In Vitro Regeneration of Valencia-type Peanut (*Arachis hypogaea* L.) from Cultured Petiolules, Epicotyl Sections and Other Seedling Explants[1]

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ABSTRACT

This study evaluated plant development via direct organogenesis from *in vitro*-cultured young seedling tissues of cultivated peanut, especially the valencia-type peanut. Complete plants were regenerated from in vitro-cultured petiolule-with-blade-attached explants, leaflet segments, and epicotyl and petiole sections. Multiple shoots arose on Murashige and Skoog medium (MS) supplemented with 6-benzylaminopurine (BA) (5-25 mg/L) plus 1-naphthaleneacetic acid (NAA) (0.5-3mg/L). After 30 d culture on 25 mg/L BA + 1 mg/L NAA, 1.6 buds or shoots/explant were regenerated from the petiolule-with-blade-attached explants. Comparable numbers of shoots were obtained from epicotyl sections of the first node region of the seedling after 60 d culture using 10 mg/L BA + 1 mg/L NAA. Leaflet segments and petiole sections were less responsive for shoot formation. Excised shoots developed roots *in vitro* upon transfer for 15 d to MS medium supplemented with NAA at 1 mg/L. Plantlets were transferred to soil and grown in a greenhouse to maturity. A wide range of cultivated peanut genotypes was evaluated for organogenic responsiveness, using the petiolule-with-blade-attached explant source. Only valencia-type cultivars, or a hybrid derivative with a valencia background, were responsive with this regeneration system.

Key Words: Peanut, seedling tissues, *in vitro*, organogenesis, regeneration.

The successful exploitation of *in vitro* techniques in plant biotechnology depends on the establishment of efficient regeneration systems (4,7). An efficient plant regeneration system is also a prerequisite for genetic transformation studies using *Agrobacterium tumefaciens* (8).

The cultivated peanut, one of the grain legume species, is recalcitrant in tissue culture, although some successful plantlet regeneration has been achieved either by organogenesis (13,14) or somatic embryogenesis (6,7, 24). A widely used explant of cultivated peanut for organogenic regeneration is the immature leaflet from very young seedling tissue. Mroginski *et al.* (15) studied shoot regeneration from primary callus of immature leaves (2-5 mm in length) of Colorado Manfredi (spanish-valencia hybrid) peanut cultured on Murashige and Skoog (MS) medium (16) supplemented with 1 mg/L each of 6-benzylaminopurine (BA) and 1-naphthaleneacetic acid (NAA), but no report was given of whole plant re-establishment. Pittman *et al.* (21) extended this study to 28 genotypes of the *Arachis* genus using the same explant. The genotypes included cultivars, species, and hybrids. All genotypes tested produced callus, and various genotypic differences were observed for callus, shoot and root production from the section *Arachis* and *Extranervosae*. Histological examination revealed shoot meristems originated from adaxial epidermal cells near the midrib of the leaflet. Seitz *et al.* (23) further extended this study to 47 commercial genotypes including virginia, spanish, spanish-valencia hybrids and valencia peanuts. Shoots were recovered from 0% to 20% of the explants. Statistical analyses

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revealed no differences for shoot formation among virginia, spanish and valencia type peanuts. A similar regeneration scheme was reported by Narasimhulu and Reddy (17). Shoots developed sporadically from peanut leaflets of 7-day-old seedlings. McKently *et al.* (14) evaluated plant development via organogenesis from *in vitro*-cultured immature leaf tissue (5 mm in length) of cultivated peanut including virginia, runner, spanish and valencia peanuts. Bud regeneration occurred from the adaxial surface of the cultured peanut explants on four concentrations of BA with 1 mg/L NAA, with the largest number produced using 5 mg/L BA.

Compared to the studies on leaflet explants of cultivated peanut, limited studies have been conducted on organogenesis using other seedling explants. Excised segments of the epicotyl, mesocotyl, cotyledon, and petiole from *in vitro*-grown seedlings have been cultured on different media (2). Buds developed from the original meristem of epicotyl segments. Limited bud development was observed from mesocotyl segments. Only callus formed from excised cotyledons and petiole segments. Shoots were induced with varying frequencies from the primary callus of seedling epicotyl, hypocotyl and cotyledon explants cultured on media containing 1 mg/L BA and 0.4 mg/L NAA, and also 1 mg/L kinetin (17). Direct shoot regeneration also was observed. These studies provided some information on the regeneration potential of young leaflets (2-5 mm in length), and limited information on the regeneration capacity of other seedling explants in some commercial genotypes. However, organogenesis from different explants with somewhat older seedling tissue (8 to 11-day old) in certain genotypes, especially the valencia-type peanuts, has been little studied.

