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Plant regeneration via organogenesis from shoot base-derived callus of *Arachis stenosperma* and *A. villosa*

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Plant regeneration via organogenesis was obtained from shoot base-derived callus cultures of wild *Arachis* species, viz. *Arachis stenosperma* and *Arachis villosa*. The callus was induced from the pulse-treated (5000 ppm IBA for five minutes) shoot bases on MS medium supplemented with 11.42 µM IAA. Green spots (meristemoids) and purple-coloured leafy shoots were observed from the shoot base-derived callus within 2–4 weeks of culture on a medium containing half strength MS nutrients and B5 vitamins supplemented with 22.20 µM BA, 2.85 µM IAA and 1.0 µM TDZ. Shoot regeneration frequency up to 75% and 68% was observed in *A. stenosperma* and *A. villosa* respectively. Anatomical study revealed the organogenic pathway of regeneration from callus cultures. The regenerated shoots of *A. stenosperma* were rooted with a frequency of 52% on media supplemented with 16.12 µM NAA and 2.32 µM kinetin. In *A. villosa*, optimum response of 25% rooting was observed on media supplemented with 11.4 µM of IAA and 2.32 µM kinetin. The rooted plantlets were transferred to soil: vermiculite (1 : 1) mixture for two weeks fortified with ½ MS nutrients and finally transferred to soil in pots with 75% survival rate. The regenerated plantlets of both the wild species flowered and set seed normally.

THE grain legumes are important group of crops with major source of dietary protein and oil. The wild genotypes of *Arachis* are valuable sources of resistant genes against several pests, pathogens besides high oil and protein content[1–4]. The groundnut (*Arachis hypogaea* L.), a major oil seed crop rich in protein, makes a substantial contribution to human nutrition. Due to lack of resistance to biotic and abiotic stresses in cultivated groundnut, the productivity remained low despite large acreage under cultivation.

The multiplication and maintenance of wild *Arachis* germplasm is very labour-intensive and involves specific protocols because many accessions are grown mostly under greenhouse/glasshouse conditions. For instance, the field-grown wild plants are uprooted and the soil has to be sifted to harvest the seeds[5]. Therefore, there is a limited supply of wild germplasm from the gene bank and it becomes difficult to maintain wild species of *Arachis* for its use in breeding programme.

Plant regeneration until the recent past in cultivated and in wild groundnut has been achieved either directly via organogenesis or indirectly through somatic embryogenesis[6–12]. However, the reports on plant regeneration with intervening callus phase are few in cultivated genotypes and less in wild *Arachis*[13–16]. The standardization of *in vitro* plant regeneration protocols with intervening callus phase would certainly help in the mass scale propagation of the wild species and also facilitate germplasm conservation *in vitro*[17]. In addition, the protocol can also be exploited for generating new genetic variability in groundnut by somatic hybridization through protoplast fusion as has been demonstrated in other legumes[18].

Further, this is an attempt to study the effect of different plant growth regulators on morphogenetic response of shoot base-derived callus. The present study deals with organogenic regeneration from shoot base-derived callus of *A. stenosperma* and *A. villosa*. *A. stenosperma* is resistant to late leaf spot and *A. villosa* confers drought tolerance and shows resistance to tikka disease, insect pests and has high oil content[1,19].

The seeds of wild species, i.e. *A. stenosperma* and *A. villosa*, ($2n = 2x = 20$) were collected from Gene Bank of ICRISAT, Patancheru, Hyderabad, India. The seeds were surface sterilized using 0.1% (w/v) mercuric chloride solution for 8 min followed by 5–6 times thorough washing with sterile distilled water. The seeds were germinated on filter paper boat on liquid half strength MS[20] media with B5[21] vitamins in culture tubes. The experiments were conducted in our lab for *in vitro* induction of rooting in wild species using a range of IBA (0–6000 ppm) solution for different time durations (1–10 min). Preliminary experiments revealed the shoots pulse-treated with 5000 ppm for 5 min were found to induce maximum callusing within 7–10 days on transfer to MS media supplemented with 11.42 µM IAA or 10.75 µM NAA compared to other concentrations of these auxins tested. Hence, in the present study, ten-day-old seedling shoots were excised and the cut ends were given pulse treatment with 5000 ppm solution of IBA for 5 min. The pulse-treated shoots were

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transferred to MS medium supplemented with 11.42 µM IAA. After four weeks, about 5 mm diameter callus induced around the pulse-treated ends of shoots was transferred to MS or ½ MS supplemented with 8.90, 13.33, 17.77, 22.20 µM BA, along with 2.85 µM IAA without or with 1.0 µM TDZ to study the morphogenetic response.

