Indian J. Plant Physiol., Vol. 10, No. 3, (N.S.) pp. 205-210 (July-Sept., 2005)

AN EFFICIENT DIRECT SHOOT REGENERATION FROM COTYLEDONARY NODE EXPLANTS OF PEANUT (*ARACHIS HYPOGAEA* L. CV. JL-24)

M.R. BEENA, S.K. JAMI, T. SRINIVASAN, T. SWATHI ANURADHA, G. PADMAJA AND P.B. KIRTI*

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

Received on 15 June, 2005

SUMMARY

The shoot regeneration response from different explants, *viz*. cotyledon, cotyledonary node and petiolule, of the most popular Indian cultivar, JL-24 of peanut (*Arachis hypogaea* L.), was evaluated by culturing on Murashige and Skoog medium containing B_5 vitamins supplemented with different growth regulators. Shoot bud initiation, proliferation and subsequent development into shoots were found to be superior from cotyledonary node explants in comparison to other explants. High frequency (69%) direct shoot regeneration from cotyledonary node was observed on MS medium with 4.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. The shoots elongated upon transfer to MS medium with lower concentrations of BAP. Elongated shoots could be continuously harvested at 15 days interval for a period of 75 to 90 days. The regenerated shoots were rooted at a high frequency of 84.5% on MS medium containing 1.0 mg l^{-1} NAA. The regenerated plantlets survived to maturity in greenhouse, flowered, set pods and seed normally. This protocol could be very efficiently used for transformation studies.

***Key words*: Cotyledon, cotyledonary node, peanut, petiolule, plant regeneration**

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is an important oilseed crop with high economic value in India and worldwide. The peanut seed has the dual advantage of being important as a source of edible oil as well as protein. In India, much of the cultivation of this crop occurs in the semi-arid tropical regions and the fate of the crop is dependent on the vagaries of nature, which are in the form of abiotic and biotic stresses. Though the genus *Arachis* contains a number of wild accessions possessing genes for resistance against different stresses, these genes could not so far utilized in peanut improvement through conventional breeding because of the association of these desirable genes with undesirable gene blocks. In such a situation, genetic transformation technology offers an efficient alternative for introducing known alien genes conferring stress tolerance into peanut. These genes could be introduced through genetic transformation into desirable cultivars of a crop species and a good number of transgenic plants could be tested for the desired level of transgene expression and improvement of the target trait. To achieve this, an efficient and repeatable regeneration protocol is a prerequisite.

Regeneration in peanut is a difficult task as it is a leguminous plant with high polyphenol content. Tissue culture at every step - shoot bud induction, elongation, rooting, acclimatization and transfer to greenhouse and establishment - is critical for this crop. Direct shoot regeneration from different explants like hypocotyl, epicotyl, leaflet, cotyledonary node etc. has been reported earlier (Mroginski *et al*. 1981, McKently *et al*. 1991,

* Corresponding author, E-mail: pbksl@uohyd.ernet.in

Cheng *et al*. 1992, Eapen and George 1993, Kanyand *et al*. 1994, Venkatachalam *et al*. 1996, Ponsamuel *et al*. 1998, Venkatachalam *et al*. 1999). But most of the protocols showed either sporadic or transient shoot regeneration with less conversion frequency. Direct shoot organogenesis from meristematic explants without callus phase is emerging as a rapid and relatively efficient method for *Agrobacterium* mediated transformation in a number of legume species as it would eliminate the negative effects of somaclonal variation. In the present study the shoot regeneration response of cotyledon, cotyledonary node and petiolule explants have been evaluated. The results obtained in the study differ from earlier reports in induction of shoot buds from cotyledonary node explants followed by continuous recovery of shoots by repeated transfer to medium containing lower levels of BAP. The protocol described here can be efficiently exploited for genetic transformation.