Our objective was to evaluate plant development via direct organogenesis, primarily with the valencia-type peanut, from four *in vitro* cultured seedling explant tissues (8 to 11-day-old): sections of epicotyls, leaflets and petioles, and the petiolule-with-blade-attached (which has not been studied previously as an explant source). The goal of these studies was to establish a suitable organogenic regeneration system for attempting peanut transformation using *Agrobacterium tumefaciens*.

Materials and Methods

Response of Petiolule-with-Blade-Attached Explants of NM Valencia A Peanut to BA and NAA Treatments

NM Valencia A peanut (9) seeds were surface sterilized with 95% ethanol for 5 min, 2.6% sodium hypochlorite (50% Clorox bleach) with a drop of Ivory detergent for 10 min, and two sterile water rinses for 10 min each. The testa were then removed aseptically. The seeds were germinated at 28C on a 16 h light/8 h dark cycle on seed germination medium described by Phillips and Luteyn (20), in 100 X 25 mm plastic petri plates. After 8-11 d, petiolule-with-blade-attached explants, 1 cm in length, were cultured *in vitro* on the medium consisting of MS salts, L2 vitamins (19), 30 g/L sucrose, 0.8% agar, with 14 combinations of BA (2-25 mg/L) and NAA (0.5-3 mg/L) in completely randomized design with 2 replications. The blades were fully expanded, and half were placed with the adaxial and half with abaxial surface in contact with medium. Twenty to 35 explants were cultured on each BA and NAA treatment. The cultures were incubated at 28C with a 16 h photoperiod. Data were collected after 30 d on the number of bud primordia, buds and shoots formed. After two months, the bud primordia were transferred to six media treatments for bud primordium development, which included four MS based media: basal medium with no hormones, 25 mg/L BA + 1 mg/L NAA, 10 mg/L BA + 1 mg/L NAA , 5 mg/l BA+ 0.5 mg/L NAA, and two L2 (19) based media supplemented with 0.01 mg/L picloram + 0.5 mg/L BA, or 0.1 mg/L NAA + 0.5 mg/L BA.

Based on the results of the first month of culture on 14 combinations of BA and NAA, four combination treatments of BA (at 5, 10 and 25 mg/L) and NAA (at 0.5 or 1 mg/L) were selected to conduct a second experiment to optimize the regeneration response of this explant. Five replications each with 10 explants were cultured on each of the four selected BA and NAA treatments.

Genotype Response

Petiolule-with-blade-attached explants from several commercial cultivars, including valencia type: NM Valencia C (10), McRan and Georgia Red (derived from a valencia X runner cross) (5); spanish-type: Pronto, Spanco and Starr; virginia type: Florigiant and NC 7; and one mixed type: PI262129 from Peru, were cultured on MS medium supplemented with three combinations of BA + NAA (5mg/L BA + 0.5 mg/L NAA, 10 mg/L BA + 1 mg/L NAA, and 25 mg/L BA + 1 mg/L NAA), with 2 replications each with 30 explants. After 30 d of culture, data were collected for the numbers of bud primordia, buds and shoots formed.

Epicotyl, Leaflet and Petiole Responses

New Mexico Valencia A peanut was used as the explant donor from 8 to 11-day-old seedlings. Leaflets about 1 cm in length were cut transversely into three pieces. Petiole sections about 0.5 to 1 cm in length were obtained without node meristems. The epicotyl stems were transversely cut 0.2 cm in length, and the serially-sectioned epicotyl stem explants were assigned to the media in serial order. This experiment used MS medium containing 25 mg/L BA + 1 mg/L NAA with four replications, each with 15 explants. After 30 d culture, data were collected for the numbers of bud primordia, buds and shoots formed.

