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Direct Multiple Shoot Induction from Different Mature Seed Explants of Groundnut (*Arachis hypogaea L.*)

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The different mature seed explants of groundnut cultivars VRI-2 and VRI-3 such as whole embryonated cotyledon (WEC), sectional embryonated cotyledon (SEC), whole de-embryonated cotyledon (WDC), sectional de-embryonated cotyledon (SDC) and whole embryonal axis (WA) were subjected to direct multiple shoot induction using KIN in combination with IAA and BAP in combination with NAA. The various explants behaved differently depending upon the kind (KIN/BAP) and concentration (5 to 25 mg/l) of cytokinin in the medium. Among the different concentrations of KIN and BAP with auxins, BAP was responded than KIN. Among the five explant types, whole embryonal axis had the highest percentage of response. Both KIN/IAA and BAP/NAA combinations produced multiple shoots at varying frequencies. In both cultivars, whole embryonated cotyledon produced more well developed shoots. The whole de-embryonated cotyledon and sectional de-embryonated cotyledon explants showed poor response. In whole de-embryonated cotyledon and sectional de-embryonated cotyledon shoot buds were produced only from the proximal half of the cotyledon. All the shoots were rooted using IBA. In general VRI-2 responded well than VRI-3.

Keywords: Seed explants, cytokinins, multiple shoot induction, root induction

Progress in plant cell, tissue and organ culture has opened up several new possibilities for the induction of genetic variability and the selection of desirable variants (Evans *et al* 1983). An efficient plant regeneration system is a prerequisite for genetic plant transformation studies using *Agrobacterium tumefaciens* (Horsch *et al* 1985). The lack of an efficient regeneration system in groundnut has slowed the improvement of this species via tissue culture selection and genetic transformation (Eapen and George, 1994). The cultivated groundnut is known to be relatively recalcitrant in tissue culture (Cheng *et al* 1992; Heatly and Smith, 1996). But several successful plantlet regenerations have been reported by several authors using seed explants of groundnut via direct organogenesis. (Sastri *et al* 1981; Atreya *et al* 1984; Banerjee *et al* 1988' Mckently *et al* 1990; Daimon and Mii 1991; Cheng *et al* 1992;).

Direct regeneration of shoots, roots and plantlets from tissue explants is fairly common in many species. Growth and development of explants in *vitro* are considerably influenced by genotype and age of the plant. Clonal propagation of favourable lines through in *vitro* culture of explants would facilitate breeding and crop improvement programme (Bajaj, 1984). Plant regeneration from seed explants has been reported for a number of legumes including mung bean *Vigna radiata* (L.) Wilczek (Gulati and Jaiswal, 1990) and pigeon pea (*Cajanus cajan* (L). (Mehta and Mohanram 1980). Plants have been successfully grown from single de-embryonated cotyledons of two strains of small Spanish type peanuts viz., 'Burpee No: 6216 and

Fields No 5322'. Most of the work has been done using 'Burpee' No 6216 peanuts (Illingworth, 1968). Atreya *et al* (1984) regenerated plants from embryo axes of the seeds of the variety TMV-2 cultured in different media, like MS, Blaydes, LS and Pe. By day 10 after culture, the explants produced plantlets with roots. The MS basal medium was found to be superior to the three others in terms of frequency of plantlets obtained and the number of roots developed per plantlet (85% regeneration and 20 roots/plant).

The direct plantlet regeneration from different parts of mature seed explants of Indian groundnut cultivars have been very limited. The present study aims to know the effect of cytokinins in combination with auxins in terms of multiple shoot induction from different parts of mature seed. Moreover, we have used all the available mature seed explants for direct organogenesis.

Materials and Methods

Two common groundnut cultivates VRI-2 and VRI-3 were obtained from the Regional Research Station (RRS), Tamil Nadu Agricultural University, Virudhachalam, Tamil Nadu, India and used as an experimental material. Seeds were soaked in tap water for 30 minutes followed by immersion in detergent solution for 5 minutes. After washing with distilled water, the seeds were washed with 70% ethanol for a few seconds and rinsed three times with distilled water. The seeds were brought to the inoculation chamber, and then surface sterilized with 0.1% $HgCl_2$ for 5-10 minutes and rinsed with sterile distilled water for 5-7 times.

The technical terms like whole embryonated cotyledon (WEC), sectional embryonated cotyledon (SEC), whole de-embryonated cotyledon (WDC), sectional de-embryonated cotyledon (SDC) and whole axes (WA) were adapted according to Mckently *et al* (1990). From the sterilized seeds the cotyledons were seperated to yield the explant types WEC and WDC. Portions of these were sliced once again longitudinally to yield SEC and SDC. WA were excised from the cotyledons to yield the fifth explant type.