The shoots (2.0–3.0 cm long) obtained from the callus after four weeks were separated and cultured for rooting on MS media supplemented with 0, 5.37, 10.75, 16.12, 21.50 and 26.88 µM NAA or 0, 5.71, 11.42, 17.13, 22.84 and 28.54 µM IAA along with 2.32 µM kinetin. The well-rooted plantlets obtained after four weeks were washed and transferred to screw cap bottles containing soil : vermiculite mixture (1 : 1 v/v) fortified with ½ strength MS nutrients. All the MS media preparations are supplemented with B5 vitamins and 3.0% (w/v) sucrose, solidified with 0.8% (w/v) agar. pH of the media was adjusted to 5.8 and autoclaved at 121°C for 20 min. The cultures were maintained at 24 ± 2°C in culture room with 16 h photoperiod for 15–30 days. The acclimatized plantlets were then transferred to soil in glasshouse and grown till maturity. Each experiment was repeated thrice with minimum of 4–5 replicates per treatment. The percentage of calluses responding with shoots was calculated after four weeks from minimum of 50–60 callus pieces. A three way ANOVA was made for the effect of media, BA and TDZ concentrations on percentage of responding calluses and number of shoots per responding calluses. Sine transformation was carried out on the percentage of the calluses responding with shoots and square root transformation for number of shoots per responding calluses. The statistical analysis was done using statistical packages: Matlab ver 5.3 and SPSS ver 10.0, Mathworks Inc. Percentage of rooting frequency and mean number of roots per shoot were calculated from all the responding shoots with roots.

The shoots with pulse treatment of 5000 ppm IBA solution for 5 min, on transfer to MS media supplemented with IAA produced maximum callus at the treated end within two weeks of culture (Figure 1 *a*). Such callus produced green-coloured spots on the same initiation medium after four weeks of culture (Figure 1 *b*). The shoot base-derived callus on transfer to half strength MS regeneration media containing 22.20 µM BA, 2.85 µM IAA and 1.0 µM TDZ produced green and purple-coloured leafy shoots within 2–4 weeks of culture. The plant regeneration frequency was 75% in *A. stenosperma* and 68% in *A. villosa* (Figure 1 *c*, Table 1). Compared to full strength MS nutrients, half strength nutrients promoted better regeneration response with maximum number of shoots, 5.66 ± 0.66 in *A. stenosperma* and 5.30 ± 0.24 in *A. villosa* (Table 1). The present work was designed on the earlier experience in our lab in groundnut, where

Table 1. Plant regeneration via organogenesis in two wild species of groundnut *A. stenosperma* and *A. villosa*

Wild species	Media[a]	PGR in µM		% of callus with shoots*	Mean no. of shoots ± SE
		BA	TDZ		
A. stenosperma					
	MS	8.90	0.0	–	–
	MS	8.90	1.0	–	–
	MS	13.33	0.0	–	–
	MS	13.33	1.0	16.6	1.11 ± 0.16
	MS	17.77	0.0	41.6	1.66 ± 0.21
	MS	17.77	1.0	42.0	2.33 ± 0.24
	MS	22.20	0.0	47.0	4.33 ± 0.24
	MS	22.20	1.0	60.0	4.50 ± 0.27
	½ MS	22.20	0.0	60.0	5.00 ± 0.36
	½ MS	22.20	1.0	75.0	5.66 ± 0.66
A. villosa					
	MS	8.90	0.0	–	–
	MS	8.90	1.0	–	–
	MS	13.33	0.0	–	–
	MS	13.33	1.0	–	–
	MS	17.77	0.0	40.0	1.33 ± 0.24
	MS	17.77	1.0	38.0	1.83 ± 0.43
	MS	22.20	0.0	48.0	3.33 ± 0.43
	MS	22.20	1.0	50.0	4.33 ± 0.24
	½ MS	22.20	0.0	58.0	4.50 ± 0.27
	½ MS	22.20	1.0	68.0	5.30 ± 0.24

[a]All MS media are supplemented with 2.85 µM IAA; PGR: Plant growth regulators.
*Data scored from 50 to 60 calluses after four weeks of culture; –, No response.

