

Establishment of efficient and rapid regeneration system for some diploid wild species of *Arachis*

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Abstract Though peanut tissue culture has advanced to a considerable extent using a number of explants with the subsequent production of transgenic plants, wild *Arachis* species appeared to be recalcitrant to using similar explants. In this study, the use of cotyledonary nodes as explants prepared from 7-day old seedlings resulted in the development of a simple and rapid regeneration protocol for five diploid wild species including *A. diogeni*, *A. stenosperma*, *A. duranensis*, *A. cardenasii* and *A. correntina* belonging to the genus *Arachis* for producing multiple shoots. Shoot bud initiation was observed 10 to 15 days after culture initiation. Responding cotyledonary nodes with shoot buds were subcultured to lower levels of cytokinin and finally to MS basal medium for further shoot development and elongation. Production of multiple shoots was observed in all the five diploid species with a maximum of 9 to 16 shoots were obtained per explant in the primary cultures. The number of shoot buds increased significantly with repeated explant subculturing with recovery up to 45 shoots from responding explants. These shoots were rooted efficiently on MS medium supplemented with 1 mg l^{-1} naphthalene acetic acid and the time taken from explanting to the transfer of shoots to potting mixture was about 12 weeks. All rooted shoots were successfully established in soil in glass house and further transferred to field. These plants survived to maturity and set seed.

Keywords Wild *Arachis* species · Cotyledonary node explants · Shoot regeneration · BA-6 · Benzyl amino purine · KIN-6 · Furfuryl amino purine · TDZ–thidiazuron

Introduction

Genus *Arachis* is exclusively of South American origin with about 69 described species (Krapovickas and Gregory 1994). Six or more of these species are cultivated; the major one being the peanut, *Arachis hypogaea* L. Peanut is the most important oilseed crop of India with equal importance world over. It is an important source of edible oil and protein for the poor of the Semi Arid Tropics. Incidentally, China is the number one producer of this crop followed by India. However, crop yields in the latter country are far less than the average yields in other countries; an obvious reason for this dismal performance is that the crop is grown under predominantly rain-fed conditions and hence, is prone to the vagaries of nature. Biotic stresses in the form of disease-causing microbes, pests and abiotic stresses in the form of drought and salinity cause serious damage to the crop. Cultivated genotypes of this crop lack resistance against various biotic and abiotic stresses. The most prevalent diseases that attack peanut are leaf spots caused by *Cercospora* and *Phaeoisariopsis* species, collectively termed as ‘tikka disease’, the rust caused by *Puccinia arachidis* and some viral diseases. Though morphological diversity is high in the cultivated accessions of the crop, genetic diversity is very low (Kochert et al. 1991). In contrast to this situation, wild *Arachis* species exhibit resistance to diseases, pests, and the genetic diversity is high making them a rich source of genes for resistance.

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Other important traits like drought resistance and early seed set are also present in them (ICRISAT Annual Report 1980). This shows that wild *Arachis* species are a source of important genes and they can be very useful for genetic improvement of *Arachis hypogaea*. Apart from the rich gene pool, wild peanuts have different uses. They are being grown as living mulch on sloping lands, for ornamental purposes and also to prevent soil erosion. Additionally, they are also useful in nitrogen fixation and as nutritious fodder (Valls and Simpson 1994; Dos Santos et al. 2003; Laxmi and Giri 2003b).

