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Research Article

RAPID *IN VITRO* PROPAGATION OF *ARACHIS HYPOGAEA* L. VAR. AK 1224: A COMPARATIVE STUDY USING DIFFERENT EXPLANTS

***Pintu Banerjee**
Department of Botany, Krishnagar Government College, Krishnagar, Nadia-741101, West Bengal, India
**Author for Correspondence*

ABSTRACTS

Clonal propagation through *in vitro* techniques using different explants viz. shoot apex, cotyledonary nodes, de-embryonated cotyledons from axenic plants, has been attempted in *Arachis hypogaea* L.var. AK 1224. 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. De-embryonated cotyledons showed swelling near proximal notch and infrequent shoot multiplication. 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.

Keywords: *Arachis hypogaea, In Vitro regeneration, N^6–benzylaminopurine*

Abbreviations*:* ANOVA- analysis of variance; BAP- N^6–benzylaminopurine; DMRT- Duncan'sMultiple 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;

INTRODUCTION

Groundnut 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 (Mehta and Ram 1980; Sastri *et al.,* 1981). Cotyledonary nodes are also used as explants for multiple shoot regeneration in Glycine max (Sastri *et al.,* 1981).

Although the tissue culture studies of this crop have been attempted by several workers (Sastri *et al.,* 1981, Banerjee *et al.,* 1988, McKently *et al.,* 1990) 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. AK 1224 using various explants like shoot-tips, cotyledonary nodes and cotyledon particularly to develop a suitable micropropagation technique.

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MATERIALS AND METHODS

Seeds of *Arachis hypogaea* L. var. AK 1224 have been procured from West Bengal State Seed Corporation, Midnapore. 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 three types of explant viz. shoot apices, cotyledonary nodes and cotyledons have been isolated and utilized for the initiation of culture. Explants were inoculated into culture tubes each containing MS basal medium (Murashige and Skoog, 1962) 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 37.5 µ 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 naphthalene 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 (1978).

RESULTS AND DISCUSSION

The proximal notch of the cotyledonary leaf explant swelled in all the treatments of BAP, except PGR-free control. However, multiple shoots were formed from the hard and swollen portion of the proximal notch only at 5 mg l^{-1} BAP (Figure l A). The frequency of multiple shoot formation was 25% and the number of shoots per explant was 2 – 4 only.

The level of cytokinin is known to be critical in shoot organogenesis (Viswanath and Jayanthi 1997). 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. AK 1224 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 (Figure l B) which showed striking similarity with mung bean (Gulati and Jaiwal 1990, 1994). Length of the new shoots was optimum in the control (mean length 62.14 ± 10.67 mm) and the use of BAP reduced the length which has been observed in other plants also (Gulati and Jaiwal 1994). 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 (1996) 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.

Therefore, a tri-directional multiplication pathway (direct shoot formation, axillary branching and shoot bud formation) was achieved by the cotyledonary node culture. The optimum number of direct shoots was obtained with 25 mg l^{-1} BAP (6.0 ± 0.39) and supra-optimal BAP proved inhibitory. The mean number of axillary branches also increased with the increase in BAP concentration up to 15 mg l^{-1} level, after which it gradually declined. The application of very high concentration of BAP (15-50 mg l^{-1}) was essential for the induction of shoot buds and optimum response recorded in the presence of 25 mg l^{-1} BAP.

