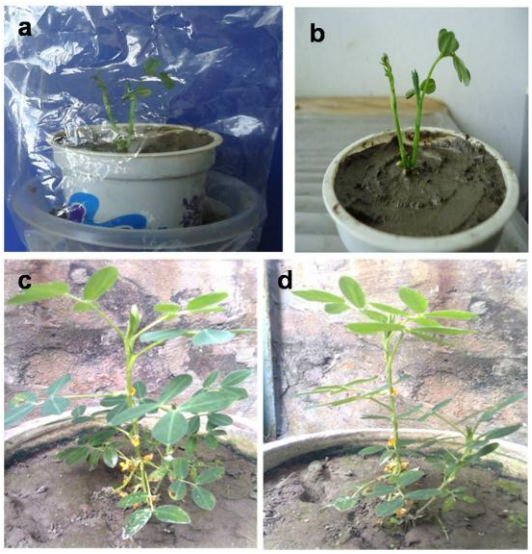

Fig. 6. Hardening and establishment of cotyledon derived mature plant in plastic/earthen pot; (a & b) hardening of the regenerated plant of Zhingabadam after transplantation in a plastic pot, and (c & d) immature cotyledon derived plant of GC(1)-35-1 after transplantation in an earthen pot at flowering and pegging stage.

DISCUSSION

Oilseeds, the most important group of crop plants, are difficult to regenerate from tissue cultures. To facilitate the development of tissue culture-based crop species, considerable effort has been devoted to developing and optimizing efficient in *in vitro* regeneration technique. Plant growth regulators have been regarded as an important parameter in determining the success of the regeneration system. A deviation from an optimum level of the hormone will have a significant impact. The combination or sole treatment of different hormones such as 2,4-D, cytokinin, BAP, IBA, IAA etc in the regeneration medium has been described as more critical for affecting the regeneration. Therefore, the present investigation was undertaken to induce callus and regenerate it for establishing as mature plants from immature cotyledons of groundnut genotypes. In our study, the genotypes and explants can induce callus when cultured on MS medium with supplemention of different concentrations of 2,4-D, BAP, cytokinin, NAA etc. We found the highest callus induction frequency (66.67%) with the application of 1 mg/L concentration of 2,4-D in genotype GC(1)-35-1. The researcher Eapen and George (1993), also recorded the highest frequency of callus induction when immature cotyledon segment cultured on MS medium supplemented with 2,4-D. Some other scientists Gill and Saxena (1992) and Radhakrishnan *et al.* (2000), also reported callus induction from cotyledon. There are significant variations in callus induction were observed among the genotypes under different hormonal concentrations. Combinations of different hormones with different doses also gave good results in callus induction from immature cotyledons. While the proliferation of callus culture also an important issue for plant establishment. In our findings, the genotypes GC(1)-35-1 showed higher degree of callus proliferation (84.62%) in combination of MS + 1.0 mg/L 2,4-D + 0.5 mg/L BAP. It is evident from the result that in general by increasing the hormonal concentrations in medium, the percentage of proliferation dropped. But there were no significant variations observed among the genotypes under same hormonal compositions. Organogenesis that means to shoot and root initiation is vital for plant regeneration. We found the best shoot regeneration (61.53%) when MS medium supplemented with

1.0 mg/L BAP + 0.5 IAA mg/L for GC(1)-3-2-1. Generally shoots developed on medium with higher BAP concentration failed to elongate and resulted in a rosette of shoot buds. This might be due to cell elongation being affected by the ratio of cytokinin-auxin ratio. McKently *et al*. (1990), reported that the number of shoots was lowest in the de-embryonated cotyledon segment of groundnut genotypes. While the higher frequency of root initiation observed in GC(1)-35-1 when half MS was supplemented with 0.5 mg/L IBA. In our study, we found fewer proportions of root system initiation. This finding was also partially supported by Rani and Reddy (1996), Venkatachalam *et al*. (1999) and Akasaka *et al*. (2000). Different researcher's *viz*. Venkatachalam and Jayabalan (1996), Venkatachalam *et al*. (1999), were used IBA for profuse rooting which confirmed our present findings. Significant variations in rooting among the genotypes were observed in our genotypes. The successful establishment of regeneration of these varieties will have several benefits in future. We found the highest frequency of (75%) plant establishment rate in genotype GC(1)-35-1 in pot culture. Almost similar results were reported by Radhakrishnan *et al.* (2000). But the epicotyl and hypocotyl explants showed improved performances than the cotyledon explants. This might be due to the presence of a meristematic cell near the cut surface of the epicotyl and hypocotyl explants.

CONCLUSION

From the above findings on the regeneration system of groundnut genotypes, the cultivar GC(1)-35-1 was found more appropriate for the regeneration from immature cotyledon. It is possible to induce shoot differentiation and complete plantlet development from immature cotyledons explants of *Arachis hypogea* (L.). The highest shoot regeneration was found when MS medium supplemented with 1.5 mg/L BAP + 1.0 mg/L NAA while the half of MS medium supplemented with 0.5 mg/L IBA showed the best result for rooting in GC(1)-35-1 genotypes.

ACKNOWLEDGEMENT

The authors express their gratitude to Dr. Abul Kalam Azad, Plant Breeding Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh-2202 for providing the groundnut germplasm used in the study.

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