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In vitro regeneration of a high yielding variety of *Arachis hypogaea* L. Var. ICG 11337 through different explants

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Abstract

In vitro propagation using different explants viz. shoot apex and cotyledonary nodes from axenic plants, has been attempted in *Arachis hypogaea* L.var. ICG 11337. The explants were cultured in Murashige and Skoog's basal medium supplemented with various concentrations (1-50 mg l^{-1}) of benzylaminopurine (BAP). Multiple shoots were induced both from shoot apices as well as from cotyledonary nodes. Shoot multiplication rate in the cotyledonary node explants was higher than shoot apical explants. However, with increasing concentrations of benzylaminopurine in the medium, both cotyledonary nodes and shoot apices exhibited multiple shoot bud formation and these tiny buds could be subsequently developed into leafy shoots by culturing in plant growth regulator free media or in presence of either low level of kinetin (5mg l^{-1}) or benzylaminopurine (5mg l^{-1}) with or without auxin supplementation (napthalene acetic acid 1mg l^{-1}). Shoots of 2-3 cm size could be quickly rooted in presence of either 2, 4-dichlrophenoxy acetic acid or napthalene acetic acid. Well grown rooted plantlets were successfully transferred to soil and after hardening established finally in the field.

***Abbreviations**:* ANOVA- analysis of variance; BAP- N^6–benzylaminopurine; DMRT- Duncan's Multiple Range Test; Kn- Kinetin; MS- Murashige and Skoog's medium; NAA- α-naphthaleneacetic acid; PGR- Plant growth regulator; 2.4-D- 2,4-dichlorophenoxyacetic acid;

Arachis hypogaea L. has established itself as one of the major oil crops in India and is extensively cultivated in southern and western provinces where the gross yield per unit area is extremely encouraging. It is not so popular among the oilseed growers of eastern provinces primarily due to prevalence of the fungal diseases like early and late leaf spot. In order to overcome this problem, spraying suitable fungicides may be a common practice. To make this crop popular among the farmers in this region, it would be necessary to bring a

number of desirable characters including the resistance to tikka disease in a particular cultivar. After achieving such clones through biotechnological methods for the improvement of this crop, rapid propagation of those lines through various *in vitro* techniques would be necessary. Moreover, ability to regenerate plants from cultured cells, tissues or organs constitutes the basis of producing transgenic crops. Legumes, in general, hardly regenerate plantlets from cultured tissues. Various explants have been tested for the regeneration of plantlets of which the cotyledon is found most suitable for obtaining a large number of plantlets in legumes.[17, 21] Cotyledonary nodes are also used as explants for multiple shoot regeneration of other leguminous species.[4, 21]

Although the tissue culture studies of this crop have been attempted by several workers[4,7,14,16,21] at different times, a detailed study on the growth and morphogenetic developmental patterns of different plant organs in relation to their multiplication potential is still inadequate. Therefore a thorough study was undertaken on rapid clonal propagation of *Arachis hypogaea* L. var. ICG 11337 using explants like shoot apices and cotyledonary nodes, particularly to develop a suitable micropropagation technique.

Seeds of *Arachis hypogaea* L. var. ICG 11337 have been procured from ICRISAT, Patancheru. Seeds were washed with liquid soap for 5-mins and surface sterilized by 90% ethanol for 2 minutes followed by treatment with 1% mercuric chloride solution for 5-6 minutes and finally washed thoroughly with sterile distilled water. The seeds were then aseptically germinated on moistened cotton bed and subsequently developed into complete seedlings. From those 12-15 days old seedlings two types of explant viz. shoot apices and cotyledonary nodes have been isolated and utilized for the initiation of culture. Explants were inoculated into culture tubes each containing MS basal medium[18] solidified with 0.8% (w/v) agar and supplemented with various concentrations of Benzylaminopurine (BAP, 1-50 mg l^{-1}. The control set did not contain any growth regulators. The pH of the medium was adjusted to 5.6-5.8 before autoclaving. The cultures were incubated at 25±2° C under 10-hour of photoperiod of 37.5µ mol. $m^{-2} s^{-1}$ light intensity. Ten replicates were used per treatment. *In vitro* produced multiple shoot buds were sub-cultured on either PGR free MS media or supplemented with comparatively low level of BAP (5mg l^{-1}) or kinetin (Kn) (5mg l^{-1}) either singly or in combination with napthalene acetic acid (NAA) (1mg l^{-1}) for subsequent leafy shoot development. *In vitro* grown shoots were rooted in MS medium containing either NAA or 2, 4 dichlrophenoxy acetic acid (2, 4-D). Well grown rooted plantlets were kept first in sterilized soil taken in plastic cups covered by transparent polyethylene bags to maintain humidity and finally transferred to pots in the experimental garden.