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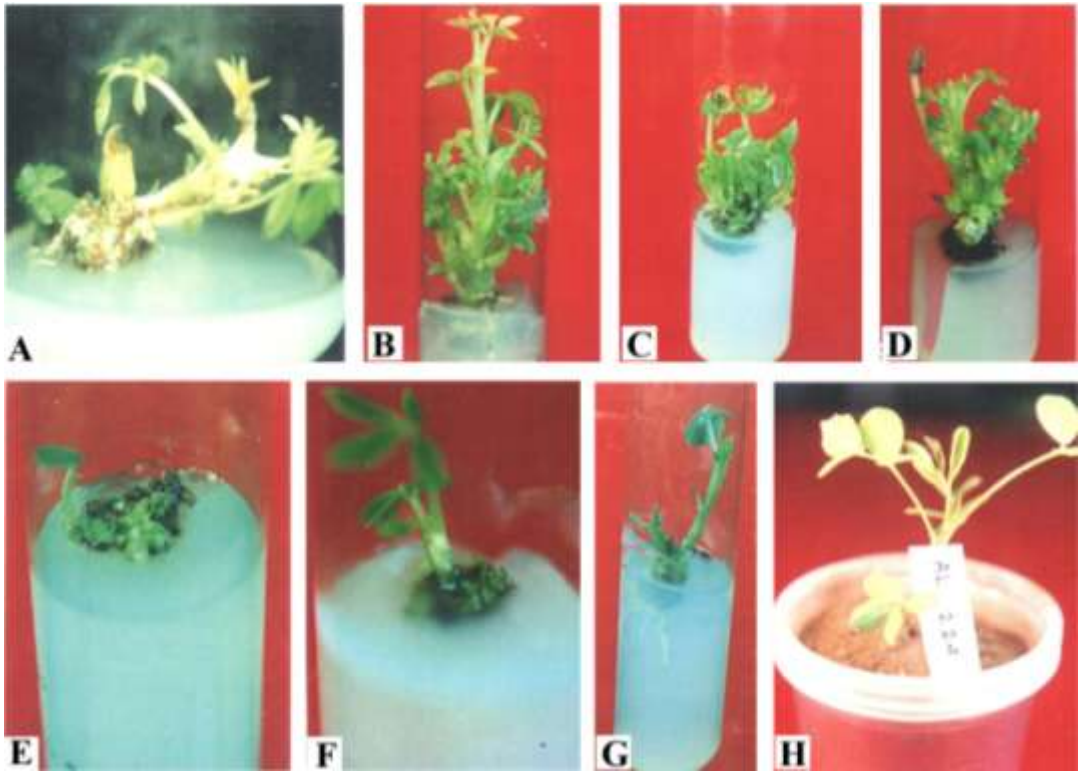

Figure1 A- H: *In vitro* propagation of *Arachis hypogaea* L. Var. Ak 1224: using different explants A. Multiple shoots from cotyledon; B. Multiple shoots from cotyledonary node; C. Multiple shoots from shoot apex; D. Shoot buds produced at higher concentrations of BAP; E. Initiation of leafy shoot emergence from shoot bud; F. Leafy shoot emergence from shoot bud; G. Induction of roots; H. Plantlet established in plastic pot

Table 1: Response of cotyledonary node of *Arachis hypogaea* L. var. AK 1224 cultured on MS agar media supplemented with various concentrations of BAP after 45 days of incubation

BAP (mg l^{-1})	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 explants ± SE
0	1.4 ± 0.16^{d}	0	0	1.4 ± 0.16^{c}	62.14 ± 10.67^{a}	7.40 ± 0.60^{c}
1	2.5 ± 0.22^{c}	0	0	2.5 ± 0.22^{c}	41.80 ± 3.37^{b}	11.6 ± 1.46^{b}
5	3.9 ± 0.27^{b}	0.3 ± 0.21^{d}	0	4.2 ± 0.35^{bc}	32.10 ± 3.03^{c}	14.9 ± 1.66^{b}
10	5.1 ± 0.31^{a}	1.6 ± 0.92^{b}	0	6.7 ± 1.05^{ab}	23.84 ± 1.58^{d}	19.4 ± 1.45^{a}
15	5.6 ± 0.61^{a}	1.7 ± 0.98^{a}	0.50 ± 0.50^{c}	7.8 ± 1.45^{a}	19.55 ± 1.65^{e}	21.4 ± 0.84^{a}
25	6.0 ± 0.39^{a}	0.8 ± 0.61^{c}	2.10 ± 1.15^{a}	8.9 ± 1.51^{a}	16.18 ± 1.39^{f}	23.3 ± 1.25^{a}
50	5.2 ± 0.38^{a}	0.7 ± 0.47^{c}	0.80 ± 0.80^{b}	6.7 ± 1.47^{ab}	15.21 ± 0.93^{f}	21.7 ± 1.57^{a}

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

The multiplication potential (total number of direct shoots + axillary branches + 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, although the multiplication potential showed no significant variations between 15 and 25 mg l^{-1} BAP, the former treatment yielded optimum axillary branches formation while the latter treatment gave more shoot buds.

