0273-2289/99/80/0193/$12.75

Rapid and High-Frequency In Vitro Plant Regeneration from Leaflet and Petiole Explants of Groundnut (*Arachis hypogaea* L.)

P. Venkatachalam,[†,1] N. Geetha,[†,1] K. Sankara Rao,[*,2] and N. Jayabalan[1]

[1]Department of Plant Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli-620 024, India; and [2]Department of Biochemistry, Indian Institute of Science, Bangalore-560 012, India

Received September 21, 1998; Accepted November 12, 1998

Abstract

An efficient protocol for regeneration of groundnut plantlets from immature and mature leaflet and petiole explants excised from axenic seedlings has been developed. The highest frequency of callus induction obtained from leaflet explants was on Murashige and Skoog (MS) medium containing 2.0 mg/L of α-naphthalene acetic acid (NAA) and 0.5 mg/L of kinetin combination. MS medium containing different auxins in combination with 6-benzylaminopurine (BAP) induced shoot buds. A BAP (2.0 mg/L) and NAA (0.5 mg/L) combination resulted in the highest frequency of shoot-bud regeneration. Subsequent shoot multiplication was obtained on MS medium supplemented with either BAP or kinetin (5.0 mg/L) in combination with NAA (1.0 mg/L). Immature leaflet explants were found to be more responsive to shoot induction than mature leaflet explants. Direct shoot-bud regeneration was also observed from petiole explants on the same regeneration medium used for leaflet callus. Regenerated shoots were rooted on MS medium containing indole-3-butyric acid (2.0 mg/L) and kinetin (0.5 mg/L). Plantlets obtained were successfully established in the field, where they grew to maturity and set viable seeds.

Index Entries: *Arachis hypogaea*; organogenesis; shoot regeneration.

*Author to whom all correspondence and reprint requests should be addressed.
[†]Present address: The Rubber Research Institute of India, Kottayam-686 009, India.

Introduction

Groundnut or peanut, *Arachis hypogaea* L., is one of the world's most important oilseed crops. For this reason, there is considerable interest in the development of tissue culture and gene transfer technologies for this species. Genetic transformation will offer exciting possibilities for quick transfer of genes controlling such traits as disease and pest resistance, as vectoring techniques are perfected and suitable genes become available. The realization of gene transfer, however, depends on successful regeneration of whole plants from single cells or tissues into which DNA has been introduced.

Groundnut has proven to be a difficult crop to manipulate in vitro and only a limited success of whole plant regeneration has been achieved in some cultivars *(1,2)*. Plant regeneration has been reported for different explants by organogenesis *(3–8)* and somatic embryogenesis *(9–15)*. Leaflets have been a more readily available explant source than other tissues in groundnut for regeneration and can be effectively utilized for *Agrobacterium*-mediated genetic transformation *(16)*. Explants from in vitro–grown plants would further facilitate availability of target tissue throughout the year. The present study describes culture conditions required for inducing callus-mediated shoot organogenesis from immature and mature leaflets and for direct shoot-bud differentiation from petiole explants of two groundnut cultivars. The objective was to develop a simple protocol for quick and efficient plant regeneration for use in transformation protocols.

Materials and Methods

Plant Material

Dry seeds from mature pods of groundnut (*Arachis hypogaea* L.) varieties VRI-2 and TMV-7 obtained from Tamil Nadu Agricultural University, Coimbatore, India, were surface-decontaminated as described by Venkatachalam et al. *(17)*, placed on Murashige and Skoog (MS) *(18)* basal medium, and incubated for germination in the dark at 24 ± 2°C.

Callus Initiation

Immature leaflets from 7-d-old seedlings and mature leaflets from 15-d-old seedlings (2–5 mm) were excised and cultured on MS medium containing B_5 vitamins *(19)*; 3% (w/v) sucrose, α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5–2.0 mg/L); and kinetin (0.5 mg/L) for callus initiation. The medium was adjusted to pH 5.8, solidified with 0.7% (w/v) agar (Hi-Media, Bombay, India), and then autoclaved at 121°C under 1.2 kg/cm^3 pressure for 15 min. All cultures were incubated at 24 ± 2°C under 16-h-d exposure to white light of 80 μE $m^{-2} \cdot s^{-1}$ intensity provided by fluorescent lamps (TL40W/54 cool daylight) for 2 wk for callus initiation.

