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Somatic embryogenesis and plant regeneration from cotyledon tissue of *Arachis hypogaea* L.

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Tissue culture techniques are useful for *ex situ* conservation of rare, endemic or threatened plant species. This report describes a protocol for somatic embryogenesis of peanut (*Arachis hypogaea* L.) through cotyledon explants. Only 10% of the explants were contaminated by using this material to start the *in vitro* culture. The highest frequency (35.4%) of embryogenic calli induction was observed on MS medium supplemented with 0.50 mg/l KIN in combination with 0.10 mg/l 2,4,-D. the percentage response for embryogenic proliferation increased in the medium supplemented with GA3(0.20 mg/l) and 1.0 mg/l BA. The regenerated embryos were successfully transferred to the embryo development medium and transferred to the trays after the development of secondary and tertiary root development.

Key words: Somatic Embryo, Cotyledon, Embryogenic calli and Hardening

Haploid plant production, protoplasmic fusion, gene transformation and exploitation of somaclonal variation are examples of *in vitro* culture techniques with potential for crop improvement. An efficient regeneration protocol either by organogenesis or somatic embryogenesis is a major prerequisite for the application of gene transfer methods. Regeneration of peanut by organogenesis from various explants, leaves, cotyledons, cotyledonary node, hypocotyl, epicotyl, and zygotic embryos, has been reported (McKently *et al*. 1990, Eapen and George 1993), however the regeneration frequency was low and plants were rarely established. A number of recent reports describe somatic embryogenesis in peanut using a variety of different explants, including leaves (Baker and Wetzstein 1992, Chengalrayan *et al*. 1994), immature cotyledons (Kim *et al*. 2004, Eapan *et al*.1993), immature embryos (Hazra *et al*.1989), mature and dry seeds (Baker *et al*. 1995) hypocotyl (Venkatachalam *et al*. 1997a), cotyledons with or without embryos, epicotyls, leaflets (Cucco and Jaume 2000) and mature epicotyl esplants(Little *et al*. 2000). Selection of plant species through somoclonal variation are known to enhance the genetic variability of crop plants and the efforts are being made to improve the characters of *Arachis hypogaea* crop for higher yield, oil content and development of cultivars resistance to disease. The present work reports a regeneration protocol for somatic embryo regeneration of peanut peanut using cotyledon explants.

Materials and Methods

In vivo germination:

The ground nut (*Arachis hypogaea* L.) cultivar Co-2 were used in the present

investigation and seeds were obtained from Tamil Nadu Agricultural University, Coimbatore, India. Two hundred seeds were sown *in vivo* under green house condition.

***In vitro* germination:**

For *in vitro* germination of seeds the seeds were immersed in distilled water with a drop of Tween 20 for 5 min and surface sterilized with 0.1% (m/v) aqueous mercuric chloride for 7-10 min followed by 5 rinses in sterile distilled water. Seeds were germinated on Murashige and Skoog (1962) basal medium under dark and temperature of 24 ±2°C.

Initiation, proliferation and selection of embryogenic callus:

The cotyledon segments were placed horizontally on medium comprising MS salts, 100 mg/l myo-inositol, 0.8 g/l agar and 30g/l sucrose and supplemented with 0.05-4.0mg/l BAP, 0.05- 0.2 IAA, 0.05-0.2 IBA, 0.05-0.2 KN and 0.05- 0.2NAA alone and in combination. After 3 weeks of culture, pro-embryonic callus was isolated and sub cultured at weekly intervals on the same medium for another 2 weeks for the induction of embryogenic calli. The embryogenic callus was identified by means of anatomical studies on the basis of its creamy white colour and presence of small less vacuolated and densely filled cytoplasmic cells.

Embryoid induction, maturation and plantlet regeneration from somatic embryos:

The embryogenic callus obtained was subcultured on medium containing MS salts, 150mg/l thiamine ABA 0.05-0.2mg/l for accelerating the induction of embryoids, weekly subcultures of embryogenic callus and the removal of dead, dark-brown cells from the callus was carried out. After 4 weeks of subculture, greenish- yellow, globular pro- embryoids were observed, and these were selected and subcultured on the same medium for maturation. Matured somatic embryos (heart- and torpedo shaped) were transferred to somatic embryo regeneration medium containing MS salts; 100mg/l myo- inositol, 0.02-0.2mg/l thiamine, 30g/l glucose together with 0.02-0.2mg/l Ga_3 and 0.05-2.0 mg/l BAP were supplemented with alone and in combination. After the complete regeneration of embryoids to plant let(40-60 days), the latter were transferred to plastic pots containing FYM, Red soil and sand in 1:1:2: ratio, for hardening. To maintain the humidity, hardened plants were completely covered with plastic bags, which were progressively removed to aid adoption to normal environmental conditions. The selected plants, then adapted to normal environmental conditions were transferred to earthen pots for further growth and development.

