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High frequency somatic embryogenesis and efficient plant regeneration from hypocotyl explants of groundnut (*Arachis hypogaea* L.)

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Embryogenic calli were obtained from hypocotyl explants of groundnut (*Arachis hypogaea* L.) cultured on a medium containing different concentrations of 2,4-D or NAA in combination with 0.5 mg/l BAP. Type of auxin, concentration and genotypes influenced somatic embryogenesis. 2,4-D was found to be the best somatic embryo inductor. As the auxin level increased beyond 20 mg/l in induction medium, both per cent as well as number of embryos were decreased. 2,4-D (20 mg/l) was found to be better than NAA for the induction of globular and heart-shaped somatic embryos. Somatic embryos developed from these calli following transfer to a medium supplemented with 0.5 mg/l 2,4-D and 2.0 mg/l BAP was found to be best for embryo maturation. The well-formed embryos germinated into plantlets on MS medium supplemented with BAP (0.5–2.0 mg/l) and NAA (0.25 mg/l). The regenerated plants were transferred to soil and grown to maturity. Hardened plantlets produced normal flowers and set viable seeds. The ability of this regeneration system to produce embryos exponentially offers potential for development of new gene transfer technology and application to synseed technology.

GROUNDNUT (*Arachis hypogaea* L.) is an important grain legume and its improvement programmes include development of varieties resistant to diseases like tikka (*Cercospora arachidicola* and *Cercosporidium personatum*), rust (*Puccinia arachidis*) and pest (red hairy caterpillar). An efficient regeneration protocol either by organogenesis or somatic embryogenesis is a major prerequisite for the application of gene transfer methods for crop improvement. Regeneration by organogenesis from various explants, viz. leaves, cotyledons, cotyledonary node, hypocotyl, epicotyl, zygotic embryos has been reported[1–6]. But regeneration frequency was low and plants were rarely established. A number of recent reports describe somatic embryogenesis in peanut using a variety of different explants, including leaves[7,8], immature cotyledons[9,10] and immature embryo axes[11,12]. In spite of these studies, efficient protocol for regeneration of plantlets via somatic embryogenesis is lacking[13]. This study describes the successful induction of somatic embryogenesis from hypocotyls and their subsequent development into plants from two important commercial cultivars.

Two popular cultivars of groundnut (*Arachis hypogaea* L. cvs VRI-2 and TMV-7) were used in this investigation. Seeds were removed from the pods and immersed in 100 ml of sterile distilled water with 5 drops of Tween 80 for 5 min. Surface sterilization with 0.1% (W/V) aqueous mercuric chloride for 7–10 min was followed by 5 rinses in sterile distilled water. Seeds were germinated on MS[14] basal medium in culture tubes and kept under dark for germination at 24 ± 2°C. Hypocotyl explants of 7-day-old seedlings were used as the sources of explants.

Eight to ten hypocotyl explants (0.5–1.0 cm length) were cultured in 250 ml conical flasks. The induction medium consisted of MS salts, B5 vitamins[15], 40 g/l sucrose with different concentrations of 2,4-D or NAA (1.0, 5.0, 10, 15, 20, 25 and 30 mg/l) in combination with BAP (0.5 mg/l). The pH of the medium was adjusted to 5.8, 0.5% (W/V) agar was added, and autoclaving was done at 121°C for 15 min. The cultures were incubated for four to six weeks at 24 ± 2°C under cool-white fluorescent light at 40 $\mu E\ m^{-2}\ s^{-1}$ with a 16 h photoperiod. The per cent of embryogenesis was calculated as follows: (Number of explants showing embryogenic calli/Total number of explants cultured) × 100.

