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In Vitro Micropropagation of Peanut (*Arachis hypogaea*) Through Direct Somatic Embryogenesis and Callus Culture

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ABSTRACT

Efficient and simple protocol for plant regeneration through direct embryogenesis and callus induction is devised here. MS basal medium supplemented with B5 vitamins, 0.8% agar, 30 g/L sucrose, 2, 4-D and picloram were used individually for direct embryogenesis. Picloram showed best results at the concentration of 19 mg/L. Callus induction was achieved by the induction of embryogenic callus from the cotyledonary explants. MS basal medium supplemented with B5 vitamins, 0.7% agar, 30 g/L sucrose and auxins (2, 4-D, NAA) were tested individually for the callus induction. Highest frequency of 87% was observed for embryogenic callus induction at the concentration of 3 mg/L 2, 4-D and in this concentration highly desirable creamy white friable callus formation was observed. MS media supplemented with B5 vitamins, BAP (0.15 mg/L) and IAA (0.20 mg/L) showed best response for regeneration. Total of 81% of plant regeneration was observed from the somatic embryos and 78% from callus. Regenerated plants showed 100% survival and fruit set in green house.

Key Words: Micropropagation; Peanut; Somatic embryogenesis; Callus induction
Abbreviations: NAA: Naphthalene acetic acid; BAP: 6-Benzylaminopurine; IAA: Indole acetic acid; PIC: Picloram; 2, 4 –D: 2, 4-Dichlorophenoxyacetic acid.

INTRODUCTION

Legumes are the most important group of crop plants next to cereals and they are very recalcitrant to tissue culture regeneration. Therefore, many efforts have been devoted to develop efficient *in vitro* regeneration system (Anuradha *et al.,* 2006). Peanut (*Arachis hypogaea* L.) is an important cash crop of farmers particularly in the semi arid tropics (Krapovickas & Gregory, 1994).

Peanuts are a rich source of edible oil (43-55%) and protein (25-28%) (Gohari & Niyaki, 2010). About 75% of the global production is used for extracting oil and remaining 25% is consumed as food. Peanut is one of the principal economic oilseed legumes and is largely cultivated in many tropical and subtropical regions of the world. Presently 108 countries in the world are growing peanut. Asia is leading with 71.7% of global groundnut production followed by Africa 18.6% production, and North-Central America 7.5% production (ICRISAT, 2009). In Pakistan peanut was first cultivated in 1949-1950 on 500 ha in Rawalpindi area of pothwar region (Khalil & Jan, 2005). During 2008-2009, total production of the country was 89 thousand tones. The major contributor in the peanut production was Punjab having 69 thousand tones along with Sindh and KPK with production of 4.7 and 11.8 thousands tones, respectively (Anonymous, 2009).

Successful generation of transgenic crops depends on the efficiency of *in vitro* micropropagation and transformation protocols (Misra & Misra, 1993; Sharma & Vanamala, 2000). The genetic improvement of crop plants using biotechnological methods requires development of suitable protocols. Plants regenerated through somatic embryogenesis are more useful than plants generated through organogenesis (Bhanumathi *et al.,* 2005). Peanut is very difficult crop to manipulate *in vitro* and only a limited success through tissue culture has been achieved (Heatley & Smith, 1996; Ponsamuel *et al.,* 1998) Tissue culture studies in peanut have been documented including direct embryoid induction and callus induction (Palanivel & Jayabalan, 2000, 2002; Palanivel *et al.,* 2001; Palanivel *et al.,* 2002) but regeneration percentage of plants is very low. Peanut is genotype specific crop so the micropropagation of peanut is tedious and requires specific protocols for each type. Somatic embryogenesis is preferred over plant multiplication because its transformation and proliferation potential is very high and the occurrence of chimeric plants can be minimized significantly (Stefaniak, 1994). The optimization of *in vitro* plant regeneration protocols through direct somatic embryo induction and callus induction would certainly help in the micropropagation of peanut cultivars.

The present study describes the effect of plant growth regulators for inducing direct somatic embryogenesis and

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callusing induction from cotyledonary node (CN) explant of peanut. The aim of this study was to develop an efficient and productive micropropagation system for peanut cultivar BARD-479 using cotyledonary nodes as explant for future use in the multiplication of in vitro genetically transformed peanut plants.

