Journal of Agricultural Technology

# A simple and improved protocol for direct and indirect somatic embryogenesis of peanut (*Arachis hypogaea* L.)

**P. Bhanumathi[1], M. Ganesan[2] and N. Jayabalan[2*]**

[1]Department of Botany, KN Government Arts College (W), Thanjavur - 15, Tamil Nadu, India
[2]Department of Plant Science, Bharathidasan University, Tiruchirappalli, 620 024, Tamil Nadu, India

Bhanumathi, P., Ganesan, M. and Jayabalan, N. (2005). A simple and improved protocol for direct and indirect somatic embryogenesis of peanut (*Arachis hypogaea* L.). Journal of Agricultural Technology 1(2): 327-344.

A simple and efficient protocol for the direct and indirect embryoid induction and plant regeneration is described here. Direct induction of somatic embryos was achieved on proximal end of embryonal axis explant in the medium fortified with MS salts, B5 vitamins, 27.5 mg/L PIC, 30 g/L sucrose and 0.8% agar. After 6 weeks of culture, the highest response to embryoid induction achieved was 97% without an intervening callus phase. About 23.6 ± 0.28 embryoids per explants were induced by the above concentration. Leaf explants were used for the indirect somatic embryogenesis. 500 mg of 30 days old embryogenic callus was used for embryoid induction through suspension culture. High frequency of embryogenic callus induction was observed on the media fortified with MS salts, B5 vitamins, 2 mg/L 2,4-D and 1 mg/L BAP. After 6 weeks, the embryoids were formed on the bottom of the suspension culture. Medium fortified with MS salts, B5 vitamins, 2 mg/L 2,4-D and 1 mg/L KIN showed best response of embryoid induction through suspension culture. The formed matured embryoids on the original medium. The embryoids induced through direct and indirect methods were regenerated on the medium fortified with MS basal salts, B5 vitamins, BAP (0.2 mg/L) and IBA (0.2 mg/l) and 75.8% of plant regeneration was observed at this concentration. The regenerated plantlets showed 100% survival rate in green house conditions. Influences of different carbohydrates, amino acids, and additives were also tested for the high frequency of somatic embryo production.

**Key words**: auxins, cytokinins, embryogenic callus, hardening, organogenesis, rooting.

**Abbreviations**

KT – Kinetin
$GA_3$ - Gibberellic acid
MS - Murashige and Skoog medium
BAP – Benzylaminopurine
B5 medium - Gamborg medium
SE - Standard error
2,4-D - 2, 4-Dichlorophenoxyacetic acid
NAA - Naphthaleneacetic acid
2, iP – 2 isopentynyl adenine
KN – Kinetin
IBA – Indole butyric acid
PGR – Plant growth regulators
PIC - Picloram

*Corresponding author: N. Jayabalan; email: jayabalan54@yahoo.co.in

## Introduction

Genetic engineering of plants to enhance the qualities of varieties requires an efficient protocol for the regeneration and production of transgenic plants (Livingstone and Birch, 1999). However, gene transfer through single cell-originated somatic embryogenesis yielded gene transfer into each cells of regenerated plants with higher transformation frequencies without chimeric variations (Wilkins *et al*., 2004). Hence, somatic embryogenesis of plants originated from single cells is highly necessary and essential for gene transfer studies. A wide range of plant species are amenable to embryogenic callus initiation and regeneration through somatic embryos. The development of suitable protocols for plant regeneration is one of the main prerequisites for the genetic improvement of crop plants using biotechnological methods. Comparatively, with respect to plant genetic transformation, plants regenerated through somatic embryogenesis are more useful than plants obtained through organogenesis.

Peanut (*Arachis hypogaea* L.) is economically the most important species of the legume family (Krapovickas and Gregory, 1994). The oil isolated from the peanut seed is widely used for cooking and is also useful for the manufacture of margarines and shortenings, and as a salad. Apart from the peanut oil, the peanut agglutinin isolated from the callus and seeds have played an essential role in blood typing, in cell separation and identification, as carriers of chemotherapeutic agents and in the study of cell membranes (D'Silva and Podder, 1994). During last decade, peanut regeneration through somatic embryogenesis has vastly increased by using different explants. Regeneration *via* somatic embryogenesis from immature cotyledon explants (Ozias Akins, 1989; Durham and Parrott, 1992; Eapen and George, 1993a,b; Wetzstein and Baker, 1993; Baker and Wetzstein, 1994; Baker *et al*., 1994), immature zygotic embryos (Sellars *et al*., 1990, Jayabalan *et al*., 2004), immature embryonic axes (Eapen and George, 1993a) mature embryo axes (Hazra *et al*., 1989; Baker *et al*., 1995), leaflets (Baker and Wetzstein, 1992; Chengalrayan *et al*., 2001), epicotyls (Little *et al*., 2000) and young leaflets from aseptically germinated embryo axes (Baker and Wetzstein, 1992) has been reported in peanut. The frequency of plantlet regeneration in all these protocols being quite low has deferred their usage for genetic transformation studies. Growth and regeneration *in vitro* is a complex phenomenon and is influenced by a number of genetic and environmental factors. Somatic embryogenesis represents a simple and very efficient alternative means of regenerating large numbers of intact plants from tissue culture (Zimmerman, 1993). However, in spite of a considerable progress in developing tissue

culture protocols in a variety of plant species, grain legumes in general have remained recalcitrant to regeneration *in vitro*.