Shoot-Bud Regeneration

Callus from leaflets and petiole segments (2–5 mm) from 7-d-old seedlings were cultured on MS medium supplemented with varying concentrations of BAP (0.5–2.0 mg/L) in combination with NAA, IAA, IBA, or 2,4-D (0.5 mg/L) individually for shoot-bud differentiation and plant regeneration. Culture conditions applied were as described for callus initiation.

Shoot Multiplication and Micropropagation

Shoots and buds formed in a cluster from callus as well as directly from petiole segments were separated into smaller clumps (of approx 20 buds) and subcultured on MS medium containing 1.0 mg/L of NAA and BAP/kinetin at varying concentrations (1.0–5.0 mg/L) for further growth and shoot multiplication. Later, shoots were separated and transferred to fresh medium for micropropagation and production of uniform-sized plantlets.

Root Induction and Transplantation

Elongated microshoots (>3 cm in length) were excised and planted on MS medium supplemented with IBA/NAA (0.5–2.0 mg/L) and kinetin (0.5 mg/L), and 3% (w/v) activated charcoal for root induction. Rooted shoots were transferred to plastic cups containing soil, sand, and compost in the ratio of 1:1:1 in a growth chamber. Subsequently, the plantlets were transferred to field conditions, where they flowered and set viable seeds.

Statistical Analysis

The cultures were observed periodically, and percentage of response was recorded on the basis of visual observations. The experimental design was completely randomized block and factorial with auxin and cytokinin as independent variables. The data pertaining to percentage of callus initiation, shoot-bud regeneration, multiple shoot production, and root initiation were subjected to analysis of variance test. Mean separation was done using Duncan's new multiple range test.

Results

Leaflet explants, immature as well as mature, enlarged twice their original size within 7 d of culture on MS medium containing IAA, NAA, IBA, or 2,4-D in the range between 0.5 and 2.0 mg/L and in combination with kinetin at 0.5 mg/L. Simultaneously, green callus began to appear at the margins of the leaflet explants. A combination of 0.5 mg/L of kinetin and 2.0 mg/L of NAA produced best callusing response from the mature (92.5%) as well as from the immature leaflets (100%) (Table 1). The frequency of callus formation was higher for leaflets of VRI-2 (100%) than for TMV-7 cultivar (87.2%) (Table 1), and the immature leaflets gave better response in both cultivars than mature leaflets.

Table 1
Comparison of Effect of Various Concentrations of NAA, IAA, IBA, or 2,4-D in Combination with 0.5 mg/L Kinetin on Callus Induction from Two Groundnut Cultivars[a]

Concentration of growth regulators (mg/L)	Callus induction frequency (percentage # mean ± SD)[b]			
	VRI-2		TMV-7	
	IL	ML	IL	ML
IAA				
0.5	60.8 ± 4.5bc	58.5 ± 3.8cd	55.2 ± 3.5de	51.8 ± 3.8f
1.0	65.5 ± 4.8bc	62.3 ± 4.9c	62.5 ± 5.8cd	59.3 ± 4.1de
1.5	72.6 ± 5.1b	70.3 ± 5.8bc	71.6 ± 5.6c	67.5 ± 5.1d
2.0	86.5 ± 6.3ab	81.2 ± 6.0b	80.5 ± 5.6b	73.3 ± 5.0c
NAA				
0.5	82.1 ± 5.9ab	75.6 ± 5.4b	73.2 ± 4.9c	64.5 ± 4.4d
1.0	92.5 ± 7.0a	80.4 ± 5.2b	81.3 ± 5.9b	74.9 ± 5.6c
1.5	96.7 ± 7.4a	86.5 ± 4.8a	87.7 ± 6.3a	82.8 ± 5.1b
2.0	100.0 ± 0.0a	92.5 ± 7.9a	91.5 ± 6.8a	88.6 ± 6.9a
IBA				
0.5	56.2 ± 3.4cd	55.2 ± 3.2cd	51.5 ± 3.9de	46.5 ± 3.0fg
1.0	67.3 ± 3.8bc	63.2 ± 4.1c	57.2 ± 4.8d	53.9 ± 3.1ef
1.5	73.5 ± 4.6b	70.1 ± 4.5bc	72.8 ± 5.6c	58.7 ± 4.1de
2.0	78.2 ± 5.0b	76.5 ± 4.7b	72.8 ± 5.1c	67.1 ± 4.2d
2,4-D				
0.5	56.5 ± 3.6cd	53.5 ± 3.1cd	49.3 ± 2.6de	45.3 ± 3.3g
1.0	64.3 ± 4.0bc	61.5 ± 4.3c	56.7 ± 4.5d	51.6 ± 4.5f
1.5	73.5 ± 4.7b	69.3 ± 3.9bc	64.3 ± 5.7cd	60.8 ± 4.9de
2.0	77.2 ± 4.9b	72.8 ± 5.1b	68.2 ± 3.9c	66.3 ± 5.1d