Only in selected regions could proembryogenic calli be identified based on the presence of dense cytoplasm and small cells (Sakhanokho *etal*. 2000). The cotyledon segments responded better than other explants with respect to proembryogenic callus induction. While the calli that developed showed variations in colour and texture- including yellowish –green, watery yellow and brownish friable- only the creamy – white calli were pro- embryogenic in nature. The selected pro- embryogenic calli were subcultured on the same medium for a second 2 week period to induce the formation of embryogenic calli, and these calli were morphologically similar to those developed by Zhang *etal*. (2001).

Statistical analysis:

Experiments were set up in a randomized block design and each experiment usually 10 replications and was repeated at least three times. Observations were recorded on the frequency of embryos from the explant and the number of

germinating embryos. Means and standard errors were used through out the study and the values were compared using Duncan's multiple range tests according to Gomez and Gomez (1976).

Table 1. Effect of BAP, KIN, 2,4- D and NAA on embryogenic callus Production in the cotyledon explants of groundnut after 3 weeks of culture

Hormone		Total no of explants	Total No. of Explants responded	No. of Callus / explants	Percentage of callusing
BAP					
0.05		50	10 e	1.7±1.26 cd	20 e
0.10		50	16 cd	2.7±1.76 ab	32 d
0.50		50	22 a	4.8±2.27 a	44 a
1.00		50	18b	2.5±1.28 bc	36 b
1.50		50	17bc	1.5±1.02 de	34 c
KIN					
0.05		50	8 de	1.6±1.2 e	16 e
0.10		50	12 bc	3.3±2.53 bc	24 c
0.50		50	19 a	4.5±2.29 a	38 a
1.00		50	13b	3.8±2.27b	26 b
1.50		50	10 cd	3.2±2.13 cd	20 d
2,4-D					
0.02		50	20 bc	4.0±1.72 d	40 c
0.05		50	29 a	5.3±2.29 bc	58 a
0.10		50	21 b	10.2±4.97 a	42 b
0.50		50	15 cd	5.6±4.02 b	30 d
1.00		50	10 de	2.9±2.84 e	20 e
BAP	**2,4-D**				
0.50	0.02	50	13 cd	3.4±1.74 de	26 d
0.50	0.05	50	21b	10.9±4.00a	42 b
0.50	0.10	50	30 a	7.6±3.44 b	60 a
0.50	0.50	50	19 c	5.4±3.23 c	38 c
0.50	1.00	50	7 e	3.8±3.21d	14 e
KIN	**2,4-D**				
0.50	0.02	50	20 d	18.3±6.18 cd	40 d
0.50	0.05	50	25 c	25.1±6.20 b	50 c
0.50	0.10	50	42 a	35.4±4.86 a	84 a
0.50	0.50	50	30 b	19.2±8.07 c	60 b
0.50	1.00	50	18 e	11.4±5.00 e	36 e

Values are mean+SD(n=10) of two independent experiments. Means followed by the same letter in a column are not significantly different as indicated by Duncan's multiple range test(P= 0.05) Mean values within a column having the same alphabet are not statically significant. Means sharing at least one letter are no significantly different at the $p<0.05$ level according to Duncan's multiple range test.

Induction of Embryogenic Callus:

Among the different combinations of growth regulators used only stem explants allowed the proliferation of embryogenic calli. Cotyledon explants developed calli three weeks after inoculation. A highest frequency (35.4%) of embryogenic calli induction was observed on MS medium augmented with 0.50 mg/l KIN in combination with 0.10 mg/l 2,4-D (Tabel-1).

The initial primary embryogenic callus was yellowish, nodular and less friable. Individual and concentrations of BAP, KIN and BAP+ 2,4-D induced nodular callus that did not become embryogenic with 4 weeks of culture. Jadhar and Hegale (2001) used tender shoots for development of somatic embryos on MS medium supplemented with 4.0 mg/l 2,4-D and 5.0 mg/l KIN.

Table 2. Effect of BAP, KIN and 2, 4-D on Embryogenic callus production from the Cotyledon explant

Growth Regulators (mg/l)	Total number of Explants	Number of explants responded	Mean Number of Embryos per explant	Percentage of Responding cultures
2,4-D				
0.05	250	61 d	4.0±1.72 cd	24.4 d
0.10	250	96 b	5.3±2.79 bc	38.4 b
0.50	250	113 a	10.2±4.97 a	45.2 a
1.00	250	81 bc	5.6±4.02 b	32.4 bc
1.50	250	46 de	2.9±2.84 de	18.4 de
2.00	250	30 ef	0.8±0.97 f	12.0 ef
BAP+2,4-D				
0.50+0.02	250	30 cd	3.4±1.74 de	24 b
0.50+0.05	250	80 a	10.9±4.00 a	32 a
0.50+0.10	250	51 b	7.6±3.44 b	20.4 bc
0.50+0.50	250	40 bc	5.4±3.23 c	16 cd
0.50+1.00	250	19 de	3.8±3.21 cd	7.6 e
0.50+1.50	250	14 ef	1.8±2.35 ef	5.6 ef
KIN+2,4-D				
0.50+0.02	250	97 d	18.3±6.18 cd	38.8 cd
0.50+0.05	250	120 c	25.1±6.20 b	48 c
0.50+0.10	250	185 a	35.4±4.86 a	74 a
0.50+0.50	250	146 b	19.2±8.07 c	58.4 b
0.50+1.00	250	72 e	11.4±5.00 e	28.8 c
0.50+1.50	250	63 ef	7.8±4.23 ef	25.2 ef

Values are mean+SD(n=10) of two independent experiments. Means followed by the same letter in a column are not significantly different as indicated by Duncan's multiple range test(P= 0.05) Mean values within a column having the same alphabet are not statically significant. Means sharing at least one letter are no significantly different at the $p<0.05$ level according to Duncan's multiple range test.