The mean values of different morphogenetic responses were shown along with their respective standard errors (SE) and were analyzed by "analysis of variance" (ANOVA). After obtaining a significant F-value ($\alpha = 0.05$) the treatment means were separated by Duncan's Multiple Range Test (DMRT). Statistical analyses were performed according to Little and Hills[13].

The level of cytokinin is known to be critical in shoot organogenesis.[4,24] Therefore, we compared response of different explants to various concentrations of BAP (1, 5, 10, 15, 25 and 50mg l^{-1}). The response of cotyledonary node explant of *Arachis hypogaea* L. var. ICG 11337 in presence of various concentrations of BAP (1-50 mg l^{-1}) has been shown in Table 1. In all the treatments, including the BAP-free control, 100% shoot development was observed from both the lateral buds of the cotyledonary node. However, in the control only a single shoot was developed from each of the nodal buds, whereas supplementation of BAP induced multiple shoot formation (Fig l A) which showed striking similarity with mung bean.[9, 10] Length of the new shoots was optimum in the control (mean length 55.55 ± 2.05mm) and the use of BAP reduced the length which has been observed in other plants also.[10] At very high BAP level (50mg l^{-1}), with the reduction of multiplication rate and shoot length, the size of leaves were also reduced. A similar response in *Hemidesmus indicus* was noted by Patnaik and Debata[19] where stunted shoots could be developed into normal ones by reducing BAP level in the medium. These shoots, in the presence of BAP, showed axillary branching. Apart from this, proliferation by means of shoot bud formation was also noted at higher level of BAP supplementation (5 mg l^{-1} -50mg l^{-1}).

Therefore, a bi-directional multiplication pathway (direct shoot formation and shoot bud formation) was achieved by the cotyledonary node culture. The optimum number of direct shoots was obtained with 10 mg l^{-1} BAP (6.7± 0.49) and supra-optimal BAP proved inhibitory. The application of very high concentration of BAP (5-50 mg l^{-1}) was essential for the induction of shoot buds and optimum response recorded in the presence of 25 mg l^{-1} BAP. The multiplication potential (total number of direct shoots + shoot buds) was enhanced with the increase in BAP concentration up to 25 mg l^{-1} and declined at very high BAP concentration (50 mg l^{-1}). However, it could be noted, the multiplication potential showed no significant variations between 10 and 25 mg l^{-1} BAP.

The results of shoot tip culture of the variety ICG 11337 in the presence of various concentrations of BAP (1-50 mg l^{-1}) is shown in Table 2 (Fig l B). As the shoot tip contains a single apical bud, a single shoot developed frequently on the BAP-free control. However, the mean number of shoots increased with the increase in BAP concentration and the optimum number (5.4 ± 0.37) was noted at 15 mg l^{-1} BAP. On the other hand, shoot buds were recorded at 5 mg l^{-1} to 50 mg l^{-1} of BAP concentration. The maximum multiplication potential was obtained with 25 mg l^{-1} BAP. The mean shoot length was highest in the control (60.10 ± 2.53) and the addition of BAP proved inhibitory.