MATERIALS AND METHODS

Plant material and culture media: Seeds of peanut (*Arachis hypogaea* L.) high yielding cultivar BARD-479 was obtained from Barani Agricultural research Institute, Chakwal. Seeds were surface sterilized by the method of (Ganesan & Jayabalan, 2004). The seeds were removed from mature pods and sterilized by soaking into 70% v/v ethanol for 1 min followed by treatment with 50% clorax for 10 min. Then several washing were made with sterile distilled water and soaked for 3 h in sterile distilled water. The cotyledons were separated out and cut down from proximal ends and were germinated on Murashige and Skoog (1962) medium supplemented with 3% (w/v) sucrose and 0.2% (w/v) Phytagel. The pH of the medium was adjusted at 5.8 and maintained under a 16-h photoperiod with a light intensity of 60 l E/m^2/s at 25±1°C.

In the case of callus induction, seed coat separated cotyledons were allowed to grow on the MS basal medium supplemented with hormones for callus induction.

Direct somatic embryogenesis and plant regeneration: Cotyledonary explants were cultured on MS basal medium supplemented with B5 vitamins (Gamborg *et al.*, 1968), 0.2% phytagel, 30 g/L sucrose, 2,4-D (1-35 mg/L) and picloram (15-22 mg/L) direct embryoid induction. The explants were incubated for 20-23 days at 26±2°C under a 16 h photoperiod with a light intensity of 60 l E/m^2/s at 25±1^{0}C.

The explants were incubated on 40 mL medium in glass jars closed with plastic bags. The sub-culturing of explants was done at an interval of 25 days for embryoid induction. The mature embryoids induced were transferred to regeneration media, composed of MS medium supplemented with B5 vitamins and with different concentrations of BAP (0.01-0.25 mg/L) and IAA (0.05-0.30 mg/L), which were tested individually and in combination. Fully developed rooted plants after 35-45 days were transferred to pots containing the 1:1:1 mixture of soil, sand and farm yard manure and incubated for one week for acclimatization (by covering with plastic bags initially & then gradually exposed to open environment) before transfer to glass house. After complete adaptation, the plants were transferred to plastic pots for further growth and development.

Callus induction and Plant Regeneration: Callus induction using cotyledonary nodes as explant was achieved using MS basal medium supplemented with B5 vitamins (Gamborg *et al.*, 1968), 0.2% phytagel, 30 g/L sucrose, 2, 4-D and NAA. The cotyledonary explants were cultured in glass Petri dishes. After 4 subcultures of 12 days each, 47-day old creamy white calli were shifted to MS basal medium supplemented with B5 vitamins, 3% sucrose, BAP (0.01-0.25 mg/L) and IAA (0.05-0.30 mg/L) for regeneration.

Statistical analysis: Means and standard errors were used during the whole study and the values were analysed using Moods median test (Snedecor & Cochran, 1989) using software MSTATC.

RESULTS

Direct somatic embryogenesis: Successful induction of somatic embryoids was achieved by using cotyledonary nodes as explant (Fig. 1A). 19 mg/L of PIC showed best response for the direct somatic embryogenesis where highest number of embryoids per explant i.e., 27. White embryos were originated from explants after 4 sub-culturing and were seen by the naked eye. No callus was seen at this concentration. Embryoid induction was also seen in all the concentrations of 2, 4-D tested but the percentage of response was significantly low as compared to that of PIC (Table I).

Callus induction: Cotyledonary nodes were used as explant for callus induction, and they produced callus on the MS medium supplemented with B5 vitamins (Gamborg *et al.*, 1968), 2, 4-D (1-4 mg/L) and NAA (1-4 mg/L) (Table II). Highest frequency of 87% was observed for callus induction at the concentration of 3 mg/L 2, 4-D and in this concentration highly desirable creamy white friable callus formation was observed (Fig. 1B). NAA also induced callus formation but of low quality and percentage.

Regeneration of direct somatic embryos and callus: Somatic embryos developed through direct embryogenesis and the well developed callus was transferred to MS basal medium fortified with BAP and IAA for regeneration (Fig. 1C). Individual response of BAP was very low as compared in combination with IAA. Among all the concentrations tested, MS media supplemented with hormonal combination BAP (0.15 mg/L) and IAA (0.20 mg/L) showed best response for regeneration (Table III). Total of 81% of plant regeneration was observed from the somatic embryos and 78% from callus. Elongated shoots were shifted to rooting induction medium comprising 0.3 mg/L NAA. After 25 days of culturing, fully developed roots were obtained (Fig. 1D). The rooted plats were then shifted to green house in pots after one week of acclimatization.