Screening of wild species showed that *A. diogeni*, *A. stenosperma*, *A. duranensis*, *A. cardenasii* and *A. correntina* are asymptomatic to rust and *A. diogeni*, *A. stenosperma* and *A. cardenasii* are highly resistant to the leaf spots. Except *A. stenosperma*, all the four species are resistant to some insects, while all the five species are also resistant to some viral diseases (ICRISAT Annual Report 1980; Foster et al. 1981). The above said features make these wild relatives of *Arachis* very important for breeding purposes. The spreading nature of the wild genotypes makes their maintenance labor intensive and difficult to use in breeding. The overexploitation and improper maintenance of the wild *Arachis* species requires in vitro conservation. With the advent of functional genomics in modern biology, wild *Arachis* species can be utilized for peanut improvement. A key component of most functional genomics is a high-throughput genetic transformation system useful for developing various gene identification strategies (Somers et al. 2003). A good regeneration protocol is an essential prerequisite for conservation as well as genetic manipulation. Earlier, explants like protoplasts, petiole, cotyledon, embryo axis, stem base callus and leaves (Li et al. 1993; Rani and Reddy 1996; Gagliardi et al. 2000; Laxmi and Giri 2003b; Mroginski et al. 2004; Vidoz et al. 2006; Pacheco et al. 2008) were used to achieve morphogenesis in vitro with limited success in wild species of *Arachis*. Cultivated peanut appears to be an easy system for in vitro culture. However, the tissue culture regeneration systems developed for different peanut explants are not applicable to the related wild *Arachis* species indicating the complexity of the system and the importance of the genotype for the success of tissue culture regeneration.

We report here the development of a simple and rapid plant regeneration system using cotyledonary node explants of the wild *Arachis* species including *A. diogeni*, *A. stenosperma*, *A. duranensis*, *A. correntina* and *A. cardenasii*. This protocol yields the highest number of shoots per explant reported to date for each of the wild species tested and also seems appropriate for large scale propagation and in vitro germplasm conservation.

Materials and methods

Plant material and explant preparation

Seeds of *A. duranensis* (ICG 8139), *A. stenosperma* (ICG 8126), *A. correntina* (ICG 8132), *A. diogeni* (ICG 8962) and *A. cardenasii* (ICG 8216) were obtained from the International Crops Research Institute for Semi Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. Seeds were thoroughly surface sterilized with 70% (v/v) ethanol for 1 min followed by a treatment with 0.1% (w/v) aqueous mercuric chloride for 10 min. They were then rinsed 3 to 4 times with sterile distilled water and germinated. Cotyledonary node explants from 7-day old seedlings were prepared as described earlier by Townsend and Thomas (1993) for soybean. In brief, germinated seeds were allowed to grow until the radicle reached about 2 cm and plumule just protruded out. The lower half of the radicle was excised and the primary shoot was completely removed. A cut was made along the embryo axis exposing the cotyledonary node explant. Axillary buds in the cotyledonary node junctions were damaged to the maximum extent without much injury to the explant by making incisions with a sharp blade.

Preparation of nutrient media

The nutrient medium consisted of MS salts (Murashige and Skoog 1962), MS vitamins, 30 g l⁻¹ sucrose, various concentrations of 6- benzyladenine (BA) (4–10 mg l⁻¹), thidiazuron (TDZ) (2–10 mg l⁻¹), 6-furfuryl amino purine (KIN) (1.5 and 2.5 mg l⁻¹), naphthalene acetic acid (NAA) (0.05–1.0 mg l⁻¹), 2,4 dichlorophenoxy acetic acid (2,4D) (0.5–1.5 mg l⁻¹) and 8 g l⁻¹ agar as a gelling agent. The pH of the medium was adjusted to 5.8 before autoclaving and the medium was autoclaved at 121°C, 15 lb/cm³ for 15 min. Explants were kept partially inserted into the medium with the cut end exposed. Cultures were maintained in a growth room at 28 ± 1°C with a photoperiod of 16 h light, supplied by white fluorescent tubes with a light intensity of about 1500 lux.

Multiple shoot induction and elongation

Shoot buds produced in media containing higher concentration of BA were subcultured to MS medium with decreased BA concentration (by 2 mg l⁻¹). This process of decreasing BA levels continued till the shoots were transferred to basal medium for rooting. Shoots above 3 cm were shifted to medium with 24 g l⁻¹ sucrose and 0.1–1.0 mg l⁻¹ NAA for rooting.

Acclimatization and transfer to green house

Rooted plantlets were removed from culture medium and shifted to liquid MS for hardening. After 1 week, plantlets were transferred to magenta boxes with a mixture of soil and vermiculite in 1:1 ratio and further transferred to greenhouse for acclimatization. The plantlets are watered every 3 days. The survival percentage was recorded 1 month after transfer.