BAP alone could induce multiple shoot formation in *Gossypium hirsutum*[5] and a low concentration of BAP was more effective in inducing multiple shoots in *Populus*.[1] These multiplications took place both by adventitious as well as by axillary shoot bud proliferation. In general, it is known that in an intact plant, the apical bud exerts an inhibitory influence on axillary buds, preventing their development into leafy shoots.[23] Moreover, the adventitious production of multiple shoot buds could be

directly controlled by the exogenous cytokinin concentration in *Rauvolfia tetraphylla*,[8,24] *Vigna radiata*,[10] *Canavalia virosa*[12] and *Lippia alba.*[11] A very high concentration of BAP in general showed an inhibitory effect on shoot bud proliferation in *Vigna radiata*[2] and in *Acacia auriculiformis.*[3] The response in terms of shoot multiplication of the peanut variety GN 2 was not encouraging in the media fortified either with NAA or BAP alone.[7] According to Banerjee *et al.*,[7] auxin: cytokinin ratio in the medium was crucial for the regeneration of multiple shoot buds in groundnut. In the present study, on the contrary, BAP alone was capable of inducing proliferation of shoot buds. However, the present findings supported the observations of Vishwanath and Jayanthi[24] and Banerjee *et al.*[5]. Further, the effect of a particular PGR depended not only on the concentrations applied, but also on the presence of the other PGRs as well as its interaction with endogenous growth regulators.[4, 20]

Cotyledonary nodes and shoot apices explants showed more or less similar response in terms of multiplication (Table 3). The multiplication potential of cotyledonary nodes was 18.6±1.4 at 25 mg l^{-1} BAP. In the case of shoot apex culture the maximum multiplication potential was recorded 18.4 ± 1.27 at 25 mg l^{-1} BAP. From this investigation it has been observed that both the explants the cotyledonary node and shoot apex are more or less equal in multiplication potential in the variety ICG 11337. However, the suitability of cotyledonary node explants, in relation to shoot multiplication potential has been reported by Banerjee *et al.*[7], Banerjee *et al.*[6], Banerjee[4] and Maity[14] in other variety of *Arachis hypogaea*.

Generally the frequency of shoot regeneration from different explants differed markedly. Although organ formation results from quantitative interactions between auxin and cytokinin.[22] The present investigation showed direct shoot regeneration in presence of cytokinin (BAP) which supports the observations of Gupta *et al.*,[11].

The slight difference in response in the regeneration capacity of cotyledonary node and shoot apex might be due to the fact that cotyledonary node contained two preformed lateral shoot buds and shoot apex contained one apical bud. Therefore, the regeneration frequency as well as the number of regenerated shoots per culture depended upon the concentration of cytokinin and also on the explant type. Although the number of shoot bud increased with the increase in the BAP level, very high concentration of BAP showed an inhibitory effect, which is in accordance with Badere *et al.*[2]

Shoot buds, produced at higher concentrations of BAP (Fig 1 C), when subcultured at low cytokinin level or without cytokinin developed leafy shoots (Fig 1 D) (Table 4). It was found that the reduction or complete omission of cytokinin from the medium was essential. Similar response was reported by Maity *et al.*[15], Banerjee *et al.*[6] and Banerjee.[4] The percentage of leafy shoot emergence in the basal medium without BAP was 47.10 ± 6.61 and at 5 mg l^{-1} BAP was 46.62 ± 1.59. The addition of NAA (1 mg l^{-1}) with BAP had no beneficial effect in leafy shoot emergence from the tiny shoot buds. On the contrary, in the presence of Kn (5 mg l^{-1}) the percentage of leafy shoot emergence was