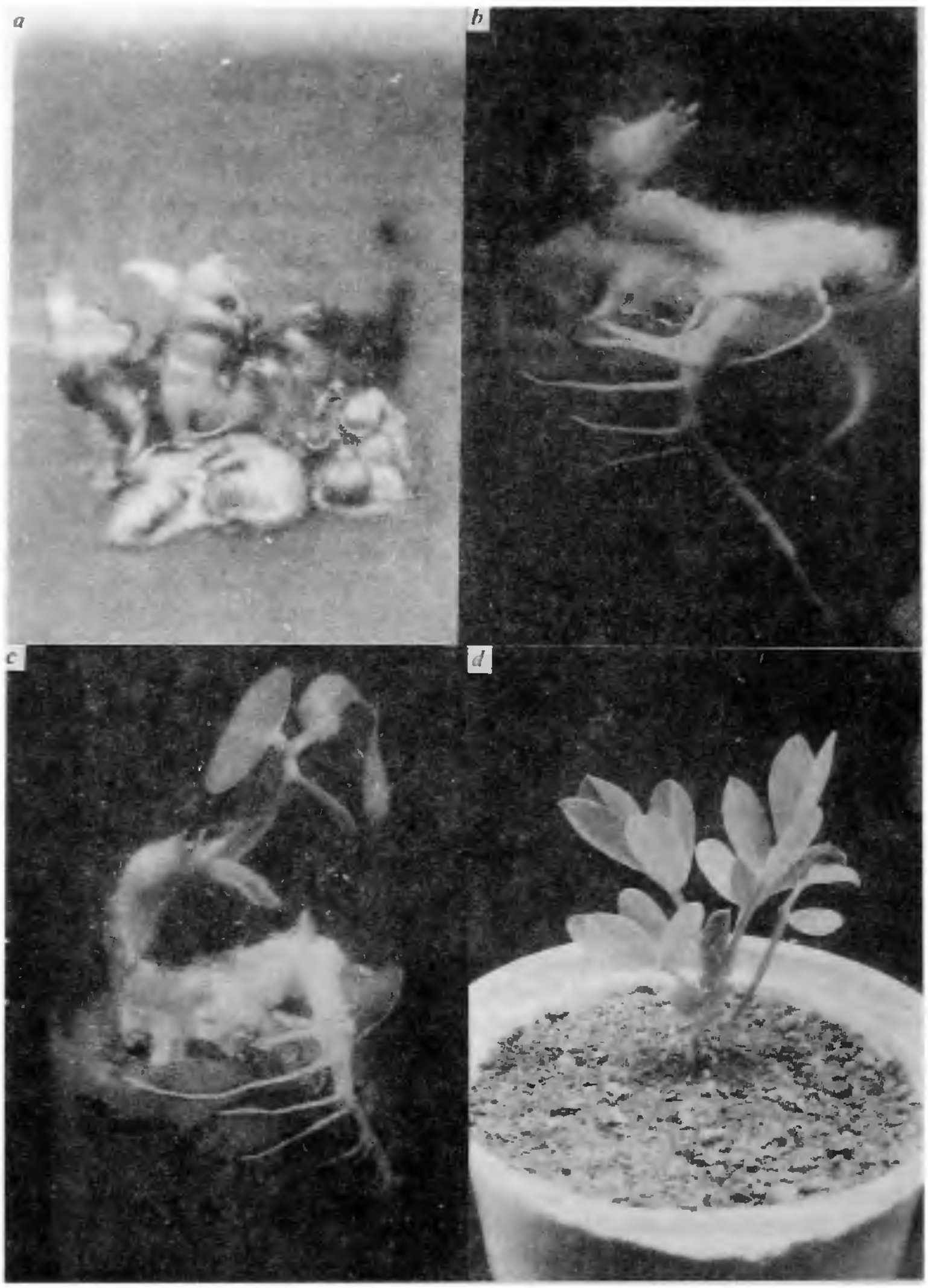

Figure 1 ***a–d***. Development of plants through somatic embryogenesis in *Arachis hypogaea*. ***a***, Differentiation of embryos from hypocotyl explant on MS medium supplemented with 20 mg/l 2,4-D and 0.5 mg/l BAP; ***b***, Germination of embryos on MS medium containing BAP (2.0 mg/l) and NAA (0.25 mg/l); ***c***, Plantlet development from somatic embryos; ***d***, Regenerated plant established in plastic cup.

After 5 weeks on induction media, embryogenic mass from the preliminary experiment was transferred to the maturation medium containing 3% (W/V) sucrose, different concentrations of BAP (0.5–2.0 mg/l) and 2,4-D (0.5 mg/l) and solidified with 0.7% (W/V) agar and adjusted to pH 5.8 prior to autoclaving. The cultures were subjected to a photoperiod of 16 h light (40 $\mu Em^{-2} s^{-1}$) and incubated at $24 \pm 2°C$. The percentage of embryogenic calli with mature embryos was calculated as follows: (Number of embryogenic calli showing mature embryos/Total number of embryogenic calli cultured) × 100.

After reaching the dicotyledonary stage, the embryos were individually transferred to MS medium containing different concentrations of BAP (0.5–2.0 mg/l) in combination with NAA (0.25 mg/l), 2% (W/V) sucrose and 3% (W/V) activated charcoal for germination and conversion into plantlets. The cultures were incubated for

two weeks at 24 ± 2°C under cool-white fluorescent light at 40 μE m^{-2} s^{-1} with 16 h photoperiod. The conversion frequency was calculated as follows: (Number of embryos germinated/Total number of embryos cultured) × 100.