Based on the results of the first experiment, a second one was conducted to optimize the regeneration response of the epicotyl stem explant taken from the region of the first node. Three sections were obtained from each epicotyl. The leaflet and petiole explants were prepared as described above. The explants were cultured in the three NAA and BA combination treatments as described in the Genotype Response section above, using a completely randomized design with four replications, each with 10 explants.

In all experiments, analyses of variance were conducted on the data to evaluate the effects of genotypes, explants and medium treatments. Fisher's protected Least Significant Difference (LSD) tests were used (P=0.05) for mean separation.

Results and Discussion

Morphogenic Response of Petiolule-with-Blade-Attached Explants in BA and NAA Combination Treatments

Multiple shoot regeneration occurred from explants cultured on 9 of the 14 BA and NAA combination treatments (Table 1). The petiolule explants began to enlarge on all medium treatments within 7 d of culture initiation. Bud primordia developed directly from some of the expanded surface of the petiolules, but not from the blade tissue, within 15-18 d of culture initiation, and some calli formed from the same region (Fig. 1). This regeneration process was different from that reported by McKently *et al.* (14) who cultured leaflets 2-5 mm in length of cultivated peanut *in vitro* for 36 d, and regenerated bud tissue directly from the adaxial leaf blade surfaces. Bud primordia developed into buds or shoots 30 d after culture initiation in our study (Fig. 2). Some buds developed abnormally in shape, and failed to grow into normal shoot structures. This feature was different from the description by Bhatia *et al.* (3) and McKently *et al.* (13). In the media with BA at or above 5 mg/L, 20% of the responsive explants formed only bud primordia in the initial one or two month culture period in our study. Calli developed from the base of these bud primordia, and additional bud primordia formed from these calli and also from the basal part of the initial bud primordia.

When these bud primordia were transferred to six different media after two months of culture in the original media, 5% of these primordia developed into buds or shoots after two months culture on MS medium supplemented with 10 mg/L BA + 1 mg/L NAA, and 2% developed on MS medium supplemented with 5 mg/L BA + 0.5 mg/L NAA. The bud primordia did not develop into buds or shoots in the remaining four medium treatments. No difference in regeneration

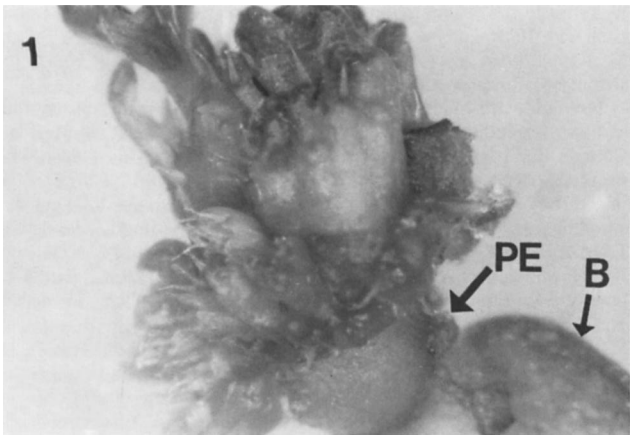

Fig. 1. Bud primordia and buds formed from a petiolule-with-blade-attached explant of NM Valencia A peanut, after 18 d culture on 25 mg/L BA + 1 mg/L NAA. (PE = Petiolule, B = Leaf blade).

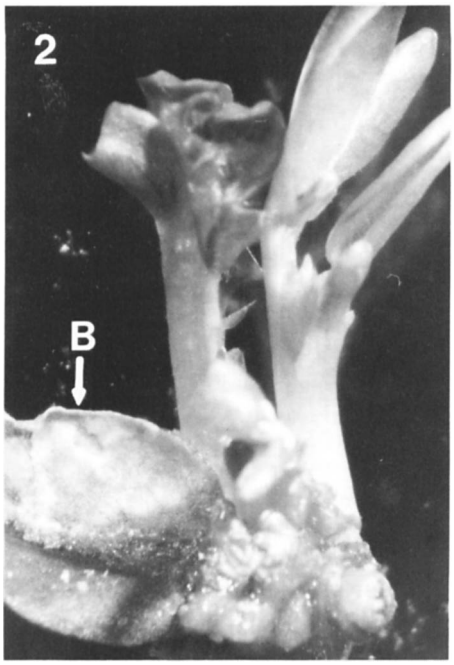

Fig. 2. Shoots developed from a petiolule-with-blade-attached explant of NM Valencia A peanut, after 30 d culture on 25 mg/L BA + 1 mg/L NAA. (B = Leaf blade).

response was noted between distal and proximal pairs of petiolule-with-blade-attached explants taken from the same leaf. There was no apparent difference in regeneration response between explants oriented with the abaxial or adaxial side of the blade in contact with the medium. However, petiolules failed to support bud or shoot formation when the blades were not attached (data not shown).