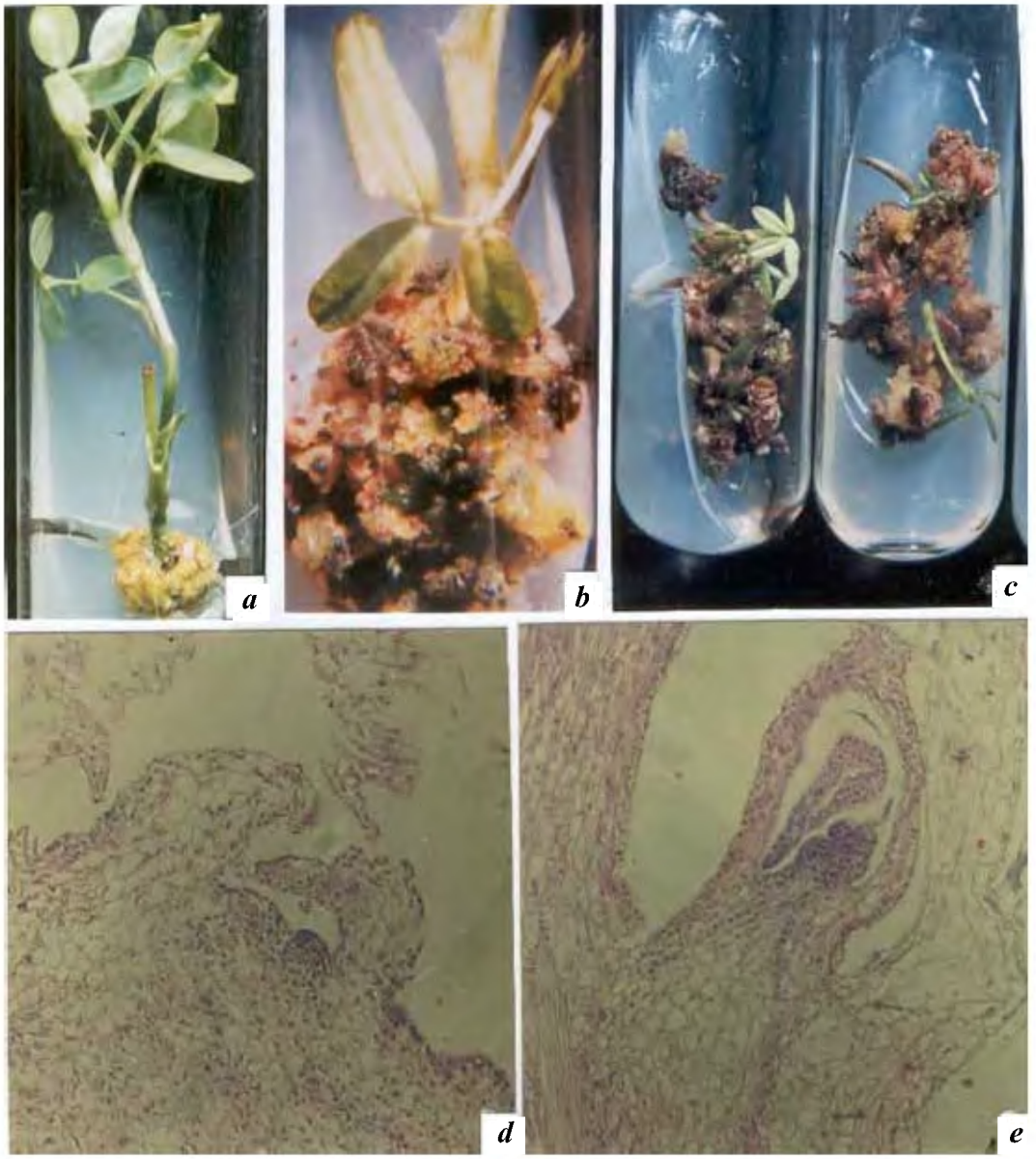

Figure 1 ***a–e.*** Plant regeneration from shoot base-derived callus of wild *Arachis* species. ***a***, Induction of callus around the base of pulse-treated shoot (with 5000 ppm/5 min) on MS + 11.42 µM IAA in *A. stenosperma* after two weeks of culture (× 1.6); ***b***, Proliferation of shoot base callus with green spots on the same callus initiation medium, MS + 11.42 µM IAA after four weeks (× 2.4); ***c***, Green to purple-coloured shoots (arrow) from the shoot base-derived callus of *A. stenosperma* and *A. villosa* on transfer to MS + 22.20 µM BA + 2.85 µM IAA and 1.0 µM TDZ (× 1); ***d***, Histological sections of shoot base-derived regenerating callus with emergence of shoot meristemoids, after one week of culture (× 400); ***e***, Histological section of callus with regenerating shoot and vascular connection (× 400).

the combination of BA and IAA gave maximum shoots from de-embryonated cotyledons[8]. However, our work with increase in BA concentration to 26.60 µM resulted in stunted and abnormal growth of the shoots.