[a]Twenty to twenty-five explants were used per treatment, and each experiment was repeated thrice.

[b]IL, immature leaflet; ML, mature leaflet. Values within column with the same letter are not significantly different at the 1% probability level according to the Duncan's new multiple range test.

Following subculture of callus on regeneration media (Table 2), shoot-bud primordia developed within 3 wk (Fig. 1C). The response of callus to different regeneration media is detailed in Table 2. MS medium containing 2.0 mg/L of BAP and 0.5 mg/L of NAA combination produced optimal shoot-bud regeneration. The highest shoot regeneration frequency obtained was 100 and 87.2% in VRI-2 (Fig. 1D) and TMV-7 cultivars, respectively, with immature leaflet callus (Table 2). The number of shoot buds produced in mature and immature leaflets of cultivar VRI-2 was higher than that of TMV-7. These buds developed into normal leafy shoots after 1 mo of culture on MS medium containing BAP or kinetin at 5.0 mg/L and NAA at 1.0 mg/L (Fig. 1A,D). Of the two cytokinins tested, BAP and NAA combination was found to be better for shoot proliferation than kinetin and NAA combination. With immature leaflet callus, the maximum number of shoots

Table 2
Effect of Different Concentrations of BAP in Combination with IAA, NAA, IBA, or 2,4-D (0.5 mg/L) on Shoot-Bud Differentiation in Groundnut Cultivars[a]

Concentration of growth regulators (mg/L)		Root-induction frequency (percentage # mean ± SD)[b]					
		VRI-2			TMV-7		
		IL	ML	LP	IL	ML	LP
BAP	IAA						
0.5	0.5	51.4 ± 3.5h	50.3 ± 2.9fg	45.6 ± 2.6f	46.4 ± 3.1l	44.5 ± 2.3l	42.0 ± 2.8g
1.0	0.5	67.5 ± 4.3f	62.1 ± 5.7de	57.3 ± 3.7de	56.1 ± 4.4i	54.6 ± 4.7g	51.5 ± 3.1e
1.5	0.5	74.3 ± 6.1h	72.3 ± 5.9c	62.5 ± 4.9d	63.8 ± 5.1g	59.8 ± 5.1ef	57.1 ± 3.9d
2.0	0.5	82.2 ± 7.5c	81.4 ± 6.3b	76.5 ± 5.5b	77.9 ± 5.5c	66.9 ± 4.3d	61.8 ± 4.5c
BAP	NAA						
0.5	0.5	66.5 ± 4.4f	64.7 ± 4.1d	60.1 ± 4.0d	57.5 ± 3.4i	55.3 ± 3.1g	53.4 ± 3.1e
1.0	0.5	75.6 ± 6.6e	76.3 ± 5.7c	65.3 ± 5.1cd	72.4 ± 4.6d	67.1 ± 4.7d	62.3 ± 4.3c
1.5	0.5	92.5 ± 7.1b	83.5 ± 6.3b	72.6 ± 5.6bc	80.1 ± 5.1b	76.2 ± 5.1d	71.2 ± 4.7b
2.0	0.5	100.0 ± 0.0a	91.5 ± 5.4a	85.8 ± 4.7a	87.2 ± 5.3a	81.2 ± 4.6a	79.1 ± 4.2a
BAP	IBA						
0.5	0.5	60.5 ± 3.7g	52.3 ± 2.7f	50.1 ± 2.1f	54.0 ± 2.7j	51.0 ± 2.3h	49.8 ± 2.2ef
1.0	0.5	74.3 ± 5.1e	65.4 ± 4.5d	59.3 ± 3.2de	65.7 ± 4.2f	60.7 ± 3.5e	57.9 ± 2.2d
1.5	0.5	80.5 ± 6.7cd	74.7 ± 4.3c	68.3 ± 3.0c	72.4 ± 4.7d	68.3 ± 3.1cd	63.5 ± 3.3c
2.0	0.5	85.4 ± 5.6c	83.5 ± 4.9b	80.5 ± 4.6b	79.0 ± 4.6b	72.5 ± 3.9c	70.0 ± 4.8b
BAP	2,4-D						
0.5	0.5	55.4 ± 3.1e	49.5 ± 2.8fg	48.5 ± 2.6f	48.5 ± 2.3k	45.6 ± 2.4i	42.4 ± 2.6g
1.0	0.5	67.4 ± 4.7f	56.6 ± 3.2f	55.6 ± 3.1de	59.4 ± 3.7h	56.5 ± 3.3g	53.5 ± 2.9e
1.5	0.5	72.5 ± 4.5e	67.5 ± 4.9d	62.7 ± 4.8d	68.7 ± 3.5e	63.4 ± 3.9e	60.4 ± 3.8c
2.0	0.5	80.3 ± 5.1cd	73.5 ± 4.5c	70.3 ± 3.6c	73.1 ± 3.1d	70.5 ± 3.5c	68.7 ± 3.3b