MATERIALS AND METHODS

Mature seeds of peanut (*Arachis hypogaea* L.) cultivar JL-24 were shelled and stored at 4°C at least 24 h before use. Seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 7 min and rinsed four to five times in sterile double distilled water. The seeds were soaked for 6 h and were germinated on filter paper wicks soaked in sterile double distilled water. Three to four-day old germinating seeds were used for the experiments; at the stage when the cotyledons turned light green and radicle of about an inch length was discernible. After removing the seed coat completely, about 70% of the radicle and the apical shoot were removed and the explants were cut vertically into two halves along the embryo axis. The nodal region that was exposed by the removal of the part of the embryo axis attached to the cotyledon was damaged vertically to inhibit the growth of any primary buds. The cotyledonary node explants so prepared was cultured on MS medium with different growth regulator combinations. For petiolule explants with the lamina attached, the unopened leaflets were excised from five to six-day old seedlings and cultured on MS media with different combinations and concentrations of growth regulators. Cotyledon explants were taken directly from the seeds that were soaked for 4 to 6 hr in sterile distilled water after surface sterilization and de-embryonation. Cotyledons and cotyledonary node explants were cut into two vertical halves and were cultured with the cut end touching medium with the proximal end inserted. A minimum of 100 explants were cultured for each treatment combination. The data obtained was analyzed by analysis of variance (ANOVA).

For the experiments on *in vitro* regeneration, various combinations of growth regulators were used with MS (Murashige and Skoog 1962) basal salts, B_5 (Gamborg *et al*. 1968) organic constituents and 3% (w/v) sucrose. The pH of the medium was adjusted to 5.7 and solidified with the addition of 0.5% (w/v) agargel (Sigma-Aldrich, St. Louis, USA). For initial experiments, different concentrations of BAP (0.5-8.0 mg l^{-1}) alone and in combination with other growth regulators like 2,4-D and NAA at concentrations ranging from 0.1 to 1.5 mg l^{-1} were tested (Table 1). All cultures were incubated at 27 ± 1°C in the culture room with 16/8 hr photoperiod maintained at about 1500 lux**.** Subcultures were done at 30-day intervals unless otherwise mentioned. Explants with shoot buds were transferred to media containing ≤ 2.0 mg l^{-1} BAP for shoot elongation. Rooting of elongated shoots was tried on nutrient media without any growth regulators and those containing 0.1, 0.5, 0.8 and 1.0 mg l^{-1} NAA.

RESULTS AND DISCUSSION

Shoot Regeneration

Direct shoot bud regeneration was observed with a high frequency from cotyledonary node in comparison to cotyledon and leaf petiolule (Fig. 1 a-d). Effectiveness of cotyledonary node in shoot regeneration was demonstrated in soybean by Olhoft and Somers (2001) and Olhoft *et al*. (2001) that was successfully used for genetic transformation. In the present study cotyledonary node explants developed numerous shoot buds only when the explant was cultured with the cotyledon attached to it. Cotyledon became shrunk once the shoot buds started developing.

The cotyledons turned green, enlarged and started developing callus at the cut end within 7 to 10 days of culture. Shoot bud initiation was observed at the proximal end of the cotyledon (Fig. 1a), where the embryonic axis was previously attached and similar observations have

Fig. 1. a-g: Regeneration in peanut (*Arachis hypogaea* L.) cultivar JL.24. Regeneration of shoot buds on a) cotyledon, b) petiolule, c and d) cotyledonary node initial stage and after 30 days of culture, e) elongated shoot buds developed on cotyledonary explants, f) well rooted shoots ready for acclimatization and glass-house transfer, g) well established and flowered plant in green house.

been made in soybean by Mante *et al*. (1989). Occasionally shoot buds developed from the cut region in contact with the medium, though profuse callus growth was prominent. When petiolule explants were cultured *in vitro*, leaflets turned green after one week of incubation and enlarged four times in size. From the petiolule cut end, small bud primordia started developing along with white friable callus.

Cotyledonary node explants enlarged and turned green in 3 days and green nodular structures differentiated at the meristematic region half way to proximal end by 5-7 days of culture, and the basal hypocotyl region attached to the explant developed callus (Fig. 1c) that probably acted as nurse cells for the developing shoot buds once the cotyledon was removed. This explains the better growth of shoot buds when this callus was retained in the culture until the shoots elongated. The shoot buds became prominent within 10 to 15 days of culture. Unlike cotyledon explants, clustering of sheath like structures (Fig. 1a) were not found in shoots developed on cotyledonary node explants (Fig. 1 c and d).