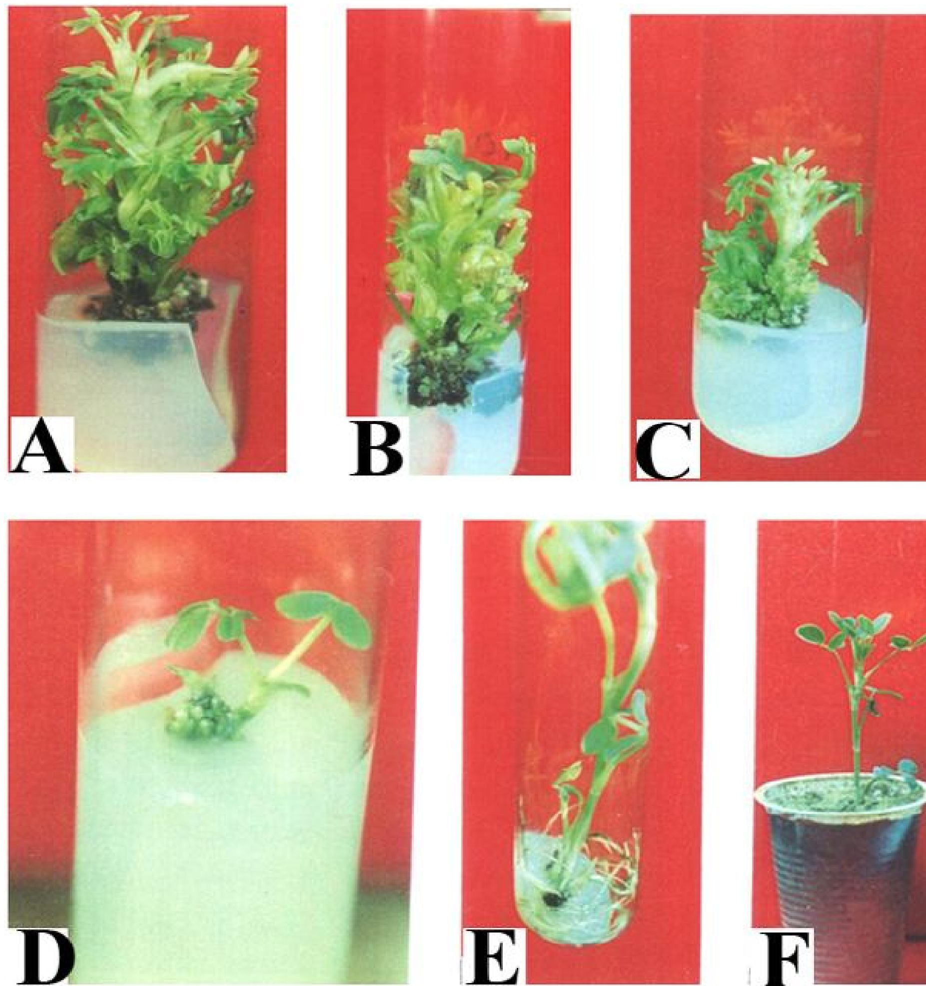

Figure 1

Figure captions

Figure1 A- F: *In vitro* propagation of *Arachis hypogaea* L. Var. ICG 11337: using different explants

A. Multiple shoots from cotyledonary node
B. Multiple shoots from shoot apex
C. Shoot buds produced at higher concentrations of BAP
D. Initiation of leafy shoot emergence from shoot bud
E. Induction of roots
F. Plantlet established in plastic pot

Table 1 Response of cotyledonary node of *Arachis hypogaea* L. var. ICG 11337 cultured on MS agar media supplemented with various concentrations of BAP after 45 days of incubation