Isolated shoots were transferred to MS basal medium supplemented with 1 mg/L NAA and the sucrose level reduced to 20 g/L. All formed roots within 15 d, and the resultant plantlets were transferred to soil. The results we obtained were similar to those from the previous reports for rooting of shoots, plantlet development, and reestablishment (1, 13).

The concentrations of BA at or above 5 mg/L and the concentrations of NAA below 3 mg/L were the best combinations for regeneration from these petiolule explants (Table 1). Because more bud primordia, buds or shoots were formed using the lower NAA concentration with higher BA levels in the previous experiment, petiolule-with-blade-attached explants of NM Valencia A peanut were placed on four BA and NAA combinations for optimizing the regeneration response during the first month of culture in the next experiment. Sixty two percent of the petiolule explants regenerated buds in the 25 mg/L BA + mg/L NAA treatment, which was significantly better ($P<0.05$) than that found among the petiolule-with-blade-attached explants cultured on the remaining three combinations. The total number of bud primordia and buds in the 25 mg/L BA + 1 mg/L NAA treatment was also significantly better than that of the other three combination treatments (Table 2). The number of shoots in the 25 mg/L BA + 1 mg/L NAA and 10 mg/L BA + mg/L NAA treatments were significantly better than in the other two treatments.

Table 1. Morphogenic response of NM Valencia A peanut petiolule-with-blade-attached explants treated with different NAA and BA concentrations for 30 d.

BA	NAA	Responsive/total explants		Buds	Shoots
mg/L		Number	%	Number	
25	1	12/25	48	46	8
	3	13/32	40	18	8
	5	10/32	32	17	9
15	1	12/31	38	26	3
	3	5/23	21	13	1
	5	6/27	22	12	0
10	0.5	21/35	60	106	3
	1	13/25	52	74	12
	3	3/25	12	13	0
5	0.5	9/20	45	42	7
	1	6/25	25	23	11
	3	10/25	40	36	3
2	0.5	4/30	13	12	0
	1	2/24	8	6	0

Genotype Response

A wide range of peanut genotypes was evaluated for organogenic responsiveness with petiolule-with-blade-attached explants cultured on three NAA + BA concentration combinations (Table 3). Although all genotypes produced callus, only the valencia type peanuts were responsive for bud and shoot formation during the first 30 d culture. For

Table 2. Regeneration response of petiolule-with-blade-attached explants of NM Valencia A peanut treated with four NAA and BA combination treatments for 30 d.

BA	NAA	Responsive/total explants	Bud primordia	Buds	Shoots
mg/L		Number	Number		
25	1	31/50A†	73A	27A	24A
10	0.5	17/50B	25B	19B	24A
10	1	12/50B	18B	3B	4B
5	0.5	10/50B	31B	6B	4B

† Means followed by the same letter within a column are not different at the P=0.05 level

Table 3. Morphogenic response of petiolule-with-blade-attached explants from various peanut genotypes treated with three NAA and BA combinations for 30 d.

Genotypes	BA	NAA	Responsive/ total explants	Bud primordia	Buds	Shoots
	mg/L		Number	Number		
PI262129 (Mixed)†	25	1	0/30	0	0	0
	10	1	0/32	0	0	0
	5	0.5	0/30	0	0	0
Pronto (sp.)	25	1	1/30	5	0	2
	10	1	1/30	5	0	0
	5	0.5	1/30	0	0	2
Spanco (sp.)	25	1	0/30	0	0	0
	10	1	2/30	15	0	0
	5	0.5	0/30	0	0	0
Starr (sp.)	25	1	0/30	0	0	0
	10	1	2/30	2	0	0
	5	0.5	0/30	0	0	0
McRan (val.)	25	1	15/30	15	18	14
	10	1	18/30	30	63	17
	5	0.5	15/30	45	33	12
Florigiant (va.)	25	1	5/30	21	0	0
	10	1	0/30	0	0	0
	5	0.5	3/30	9	0	0
NC 7 (va.)	25	1	0/30	0	0	0
	10	1	0/30	0	0	0
	5	0.5	0/30	0	0	0
Georgia Red (val.-rr.)	25	1	15/30	133	0	0
	10	1	10/30	34	6	15
	5	0.5	11/30	24	5	2
Valencia C (val.)	25	1	15/30	40	21	17
	10	1	10/30	20	12	10
	5	0.5	6/30	16	13	5