Cotyledons cultured on MS medium with BAP (0.5-8.0 mg l^{-1}) alone developed shoot buds. Green hard callus developed on medium containing low concentrations of BAP (0.5-3.0 mg l^{-1}) and the number of shoots produced was found to be less. On medium containing higher concentrations of BAP (6.0-8.0 mg l^{-1}) the number of shoot buds decreased and the shoots formed were very short with dark green brittle leaves. Large number of shoot buds proliferated on medium with 4. 0 mg l^{-1} BAP; however, on an average, 7.5 shoot buds elongated into shoots after 30 days of culture on the same medium. Shoot regeneration was higher on medium containing 4.0 mg l^{-1} BAP in combination with 0.2 mg l^{-1} NAA. An average of 6 shoot buds elongated following sub-culture to the same medium. When clumps of shoot buds were transferred to medium containing 2.0 mg l^{-1} BAP, 4 to 5 shoot buds elongated accounting to 75% shoot recovery and as many as 40 shoots could be harvested from a single cotyledonary node explant in 90 days time following this method. Similar situation has been encountered while culturing petiolule explants on MS medium containing

different concentrations of BAP alone and in combination with NAA but with induction of less number of shoot buds and low frequency of shoot bud elongation.

Among the various concentrations of BAP tested on cotyledonary node explants 4.0 mg l^{-1} BAP gave maximum number of shoot buds (35-40) per explant (Table 1). The shoot buds were clustered and fasciation was occasionally noticed at higher concentration of BAP and similar observations have been made earlier in peanut (Baker and Wetzstein 1998). Higher concentration of BAP stimulated bud formation but inhibited further development and growth of shoot buds as also observed by Venkatachalam *et al*. (1999). Relatively low number of shoot buds were produced at lower concentration of BAP (0.5-3.0 mg l^{-1}). Addition of auxin along with BAP favoured induction of more shoot buds (50-60) per cotyledonary node explant (Table 1*)*. Among the growth regulators tested, 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} 2,4-D along with 4.0 and 5.0 mg l^{-1} BAP, respectively gave better results (Table 1 and 2). Cytokinins always showed this synergistic effect when used along with lower concentration of auxins.

When similar growth regulator combinations were tried with slight variations on petiolule explants, the regeneration response was poor. Though few shoot buds differentiated, only 2% of them elongated to shoots even after 2-3 sub-cultures. Cheng *et al*. (1992) reported regeneration from petiolules at a very high concentration of BAP (25.0 mg l^{-1}) in combination with 1.0 mg l^{-1} NAA. Differences in the regeneration potential among the three explants used could be due to physiological status and extent of tissue differentiation. This also explains the fact that younger unopened leaves responded faster and positively compared to the older leaves. Mroginski *et al*. (1981) and Baker and Wetzstein (1998) reported that young unopened leaves have high

Table 1. Effect of different growth regulators on direct shoot regeneration from cotyledonary node (Cn), cotyledon and petiolule explants of peanut after 20 days of culture