BAP (mg/l)	Mean no. of shoots ± SE(A)	Mean no. of axillary branches ± SE (B)	Mean no. of shoot buds ± SE(C)	Multiplication potential per explant ± SE(A+B+C)	Shoot length ± SE (mm)	Mean no. of leaves per explant ± SE
0	2.0 ± 0[d]	0	0	2.0 ± 0[c]	55.55 ± 2.05	7.0 ± 0.29
1	2.3 ± 0.15[d]	0	0	2.3 ± 0.15[c]	38.39 ±1.23	8.4 ± 0.92
5	3.9 ± 0.17[c]	2.3 ± 0.36	2.7± 0.3	8.9 ± 0.58[b]	29.66 ± 1.17	15.6 ± 0.97
10	6.7 ± 0.49[a]	3.5 ± 0.5	6.1 ± 0.53	16.3 ± 0.51[a]	21.8 ± 1.18	18.5 ± 1.02
15	5.4 ± 0.22[b]	2.0 ± 0.36	9.8 ± 0.91	17.2 ± 0.77[a]	16.98 ± 0.81	17.3 ± 0.85
25	4.4 ± 0.26[c]	3.1 ± 0.27	11.1 ± 1.32	18.6 ± 1.4[a]	13.63 ± 1.1	14.6 ± 1.53
50	2.5 ± 0.22[d]	0.6 ± 0.22	7.3 ± 1.43	10.4 ± 1.55[b]	9.52 ± 0.93	6.5 ± 0.61

Mean values followed by same letter are not significantly different at 0.05 level (DMRT)

Table 2 Response of shoot apex of *Arachis hypogaea* L. var. ICG 11337 cultured on MS agar media supplemented with various concentrations of BAP after 45 days of incubation

BAP (mg/l)	Mean no. of shoots ± SE(A)	Mean no. of axillary branches ± SE (B)	Mean no. of shoot buds ± SE(C)	Multiplication potential per explant ± SE(A+B+C)	Shoot length ± SE (mm)	Mean no. of leaves per explant ± SE
0	1.0 ± 0	0	0	1.0 ± 0	60.10 ± 2.53	4.6 ± 0.26
1	1.2 ± 0.13[d]	0	0	1.20 ± 0.13[d]	52.91 ± 2.63	5.4 ± 0.37
5	2.3 ± 0.21[c]	1.3 ± 0.21	5.30 ± 0.83	8.90 ± 0.83[c]	40.13 ± 2.40	8.0 ± 0.51
10	3.8 ± 0.41[b]	2.6 ± 0.49	8.10 ± 1.05	14.5 ± 0.95[b]	28.21 ± 1.18	13.3 ± 1.21
15	5.4 ± 0.37[a]	3.1 ± 0.48	8.60 ± 0.97	17.1 ± 1.19[ab]	18.87 ± 0.79	19.3 ± 1.26
25	4.0 ± 0.33[b]	2.9 ± 0.43	11.5 ± 1.00	18.4 ± 1.27[a]	15.45 ± 0.89	16.0 ± 1.0
50	2.3 ± 0.26[c]	0.6 ± 0.26	7.20 ± 1.35	10.1 ± 1.51[c]	11.86 ± 0.87	7.5 ± 0.99

Mean values followed by same letter are not significantly different at 0.05 level (DMRT)

Table 3 Comparative response of cotyledonary node and shoot apex explants of *Arachis hypogaea* L. var. ICG 11337 in terms of multiplication potential after 45 days of incubation

MS + BAP (mg/l)	Multiplication potential per explant ± SE*	
	Cotyledonary node	Shoot apex
0	2.00 ± 0[c]	1.0 ± 0[d]
1	2.30 ± 0.15[c]	1.20 ± 0.13[d]
5	8.90 ± 0.58[b]	8.90 ± 0.83[c]
10	16.30± 0.51[a]	14.50 ± 0.95[b]
15	17.20 ± 0.77[a]	17.10 ± 1.19[ab]
25	18.60 ± 1.40[a]	18.40 ± 1.27[a]
50	10.40 ± 1.55[b]	10.10 ± 1.51[c]

*Multiplication potential = (Number of shoots + Number of shoot buds)
Mean values followed by same letter are not significantly different at 0.05 level (DMRT)

Table 4 Response of shoot buds of *Arachis hypogaea* L. var. ICG 11337 on MS agar media supplemented with different plant growth regulators after 45 days of incubation