The five different mature seed explants were inoculated onto MS basal medium (Murashige and Skoog, 1962) containing 30g/L of sucrose, 8g/L agar and varying concentrations of cytokinins and auxins supplemented with B5 vitamins (Gamborg et al 1968). Auxins NAA and IAA at 0.5mg/l and cytokinins, BAP and KIN at 5mg/l to 25mg/l were tested. The pH of the medium was adjusted to 5.8 prior to autoclaving. The cultures were maintained at $25^0C \pm 2^0C$ under 16:8 hour light and dark period. All experiments were carried out in triplicate. After 10 days, the cultures with fungal or bacterial contamination were discarded. Thirty days after inoculation, the data on the responsive explants, number of shoots/culture, shoot length were collected. For better results, the responsive cultures were transferred to fresh media after 2-3 weeks. The influence of explant type, effect of BAP and KIN on multiple shoot induction and influence of IBA on root induction have been analysed and the influence of genotypes also noted.

All the regenerated shoots were rooted with IBA. The concentrations of IBA ranged from 1 mg/L to 5 mg/L. The rooted plantlets were transferred to plastic cups containing autoclaved soil and subsequently transferred in the field for further evaluation. Each experiment was repeated three times. Wherever an appropriate standard deviation and mean separation were carried out using Duncan's Multiple Range Test (DMRT).

Results and Discussion

Multiple shoots were induced from all the five mature seed explants depending upon the concentration of BAP or KIN. All the four cotyledon derived explants like WEC, SEC, WDC and SDC expanded in their size and turned green within 8-12 days. The fifth explant type, WA began to initiate multiple shoots within 4 to 6 days. So far, there was no detailed information in connection with multiple shoot induction from seed explants of commonly cultivated Indian cultivars. Multiple shoots were observed quickly from WEC and SEC when compared to WDC and SDC. Generally, in WDC and SDC the multiple shoot induction took a longer period. In WDC and SDC, multiple shoot induction was associated with callus formation and shoots were developed only from the proximal half of the cotyledon. Among the two different combinations: NAA/KIN and IAA/BAP, the explants turned green within a short time in IAA\BAP combination. Regeneration and induction of multiple shoots were accomplished in a single step when the explants were cultured on medium supplemented with combinations of plant growth regulators.

The morphogenetic responses of different seed explants with KIN and IAA are shown in Tables 1 and 2. Mature seed explants from both VRI-2 and VRI-3 cultivars were cultured onto the medium containing different concentrations of KIN (5.0 mg/L to 25.0 mg/l) and IAA (0.5 mg/L) for multiple shoot induction. Among the different concentrations and combinations, the combination of 25 mg/L of KIN with 0.5 mg/L IAA was found to be the best having the highest frequency of multiple shoot indcution. The maximum percentage of responsive explants (91.2%) was observed in WA followed by WEC, WDC and SDC showed low frequency (63.4% respectively). But the maximum

Table 1. Effect of different concentrations of KIN in combination with 0.5mg/L of IAA on multiple shoot induction using different mature seed explants of groundnut cv VR1-2.

[illegible]	[illegible]					[illegible]					[illegible]				
	WEC	SEC	WDC	SDC	WA	WEC	SEC	WDC	SDC	WA	WEC	SEC	WDC	SDC	WA
5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
20	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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25	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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WA - Whole Embryonal Axis
SEC - Sectional Embryonated Cotyledon
SDC - Sectional De-embryonated Cotyledon
Values with the same superscript are not significantly different at the 0.5% probability level according to Duncan's multiple range test (within the explant).

Table 2. Effect of different concentrations of KIN in combination with 0.5/L of IAA on multiple shoot induction using different mature seed explants of groundnut cv VR1-3.

[illegible]	[illegible]					[illegible]					[illegible]				
	WEC	SEC	WDC	SDC	WA	WEC	SEC	WDC	SDC	WA	WEC	SEC	WDC	SDC	WA
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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number of shoots/explant was recorded in WEC (13.4). The highest shoot length was also observed in WEC.

The influence of BAP in combination with NAA were presented in Tables 3 and 4. Among the different concentrations of BAP in combination with 0.5 mg/L of NAA, 25 mg/L of BAP was found to be the best. This applies to percentage of responsive explants, mean number of shoots produced and mean shoot length. Among the two varieties, VRI-2 was the most suitable for multiple shoot production than VRI-3. Based on the data collected, BAP performed well then KIN in all characteristics studied. After 30 days, the shoots reached the minimum height for root induction and this led to ignoring addition of GA for shoot elongation. The highest mean number of shoots were comparatively more in BAP derived cultures. BAP induced multiple shoots quickly than KIN. The varietal differences also highly influenced the above parameters. Shoot length also varied in both cultivars.