Sl no.	Plant growth regulators (mg l^{-1})			Av. number of shoot buds ± SD			Per cent response		
	BAP	NAA	2,4-D	Cotyledon	Cn	Petiolule	Cotyledon	Cn	Petiolule
1	3.0	—	—	13.9 ± 1.37	30.6 ± 2.16	15 ± 1.0	60	63	50
2	4.0	—	—	20.3 ± 1.18	34.8 ± 0.66	10 ± 0.33	58	76	49
3	5.0	—	—	18.1 ± 1.51	32.2 ± 2.19	10 ± 0.33	35	58	30
4	3.0	0.1	—	17.8 ± 1.4	28.4 ± 0.66	19 ± 0.89	12	50	43
5	4.0	0.1	—	22.4 ± 1.95	39.7 ± 2.10	10 ± 0.6	30	25	50
6	5.0	0.1	—	19.5 ± 2.8	25.2 ± 4.04	11 ± 1.2	50	65	40
7	3.0	0.2	—	18.1 ± 1.3	31.1 ± 0.94	16 ± 0.04	48	25	50
8	4.0	0.2	—	24.7 ± 1.61	52.2 ± 4.52	10 ± 0.93	64	79	40
9	5.0	0.2	—	17.9 ± 1.44	27.3 ± 1.41	10 ± 1.14	45	50	45
10	3.0	—	0.5	—	21.1 ± 1.22	—	—	67	—
11	4.0	—	0.5	—	18.8 ± 1.09	—	—	75	—
12	5.0	—	1.0	—	29.8 ± 1.77	—	—	60	—
13	3.0	—	1.5	—	25.8 ± 1.06	—	—	50	—
14	4.0	—	1.5	—	17.2 ± 0.9	—	—	20	—
15	5.0	—	1.5	—	15.0 ± 1.67	—	—	40	—

Table 2. Regeneration percentage and average number of shoot buds per explant of cotyledon and cotyledonary nodal (Cn) explants of *Arachis hypogaea* cv. JL-24 on MS medium containing BAP and NAA (after 60 days)

S.No.	Growth regulators (mg l^{-1})		Av. no. of shoot buds/ explant ± SD		Per cent regeneration	
	BAP	NAA	Cotyledon	Cn	Cotyledon	Cn
1	3.0	0.1	22 ± 0.08	25 ± 1.2	22	25
2	3.0	0.2	28 ± 1.6	46 ± 2.8	36	37
3	3.0	0.5	25 ± 0.95	55 ± 2.19	35	39
4	4.0	0.1	43 ± 1.4	84 ±1.79	40	41
5	4.0	0.2	54 ± 1.5	104 ± 13.8	48	69
6	4.0	0.5	25 ± 0.1	77 ± 1.23	19	42
7	5.0	0.1	20 ± 1.3	—	22	—
8	5.0	0.2	15 ± 0.66	—	18	—
9	5.0	0.5	19 ± 0.52	—	5	—

potential for shoot regeneration in comparison to older leaves. Culture of petiolule explants on 2,4-D containing medium resulted in more callus along with shoot buds, which started re-callusing upon subculture to medium devoid of auxin. While reporting shoot regeneration in peanut, similar observations were also made by Narasimhulu and Reddy (1983). This could be due to the higher levels of endogenous auxins in the explants or imbalance between the endogenous hormone levels and the growth regulators supplied externally.

Initial culture of cotyledonary node explants on 4.0 mg l^{-1} BAP with 0.2 mg l^{-1} NAA gave an average of 52.2 shoot buds. An average of three shoot buds per cotyledonary node explant developed into shoots of 1.0-2.0 cm length in the same medium. Efficient shoot development (69%) was observed from cotyledonary node explants and the number of shoot buds almost doubled at the end of 60 days of culture (Table 2). Remaining 31% of the cotyledonary node explants showed callus formation along with few shoot buds. On medium containing 2.0 mg l^{-1} BAP, 4-5 shoot-buds per explant elongated per cluster (Fig. 1e). Elongation of shoot buds from the shoot buds that failed to elongate was achieved by gradual reduction of BAP from 1.5 to 0.5 mg l^{-1}. This cycle of harvesting shoots and sub-culturing on to medium containing lower concentration of BAP could be done till it was cultured on to MS basal medium without any cytokinin. Thus at the end of 5 to 6 sub cultures at 15 days interval about 65 shoots were obtained. This result is in corroboration with the study of Kanyand *et al*. (1994) that showed the suitability of such regeneration system for gene transfer because of the ease in imposing antibiotic selection on transformed tissues and the *de novo* induction of shoot buds from transformed tissues without the intervention of callus phase.