In this communication, we describe here a comparative study of direct and indirect somatic embryogenesis of *Arachis hypogaea*. Even though there are several reports presented for direct and indirect somatic embryogenesis, there are no reports presented for the comparative studies of peanut somatic embryogenesis. Hence, this present research work was aimed to standardize the simple reliable protocol for direct and indirect somatic embryogenesis of peanut. Mean while we also measured the factors influencing the somatic embryogenesis of peanut.

## Materials and methods

### *Seed germination*

Seeds of peanut variety VRI 4 (*Arachis hypogaea* cv VRI 4) were obtained from Regional Research Station, Tamil Nadu Agricultural University, Virudhachalam, Tamil Nadu. The seeds were surface sterilized by the method of Ganesan and Jayabalan (2004).

The cotyledons were removed from the surface sterilized seeds and matured embryo axes were collected. The radical end was removed and only proximal end of embryo axes was selected as explant for the induction of direct somatic embryos. In the case of indirect somatic embryogenesis, the surface sterilized seeds were allowed to grow on the MS basal medium. After 7 days of germination, the leaf explants were isolated and allowed for embryogenic callus induction and embryoids.

### *Direct embryoid induction and plant regeneration*

Medium supplemented with MS basal salts (Murashige and Skoog, 1962) with $B_5$ vitamins (Gamborg *et al*., 1968), 0.8% agar, 30g/L sucrose, 2,4-D (10-45 mg/L) and picloram (26-29 mg/L) were used individually for direct embryoid induction. The pH of the media was adjusted between 5.75 to 5.8 with NaOH or HCl prior to autoclaving at 121°C for 15 minutes with 1.06 Kg $cm^{-2}$ pressure. They were incubated for 15-25 days at 25 ± 2°C under TL 40 W/54 cool white fluorescent light at 40µ $Em^{-2}s^{-1}$ with 16 hour photoperiod. The explants were cultured on 15 ml medium in glass test tubes closed with non-absorbent cotton plugs. The explants were sub-cultured at an interval of 30 days for the induction of somatic embryos. At the end of 3rd subculture, the somatic embryos directly developed from the explants were observed by the

unaided eye. The induced embryoids were then allowed to reach maturation in the same medium. After another 3 subcultures all the induced white transparent globular embryos were turned into green torpedo staged embryoids. These green torpedo staged embryoids were considered as matured embryos; they were detached and further transferred to embryoid regeneration medium for further growth and development. The medium supplemented with MS salts and B5 vitamins and with different concentrations of BAP (0.05-0.35 mg/L) and IBA (0.05-0.35 mg/L) were tested individually or in combination for regeneration of somatic embryos. After the complete regeneration of embryoids to plantlets (30-40 days), the latter were transferred to plastic pots containing sand, soil and vermiculite in 1:1:1 ratio for hardening. To maintain the humidity, hardened plants were completely covered with plastic bags, which were progressively removed to aid adaptation to normal environmental conditions. After adaptation, the selected plants were transferred to earthen pots for further growth and development.

***Indirect embryoid induction***

Indirect somatic embryogenesis was achieved after the induction of embryogenic callus from the leaf explants. MS basal medium containing $B_5$ vitamins, 0.8% agar, 30g/L sucrose and auxins (2,4-D, NAA, IBA) were tested individually and in combination with BAP (0.25-1.5 mg/L), for the induction of embryogenic callus. The leaf explants were placed in 25 × 150 mm tubes containing 15 ml medium slants. The induced callus was sub-cultured at an interval of 10 days on the MS medium with different concentrations of BAP (0.25-1.5 mg/L) and 2,4-D (2 mg/L).

After 3 subcultures, 30-day old creamy white friable calli (approximately 500 mg) was transferred to MS liquid medium to initiate cell suspension culture. They were aseptically transferred to 250 ml conical flasks (Borosil-India) containing 50 ml of medium per flask which contained MS salts, B5 vitamins 2,4-D (0.5-3.5 mg/L), NAA (0.5-3.0 mg/L), IBA (1-3 mg/L) individually or along with KIN (0.25-1.5 mg/L) and 3% sucrose. Further sub cultures involved the replacement of half of the medium with fresh medium of the same formulation at 10 day intervals. Cultures were filtered through 125 µm stainless steel sieves to separate individual cells and small cell clumps. Cells from the suspension were observed with a microscope during the culture period and the growth rate of cells was monitored from the $12^{th}$ day by determining the packed cell volume (PCV) of samples from 10 replicates. PCV was measured after centrifuging the suspension at 2000 rpm for 10 minutes in a graduated centrifuge tube (Kumar *et al*., 1988).

***Development of plantlets***

Maintenance of clusters for 4 weeks yielded high frequency of pro-embryoids. From the pro-embryoids only the embryoids were developed. Globular staged embryos were sub cultured in the same medium for further maturation. After 12 days of sub culture, heart and torpedo shaped embryos were formed simultaneously from globular staged embryos. After a period of further growth, torpedo shaped embryos were separated and transferred to embryoid regeneration medium. The media used for the regeneration of somatic embryos is similar to the somatic embryo regeneration medium of direct embryogenesis. The cultures in liquid medium were examined everyday during the experimental period to trace the ontogeny of somatic embryos. Samples from 20 replicate flasks per treatment were taken at random in order to determine the frequency of embryogenic initials and different stages of somatic embryos during somatic embryogenesis. The torpedo-shaped embryos were transferred to medium supplemented with MS salts, B5 vitamins, 3% sucrose, BAP (0.05-0.35 mg/l) and IBA (0.05-0.35 mg/l) for germination.