All the experiments were repeated thrice and data collected at regular intervals, were analyzed with Sigma-plot statistical software version 9.0. Significance of difference was analyzed by repeated measures ANOVA and Tuckey's test.

Results

Explants that usually respond well in peanut, like de-embryonated cotyledons, hypocotyls and leaf petioles were not responsive in the diploid wild *Arachis* species. In case of *A. duranensis*, however, embryo axes explants were successful to a limited extent (data not shown). Cotyledonary node explants obtained from the germinated seedlings of the five different species were subsequently cultured on different shoot induction media. Explants enlarged and became thick within 7 to 10 days of culture. The undamaged explants produced a maximum of two or sporadically three thick shoots per explants (Fig. 1). Some callusing was observed rarely at the cut ends, but multiple shoot buds arose directly from explant tissue rather than callus.

Explants from *A. stenosperma* did not proliferate shoots on media with BA alone or in combination with NAA or



Fig. 1 The undamaged explants of *A. correntina* with a maximum of three shoots

2,4-D. However, they developed a mixture of greenish brown callus with loose texture. Multiple shoot induction was observed only when the medium was supplemented with TDZ or with BA and KIN in combination. The media supplemented with 2 or 3 mg l⁻¹ TDZ and the two tested combinations of BA and KIN were not effective (Table 1) in the species, and the maximum number of shoots was obtained on a medium containing 5 mg l⁻¹ TDZ. With an increasing concentration of TDZ, explant necrosis was observed. In contrast to this, *A. duranensis* developed callus at a lower TDZ concentration and showed necrosis at higher concentrations of TDZ and BA (Table 1). A maximum number of 11 shoots were obtained on a medium supplemented with BA and KIN (2.5 mg l⁻¹ each). Only up to 3 shoots were obtained on other BA supplemented culture media (Table 1). Culture media with a combination of BA and KIN induced low callus proliferation compared to BA or TDZ.

Cotyledonary node explants of *A. correntina*, *A. cardenasii* and *A. diogoi* were cultured on media with similar combinations of growth regulators (Table 1). Though the response of these three species overlapped in different media, each species showed a specific requirement for growth regulators (Table 1). All the three species responded well in a medium supplemented with 2.5 mg l⁻¹ BA and 2.5 mg l⁻¹ KIN and this was followed by 1.5 mg l⁻¹ BA and 1.5 mg l⁻¹ KIN, 4 mg l⁻¹ or 6 mg l⁻¹ BA. BA in combination with NAA was effective only in *A. correntina*, in which a combination of NAA (0.05 mg l⁻¹) and 5 mg l⁻¹ BA yielded 16 shoots in primary cultures, which in itself was the maximum (Fig. 2). As the NAA concentration doubled keeping BA concentration constant, there was a 38% decrease in the shoot proliferation. With the increase of NAA to 0.5 mg l⁻¹, there was a further decrease (37%) in the number of shoots. Callus induction was negligible on media with NAA as compared to 2,4-D. There was not much impact of 2,4-D on the number of shoots developed in culture, when it substituted NAA in the above-mentioned media (Table 1). The number of shoots per explant ranged from 2.3 to 3.6 and there was a significant increase in callus development with an increase in 2,4-D concentration.

For *A. cardenasii*, the presence of an auxin in the culture medium was not effective in inducing shoot proliferation except in 0.5 mg l⁻¹ 2,4-D and 5 mg l⁻¹ BA. A maximum number of 9.2 shoots were obtained on a medium supplemented with BA and KIN (2.5 mg l⁻¹ each). Lower concentrations of BA and KIN were not as effective as higher concentrations. BA at 4–6 mg l⁻¹ in the culture medium gave considerably lower shoot proliferation. Combination of NAA and BA has marginal impact on this species, which was contrasting when compared to *A. correntina*. In *A. cardenasii*, the zone of shoot proliferation on the explant

Table 1 Mean number of shoots induced from cotyledonary nodes of five *Arachis* wild species