[a]Twenty to twenty-five explants were used per treatment, and each experiment was repeated thrice.

[b]IL, immature leaflet; ML, mature leaflet; LP, leaflet petiole. Values within column with the same letter are not significantly different at the 1% probability level according to the Duncan's new multiple range test and the letters indicate the significance of the percentage.

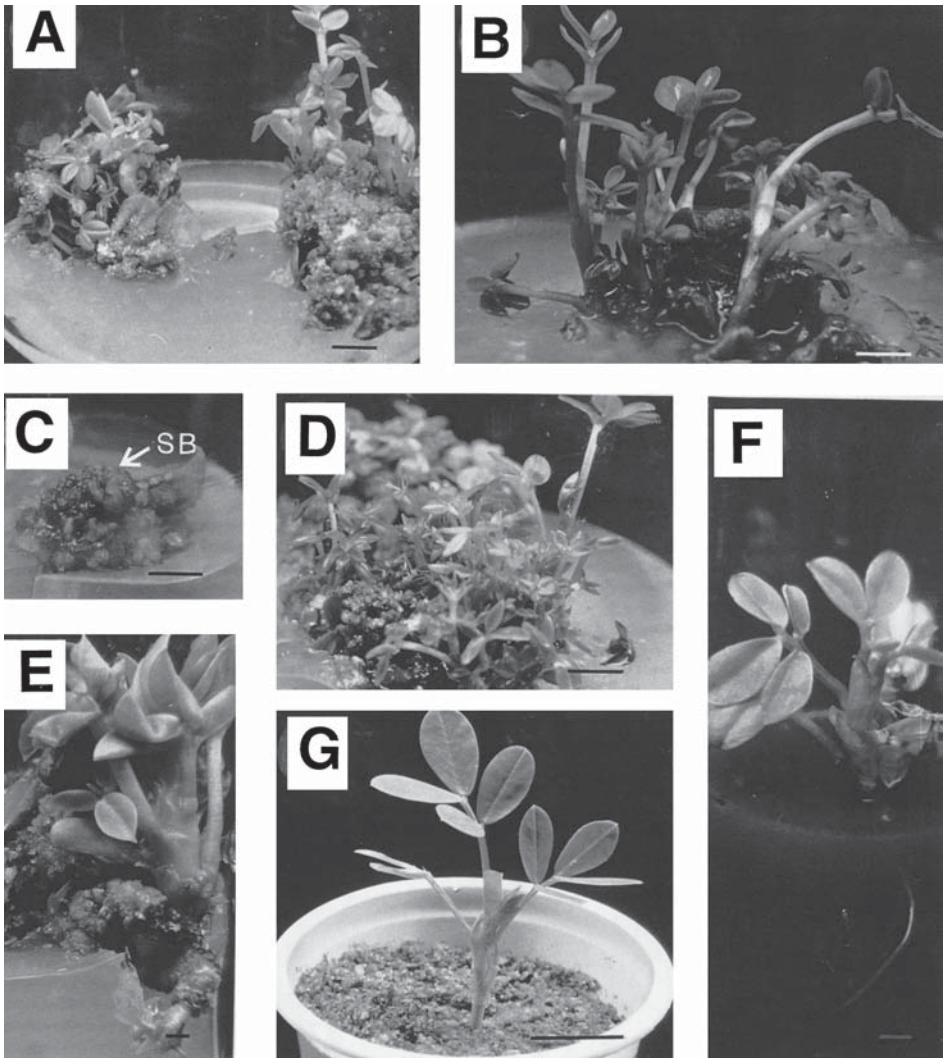

Fig. 1. Morphogenesis in organ cultures of groundnut (*Arachis hypogaea* L. cv.VRI-2) **(A)** Regenerating callus from mature leaflet explant; **(B)** proliferation and elongation of shoots from mature leaflet–derived callus; **(C)** shoot-bud regeneration in immature leaflet–derived callus (SB, shoot buds); **(D)** differentiation of shoots from immature leaflet–derived callus; **(E)** direct shoot development from a petiole explant; **(F)** root induction; **(G)** regenerated plant established in plastic cup containing soil. (Bars: 0.5 cm for (A) and (D); 1.0 cm for (B), (C), (F), and (G); 0.25 cm for (E).

was 45.9 and 36.6 per culture in VRI-2 and TMV-7, respectively (Table 3; Fig. 1D). Shoot development from all buds on a callus, however, was not simultaneous. Segmentation and subculture of regenerating shoots not only caused elongation of all shoot buds but also resulted in the differentiation of more and more shoot buds from the callus and consequent multiple shoot formation. Shoot-bud multiplication was augmented when segments