***Effect of media, carbohydrates and additives on somatic embryogenesis***

Different concentrations and forms of media, amino acids and additives were analysed in both the explants for the enhanced somatic embryos induction and germination. During embryogenesis, selected concentrations of above components were directly added to the medium before autoclaving. The efficiency of different media [MS, MS+$B_5$, mMS, WM (Nitsch, 1969)], carbohydrates (10 to 50 g/L of Sucrose, Maltose, Fructose and Glucose) and additives [charcoal (0.5-3 mg/l) and 10-50 mg/l of PVP, ASC, CA) were also evaluated in both type of embryoid induction methods. The influence of amino acids (10-60 mg/L of alanine, glutamine, serine and proline) at different concentrations was also examined during embryogenic callus induction from leaf explants.

***Media composition, preparation and culture conditions***

MS, modified MS (mMS), MS + $B_5$ and White's media were prepared according to the authors. For medium preparation, prepared stock solutions were mixed in proportion in each medium and according to the requirement for growth hormones, vitamin sources, carbon source and agar. The pH of the medium was adjusted to between 5.7 and 5.8 using 0.1N NaOH and 0.1N HCl before autoclaving at 121°C for 15 min. The hormones BAP and KIT were

prepared by dissolving 10 mg in 0.1N HCl (2 ml) and diluted with double-distilled water to make 1 mg/2 ml stock. Likewise, 10 mg of 2,4-D, NAA, PIC and GA3 was dissolved individually in 0.1N NaOH (1 ml) and diluted with double distilled water to make a 1 mg/2 ml stock. All of the prepared stock solutions were stored in the refrigerator for up to 20 days. Vitamins, such as T.HCL and myo-inositol, were freshly added to the medium, as required, in the form of a solution in double-distilled water. In all the types of media pH was adjusted to 5.75-5.8. All the cultures were maintained at 25 ± 2°C under a 16/8-hour (light/day) photoperiod with light supplied at an intensity of 2,500 lux.

### *Statistical analysis*

Means and standard errors were used throughout the study and the values were assessed using a parametric Moods median test (Snedecor and Cochran, 1989). The data were analysed for variance by Duncan's multiple range test (DMRT) using the SAS programme (SAS Institute, Cary, N.C.).

## Results and discussion

### *Embryoid induction*

In direct somatic embryogenesis, successful induction of embryoid was achieved by using proximal end of embryonal axis. 27.5 mg/L of PIC showed best response for the direct induction of embryoids. After 3 weeks of culture, white and transparent embryoids were emerged on the surface of explants (Figs 1a-c). These embryoids were identified by the unaided eye. Before embryoid induction, bulging of explants was clear. Formation of embryogenic callus or callus was absent in this case. The highest number of 23.6 ± 0.28 embryoids per explant was initiated at the above concentrations. In the case of 2,4-D, embryoid induction was observed in all the concentrations tested. The percentage of response for the induction of embryoids and the number of embryoids per explants was significantly low (Table 1). Usually picloram at low concentration induced the development of embryos from embryo axes and is a very potent phytohormone capable of inducing somatic embryogenesis in many plants (Eapen and George, 1990). In the majority of reports available in literature, somatic embryos are described as white structures (Ganesan and Jayabalan, 2004). In the present study also white and transparent embryos were observed in the culture containing 27.5 mg/l picloram. When somatic embryos from embryonal explants were tested for plant conversion frequency, picloram

at all concentrations favored higher plant differentiation in comparison with 2,4-D.

**Table 1.** Influence of different concentrations of auxins (PIC, 2,4-D) on induction of somatic embryos from proximal end of embryonal axis cultured on the medium fortified with MS salts and B5 vitamins.

| Concentrations of growth regulators (mg/L) | No. of explants responded | Percentage of response | Mean no. of Embryoids / explant | Mean no. of abnormal embryoids / explant |
|---|---|---|---|---|
| **2,4-D** | | | | |
| 10.0 | 112 | 56.0 h | 2.4 ± 0.18 hi | 6.4 ± 0.22 cd |
| 15.0 | 125 | 62.5 fg | 3.6 ± 0.18 h | 6.7 ± 0.28 c |
| 20.0 | 134 | 67.0 ef | 6.3 ± 0.21 g | 7.4 ± 0.26 b |
| 25.0 | 140 | 70.0 e | 7.5 ± 0.22 f | 7.9 ± 0.22 a |
| 30.0 | 130 | 65.0 f | 2.1 ± 0.24 i | 7.1 ± 0.20 bc |
| 35.0 | 122 | 61.0 g | 1.2 ± 0.20 ij | 6.4 ± 0.28 cd |
| 40.0 | 112 | 56.0 h | 1.0 ± 0.18 j | 5.9 ± 0.22 d |
| 45.0 | 102 | 51.0 hi | 0.7 ± 0.18 jk | 5.0 ± 0.16 de |
| **PIC** | | | | |
| 26.0 | 156 | 78.0 d | 12.4 ± 0.28 d | 4.2 ± 0.26 ef |
| 26.5 | 172 | 86.0 c | 16.7 ± 0.30 c | 3.9 ± 0.29 g |
| 27.0 | 185 | 92.5 b | 19.5 ± 0.32 bc | 3.7 ± 0.34 h |
| 27.5 | 194 | 97.0 a | 23.6 ± 0.28 a | 2.9 ± 0.22 hi |
| 28.0 | 180 | 90.0 bc | 20.4 ± 0.29 b | 3.8 ± 0.18 gh |
| 28.5 | 168 | 84.0 cd | 16.2 ± 0.30 cd | 4.1 ± 0.24 f |
| 29.0 | 149 | 74.5 de | 12.1 ± 0.26 e | 4.6 ± 0.26 e |

Number of explants tested – 200;

Values are means ± SE of three repeated experiments with three replicates.