Combination of growth regulators mg l ⁻¹	<i>A. stenosperma</i>	<i>A. duranensis</i>	<i>A. correntina</i>	<i>A. cardenasii</i>	<i>A. diogoi</i>
2 TDZ	3.6 ± 0.5	0	0	0	0
3 TDZ	4.6 ± 0.5	0	0	0	0
5 TDZ	9.6 ± 0.5	0	0	0	0
10 TDZ	0	0	0	0	0
4 BA	0	2.3 ± 0.5	3.0 ± 0.5	2.0 ± 1.0	9.0 ± 1.0
6 BA	0	3.0 ± 0.5	4.3 ± 1.5	1.6 ± 0.5	3.6 ± 0.5
10 BA	0	0	0	0	0
1.5KIN–1.5 BA	2.6 ± 0.5	4.6 ± 1.1	4.6 ± 0.5	5.6 ± 0.5	2.6 ± 0.5
2.5KIN–2.5 BA	4.3 ± 0.5	11.3 ± 0.5	6.0 ± 1.0	9.3 ± 0.5	5.0 ± 1.0
0.05NAA–5 BA	0	0	16.0 ± 2.0	1.3 ± 0.5	1.0 ± 0.5
0.1NAA–5 BA	0	0	11.0 ± 1.0	4.6 ± 0.5	4.6 ± 0.5
0.5NAA–5 BA	0	0	4.3 ± 0.5	2.3 ± 0.5	2.0 ± 1.1
0.5 2,4 D–5 BA	0	0	3.3 ± 0.5	7.6 ± 0.5	1.0 ± 0.5
1.0 2,4 D–5 BA	0	0	2.3 ± 0.5	3.0 ± 0.5	4.3 ± 0.5
1.5 2,4 D–5 BA	0	0	2.6 ± 0.5	1.6 ± 0.5	14.3 ± 1.5

Values indicated in bold face correspond to the highest number of shoots developed in primary cultures for the respective species

Experiments were repeated three times and means ± SE was plotted ($P < 0.05$, $n = 3$)

did not expand comparatively with the number of subcultures. 2,4-D at 0.5 mg l⁻¹ concentration was effective when it replaced NAA in combination with BA. This combination of 2,4-D and 5.0 mg l⁻¹ BA generated an average of 7.6 shoots per explant. With the doubling of 2,4-D concentration, there was 60% reduction in shoot proliferation. A further 0.5 mg l⁻¹ increase in 2,4-D resulted in further decrease in shoot development by 20% (Table 1).

Similar to *A. cardenasii*, shoot proliferation in *A. diogoi* was not efficient when a combination of NAA and BA was used. At 0.1 mg l⁻¹ NAA and 5.0 mg l⁻¹ BA, there was an average of 4.6 shoots per explant. Increase or decrease in NAA concentration resulted in reduced shoot proliferation (Table 1). When NAA was substituted by 2,4-D, shoot proliferation increased with increase in concentration of 2,4-D. Simultaneously, there was also increased callus proliferation. On a medium supplemented with 0.5 mg l⁻¹ 2,4-D and 5.0 mg l⁻¹ BA, there was an average of 1.0 shoot. When 2,4-D was doubled keeping BA concentration constant, the number of shoots increased to 4.3, which was three fold higher. With a further increase in 2,4-D concentration by an additional 0.5 mg l⁻¹, there was a further increase in the number of shoots up to 14.3 per explant. The amount of callus produced also increased moderately. The impact of cytokinin alone on this explant was quite varying. Formation of callus was higher on media containing BA and 2,4-D compared to media with BA and KIN. Media with 4 mg l⁻¹ BA generated 9.0 shoots while 6 mg l⁻¹ BA generated only 3.6 shoots. Supplementation of the medium with 2.5 mg l⁻¹ BA and 2.5 mg l⁻¹ KIN yielded an average of 5 shoots per explant. Over all, *A. correntina* and *A. diogoi* yielded the higher number of