Table 3
Mean Number of Shoots Recovered per Culture After 3 to 4 wk on MS Medium Supplemented with Different Concentrations of BAP or Kinetin in Combination with NAA at 1.0 mg/L in Groundnut Cultivars[a]

Concentration of growth regulators (mg/L)	Mean number of shoots/culture ± SD[b]					
	VRI-2			TMV-7		
	IL	ML	LP	IL	ML	LP
BAP						
1.0	15.2 ± 1.8h	12.5 ± 1.4i	10.5 ± 1.2i	10.2 ± 1.3h	8.4 ± 1.1g	7.3 ± 1.0c
2.0	23.3 ± 1.6f	18.5 ± 1.5g	17.5 ± 1.4g	24.5 ± 1.8e	22.1 ± 1.9e	20.2 ± 2.0ab
3.0	36.1 ± 2.9c	26.3 ± 2.4e	21.4 ± 2.3e	30.0 ± 2.5c	28.5 ± 2.6c	24.7 ± 2.2a
4.0	41.7 ± 3.0b	38.5 ± 2.7b	32.5 ± 2.5c	32.4 ± 3.1b	31.4 ± 2.9b	29.5 ± 2.4a
5.0	45.9 ± 3.6a	42.1 ± 3.1a	40.7 ± 2.8a	36.6 ± 3.3a	35.3 ± 3.2a	30.3 ± 2.5a
Kinetin						
1.0	15.6 ± 1.3h	9.3 ± 1.0j	8.4 ± 0.9j	9.9 ± 0.8i	7.5 ± 0.9g	6.2 ± 0.7c
2.0	20.5 ± 1.7g	16.5 ± 2.1h	16.4 ± 1.8h	18.1 ± 1.6g	16.5 ± 1.8f	15.5 ± 1.5b
3.0	28.5 ± 2.4e	20.5 ± 2.2f	20.4 ± 2.0f	21.8 ± 2.1f	20.1 ± 2.3e	20.4 ± 2.5ab
4.0	32.3 ± 2.9d	30.1 ± 2.7d	30.4 ± 3.5d	26.4 ± 2.4d	25.5 ± 2.2cd	24.3 ± 2.6a
5.0	35.4 ± 3.1c	34.5 ± 2.9c	33.5 ± 2.5b	30.5 ± 2.7c	28.4 ± 2.4c	25.3 ± 2.2a

[a]Fifteen to twenty shoot-bud clusters were used per treatment, and each experiment was repeated thrice.