In addition to BA and IAA, TDZ has been found beneficial in groundnut to induce more number of shoots. The groundnut explants cultured initially on media fortified with TDZ, reported to produce repetitive cycles of shoots[6]. In the present study, addition of lower concentration of TDZ (1.0 µM) along with BA and IAA showed moderate increase in the percentage of plant regeneration and also the mean number of shoots per responding callus (Table 1). However, with decrease in BA concentration up to 8.90 µM, the morphogenetic response of the callus was either decreased or absent in both the species. The histological studies carried out with the regenerating callus at different stages of growth revealed the indirect organogenic regeneration pathway. The anatomical sections of regenerating callus showed emergence of shoot primordia/meristemoids (Figure 1 *d*) and the developing

Figure 2 *a–c*. Induction of rooting and establishment of plants in glass house. ***a***, Induction of roots from regenerated shoots of *A. stenosperma* and *A. villos* on MS + 16.11 µM NAA + 2.32 µM kinetin (× 0.8); ***b***, Regenerated plant of *A. stenosperma* in glass house (× 0.01); ***c***, Regenerated plant of *A. villosa* in glass house (× 0.01).

shoots with clear vascular connection with the callus (Figure 1 *e*).

The elongation of the regenerated shoots of *A. stenosperma* and *A. villosa* was observed on MS media supplemented with 8.90 µM BA and 2.85 µM IAA devoid of TDZ. Addition of TDZ in the subculture media along with BA and IAA was found to inhibit further development of the shoots, and sometimes stunted adventitious shoots were observed. The regeneration potential of the callus was retained up to ten months in *A. stenosperma* and more than one year in *A. villosa*. From three-way ANOVA, it was found that the interaction between media and BA, with respect to percentage of callus with shoots was not significant in *A. stenosperma*, but the response was significant in *A. villosa*. The interaction between media, BA and TDZ to the number of shoots per callus was significant in both *A. stenosperma* and *A. villosa* at 5%. The regenerated shoots (2–3 cm in height) of *A. stenosperma* developed roots within ten days of transfer to MS rooting media supplemented with 16.12 µM NAA and 2.32 µM kinetin (Figure 2 *a*). A maximum of 52% rooting was observed with a mean number of 4.2 ± 0.90 roots/shoot followed by 50%, with 7.3 ± 0.52 roots/shoot in MS with 21.50 µM NAA and 2.32 µM kinetin (Table 2). However, 7–8 roots/shoot were observed in media supplemented with 26.88 NAA and 2.32 µM kinetin at 40% frequency but the roots were short and thick. When IAA was evaluated for induction of roots from regenerated shoots, no rooting response was obtained. Non-proliferative restricted callus without further growth was observed at the cut end of the shoots (Table 2).

The regenerated shoots of *A. villosa* on transfer to rooting media, i.e. MS supplemented with 5.37 to 26.88 µM NAA along with 2.32 µM kinetin induced callus and no rooting was observed. However, when NAA was replaced with IAA, 1–2 roots/shoot was observed within 12–15 days in *A. villosa* (Figure 2 *a*). Regenerated shoots of *A. villosa* showed rooting response of 25% on MS media supplemented with 11.42 µM IAA and 2.32 µM kinetin followed by 20% and 16% using 17.13 µM and 22.84 µM

Table 2. Effect of NAA and IAA on rooting efficiency of *in vitro* regenerated shoots in *A. stenosperma* and *A. villosa*