† Mixed = Mixed type peanut
sp. = spanish peanut
va. = virginia peanut
val. = valencia peanut
val.-rr. = valencia-runner hybrid derivative peanut

the valencia-runner hybrid derivative peanut, the regeneration response in these three media was similar to that for valencia type peanuts; however, in the 25 mg/L BA and 1 mg/L NAA treatment, numerous bud primordia formed without further development. For bud primordia formation, very little positive response occurred in the cultivars Pronto, Spanco, Starr and Florigiant. There were no positive responses in NC 7 or PI 262129. Thus, among the genotypes tested, only valencia-type or a hybrid derivative of valencia peanuts were capable of regeneration using this explant source. Variation in bud or shoot formation among genotypes was also reported by Pittman *et al.* (21), Seitz *et al.* (23) and McKently *et al.* (13, 14) with immature leaflets or seed explants; however, this genotypic variation was not correlated with market type.

Morphogenic Response of Epicotyl, Leaflet and Petiole Explants of NM Valencia A Peanut on BA and NAA Combination Treatments

A. Epicotyl explant response

Within 8 d of culture initiation, two or three serial sections from each epicotyl explant produced adventitious buds along the cut surfaces. The explants expanded to twice their original size, and also formed white callus at the edge of the explants where the bud primordia did not occur. These calli did not differentiate into bud primordia after three months culture in MS medium supplemented with 25 mg/L BA and 1 mg/L NAA. The rest of the epicotyl sections distal to the first node did not regenerate, but 100% of the explants formed calli in this medium in the first month of culture. Thirty five percent of the bud primordia developed into normal buds after one month culture (Fig. 3). Fifty percent of these buds developed into shoots after two months culture in that medium. When these shoots were transferred to MS medium supplemented with 1 mg/L NAA and 20 g/L sucrose, all formed roots within 15 d, and the resulting plantlets were transferred to soil. Of 30 plantlets transferred to the greenhouse, 25 (83%) survived the initial 60 d transfer period, developed successfully to maturity and set seeds.

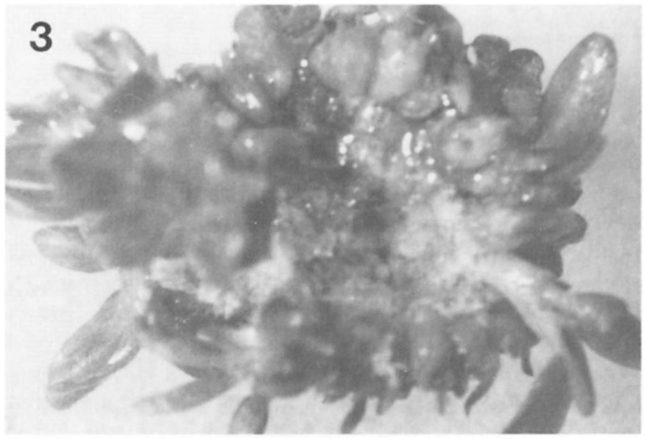

Fig. 3. Bud primordia and shoot buds formed from an epicotyl section of NM Valencia A peanut, after 30 d culture on 10 mg/L BA + 1 mg/L NAA.

Narasimhulu and Reddy (17) cultured epicotyl explants on a medium with 2 mg/L IAA and 2 mg/L kinetin, and also observed the direct regeneration of shoots, although pre-existing meristems were suspected to be responsible. Bajaj *et al.* (2) cultured epicotyl and mesocotyl explants, and observed shoot meristem development directly from the epicotyl explants. These cultures of epicotyl segments with the shoot meristem tissue included were very similar to the shoot tip cultures reported by Kartha *et al.* (11) and Russo and Varnell (22), who propagated multiple shoots from shoot tip explants. In our study, shoot regeneration occurred only from epicotyl sections obtained from the region of the first node, but pre-existing shoot meristems were not present in the explants. Most of the regenerated shoots and buds originated from cortex cells, some originated from epidermal tissues, and fewer originated from vascular cambium tissues (histological data not shown).