[b]IL, immature leaflet; ML, mature leaflet; LP, leaflet petiole. Values within column with the same letter are not significantly different at the 1% probability level according to the Duncan's new multiple range test and the letters indicate the significance of the percentage.

of organogenic callus were incubated for 2 mo on the same medium on which bud elongation had been obtained, i.e., MS medium with 5.0 mg/L of BAP and 1.0 mg/L of NAA (Fig. 1B).

Petiole explants on MS medium supplemented with 2.0 mg/L of BAP and 0.5 mg/L of NAA enlarged to about one-half their size and formed shoot buds as protuberances on the edges of the cut ends within 3 wk (Table 2). Shoot buds emerged from the protuberances in both cultivars (86% in VRI-2 and 79% in TMV-7). A quantitative increase in shoot-bud formation occurred on MS medium containing 5.0 mg/L of BAP and 1.0 mg/L of NAA (Table 3). The maximum number of shoots produced was 40.7 and 30.3 per culture in VRI-2 and TMV-7 cultivars, respectively. Accessory buds developed from the region of the protuberance and developed into shoots after 30 d of culture (Fig. 1E).

Elongated shoots obtained by direct and callus-mediated regeneration formed roots within 2 wk on MS medium containing NAA or IBA (0.5–2.0 mg/L) and 0.5 mg/L of kinetin (Fig. 1F). Roots emerged from the cut end of the shoots. The IBA (2.0 mg/L) and kinetin (0.5 mg/L) combination, however, induced roots that were thick, bearing numerous lateral roots. The highest frequency of rooting was 84.5% and 78.5% in VRI-2 and TMV-7 cultivars, respectively, with immature leaflet-derived shoots (Table 4). The rooted plantlets were transferred to soil and later established in the field, where 95% survived and resumed growth to maturity (Fig. 1G).

Discussion

The present study reveals that callus formation and shoot-bud regeneration responses in groundnut vary between cultivars. Furthermore, the responses also depended on the maturity status of the explants. Of the four auxins used, NAA was, in general, observed to be more suitable than IAA, IBA, or 2,4-D for callus initiation. The efficiency of callus formation was better with 2.0 mg/L of NAA and 0.5 mg/L of kinetin combination. A combination of higher auxin and a lower level of cytokinin resulted in effective callus formation. Therefore, kinetin at 0.5 mg/L was added to increase the frequency of callusing. These results are in agreement with the findings of Bajaj et al. *(20)*, Narasimhulu and Reddy *(3)*, and Venkatachalam et al. *(17)* in groundnut.

The shoot buds obtained from calluses of immature and mature leaflet explants was from a higher cytokinin (BAP) with low auxin-containing media. Likewise, direct shoot-bud differentiation from petiole explants was also obtained on a medium with similar hormone ratio. One of the strategies used for obtaining shoot-bud regeneration from undifferentiated callus was to maintain the callus on the basal medium with a low concentration of auxins together with various concentrations of BAP. The fixed concentration of auxin was 0.5 mg/L, which by itself was capable of sustaining a good rate of callus growth. The ratios of cytokinin (BAP) and auxins used here that enhanced shoot-bud primordia development, shoot elongation, and multiple shoot formation were also used previously to

Table 4
Percentage of Root Formation on MS Medium Supplemented with Different Concentrations of NAA or IBA with 0.5 mg/L of Kinetin for Groundnut Cultivars[a]