Plant growth regulators (mg/l)	Percentage of leafy shoot emergence ± SE	Shoot length (mm) ± SE	Number of leaves per shoot
0	47.10 ± 6.61[c]	8.04 ± 0.31	1.64 ± 0.12
5 BAP	46.62 1.59[c]	8.95 ± 0.31	2.13 ± 0.10
5 BAP + 1 NAA	47.11 ± 2.57[c]	9 28 ± 0.26	2.19 ± 0.14
5 Kn	71.25 6.77[a]	6.35 ± 0.22	2.02 ± 0.11
5Kn +1 NAA	59.43 ± 2.89[b]	7.90 ± 0.30	2.00 ± 0.06

Mean values followed by same letter are not significantly different at 0.05 level (DMRT)

Table 5 Induction of root in the regenerated shoots of *Arachis hypogaea* L. var. ICG 11337 after 45 days of incubation

Plant growth regulators (mg/l)	Frequency of root induction (%)	Number of roots ± SE	Mean root length (cm) ± SE
0	0	0	0
NAA 0.5	50	0.8 ± 0.32[c]	0.9 ± 0.37[d]
NAA 1.0	100	6.3 ± 1.20[b]	7.7 ± 1.29[a]
NAA 2.0	100	5.2 ± 0.77[b]	8.9 ± 1.31[a]
NAA 4.0	100	5.4 ± 3.95[b]	8.0 ± 0.95[a]
2,4-D 0.5	100	6.6 ± 0.89[b]	6.49 ± 0.78[b]
2,4-D 1.0	100	13.4 ± 1.17[a]	6.75 ± 0.44[a]
2,4-D 2.0	100	12.2 ± 2.03[a]	3.19 ± 0.68[c]
2,4-D 4.0	0	0	0

Mean values followed by same letter are not significantly different at 0.05 level (DMRT).

found 71.25 ± 6.77 that was higher than the control media. The addition of NAA (1 mg l^{-1}) with Kn had also no beneficial effect in terms of leafy shoot emergence; rather it exhibited slight inhibitory effects which corroborate the findings of Banerjee *et al.*[6] and Banerjee.[4]

It is observed that the presence of auxin (either NAA or 2, 4-D) is essential for the induction of roots (Fig l E) from the base of the isolated shoots, which was initiated after 20-25 days of incubation (Table 5). Moreover, the concentration of auxin application was found to be critical, as relatively higher level of 2, 4-D (4 mg l^{-1}) completely inhibited root induction. As the development of well ramified root system is an essential prerequisite for successful transfer of plantlets to the field condition, the number of roots and the length of the roots were recorded after 45 days of incubation. The frequency of root induction in 0.5 mg l^{-1} NAA was only 50% where as 100% root induction was observed in the presence of 1- 4 mg l^{-1} NAA and 0.5- 2 mg l^{-1} 2, 4-D. The general characteristics of the roots induced by same concentration of 2,4-D (2 mg l^{-1}) and NAA (2 mg l^{-1}) was that the roots formed in the presence of 2,4-D were characteristically shorter in length with greater girth than the roots generated with NAA. It could be mentioned that the roots developed at the base of the isolated shoots did not turn into callus.

Standard hardening process had been followed for transfer of *in vitro* grown plantlets to the field condition. During the hardening process of *in vitro* grown plantlets it was observed that fungal infection and wilting were two serious problems. The problem of infection of cultured plantlets was tackled by thorough washing with excess amount of sterile water to completely remove the agar medium adhered to the base of the plantlets. Wilting of plantlets was managed through maintaining a high humidity by covering the plantlets in plastic pots containing sterile sand soil mixture with transparent polyethylene bags (Fig l F). The first sign of emergence of leaf occurred within four weeks of field transfer. The survival percentage was 81.24%.

In conclusion, the findings of the present study are of considerable significance, since data available on direct induction of multiple shoot buds as well as multiple shoots on Indian cultivars of groundnuts are inadequate and the present study described *in vitro* regeneration technique using cotyledonary node and shoot apex explants of this high yielding variety in a single medium, which has not previously been reported. Therefore, this efficient and reliable micropropagation technique can now be exploited for genetic improvement of this economically viable crop.

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