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The results of shoot tip culture of the variety AK 1224 in the presence of various concentrations of BAP (1-50 mg l^{-1}) is shown in Table 2 (Figure l C). As the shoot tip contains a single apical bud, a single shoot developed on the BAP-free control. However, the mean number of shoots increased with the increase in BAP concentration and the optimum number (5.0 ± 0.81) was noted at 10 mg l^{-1} BAP. On the other hand, the optimum axillary branches and shoot buds were recorded at 25 mg l^{-1} of BAP concentration. The maximum multiplication potential was also obtained with 25 mg l^{-1} BAP. The mean shoot length was highest in the control (80.8 ± 11.78) and the addition of BAP proved inhibitory.

Table 2: Response of shoot apex of *Arachis hypogaea* L. var. AK 1224 cultured on MS agar media supplemented with various concentrations of BAP after 45 days of incubation

BAP (mg l^{-1})	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^{e}	0	0	1 ± 0^{e}	80.8 ± 11.78^{a}	6.0 ± 0.68^{b}
1	1.3 ± 0.3^{d}	0	0	1.3 ± 0.3^{d}	54.23 ± 10.04^{b}	7.2 ± 1.53^{b}
5	1.8 ± 0.24^{d}	0.6 ± 0.40^{c}	0	2.4 ± 0.49^{cd}	36.50 ± 3.32^{c}	10.1 ± 2.06^{ab}
10	5.0 ± 0.81^{a}	0.5 ± 0.22^{c}	1.80 ± 1.0^{c}	7.3 ± 1.19^{a}	23.12 ± 1.61^{d}	14.4 ± 1.49^{a}
15	3.8 ± 0.64^{b}	0.8 ± 0.80^{b}	2.6 ± 0.97^{b}	7.2 ± 1.27^{ab}	17.47 ± 1.35^{e}	13.1 ± 2.43^{a}
25	3.3 ± 0.85^{c}	1.0 ± 0.63^{a}	3.3 ± 1.75^{a}	7.6 ± 1.6^{a}	15.06 ± 1.82^{f}	12.1 ± 2.23^{a}
50	2.9 ± 0.34^{c}	0	1.7 ± 0.63^{c}	4.6 ± 0.54^{c}	10.48 ± 1.26^{g}	7.9 ± 0.69^{b}

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

BAP alone could induce multiple shoot formation in *Gossypium hirsutum* (Banerjee *et al.*, 1999) and a low concentration of BAP was more effective in inducing multiple shoots in *Populus* (Agarwal and Gupta 1999). 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 (Street and Opik 1986). Moreover, the adventitious production of multiple shoot buds could be directly controlled by the exogenous cytokinin concentration in *Rauvolfia tetraphylla* (Vishwanath and Jayanthi 1997, Ghosh and Banerjee 2003) *Vigna radiata* (Gulati and Jaiwal 1994), *Canavalia virosa* (Kathiravan and Ignacimuthu 1999) and *Lippia alba* (Gupta *et al.*, 2001) A very high concentration of BAP in general showed an inhibitory effect on shoot bud proliferation in *Vigna radiata* (Badere *et al.*, 2002) and in *Acacia auriculiformis* (Banerjee 2013). 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 (Banerjee *et al.*, 1988). According to Banerjee *et al.*, 1988 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 (1997) and Banerjee *et al.*, (1999). 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 (Roy and Banerjee 2000).

Cotyledonary nodes proved its supremacy over shoot apices explants in terms of multiplication (Table 3). The multiplication potential of cotyledonary nodes was 8.9 ± 1.51 at 25 mg l^{-1} BAP. On the contrary, in the case of shoot apex culture the maximum multiplication potential was recorded 7.6 ± 1.6 at 25 mg l^{-1} BAP. From this investigation it has been observed that so far as the high multiplication of shoot is concerned in the variety AK 1224, cotyledonary nodes would be the best explant which should be cultured in presence of 25mg l^{-1} BAP (Table 3). The suitability of cotyledonary node explants in relation to shoot multiplication has also been reported by Banerjee *et al.*, 1988 and Banerjee *et al.*, 2011.