DISCUSSION

In this study, we have evolved an efficient protocol to obtain a high frequency of regeneration from directly developed somatic embryoids as well as from callus. Somatic embryoid induction has been successfully carried out in more than one hundred species (Tautorus *et al.*, 1991) and auxins were majorly used hormones especially 2,4-D,

Table I: Effect of different concentrations PIC and 2, 4-D on induction of somatic embryoids from cotyledonary nodes

Concentration of Hormones (mg/L)	Explants responded	Responsive explants (%)	Means of embryos/explant
2,4-Dichlorophenoxy acetic acid			
1	90	30	1±0.15
5	108	36	2±0.17
10	120	40	4±0.19
15	177	59	6±0.21
20	234	78	9±0.23
25	213	71	6±0.20
30	186	62	5±0.19
35	162	54	4±0.19
Picloram			
15	186	62	8.0±0.21
16	204	68	13±0.24
17	228	76	18±0.25
18	240	80	20±0.26
19	273	91	27±0.29
20	246	82	22±0.25
21	228	76	17±0.26
22	180	60	10±0.21

Total no. Of cultured explants =300

Table II: Effect of different concentrations of 2, 4-D and NAA on callus induction from cotyledonary nodes

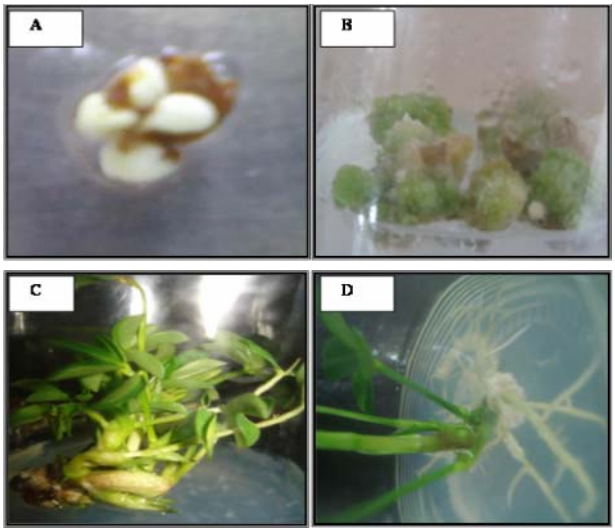
Concentration of hormones (mg/lit)	Callus induction (%)	Concentration of hormones (mg/lit)	Callus induction (%)
2,4-Dichlorophenoxy acetic acid		Naphthalene Acetic Acid	
1	55	1	23
2	71	2	42
3	87	3	56
4	75	4	69
5	53	5	77

Table III: Effect of different concentrations of BAP and IAA on regeneration of somatic embryos and callus cultured

Concentrations of Hormones (mg/L)	No. of Embryos cultures	No. of Responded embryos	Mean no. of Plantlets regenerated	Percentage of plantlets regenerated (%)
6-BAP				
0.01				
0.05	78	30	15±0.18	50
0.10	78	35	18±0.21	51
0.15	81	39	22±0.23	56
0.20	77	45	26±0.28	57
0.25	77	39	21±0.20	53
	79	36	19±0.21	52
BAP+IAA				
0.15+0.05	50	40	25±0.27	62
0.15+0.10	50	42	27±0.28	64
0.15+0.15	50	43	29±0.28	67
0.15+0.20	50	45	35±0.33	77
0.15+0.25	50	43	30±0.33	69
0.15+0.30	50	41	26±0.28	63

Values are means ± SE of three experiments with three replicates

Fig. 1: Different developmental stages of peanut micropropagation through cotyledonary node mediated direct regeneration; (A) somatic embryoid development (B) callus induction (C) regenerated shoots (D): Rooted plants containing the secondary and tertiary roots



that determines the embryoid induction. Several protocols of micropropagation of peanut have been reported during last decade but due to its highly recalcitrant nature, peanut tissue culture is very difficult. During present investigation, an efficient protocol was derived to obtain mass scale production of plants from both direct somatic embryogenesis as well as through callus induction.

In direct somatic embryogenesis, 2, 4-D was observed not to produce high no of somatic embryoids as compared to Picloram, as most of explants turned to callus phase and turned brown. The percentage for induction and number of embryoids was low in case of 2, 4-D as was also seen in a previous study (Bhanumathi *et al.*, 2005). Picloram efficiently produced embryoids as it was confirmed previously that it efficiently produces embryoids from embryo axes at lower consentrations (Eapen & George, 1990). In present study, highest number of white mature embryoids was produced at the concentration of 19 mg/L Picloram.