Both KIN and BAP induced multiple shoots at varying frequencies in all the five different explants. But BAP produced the highest mean number of shoots. Addition of BAP or KIN above 25 mg/l suppressed multiple shoot induction. In both the cultivars, WEC produced more number of well-developed shoots. Even though a higher percentage of responsive explants were recorded in WA, further development of shoot buds into well developed shoots were reduced. The WDC and SDC explants showed poor response in terms of all the parameters studied in both varieties. In WEC, SEC and WA, no callus formation was observed. From the proximal end, the explants produced shoot buds in highest frequency. WDC and SDC produced multiple shoots along with callus formation. The distal end of the cotyledon also produced callus but there was no multiple shoot formation in both the varieties. In all the seed explants, as the multiple shoots were well developed, the shoot length was also considered as one of the parameters to assess the effect of BAP/ KIN. Vigorous growth and highest mean shoot length was observed in WEC in both the cultivars, with either BAP/KIN as an inducing agent.

In *vitro* morprogenetic responses of five different seed explants were studied using high concentrations of cytokinins and low concentration of auxins. In general, seed explants required higher level of cytokinins. The plantlet regeneration was a two step process with the early shoot induction followed by root induction. There are very few reports similar to our study using various seed explants. The requirements of high concentrations of cytokinins, especially BAP, was supported by several authors. The response of seed explants cultured in medium containing high concentrations of BAP was reported by Mckently et al (1990). The embryonated and de-embryonated cotyledons when cultured on MS medium supplemented with 25 mg/l of BAP +gly (2 mg/l) + NI 0.5 mg/l + PH 0.1 mg/l produced respectively 12 and 22.2 multiple shoots per explant. Illingworth (1968) pointed out that cotyledons have been used most frequently as explants for regeneration studies and reported regeneration from liquid nitrogen frozen cotyledons into full plants.

There are several earlier observations confirming our study. Guy *et al* (1980), reported rhizogenesis from cultured de-embryonated cotyledons. Sastri et al (1981), on culturing de-embryonated cotyledons on MSZ_4 medium obtained multiple shoots. These shoots were rooted in another step to obtain plants. Mhatre et al (1985), found that a combination of MS + BAP (1 mg/L) was the optimum for multiple shoot induction in groundnut. Rooting was induced in the regenerated shoots by growing them on MS medium (at half the strength of the salts) supplemented with IAA (1 mg/L). Whole and sectional de-embryonated cotyledons produced calli on both the cut ends and more shoot buds arose from only the proximal half of the cotyledons. In legumes, shoot development usually occurs from the proximal end of the cotyledon at the nodal region by development of multiple buds (Illingworth, 1968, Kumar *et al* 1983).

In chick pea, the whole regeneration was a single step process with shoot bud/shoot formation followed by rooting but in groundnut it was a two step process. The results indicated that the excised embryo of both varieties when cultured on MS medium supplemented with different concentrations of hormones showed differences in regeneration. This could probably be due to a relative sensitivity of excised embryo from different genotypes and their response towards different levels of growth regulators supplemented with the media. Like our present study, (Agnihorti *et al*, 2001) multiple shoots were induced in *Vigna mungo* using various seed explants.

After 40 days of incubation, the well-developed shoots were transferred to root induction medium containing $MS+B_5$+IBA. The IBA concentration ranged from 1 mg to 5 mg/L. Maximum percentage of root formation was observed at 3 mg/L of IBA (Data not shown). Above 3 mg/l of IBA, root induction was followed by callus formation. The well developed rooted plantlets were grown in plastic cups with autoclaved soil, sand and manure mixture in the ratio 1:1:1 for 30 days under green house conditions and then transferred to the field. All the regenerated shoots were rooted using IBA. IBA is a potent auxin to induce rooting. It was confirmed by several earlier reports (Mroginski *et al* 1990; Mckently *et al* 1991; Cheng *et al* 1992; Palanivel and Jayabalan 2000 and 2001.)

Direct multiple shoot induction from different seed explants of groundnut was much useful in transformation studies and also in clonal propagation and selection of desirable genotypes in breeding

Table 3. Effect of different concentrations of BAP in combination with 0.5mg/L of NAA on multiple shoot induction using different mature seed explants of groundnut cv

[illegible]	Response of explants (%)					Mean number of shoots/explant					Mean shoot length (cm)				
	WEC	SEC	WDC	SDC	WA	WEC	SEC	WDC	SDC	WA	WEC	SEC	WDC	SDC	WA
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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Values with the same superscript are not significantly different at the 0.5% probability level according to Duncan's multiple range test (within the explant).

Table 4. Effect of different concentrations of BAP in combination with 0.5mg/L of NAA on multiple shoot induction using different mature seed explants of groundnut cv

[illegible]	[illegible]					[illegible]					[illegible]				
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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programmes. Moreover, the present study has demonstrated successful plant regenerations from seed explants with higher survival percentage. We have described a suitable and reproducible *in vitro* culture technique using different seed explants via direct plantlet regeneration. The advantage of the present study is the availability of experimental material thoughout the year. Based on the responses of various explant sources, it is possible to speed up the breeding programes of groundnut. Moreover, we can use these techniques for genetic transformation and *In vitro* mutagenic studies of groundnut.

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