Means within a column followed by the same letters are not significant at $P$=0.05 according to DMRT

In the case of indirect somatic embryogenesis, 7 day old leaf explants produced embryogenic callus on the medium fortified with MS salts, B5 vitamins, 2,4-D (2 mg/l) and BAP (1 mg/l) combination (Fig. 2a). At this concentration highest of 84% was observed for embryogenic callus induction and in this concentration only the creamy white friable embryogenic callus formation was observed. A similar result on embryogenic callus induction was observed in *Camellia* spp. (Wachira and Ogada, 1995). In their studies they reported that the combination of auxin with cytokinin (2,4-D with BAP) gave a significant response for embryogenic callus induction. Commonly, embryogenic callus induction has been effectively achieved by the combined treatment with auxin and cytokinin (Hernandez *et al*., 2003; Aly *et al*., 2002;

Ma and Xu, 2000). In the present study, the individual effect of 2,4-D and NAA were also tested and they showed a poor response for the induction of embryogenic callus. In contradiction to the above, individual auxin treatment to explants produced embryogenic callus and somatic embryos (Kim *et al*., 2003). These results show that 2,4-D is the essential plant growth regulator for the induction of embryogenic callus (Maureen *et al*., 1990).

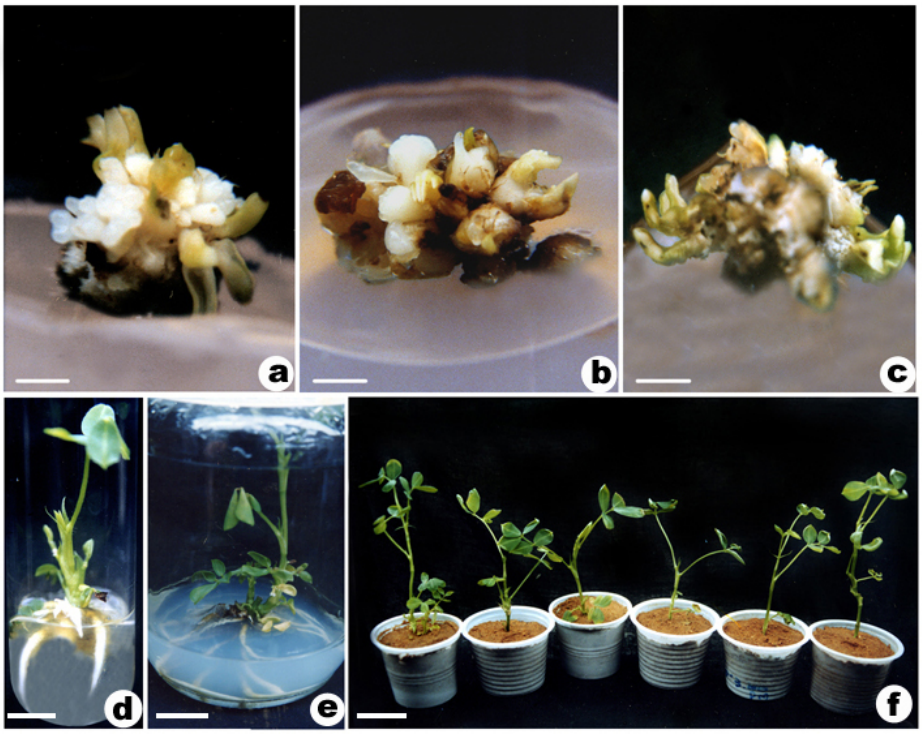



**Fig. 1.** a-f. Different developmental stages of direct somatic embryogenesis of peanut (*Arachis hypogaea* L.): **a**. Direct embryoid induction from the explant with heart and globular shaped embryoids. Bar: 2 cm. **b**. Four week - old somatic embryos. Bar: 5 mm. **c**. Regeneration of somatic embryos. Bar : 1 cm **d**. Regenerated somatic embryos . Bar : 1 cm **e**. Regenerated somatic embryo with tertiary root. Bar : 2 cm **f.** Hardened plantlets on plastic pots.

In the case of amino acids, among the different concentrations tested, glutamine 30 mg/L showed best response for embryogenic callus induction. 99.5% of embryogenic callus induction was observed in this concentration. In other amino acids assays (alanine, serine and proline) the percentage of embryogenic callus induction was reduced compared with control. High frequency of abnormalities were observed in serine and proline treated explants. Generally, glutamine has been used for the induction of embryogenic callus and direct and indirect induction of somatic embryos (Hazal *et al*.,

1993). In our study also enhanced percentage of embryogenic callus induction was observed by the supplementation of glutamine to embryogenic callus induction media.