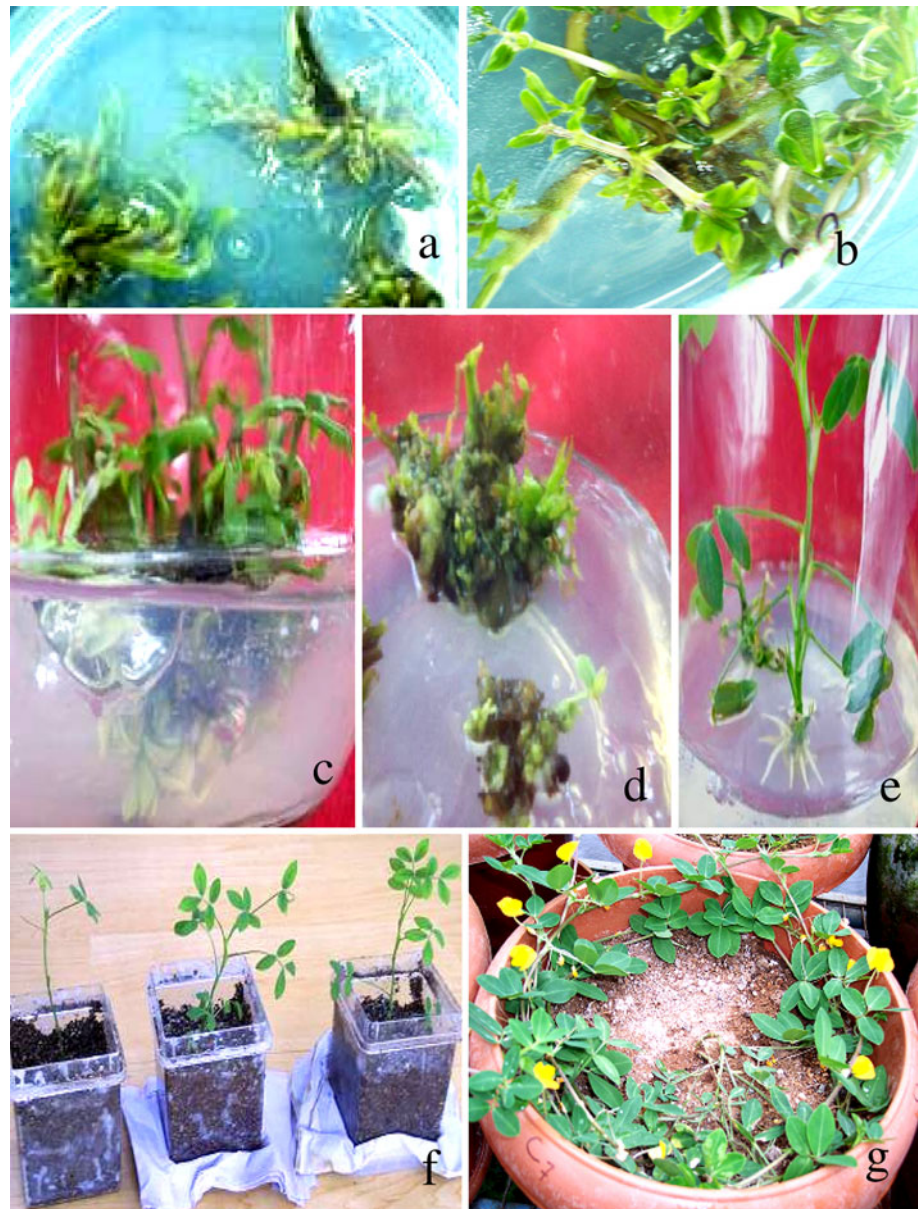
shoots, followed by *A. duranensis*, *A. stenosperma* and *A. cardenasii*. Harvest of shoots from the explants of different species and their subsequent maintenance on a BA containing medium resulted in further production of shoots in different subcultures. The whole number of shoots produced by the explants reached up to 45 in different wild species (data not shown).

Rooting

Elongated shoots of all the tested species were easily rooted on a rooting medium containing 1.0 mg l⁻¹ NAA. The rooting percentage in all the five wild species ranged from 87 to 100. Rooting was generally profuse, which has led to comfortable survival of the in vitro regenerated plantlets.