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Table 3: Comparative response of cotyledonary node and shoot apex explants of *Arachis hypogaea* L. var. AK 1224 in terms of multiplication potential after 45 days of incubation

MS + BAP (mg l^{-1})	Multiplication potential per explant ± SE*	
	Cotyledonary node	Shoot apex
0	1.4 ± 0.16^{d}	1 ± 0^{d}
1	2.5 ± 0.22^{c}	1.3 ± 0.3^{d}
5	4.2 ± 0.35^{bc}	2.4 ± 0.49^{cd}
10	6.7 ± 1.05^{ab}	7.3 ± 1.19^{a}
15	7.8 ± 1.45^{a}	7.2 ± 1.27^{ab}
25	8.9 ± 1.51^{a}	7.6 ± 1.6^{a}
50	6.7 ± 1.47^{ab}	4.6 ± 0.54^{bc}

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

The frequency of shoot regeneration from different explants differed markedly. Although organ formation results from quantitative interactions between auxin and cytokinin (Skoog and Miller, 1957), the present investigation showed direct shoot regeneration in presence of cytokinin (BAP) which supports the observations of Gupta *et al.,* (2001).

The differential response in the regeneration capacity of cotyledonary node, shoot apex and cotyledonary leaf might be due to the fact that cotyledonary node contained two preformed lateral shoot buds, shoot apex contained one apical bud while in cotyledonary leaf, meristemoids need to be formed adventitiously for development of shoot buds. 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 axillary branch and 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.,* (2002). Shoot buds, produced at higher concentrations of BAP (Figure l D), when subcultured at low cytokinin level or without cytokinin developed leafy shoots (Figure l E & F) (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.,* (2005) and Banerjee *et al.,* (2011). The percentage of leafy shoot emergence in the basal medium without BAP was 52.35 ± 2.38 and at 5 mg l^{-1} BAP was 50.97 ± 2.18. 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 found 57.83 ± 2.48 that was higher than both the control and BAP supplemented 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.,* (2011).

Table 4: Response of shoot buds of *Arachis hypogaea* L. var. AK 1224 on MS agar media supplemented with different plant growth regulators after 45 days of incubation

Plant growth regulators (mg l^{-1})	Percentage of leafy shoot emergence ± SE	Shoot length (mm) ± SE	Number of leaves per shoot
0	52.35 ± 2.38^{b}	7.82 ± 0.56	1.83 ± 0.07
5 BAP	50.97 ± 2.18^{b}	11.05 ± 0.36	2.30 ± 0.15
5 BAP + 1 NAA	50.08 ± 2.21^{b}	13.50 ± 0.66	2.50 ± 0.16
5 Kn	57.83 ± 2.48^{a}	10.15 ± 0.39	2.10 ± 0.05
5Kn +1 NAA	52.48 ± 2.52^{b}	13.78 ± 0.33	2.32 ± 0.10

It is observed that the presence of auxin (either NAA or 2, 4-D) is essential for the induction of roots (Figure l G) 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

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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 0.5-1 mg l^{-1} 2. 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 (Table 5). It could be mentioned that the roots developed at the base of the isolated shoots did not turn into callus.

Table 5: Induction of root in the regenerated shoots of *Arachis hypogaea* L. var. AK 1224 after 45 days of incubation

Plant growth regulators (mg l^{-1})	Frequency of root induction (%)	Number of roots ± SE	Mean root length (cm) ± SE
0	0	0	0
NAA 0.5	50	1.4 ± 0.49^{c}	1.5 ± 0.53^{d}
NAA 1.0	100	5.9 ± 0.50^{a}	7.14 ± 0.47^{a}
NAA 2.0	100	6.8 ± 0.32^{a}	7.68 ± 0.27^{a}
NAA 4.0	100	6.3 ± 0.49^{a}	5.67 ± 0.46^{b}
2,4-D 0.5	100	6.1 ± 0.54^{a}	6.06 ± 0.42^{b}
2,4-D 1.0	100	6.5 ± 0.40^{a}	5.63 ± 0.34^{b}
2,4-D 2.0	90	2.6 ± 0.40^{b}	2.57 ± 0.30^{c}
2,4-D 4.0	0	0	0

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

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 (Figure l H). The first sign of emergence of leaf occurred within four weeks of field transfer. The survival percentage was 83.88%.

In conclusion, the findings of the present study are of considerable significance, since data available on direct induction of multiple shoot buds, axillary branches as well as multiple shoots on Indian cultivars of groundnuts are inadequate and the present study described a tri-directional micro propagation technique in a single medium, which has not previously been reported. Therefore, this efficient and reliable tri-directional micro propagation technique can now be exploited for genetic improvement of this economically viable crop.

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