Approximately 250 mg of embryogenic callus was transferred to liquid medium for the cell suspension culture induction to induce embryoids. Media supplemented with MS salts, B5 vitamins, 30 g/L sucrose, 2 mg/L 2,4-D and 1 mg/L KIN showed best response for cell suspension culture initiation and embryoid induction. The embryogenic nature of the cell cluster was confirmed by the procedure of Tsay and Huang (1998). A similar tendency was found with cell suspension-cultures of Carrot (Bayliss, 1977) and *Nicotiana* spp. (Evans and Gamborg, 1982) and these authors confirmed that cell cluster formation positively influenced the somatic embryo induction and formation (Fig. 2a-e) (Toonen *et al*., 1996). At each subculture, the embryogenic masses were divided into clumps consisting of embryos. Microscopic examination of liquid cultures revealed numerous aggregates of somatic embryos. After 6 weeks of culture on the same medium, formation of pro-embryoids occurred and highest of 190.2 ± 2.06 pro-embryoids were initiated in the above concentration (Table 2) (Fig. 2f). The formed embryoids were then subcultured on the same medium for another 6 weeks for maturation. In the case of *Capsicum annum*, a similar trend was observed for the somatic embryo induction (Kintzios *et al*., 2001) and 9 µM 2,4-D with 12.9 µM BAP was used for the induction of somatic embryos at high frequency. Further subculture of globular embryoids in the mother medium for another 3 weeks yielded matured green somatic embryos. By using the formation of heart and torpedo-shaped embryos, we confirmed the embryo maturation process (Fig. 1c) (Figs 2 g-j) (Varisai Mohamed *et al*., 2004). At the end of $12^{th}$ week 189 ± 2.5 matured somatic embryos were obtained. Here also sucrose 30 g/L showed best response for embryoid induction.

Incorporation of PVP in the medium reduced leaching to lesser extent. The results from present study therefore suggest that MS medium with $B_5$ vitamins, 2,4-D and BAP along with Glutamine and ASC were essential for establishment and maintenance of embryogenic callus and embryoids. 2,4-D with KIN was required for embryoids induction. Thus establishment of embryogenic cultures in liquid medium extends the information previously available on peanut somatic embryogenesis.

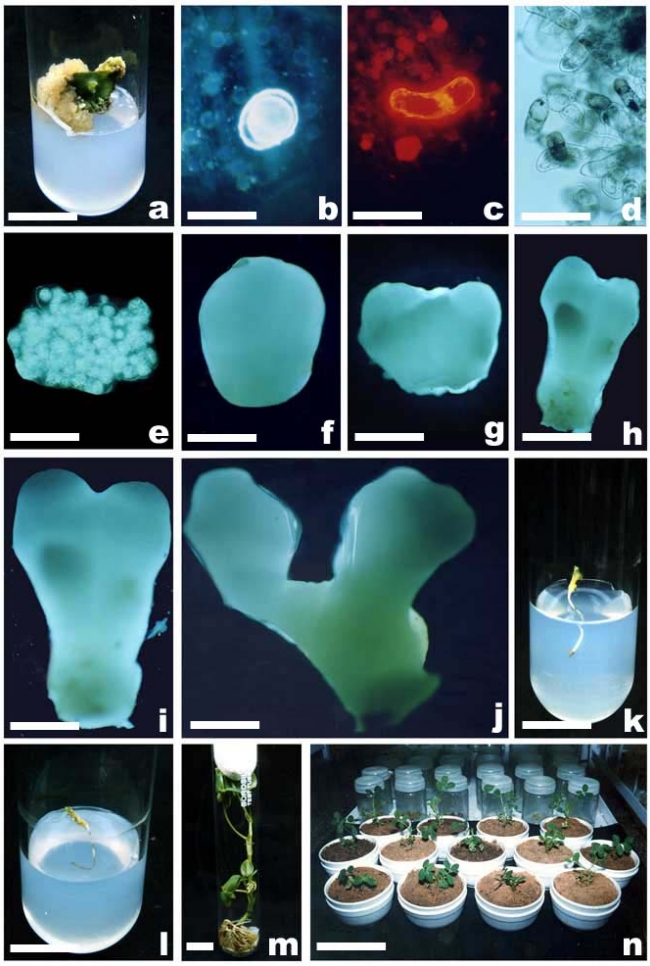



**Fig. 2.** a-r. Embryogenic callus induction and somatic embryogenesis of peanut (*Arachis hypogaea* L.) **a.** Embryogenic callus induction from leaf explants. Bar = 2 cm. **b.** Single cells present in the suspension culture. Bar = 250 µm **c.** Double celled stage. Bar : 250 µm **d, e.** Cell clusters in the suspension culture. Bar = 750 µm **f-j.** Various stages of somatic embryos from pro-embryoids to torpedo stage. **f,** Globular embryoid. Bar = 1.5 mm **g, h**. Heart-shaped embryoids Bar = 1.5 mm **i.** Matured torpedo-shaped embryo Bar = 1.5 mm **j**. Torpedo-shaped embryo before germination. Bar =: 1.5 mm **k, l.** Somatic embryo germination. Bar = 1.5 cm **m.** Germinated somatic embryo. Bar = 2.5 cm. **n.** Regenerated plantlets on plastic pots. Bar = 10 cm.

Histological observations made on regenerating explants confirmed the presence of somatic embryos. The proembryos, consisting of balls of compactly arranged, actively dividing cells, were visible after 2 weeks of culturing. Proembryos further developed into globular somatic embryos. These globular somatic embryos developed into heart shaped somatic embryos and then it underwent further maturation and development.