Three BA and NAA combination treatments were tested to optimize the regeneration from epicotyl sections from the region of the first node. Fifty percent of the epicotyl sections of NM Valencia A peanut responded within one month of culture on 10 mg/L BA + 1 mg/L NAA (Table 4), which was not significantly different from epicotyl sections cultured on the remaining two treatments. The analysis of variance for total bud primordia and buds indicated that the main effects of medium treatment were also not significant ($P>0.05$). The medium with 10 mg/L BA + 1 mg/L NAA was chosen for routine use in subsequent experiments.

B. Leaflet explant response

Leaflets about 1 cm in length (from 8 to 11 d old seedlings) were cut into three sections, and cultured in three BA and NAA combinations. Within 8 d of culture initiation, the cut surface of the main vein region of the leaflet segments began

Table 4. Morphogenic response of epicotyl, leaflet and petiole sections of NM Valencia A peanut treated with optimal NAA and BA combination.

Type of explants	BA	NAA	Responsive/total explants	Bud primordia	Buds
	mg/L		Number	Number	
Epicotyl†	10	1	20/40	50	25
Leaflet†	25	1	25/50	10	56
Petiole‡	25	1	43/50	15	62

† Explants were cultured for 30 d

‡ Explants were cultured for 35 d

to expand, and form green protuberances (Fig. 4A). At 15 d, bud primordia formed from the protuberances, which developed further into shoots (Fig. 4B). Regeneration of plantlets from peanut leaflets was also achieved by Mroginski *et al.* (15) from immature leaves 2-5 mm in length from 3 to 5-day-old peanut seedlings. Pittman *et al.* (21) achieved regeneration from the epidermal cells of the cut surface of the leaflet blade, with McKently *et al.* (14) obtained regeneration from adaxial leaf surfaces. In the treatment containing a high level of BA (10 or 25 mg/L + 1 mg/L NAA), 25% of the buds were abnormal with only elongated leaflets, which never developed an apical meristem or elongated into shoots in our study. In the 5 mg/L BA + 0.5 mg/L NAA treatment, 12% of the leaflet segments formed roots from the region of the green protuberances. The organogenic formation of buds and roots from a single cultural treatment was also described by Mroginski *et al.* (15).

Within 30 d culture initiation, 50% of all leaflet explants formed bud primordia or buds (Table 4). Analysis of variance for the responsive explants and bud formation with leaflet explants indicated that the main effects of medium treatment were not significant ($P>0.05$). Considering the number of bud primordia and bud formation from responsive explants together, the 25 mg/L BA and 1 mg/L NAA combination treatment had a positive influence on regeneration efficiency, and this treatment was chosen for routine use in subsequent experiments.

C. Petiole explant response

Fig. 4A. Green protuberance formed from a leaflet segment of NM Valencia A peanut, after 13 d culture on 10 mg/L BA + 1 mg/L NAA. (G = Green protuberance, B = Leaf Blade).

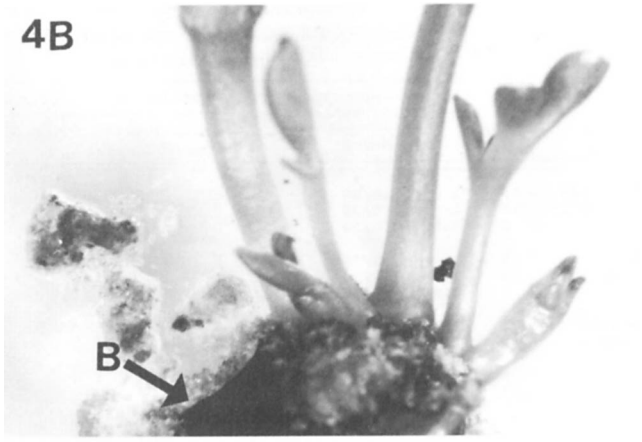

Fig. 4B. Shoots developed from a leaflet segment of NM Valencia A peanut, after 30 d culture on 10 mg/L BA + 1 mg/L NAA. (B = Leaf Blade).