Rooting of in vitro shoots

Elongated shoots when cultured on MS salts alone gave rise to less number of roots that were thin and unbranched. On MS medium containing 1.0 mg l^{-1} NAA 84.5% shoots rooted readily with induction of four to eight healthy roots from the base of each shoot. Shoots developed from cotyledon explants took more than ten days to root while shoots from cotyledonary node rooted relatively easily. Roots developed were thick and long with more than 10 branches (Fig. 1f). Plantlets survived on sterile vermiculite-sand mixture (1:1), grew well and acclimatized within 15 days under culture room conditions and were subsequently transferred to the glass house, where they were covered with polythene bags to maintain high humidity initially. Once in a week, they were supplied

with MS salts and vitamins for one month or until they produced new leaves and started growing normally. About 60% of the plantlets developed from cotyledon and 98% of the plantlets from cotyledonary node grew well and established under glass house conditions, where they flowered (Fig. 1g) and set viable seeds, which showed 100% germination and produced phenotypically normal R_1 generation. The present study showed that cotyledonary node is the most regenerative explant for producing large number of shoots within short period of time and can be utilized for regeneration and transformation of peanut with good efficiency.

ACKNOWLEDGEMENTS

Financial support from Andhra Pradesh-Netherlands Biotechnology Programme and the National Agricultural Technology Project - Indian Council of Agricultural Research is greatly acknowledged. MRB and SKJ acknowledge the Senior Research Fellowship from the Council of Scientific and Industrial Research, New Delhi.

REFERENCES

Baker, C.M. and Wetzstein, H.Y. (1998). Leaflet development, induction time and medium influence somatic embryogenesis in peanut (*Arachis hypogaea* L.). *Plant Cell Rep*. **17**: 925-929.

Cheng Ming, David, C.H. Hsi and Gregory, C. Philips (1992). *In vitro* regeneration of valencia-type peanut (*Arachis hypogaea* L.) from cultured petiolules, epicotyl sections and other seedling explants. *Peanut Sci*. **19**: 82-87.

Eapen Susan and George, L. (1993). Plant regeneration from leaf discs of peanut and pigeon pea: Influence of benzyl adenine, indole acetic acid and indole acetic acid-amino acid conjugates. *Plant Cell, Tiss. Org. Cult*. **35**: 223-227.

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

Kanyand Matand, Ananta Probo Desai and Channapatna, S. Prakash (1994). Thidiazuron promotes high frequency regeneration of peanut (*Arachis hypogaea*) plants *in vitro*. *Plant Cell Rep*. **14**: 1-5.

Mante, S. Scorza, R. and Cordts, J. (1989). A simple, rapid protocol for adventitious shoot development from mature cotyledons of *Glycine max* cv Bragg. *In Vitro Cell. Dev. Biol*. **25**: 385-388.

McKently, A.H., Moore, G.A. and Gardner, F.P. (1991). Regeneration of peanut and perennial peanut from cultured leaf tissue. *Crop Sci*. **31**: 833-837.

Mroginsky, L.A., Kartha, K.K. and Shyluk, J.P. (1981). Regeneration of peanut (*Arachis hypogaea*) plantlets by *in vitro* culture of immature leaves. *Can. J. Bot*. **59**: 826-830.

Murashige, T. and Skoog, F.A. (1962). Revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant*. **15**: 473-497.

Narasimhulu, S.B. and Reddy, G.M. (1983). Plantlet regeneration from different callus cultures of *Arachis hypogaea* L. *Plant Sci. Lett*. **31**: 157-163.

Olhoft, P.M. and Somers, D.A. (2001). L-Cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. *Plant Cell Rep*. **20**: 706-711.

Olhoft, P.M., Lin, K., Galbraith, J. and Nielsen, N.C. (2001). The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary-node cells. *Plant Cell Rep*. **20**: 731-737.

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

Venkatachalam, P., Geetha, N., Rao, K.S., Jayabalan, N. and Saravanbabu, S. (1999). BAP-regulated direct shoot organogenesis from cultured seedling explants of groundnut (*Arachis hypogaea* L.). *Ind. J. Exp. Biol*. **37**: 807-812.

Venkatachalam, P., Subramaniampillai, A. and Jayabalan, N. (1996). *In vitro* callus culture and plant regeneration from different explants of groundnut (*Arachis hypogaea* L.). *Breeding Sci*. **4**: 315-320.