**Table 2.** Effect of different concentrations of auxins (2,4-D and NAA) with cytokinin (KIN) on embryoids induction from immature leaf derived embryogenic callus cultured on the liquid medium fortified with MS salts and B5 vitamins.

| Concentrations of growth regulators | Mean no. of proembryoids / 500 ml (After 6 weeks) | Mean no. of embryoids / 500 ml (After 8 weeks) | Mean no. of matured embryoids / 500 ml (After 10 weeks) | Percentage of somatic embryo maturation from embryoids |
|---|---|---|---|---|
| **2,4-D** | | | | |
| 0.5 | 125.6 ± 1.98 g | 118.4 ± 1.22 h | 90.4 ± 0.82 hi | 76.3 g |
| 1.0 | 130.4 ± 1.89 fg | 119.0 ± 1.24 g | 91.5 ± 0.84 h | 76.8 fg |
| 1.5 | 140.0 ± 1.92 e | 121.0 ± 1.22 ef | 93.4 ± 0.80 fg | 77.1 ef |
| 2.0 | 145.2 ± 1.84 d | 121.6 ± 1.26 e | 94.0 ± 0.86 f | 77.3 e |
| 2.5 | 140.5 ± 1.88 de | 120.4 ± 1.25 f | 92.9 ± 0.84 g | 77.1 ef |
| 3.0 | 130.5 ± 1.94 f | 119.6 ± 1.28 fg | 92.0 ± 0.86 gh | 76.9 f |
| 3.5 | 120.2 ± 1.92 h | 118.5 ± 1.22 gh | 90.4 ± 0.84 hi | 76.2 gh |
| **NAA** | | | | |
| 0.5 | 65.2 ± 0.78 jk | 32.4 ± 0.22 j | 12.9 ± 0.18 jk | 39.8 k |
| 1.0 | 65.5 ± 0.82 j | 33.5 ± 0.24 ij | 13.4 ± 0.16 j | 40.0 ij |
| 1.5 | 70.4 ± 0.76 i | 34.5 ± 0.22 hi | 14.6 ± 0.17 i | 42.3 h |
| 2.0 | 60.5 ± 0.80 k | 33.9 ± 0.28 i | 13.9 ± 0.22 ij | 41.0 hi |
| 2.5 | 55.5 ± 0.84 kl | 31.8 ± 0.25 jk | 12.9 ± 0.20 jk | 40.5 i |
| 3.0 | 55.2 ± 0.82 l | 30.9 ± 0.24 k | 12.1 ± 0.19 k | 39.1 j |
| **2,4-D + KIN** | | | | |
| 2.0 + 0.25 | 160.1 ± 2.10 cd | 151.5 ± 2.12 de | 148.5 ± 2.42 e | 98.0 cd |
| 2.0 + 0.50 | 175.5 ± 2.06 bc | 172.0 ± 2.18 cd | 171.5± 2.48cd | 99.7 c |
| 2.0 + 0.75 | 185.5 ± 2.08 ab | 183.5 ± 2.24 b | 183.2 ± 2.46 b | 99.8 b |
| 2.0 + 1.00 | 190.2 ± 2.06 a | 189.0 ± 2.26 a | 189.0 ± 2.50 a | 100 a |
| 2.0 + 1.25 | 180.4 ± 2.10 b | 176.0 ± 2.25 c | 174.0 ± 2.40 c | 98.8 bc |
| 2.0 + 1.50 | 170.4 ± 2.10 c | 168.3 ± 2.26 d | 164.3 ± 2.45 d | 97.6 d |

Number of explants tested – 250. Values are means ± SE of three repeated experiments with three replicates. Means within a column followed by the same letters are not significant at *P*=0.05 according to DMRT

### *Influence of carbohydrates on embryogenic callus and embryoid induction*

The influence of different concentrations of carbohydrates were also tested, among them sucrose 30 g/L showed best response for embryoid and maturation in the both the type of embryoid induction. The frequency of plant development decreased considerably in the presence of maltose and fructose

and was completely inhibited by glucose. Germination of embryos was also depending upon the type of carbohydrates used. However higher concentrations of sucrose (<3%) inhibited embryogenesis. When different concentrations of sucrose were incorporated into the basal medium, the highest average number of somatic embryos and plantlets were obtained at 3% followed by 4% and 2%. In the absence of sucrose no plantlet development was observed. Sucrose is generally known to be the most useful sugar for plant tissue culture though in some instances it may not be effective or even inhibitory. Among the different sugars, sucrose was most favorable for somatic embryogenesis producing the highest frequency and average number of somatic embryos. In the presence of maltose and fructose, the frequency was reduced while glucose was completely ineffective for somatic embryogenesis. Increased concentrations of glucose seem to have inhibitory effect on the emergence of somatic embryos. It was less effective than sucrose in inducing embryo formation. Among the different carbon sources tested sucrose and maltose were highly effective in the induction and development of somatic embryos while others were partially or completely inhibitory. Sucrose and maltose produced the highest average number of germinated plantlets. The frequency of plant development decreased considerably in the presence of fructose and was completely inhibited by glucose. In the absence of sucrose no plantlet development was observed.

The most commonly used carbohydrate for plant tissue culture is sucrose. In nature, carbohydrate is transported within the plant as sucrose and the tissue may have the inherent capacity for uptake, transport and utilization of sucrose. In tissue culture of egg plant sucrose was the best carbon source, while in *Medicago sativa*, maltose induced the highest embryo yield (Strickland *et al*., 1987). In the present study, carbohydrates other than sucrose and maltose inhibited embryo growth and plant production either partially or completely. The most commonly used carbohydrate for plant tissue culture is sucrose. While fructose supported embryogenesis in *M. sativa* (Strickland *et al.*, 1987), they had an inhibitory effect on finger millet as peanut. The inhibitory effect of several carbohydrates on tissue culture may be due to the lack of proper uptake and transport and subsequent utilization of the substances by the tissue. Sucrose at 3% gave the best response, while at lower concentration, the frequency of response and average number of somatic embryos per responding culture was less. However higher concentrations of sucrose (3.5, 4, 4.5 and 5.5%) inhibited embryogenesis.