Petioles about 0.5 and 1.0 cm in length were cultured on three BA + NAA combinations. Within one week, the petiole explants started to elongate and expanded to about one and half times the size of the original explants. Protuberances formed on the edge of the cut surface within 15 d. Multiple bud primordia developed from the region of the protuberance, which developed further into shoots after 30 d culture (Fig. 5). The calli usually formed at the basal part of the bud after 20 d culture of the explants. Some calli also formed on the surface of the original explants within one month. In the 25 mg/L BA + 1 mg/L NAA and 10 mg/L BA + 1 mg/L NAA treatments, about 65% of the regenerated buds were thin and weak, and subsequently failed to develop into normal shoots. The isolated shoots were transferred to the rooting medium described previously, and all formed roots. The resultant plantlets were transferred to soil. Bajaj *et al.* (2) cultured the excised petiole, however, only a mass of callus was recovered from this explant.

Analysis of variance for the responsive explants and bud formation with petiole explants indicated that the main effects of medium treatments were significant ($P<0.05$). At the end of 35 d culture, 86% of the explants in the 25 mg/L BA + 1 mg/L NAA treatment (Table 4), and 64% of the explants in the 10 mg/L BA + 1 mg/L NAA treatment formed

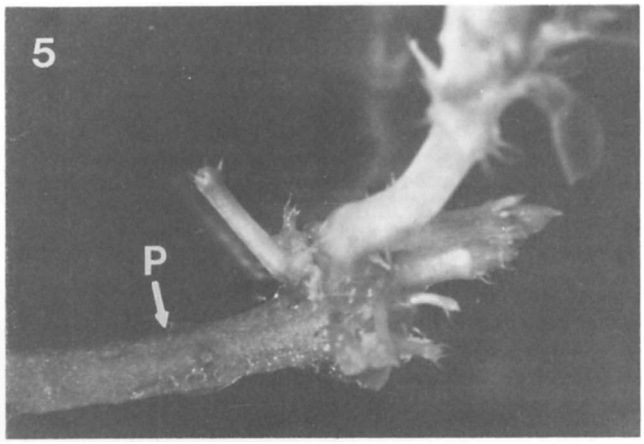

Fig. 5. Shoots developed from a petiole explant of NM Valencia A peanut, after 35 d culture on 10 mg/L BA + 1 mg/L NAA. (P = Petiole).

bud primordia or buds, which were significantly better than that formed in the 5 mg/L BA + 0.5 mg/L NAA treatment. Considering only the number of buds formed, the treatments of 25 mg/L BA + 1 mg/L NAA and 10/mg/L BA + 1 mg/L NAA were significantly better than the treatment of 5 mg/L BA + 0.5 mg/L NAA.

Conclusions

These experiments demonstrated successful organogenic regeneration of peanut plantlets by *in vitro* culture of seedling tissues: petiolule-with-blade-attached, and epicotyl, leaflet and petiole sections. In this regeneration system the valencia-type peanut developed shoots directly from these explants without an intervening callus. Maximum regeneration responses were obtained with petiolule-with-blade-attached explants cultured on MS medium supplemented with 25 mg/L BA + 1 mg/L NAA, however, this regeneration system was only effective in valencia-type peanuts. Epicotyl sections from the region of the first node, leaflet segments and petiole sections showed approximately similar regeneration responses in MS medium supplemented with BA (5, 10, 25, mg/L) and NAA (0.5, 1 mg/L).

The seedling tissues of cultivated peanut used for plant regeneration via organogenesis were previously focused on very young leaflets (2-5 mm) (14, 15, 21). This small explant provides only limited wound sites for *Agrobacterium* infection in the peanut transformation system. Seed explants such as cotyledons and embryo axes (13) were utilized for organogenesis successfully, and somatic embryogenesis from immature zygotic embryos has also been reported (7, 24). However, these explants may not be suitable for peanut transformation due to low susceptibility to *Agrobacterium*, as has been shown in soybean transformation studies (18).

This regeneration protocol may be useful for genetic improvement of valencia type peanut by *Agrobacterium*-mediated gene transfer. Because this regeneration system does not involve any undifferentiated tissue stage, i.e callus or protoplast, the likelihood of regenerating genetically normal plants should be high (12).

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