In case of callus induction, callus of high quality and percentage was observed at the concentration of 3 mg/L 2, 4- D. Cotyledonary nodes were used as explant. A good quality callus was produced at the above concentration. Cotyledonary nodes produce good quality callus as they contain embryo axes (Rani & Reddy, 1997). Very high concentration of 40-50 mg/L reported previously for callus induction in peanut (Cucco & Jaume, 2000). In the present study, the individual effect of 2, 4-D and NAA were tested and the NAA showed a poor response for the callus induction. That individual treatment of auxins induces somatic embryoids in a previous study (Kim *et al.*, 2003). Data revealed that 2, 4-D has critical role for callus induction (Misra, 1996; Misra *et al.*, 1994).

Mature embryoids developed through direct somatic embryogenesis and fully developed calli were transferred to MS medium supplemented with BAP and IAA for regeneration. Best results were obtained at the concentration of BAP (0.15 mg/L) and IAA (0.20 mg/L). About 81% regeneration of somatic embryos was observed at this concentration. Whilst the individual effect of BAP and IAA was very low. This low frequency at individual treatment was very low in earlier experiments where this was reported only 0-18% (Ozias-Akins, 1989). This effect of BAP to enhance regeneration of somatic embryo was also observed in cotton (Misra *et al.*, 1994; Chengalrayan *et al.*, 2001; Ganesan & Jayabalan, 2004). In this study, same media was seen equally responsive for plantlet regeneration through somatic embryos as well as callus. Low concentration of auxins along with higher level of cytokinins proved better to get regeneration from calli. High cytokinins level resulted in increased differentiation of shoots (Venkatachalam *et al*., 2000).

Root induction was achieved at 0.3 mg/L while in a previous study, roots were induced at 0.1 mg/L (Verma *et al*., 2009). At the higher concentration of NAA, malformed callus was observed rather than roots on the base of shoots. Roots were induced within 15-20 days. Well developed rooted plants were taken out from jars and transferred into polythene bags for hardening.

In conclusion, the protocol developed here has provided efficient approaches for the micropropagation of peanut through mature embryoids as well as callus. The results obtained with cotyledonary nodes confirmed that embryos are suitable explants for somatic embryoid induction as well as callus induction and this methodology could be extended to other species not manipulated.

REFERENCES

Anonymous, 2009 available at http://www.statpak.gov.pk/depts/fbs/statistics/agriculture_statistics/area_production_crops.pdf

Anuradha, T.S., S.K. Jami, R.S. Datla and P.B. Kirti, 2006. Genetic transformation of peanut (*Arachis hypogaea* L.) using cotyledonary node as explant and a promoterless gus nptII fusion gene based vector. *J. Biosci*., 31: 235–246

Bhanumathi, P., M. Ganesan and N. Jayabalan, 2005. A simple and improved protocol for direct and indirect somatic embryogenesis of peanut (*Arachis hypogaea* L.). *J. Agric. Technol*., 1: 327–344

Chengalrayan, K., S. Hazra and M. Gallo Meagher, 2001. Histological analysis of somatic embryogenesis and organogenesis induced from mature zygotic embryo derived leaflets of peanut (*Arachis hypogaea* L.). *Plant Sci.*, 161: 415–421

Cucco, M.F. and A.D.R. Jaume, 2000. Protocol for regeneration in vitro of Arachis hypogaea L. *EJB Elect. J. Biotechnol.*, 3: 154–160

Eapen, S. and L. George, 1990. Influence of phytohormones, carbohydrates, aminoacids, growth supplements and antibiotics on somatic embryogenesis and plant differentiation in finger millet. *Plant Cell Tiss. Org. Cult.*, 22: 87–93

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res.*, 50: 151–158

Ganesan, M. and N. Jayabalan, 2004. Evaluation of haemoglobin (erythrogen): for improved somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L. cv. SVPR 2). *Plant Cell Rep.*, 23: 181–187

Gohari, A.A. and A.N. Niyaki Syed, 2010. Effects of Iron and Nitrogen Fertilizers on Yield and Yield Components of Peanut (*Arachis hypogaea* L.) in Astaneh Ashrafiyeh, Iran. *American-Eurasian J. Agric. Environ. Sci.*, 9: 256–262