**Table 3.** Influence of different concentrations of BAP and IBA on regeneration of somatic embryos cultured on the medium fortified with MS salts and B5 vitamins.

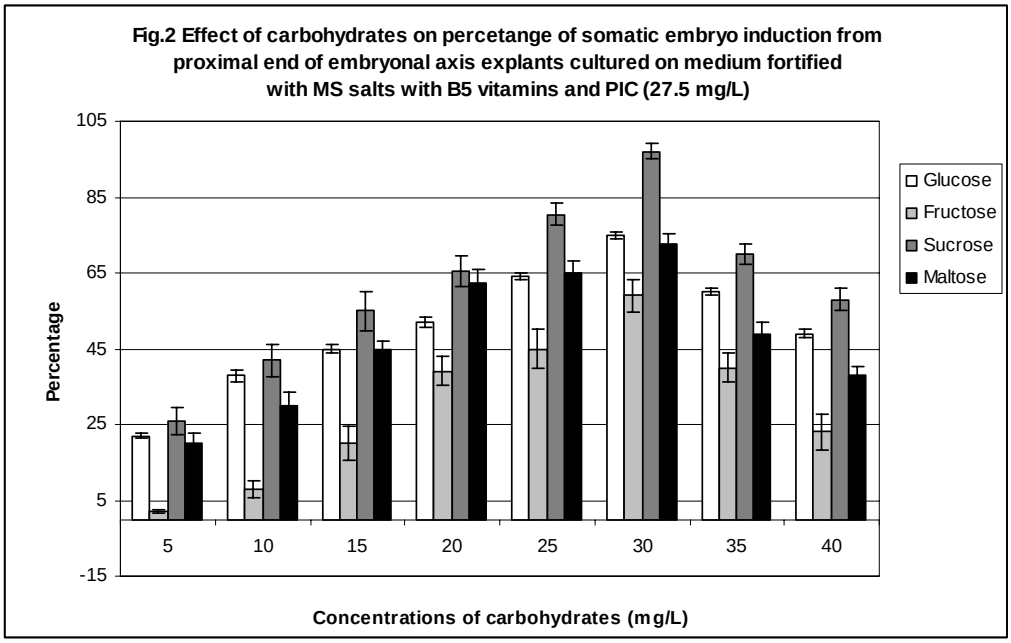
| Concentrations of growth regulators (mg/L) | No. of somatic embryos tested | No. of somatic embryos responded | Mean no. of matured somatic embryos | Mean no. of regenerated plantlets | Percentage of plant regeneration |
|---|---|---|---|---|---|
| **BAP** | | | | | |
| 0.05 | 62 | 38.5 ± 0.42 fg | 38.5 ± 0.46 gh | 23.5 ± 0.28 gh | 61.0 h |
| 0.10 | 66 | 39.4 ± 0.38 ef | 39.4 ± 0.48 fg | 24.5 ± 0.29 fg | 62.18 g |
| 0.15 | 71 | 40.2 ± 0.26 e | 40.2 ± 0.44 f | 25.2 ± 0.30 ef | 62.68 fg |
| 0.20 | 72 | 40.8 ± 0.28 de | 40.8 ± 0.46 ef | 26.0 ± 0.26 e | 63.72 ef |
| 0.25 | 71 | 39.2 ± 0.34 f | 39.2 ± 0.42 g | 25.1 ± 0.25 f | 64.0 e |
| 0.30 | 70 | 38.1 ± 0.36 g | 38.1 ± 0.40 h | 24.3 ± 0.27 g | 63.7 f |
| 0.35 | 69 | 37.0 ± 0.32 gh | 37.0 ± 0.44 hi | 22.9 ± 0.24 h | 61.8 gh |
| **BAP + IBA** | | | | | |
| 0.20 + 0.05 | 68 | 62.5 ± 0.75 cd | 62.5 ± 0.72 d | 43.5 ± 0.52 de | 69.6 de |
| 0.20 + 0.10 | 67 | 63.0 ± 0.76 bc | 63.0 ± 0.76 c | 44.2 ± 0.54 cd | 70.1 d |
| 0.20 + 0.15 | 68 | 64.2 ± 0.70 ab | 64.2 ± 0.72 b | 45.0 ± 0.53 bc | 70.1 d |
| 0.20 + 0.20 | 67 | 65.8 ± 0.68 a | 65.8 ± 0.70 a | 49.9 ± 0.58 a | 75.8 a |
| 0.20 + 0.25 | 67 | 64.0 ± 0.64 b | 64.0 ± 0.68 bc | 47.2 ± 0.56 b | 73.8 b |
| 0.20 + 0.30 | 67 | 62.9 ± 0.72 c | 62.9 ± 0.64 cd | 44.7 ± 0.50 c | 71.1 c |
| 0.20 + 0.35 | 68 | 62.0 ± 0.76 d | 62.0 ± 0.70 e | 43.9 ± 0.54 d | 70.8 cd |

Values are means ± SE of three repeated experiments with three replicates.
Means within a column followed by the same letters are not significant at *P*=0.05 according to DMRT