For *A. correntina*, an exclusive study on the influence of NAA present in shoot induction medium on rooting was studied in detail. The regenerated shoots were taken from three different shoot regeneration media that varied only in the concentration of NAA. These media were supplemented with 5 mg l⁻¹ BA along with 0.05 or 0.1 or 0.5 mg l⁻¹ NAA. Healthy shoots were excised and transferred to rooting media with varying concentrations of 0.6, 0.8 and 1.0 mg l⁻¹ NAA alone. With the increase of NAA concentration in shoot induction medium, there was a decrease in percentage of rooting and number of roots per plantlet (Table 2). The shoots developed on the lowest concentration of NAA showed a high rate of rooting on 1 mg l⁻¹ NAA, which was the maximum. A higher concentration of NAA (>1 mg l⁻¹) induced callus growth at the cut end of shoots in rooting medium. There was a minimum of 30% decrease in rooting, when NAA

Fig. 2 **a** Multiple shoot induction in *A. correntina*, **b** Multiple shoot induction in *A. diogeni*, **c** Shoot elongation in *A. correntina*, **d** Fresh shoot bud induction after harvest of shoots in primary cultures, **e** Plantlet of *A. correntina* regenerated in vitro, **f** Acclimatization of plantlets g. In vitro regenerated plant of *A. correntina* in the green house



concentrations were altered in either one of the media tested. The rooting data of *A. correntina* showed the impact of NAA concentration in regeneration medium on subsequent root induction (Table 2).

Regenerated plantlets of all the wild species acclimatized easily and the survival rate of all these genotypes was around 90%. The un-rooted shoots on repeated sub cultures rooted sporadically and some callused without proper root induction.

Discussion

Though there is a great deal of information available on tissue culture studies in peanut using different explants,

only scanty information is available on the wild *Arachis* species, comparatively. In peanut, a number of explants like cotyledons, de-embryonated cotyledons (Gill and Saxena 1992), hypocotyls (Kanayand and Prakash 1994), embryo axes (Mckently 1991) and leaves (Eapen and George 1993) were used with reasonable efficiency and transgenic plants were produced using some of these explants. However, most of the explants like de-embryonated cotyledons, hypocotyls, embryo axes, leaf petioles, and internodal segments from the wild *Arachis* species were not effective. This required the use of cotyledonary node as an explant as used by Meurer et al. (1998) in soybean.

The present investigation has been undertaken with the objective of developing an efficient and rapid protocol for

Table 2 Frequency of rooting and mean number of roots induced on shoots of *A. correntina* in response to different levels of NAA

NAA (mg l ⁻¹)	Shoots induced from 0.05 NAA-5 BA (mg l ⁻¹)		Shoots induced from 0.1 NAA-5 BA (mg l ⁻¹)		Shoots induced from 0.5 NAA-5 BA (mg l ⁻¹)	
	No. of roots	Percentage of rooting	No. of roots	Percentage of rooting	No. of roots	Percentage of rooting
0.6 NAA	5.3 ± 1.1	37.7 ± 4.0	4.3 ± 0.5	11 ± 1.7	0	0
0.8 NAA	8.0 ± 1.0	71.7 ± 2.8	6.3 ± 2.0	59.3 ± 5.7	4.6 ± 1.5	37.0 ± 6.9
1.0 NAA	14 ± 2.0	100 ± 0.0	9.0 ± 2.0	62.0 ± 3.4	6.3 ± 1.5	45.0 ± 4.3

Experiments were repeated three times and means ± SE was plotted ($P < 0.05$, $n = 3$)

shoot regeneration from the selected wild *Arachis* species, in which cotyledon and embryo axis were the preferred explants for regeneration followed by stem base and leaflets in the earlier studies (Rani and Reddy 1996; Laxmi and Giri 2003a, b). The regeneration efficiency using these explants in wild *Arachis* was low compared to the cotyledonary node explants tested in the present investigation.

Preliminary studies were performed with different growth regulators either individually or in combination from basal level to higher concentrations until there was an affirmative response. The effective hormonal combinations were repeated in the main experiment. Whereas the undamaged explants produced a maximum of three thick shoots per explant, excision of the primary shoot followed by damage of preformed buds and incision of the meristematic zone has resulted in increased number of meristematic points that were able to regenerate new shoots. Each *Arachis* species needed a specific combination of growth regulators for optimum shoot proliferation, which shows that every species has its own internal hormonal balance for this kind of response.