Heatley, M.E. and R.H. Smith, 1996. Whole plant regeneration from the shoot apex of Arachis hypogaea L. *In Vitro* Cell. *Dev. Biol.*, 32: 115–118

ICRISAT, 2009. http://www.icrisat.org/GroundNut/GroundNut.htm

Khalil, I.Q. and A. Jan, 2005. *Cropping Technology*, pp: 242–247. National Book Foundation

Kim, S.W., T.J. Kim and J.R. Liu, 2003. High frequency somatic embryogenesis and plant regeneration in petiole and leaf explant cultures and petiole-derived embryogenic cell suspension cultures of Hylomecon vernalis. *Plant Cell Tiss. Org. Cult.*, 74: 163–167

Krapovickas, A. and W. Gregory, 1994. Taxonomia del genero Arachis (Leguminosae). *Bonplandia*, 8: 1–186

Misra, M. and A.N. Misra, 1993. *Genetic Transformation of Grass Pea*, pp: 246–251. In: DAE Symposium on Photosynth. and Plant Molecular Biology, BRNS/DAE, Bombay

Misra, M., 1996. *In vitro* micropropagation of Pogostemon cablin Benth. through callus culture. *Plant Cell Rep.*, 15: 991–994

Misra, M., G. Addis and R.K.J. Narayan, 1994. Methods for callus induction and differentiation of *Lathyrus sativus* and embryo rescue in interspecific crosses by tissue culture. *J. Agric. Soc. University Wales*, 74: 129–144

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, 15: 473–497

Ozias-Akins, P., 1989. Plant regeneration from immature embryos of peanut. *Plant Cell Rep*., 8: 217–218

Palanivel, S. and N. Jayabalan, 2000. Correlative effect of Adenine sulphate and Benzylamino purine on the regeneration potentiality in cotyledonary explants of groundnut (*Arachis hypogaea* L.) *J. Plant Biotechnol*., 2: 21–24

Palanivel, S. and N. Jayabalan, 2002. Direct multiple shoot induction from different mature seed explants of groundnut (*Arachis hypogaea* L.) *Philippines J. Sci.*, 131: 127–131

Palanivel, S., S. Parvathi and N. Jayabalan, 2001. In vitro culture of mature embryo axes of groundnut (*Arachis hypogaea* L.). *J. Indian Bot. Soc*., 80: 15–19

Palanivel, S., S. Parvathi and N. Jayabalan, 2002. Callus induction and plantlet regeneration from mature cotyledonary segments of groundnut (*Arachis hypogaea* L.). *J. Plant Biol.*, 45: 22–29

Ponsamuel, J., D.V. Huhman, B.G. Cassidy and D. Post-Beittenmiller, 1998. *In vitro* regeneration via caulogenesis and brassin-induced shoot conversion of dormant buds from plumular explants of peanut (*Arachis hypogaea* L. cv `Okrun'). *Plant Cell Rep*., 17: 373–378

Rani, A.S. and G.M. Reddy, 1997. Plant regeneration from different seedling explants of groundnut, *Arachis hypogaea* L. *In Vitro*., 33: 3 (Conference abstract Nº 54)

Sharma, K.K. and A. Vanamala, 2000. An efficient method for the production of transgenic plants of peanut through Agrobacterium tumefaciens-mediated genetic transformation. *Plant Sci.*, 159: 7–19

Snedecor, G.W. and W.G. Cochran, 1989. *Statistical Methods*, 8th edition. Iowa State University, Ames, Iowa

Stefaniak, B., 1994. Somatic embryogenesis and plant regeneration of gladiolus (*Gladiolus hort*). *Plant Cell Rep*., 13: 386–389

Tautorus, J.E., L.C. Fowke and D.I. Dunstan, 1991. Somatic embryogenesis in conifers. *Canadian J. Bot.*, 69: 1873–1899

Venkatachalam, P., N. Geetha, A. Khandelwal, M.S. Shaila and G. Lakshmi Sita, 2000. Agrobacterium-mediated genetic transformation and regeneration of transgenic plants from cotyledon explants of groundnut (*Arachis hypogaea* L.) via somatic embryogenesis. *Curr. Sci.*, 78: 1130–1136

Verma, A., 2009. Response of groundnut varieties to plant growth regulator (BAP) to induce direct organogenesis. *World J. Agric. Sci.*, 5: 313–317

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