### *Germination of mature somatic embryos*

Torpedo-shaped embryos developed through direct and indirect somatic embryogenesis were separated and transferred to MS basal medium supplemented with BAP and IBA for further development and regeneration of somatic embryos, while MS basal medium lacking growth regulators served as the control. Among different concentrations tested, media fortified with MS salts, B5 vitamins, BAP (0.2 mg/L) and IBA (0.2 mg/L) showed best response for somatic embryo regeneration (Table 3). In this concentration 75.8% of plant regeneration was observed from the matured somatic embryos (Figs 1d-e) (Figs 2k-m). The individual effect of BAP and IBA showed very less response for somatic embryo regeneration. Finally highest of 49.9 ± 0.58 plantlets were regenerated form the 67 somatic embryos tested. Sellars *et al*. (1990) found that an average of 80% of somatic embryos of peanut produced shoots, while an average of 61% produced roots. In the experiments of Ozias-Akins (1989) the conversion frequency ranged from 0-18% overall experiments. Some times

NAA favored subsequent conversion of somatic embryos into plantlets. This type of BAP mediated enhanced somatic embryo regeneration was also observed in cotton (Ganesan and Jayabalan, 2004; Chengalrayan *et al*., 2001). In the case of peanut different forms and types of media with PGR ($GA_3$, ABA, KN, BA, Zeatin, 2-iP and TDZ ) were used for the regeneration of somatic embryos (Baker and Wetzsten, 1994; Sellars, 1990; McKently *et al*., 1991).




Number of explants tested – 200;
Values are means ± SE of three repeated experiments with three replicates.
Means within a column followed by the same letters are not significant at $P = 0.05$ according to DMRT

Usually, somatic embryos induced through direct and indirect method requires separate medium for germination (McKently *et al*., 1991). The advantage of this study is the embryos developed through direct and indirect method did not required separate medium for regeneration. They showed highest percentage of embryo conversion in the medium fortified with BAP and IBA. Development of abnormal somatic embryos is main problem during somatic embryogenesis. In this present investigation, it was highly reduced by 2%. Fully germinated embryos with well developed root and shoot systems were counted. From the fully regenerated plantlets the tertiary root formation was observed after 25 days of incubation. The plantlets with tertiary roots were allowed to harden. Then the plantlets were removed from the tubes and washed

in running water and were transferred to plastic pots. Initially the plastic pots were covered with plastic bags that were progressively removed to reach normal atmospheric conditions and the hardened plants showed 100% survival

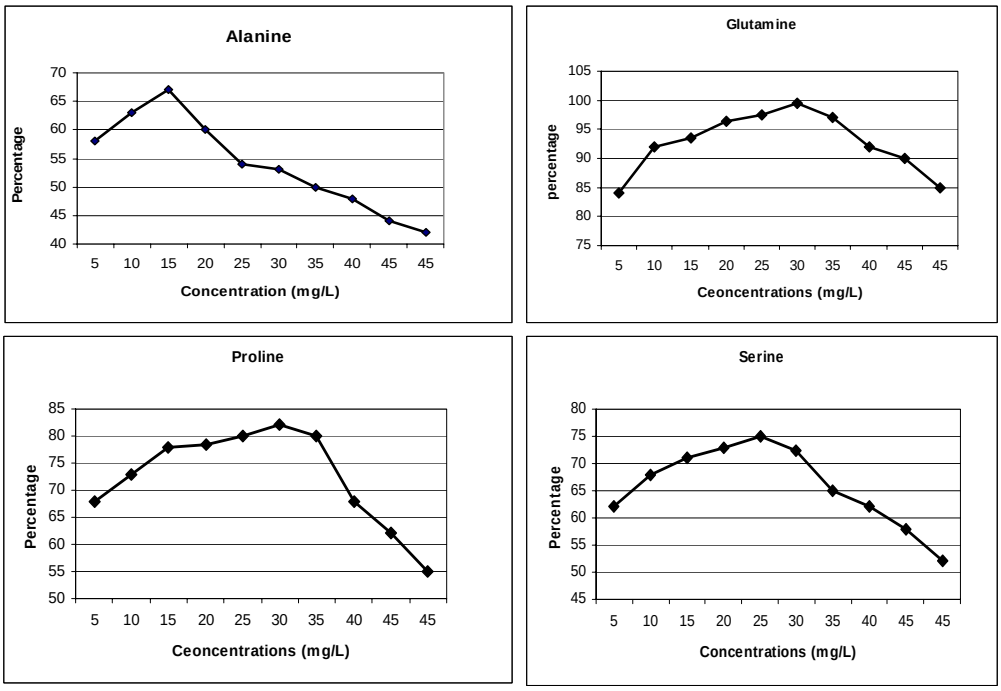



**Fig. 3.** Effect of various concentration of amino acids on the percentage of embryogenic callus induction in MS medium fortified with 2,4-D(2.0 mg/L) and BAP (1 mg/L). Number of explants tested – 250; Values are means ± SE of three repeated experiments with three replicates.
Means within a column followed by the same letters are not significant at $P = 0.05$ according to DMRT

rate at green house condition (Figs 1f, 2n). Regeneration of plantlets from somatic embryos on simple medium might be used for the production of somaclones of plants and for the storage and the maintenance of germplasm. It could be of practical application for raising hybrid seedlings of difficult crosses and mutagenesis in vitro. It can also be applied to plant transformation, either by particle bombardment or Agrobacterium-mediated gene transfer technology.

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(Received 27 June 2005; accepted 15 December 2005)