Development of Somatic Embryos:

Embryogenic calli which were separated from the primary culture and subcultured on fresh medium containing MS basal medium supplemented with combinations of BAP(1.50), KIN (0.50) and (0.02-1.00) 2, 4- D. It was reported that in other studies that 2,4-D has been the most commonly used auxin for induction of somatic embryogenesis (Muthusamy *et al*.,

2007). The percentage response for embryogenic proliferation increased to 75% in the medium supplemented with KIN(0.50) and 2,4-D(0.10) on medium with 0.50 mg/l KIN and 0.10 mg/l 2,4- D numerous globular and bipolar somatic embryos developed on the surface of the embryogeneic calli within 2 weeks of culture repeated secondly. Somatic embryogenesis was achieved by regular subculture. In normal somatic embryogenesis, only part of the callus embryogenic and the non-embryogenic was progressively removed, otherwise if converted the embryogenic calli into non-embryogenic calli.

Table 3. Effect of growth regulators on germination of Somatic embryos

Growth Regulators (mg/l)	Total number of Explants	Number of explants responded	Mean Number of Embryos per explants	Percentage of Responding cultures
½ MS	250	6 b	0.9±0.83 b	2.4 b
MS Basal	250	10 a	6.3±2.83 a	184 a
BAP				
0.02	250	18 b	3.3±1.73 b	7 b
0.05	250	80 a	5.4±1.42 a	32 a
0.10	250	7 c	2.7±1.79 bc	2.8 bc
0.50	250	3.5 d	1.1±0.83 d	1.4 cd
1.00	250	2 de	0.6±1.01 de	0.8 de
BAP+GA_3				
0.05+0.02	250	18 c	5.2±1.46 bc	7.2 c
0.05+0.05	250	52 b	7.1±2.16 b	20.8 b
0.05+0.10	250	88 a	10.3±4.1 a	35.2 a
0.05+0.50	250	5 cd	2.0±2.23 d	2 cd
0.05+1.00	250	3 de	0.8±1.97 de	1.2 de
BAP+ABA				
0.05+0.02	250	21 de	3.2±1.83 cd	8.4 cd
0.05+0.05	250	104 b	10.2±3.09 b	41.6 b
0.05+0.10	250	167 a	18.0±2.36 a	66.8 a
0.05+0.50	250	58 c	6.5±2.80 bc	23.2 c
0.05+1.00	250	5 f	1.4±1.35 de	2 cd

Values are mean+SD(n=10) of two independent experiments. Means followed by the same letter in a column are not significantly different as indicated by Duncan's multiple range test(P= 0.05) Mean values within a column having the same alphabet are not statically significant. Means sharing at least one letter are no significantly different at the $p<0.05$ level according to Duncan's multiple range test.

The induced embryoids were subcultured for another 3 weeks for complete maturation and the formation of globular heart and torpedo shaped embryos demonstrated the embryo maturation process. The mature somatic embryos were transferred to somatic embryo regeneration medium containing MS salts, B5 Vitamins, 0.2mg/l GA_3 and 1.0 mg/l BA (Table 3) and observed 53% plant

regeneration from mature somatic embryos after 8 weeks of culture with subculture at 8 day intervals.

During somatic embryo germination, some abnormal growth occurred in which there was only shoot or root formation the occurrence of abnormal somatic embryo genesis during somatic embryogenesis reduces the percentage of plant recovered.

Regenerated plantlets having secondary and tertiary roots were considered to be fully regenerated and were transferred to plastic pots for hardening in a mixture containing Red soil sand and vermiculate(1:1:1) ratio. The plastic pots were covered with plastic bags that were progressively removed to reach normal atmospheric conditions. The regenerated plants were phenotypically similar, although smaller to the parental lines and in *in vitro* seed derived plantlets. The *in vitro* regenerated plants showed a 100% similar rate after hardening and after 3 weeks, were transferred to earthen pots for the further growth. An increase in the number of somatic embryos and the regeneration potential of embryoids was also observed.

We also conclude that the protocol for *Emilia zeylanica* somatic embryogenesis described here in reduces the difficulties involved in plant regeneration and will be useful for medicinal plant germplasm improvement. We suggest the further research can be undertaken to study the effect of this protocol for Agrobacterium mediated transformation studies.

In conclusion, further investigations are necessary enhance the germination of somatic embryos and subsequent establishment in the green house. This protocol will help in rapid propagation of *Emilia* and other aster members.

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