A maximum of 5.6 shoots was reported in *A. stenosperma* till date (Laxmi and Giri 2003a, b; Pacheco et al. 2008). Rani and Reddy (1996) reported sporadic regeneration in *A. duranensis* and *A. cardenasii*; whereas a mean of 9.3 shoots per explant was obtained from *A. cardenasii* in this study. Recently, studies on regeneration of *A. correntina* by Mroginski et al. (2004) and Vidoz et al. (2006) yielded a maximum of 3.0 shoots per explant. In the present investigation, a maximum of 16.0 shoots per explant was obtained in *A. correntina* (Table 1) and the maximum number of shoots in the rest of the wild species ranged from 9.6 to 14.3 per explant in the primary cultures. Shoots were subcultured to lower levels of BA regularly for proper development and elongation. TDZ has been described as a potent growth regulator and more effective than BA in peanut (Gill and Saxena 1992; Kanayand and Prakash 1994). However, *A. stenosperma* alone responded positively to TDZ in the present investigation. According to Akasaka et al. (2000) prolonged exposure of explants to TDZ containing medium inhibited shoot formation and at times, rooting of developed shoots. Hence, the explants that

produced shoot buds on TDZ containing medium were transferred to BA (2 to 4 mg l⁻¹) containing media for further shoot development. While lower concentrations of TDZ were not effective in *A. duranensis* and highly effective in *A. stenosperma*, other three species did not respond to TDZ in the culture medium in the present work.

Sporadic regeneration of one or two shoots with varying volume of callus was observed in *A. stenosperma* and *A. duranensis* using cotyledonary node as an explant, when tested in different combinations of NAA and BA. The use of 2,4-D induced profuse callusing and hence, it was not tried in further studies in these two species. The other three species including *A. correntina*, *A. cardenasii* and *A. diogeni* responded with shoot proliferation to varying extent in all concentrations of growth regulators tested except TDZ. Hence, the present work shows that different wild *Arachis* species have different growth regulator regimes and responding explants unlike in peanut, in which embryo axes, hypocotyls and de-embryonated cotyledons were the preferred explants in regeneration studies. This shows the strong background influence of the genotype on in vitro regeneration in wild *Arachis* species. It has been shown earlier that genotype plays a crucial role in in vitro regeneration in different species (Bailey et al. 1993).

IAA was not effective in root induction with a lot of callus proliferation at the cut ends of shoots (Laxmi and Giri 2003b). NAA has been shown to be effective in rooting of wild *Arachis* species at 0.5 and 1.0 mg l⁻¹ (Still et al. 1987; Gagliardi et al. 2000). Hence, different levels of NAA were tried for effective root induction in the present investigation. No auxin-like substance was included in the shoot induction media for *A. stenosperma* and *A. duranensis* in the present study. Root induction was observed in low concentration of NAA in the rooting medium, but optimum rooting was observed at 1.0 mg l⁻¹ NAA, which was the same for all other wild species tested. Higher levels of NAA induced callusing at the cut end of shoots kept for rooting.

Study of rooting in *A. correntina* showed that lower level of NAA in the shoot induction medium induced efficient rooting indicating that judicious use of auxin-like substances in the shoot regeneration medium is important

for obtaining intact plantlets. At the end of first and second subculture, it was observed that the shoots from lower concentrations of NAA yielded profuse and thick rooting (Table 2).

Our observations showed that cotyledonary node is an explant that can be profitably utilized in shoot regeneration of these five wild *Arachis* species, as compared to other explants reported earlier. Though the mean number of shoots from primary cultures was around 9 to 16, subculture of explants with shoot buds to media with lower cytokinin levels resulted in profuse shoot proliferation yielding up to 45 shoots per explant. A good regeneration yield is required for conservation of the species or for isolation of important genes using insertional mutagenesis and transposon tagging. Isolation of novel genes will help improve the genomic resources of any species and is a very good supplement to crop breeding.

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