Concentration of growth regulators (mg/L)		Root-induction frequency (percentage # mean ± SD)[b]					
		VRI-2			TMV-7		
		IL	ML	LP	IL	ML	LP
NAA	Kinetin						
0.5	0.5	43.7 ± 2.1g	38.3 ± 1.8g	35.5 ± 1.9f	41.5 ± 2.0g	36.5 ± 1.4e	35.3 ± 1.9cd
1.0	0.5	56.5 ± 3.4e	46.7 ± 2.7e	42.0 ± 2.1e	52.6 ± 2.6e	42.5 ± 2.4d	40.5 ± 1.8c
1.5	0.5	67.4 ± 3.8d	53.2 ± 2.1d	50.6 ± 2.2d	64.7 ± 4.2c	56.5 ± 3.5c	48.9 ± 2.8b
2.0	0.5	72.3 ± 4.7c	61.7 ± 3.3c	57.4 ± 3.1c	71.2 ± 4.9b	60.5 ± 3.6ab	54.3 ± 2.7ab
IBA	Kinetin						
0.5	0.5	48.5 ± 2.4f	42.3 ± 2.4f	40.3 ± 2.9e	45.3 ± 2.1f	42.4 ± 1.6d	45.2 ± 1.9c
1.0	0.5	59.3 ± 3.5e	52.6 ± 3.7d	50.2 ± 3.4d	60.5 ± 3.7d	56.5 ± 2.8c	51.6 ± 3.5b
1.5	0.5	76.3 ± 4.2b	67.3 ± 3.9b	63.4 ± 3.1b	72.3 ± 4.6b	62.3 ± 3.4a	59.3 ± 3.3a
2.0	0.5	84.5 ± 4.0a	72.0 ± 3.3a	69.5 ± 2.9a	78.5 ± 4.3a	65.5 ± 3.8a	62.5 ± 3.9a

[a]Fifteen to twenty shoots were used per treatment, and each experiment was repeated thrice.

[b]IL, immature leaflet; ML, mature leaflet; LP, leaflet petiole. Values within column with the same letter are not significantly different at the 1% probability level according to the Duncan's new multiple range test and the letters indicate the significance of the percentage.

elicit similar responses from other grain legumes *(21–23)* and leguminous trees *(24)*. Of the three explants used in our experiments, immature leaflet–derived callus was found to be better for shoot-bud differentiation than that of mature leaflet. Regeneration was also achieved from immature leaflet from 3- to 5-d-old peanut seedlings by Mroginski et al. *(25)*, although the frequency obtained was considerably low when compared with the regeneration in the present protocol.

The choice of explant and method of excision may have resulted in lowered regeneration response in earlier reports. Pittman et al. *(26)* and McKently et al. *(4)* obtained shoot regeneration from the cut ends of the leaf blade and from the adaxial leaf surface of groundnut, respectively. Bajaj et al. *(20)* also cultured leaflet petioles; however, only a mass of callus was recovered from this type of explant without the accompanying regeneration. Although the protocols used by earlier investigators worked efficiently for the cultivars reported, neither of these methods was successful with VRI-2 and TMV-7, popular Indian cultivars. The percentages of explants bearing buds and shoots were comparatively higher when following the method shown here. Also a higher percentage of conversion of buds into well-developed shoots was possible. McKently et al. *(4)* demonstrated that a portion of embryonic axes remaining attached to the explants was essential for inducing a higher number of multiple shoots in peanut. However, in experiments here, leaflets without the embryonic axes or pre-existing meristem were used. More than 85% of regenerated shoots were rooted on media with IBA. Similar observations were made by Narasimhulu and Reddy *(3)*, Moss et al. *(27)*, and Cheng et al. *(5)*, but the percentage of rooting obtained was low.

Because the regenerative ability is under the control of the genotype, the protocols established for a particular genotype may not be effective with others. Variation in bud or shoot formation among genotypes in groundnut was also reported by Pittman et al. *(26)*, Seitz et al. *(28)*, and McKently et al. *(29)* with immature leaflets or seedling explants. The procedure we have developed for both direct and callus-mediated regeneration of shoots from leaflets and petioles of groundnut is simple, rapid, and reproducible. These features make this an ideal regeneration system to be adopted in genetic transformation of groundnut through either *Agrobacterium*-mediated transformation or particle bombardment method.

Acknowledgment

The first author (P. V.) acknowledges the Council of Scientific and Industrial Research for the award of Senior Research Fellowship during the course of this investigation.

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