Wild species	PGR in µM		No. of shoots		% of rooting*	Mean no. of roots/ shoot ± SE
	NAA	IAA	Cultured	Responded		
A. stenosperma						
	0.0	0	12	1	16.6	1.0
	5.37	0	12	2	25.0	1.0
	10.75	0	15	5	33.3	3.4 ± 0.5
	16.12	0	25	13	52.0	4.2 ± 0.9
	21.50	0	20	10	50.0	7.3 ± 0.5
	26.88	0	15	7	40.0	7.5 ± 0.8
	0	5.71	10	2	NPC	–
	0	11.42	10	4	NPC	–
	0	17.13	12	5	NPC	–
	0	22.84	12	8	NPC	–
	0	28.54	10	6	NPC	–
A. villosa						
	0.0	0	10	–	–	–
	5.37	0	10	2	C	–
	10.75	0	10	5	C	–
	16.12	0	12	6	C	–
	21.50	0	12	10	C	–
	26.88	0	12	10	C	–
	0	5.71	10	–	–	–
	0	11.42	20	5	25.0	2.0
	0	17.13	20	4	20.0	2.0
	0	22.84	25	4	16.0	1.0
	0	28.54	15	–	–	–

All MS media supplemented with either NAA or IAA has 2.32 µM kinetin.
*Data scored after four weeks of culture. PGR, Plant growth regulators; NPC, Non-proliferative callus; C, Callus; –, No response.

IAA respectively (Table 2). The rooting frequency was found to decrease with increase in IAA concentration. Out of 32 rooted plants of *A. stenosperma* (Figure 2 *b*) and *A. villosa* (Figure 2 *c*), 24 of them (75%) survived in the field and set seed.

The present study was made to evaluate the morphogenetic potential of shoot base-derived callus. Inclusion of TDZ for short duration in the media promoted shoot formation and plant regeneration via organogenesis or somatic embryogenesis in cultivated peanut. It has also been reported that, the exposure of the explants to TDZ (10.0 µM) for one week promoted initiation of adventitious shoots and with decrease in TDZ concentrations in subculture medium reduced the frequency of morphological abnormalities[6,7,22]. The cytokinins, i.e. BA and TDZ promoted shoot regeneration from explants of most of the grain legumes[23]. The callus regenerated for four weeks on MS media supplemented with BA, IAA and lower concentrations of TDZ (1 µM) and retained its regeneration potential up to ten months and one year in *A. stenosperma* and *A. villosa* respectively. The rooting response of the two species differed with two hormones (NAA/IAA) tested.

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Histology of loose-shell affected *Penaeus monodon*

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The loose-shell affected *Penaeus monodon* was studied for induced infection, recovery and histology under the laboratory condition and grow-out system. The maximum percentage of loose-shell affected animals was 23.3% in summer and 14.15% in winter culture ponds. Mortality pattern of experimentally infected animals varied with the mode of infection. The cumulative mortality reached 100% within 9 to 10 days through the water infection, 11 to 12 days through oral feeding, 12th day at 0.01 ml, 8th day in 0.05 ml and 4th day at 0.1 ml through the intramuscular injection. A marked recovery of loose-shell *P. monodon* fed with frozen clam, mussel meat at 12% of body weight daily was observed. The data suggested that improved survival, up to 64% shell recovery and 17 to 47.5% of weight gain in the case of clam meat experiment, and up to 66.6% shell recovery and 16.2 to 48.4% of weight gain in mussel meat experiment. The histology of the loose-shell-affected animals showed the presence of inclusion bodies, gill cuticle separated from the epithelial cells, degeneration of basement membrane in the gut.

THE chronic soft-shell syndrome in the giant tiger shrimp *Penaeus monodon* is a significant threat to production in some brackishwater ponds in India as well as in other countries. Cook and Rabanal[1] have suggested the syndrome as a papery shell condition and subsequent inability of the shrimps to synthesize sufficient quantity of chitin. This syndrome results from nutritional deficiency, exposure to chemical pesticides, poor soil and water conditions, and is highly correlated with some management practices in the pond[2]. It has been reported that pesticides may inhibit the chitin synthesis[3,4].

The exoskeleton and hepatopancreas play important roles in shrimp shell hardening *vis-à-vis* the hemolymph and residual tissues[2]. Apart from the fact that abnormal loose-shelled shrimps have shells that remain soft or papery for several weeks, nothing is known about the extent of damage in shrimp tissues. Soft-shelled shrimp are more susceptible to cannibalism. As they are relatively weaker organisms with surface fouling by *Zoothamnium* spp., they suffer higher mortality and fetch lower market values than hard-shelled prawns upon harvest. The principal means of control is early recognition and subsequent elimination of infected animals. No other controlling mea-

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