Fully germinated embryos were transferred to growth regulator free MS medium with 0.7% (W/V) agar for further elongation of shoots and roots. Cultures were incubated for 10 to 15 days in test tubes with 15 ml medium, under conditions described for germination of somatic embryos. Plants with well-developed roots were transferred to plastic cups containing a 1:1:1 mixture of sand, red soil and manure. Each plantlet was covered with a polyethylene bag and the cups were maintained in controlled temperature at 60% relative humidity. Well established plants from cups were transferred to the field. Regenerated plants were grown to maturity that produced normal flowers and set viable seeds in pods.

Statistical computations were performed using computer software. The cultures were observed periodically and the somatic embryo induction, maturation and germination percentages were recorded on the basis of visual observations. Wherever possible, the effects of different treatments were quantified on the basis of percent cultures showing the response per culture. The experimental design was random and factorial with auxin and cytokinin as independent variables. The data pertaining to percentage of embryo induction, maturation and conversion were subjected to analysis of variance (ANOVA) test. Mean separation was done using Duncan's New Multiple Range Test (DNMRT).

Highly organized, round, creamish to white coloured protuberances differentiated from all over the surface of the hypocotyls when cultured on MS medium containing different concentrations of 2,4-D or NAA (1–30 mg/l) in combination with BAP (0.5 mg/l). The epidermal cell layer of the hypocotyl split up and organized structures emerged from the subepidermal layers. The number of organized structures was higher at the cotyledonary nodal region compared to the rest of the hypocotyl. These organized structures closely resembled globular and heart-shaped somatic embryos (Figure 1 *a*). 2,4-D at a concentration of 20 mg/l produced the highest frequency of cultures showing somatic embryogenesis (62.7%) and highest average number of somatic embryos per hypocotyl explant in VRI-2 which was statistically significant at 1% level (Table 1). However, in cv TMV-7, 20 mg/l of 2,4-D induced the highest frequency of responding cultures (50.3%) and induced the highest number of somatic embryos (Table 1). When NAA was used at 1, 5, 10, 15, 20, 25 and 30 mg/l, the frequency of response varied from 9.2 to 40.5% and 7.2 to 34.2% in VRI-2 and TMV-7 cultivars respectively and average number of somatic embryos per hypocotyl ranged from 3 to 11 and 2 to 9 in VRI-2 and TMV-7 cultivars respectively (Table 1).

Table 1. Frequency of somatic embryos from hypocotyl explants of *Arachis hypogaea* cultured on a medium containing different concentrations of 2,4-D and NAA in combination with 0.5 mg/l of BAP

Hormonal concentration (mg/l)	Per cent of somatic embryogenesis		Mean no. of embryos/explant ± SD	
	VRI-2	TMV-7	VRI-2	TMV-7
2,4-D				
0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5.0	10.4 ± 1.3^{f}	11.3 ± 1.4^{f}	4.0 ± 0.5^{e}	4.0 ± 0.5^{d}
10.0	24.7 ± 2.4^{e}	22.6 ± 2.1^{de}	8.0 ± 1.0^{d}	7.0 ± 0.8^{bc}
15.0	44.5 ± 3.2^{b}	41.7 ± 3.6^{b}	11.0 ± 1.2^{c}	10.0 ± 1.1^{b}
20.0	62.7 ± 5.3^{a}	50.3 ± 4.7^{a}	18.0 ± 1.9^{a}	15.0 ± 1.4^{a}
25.0	40.2 ± 3.6^{c}	37.4 ± 3.2^{c}	14.0 ± 1.2^{b}	12.0 ± 1.1^{b}
30.0	30.6 ± 2.8^{d}	26.9 ± 2.4^{cd}	12.0 ± 1.1^{c}	10.0 ± 1.2^{b}
NAA				
0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
10.0	9.2 ± 1.3^{f}	7.2 ± 1.1^{f}	3.0 ± 0.6^{e}	2.0 ± 0.5^{d}
15.0	21.3 ± 2.8^{e}	18.4 ± 1.8^{de}	7.0 ± 0.9^{d}	4.0 ± 0.8^{d}
20.0	40.5 ± 3.6^{c}	34.2 ± 3.0^{c}	11.0 ± 1.4^{c}	9.0 ± 1.1^{b}
25.0	32.4 ± 2.9^{d}	29.3 ± 2.6^{c}	8.0 ± 0.6^{d}	7.0 ± 0.8^{bc}
30.0	21.4 ± 2.3^{e}	23.6 ± 2.1^{c}	8.0 ± 0.7^{d}	8.0 ± 0.9^{b}

Mean separation using Duncan's New Multiple Range Test, means within column with different letters are significant at 1% level.

For further development, globular and heart-shaped somatic embryos obtained from hypocotyls were transferred to MS medium supplemented with 3% (W/V) sucrose, different concentrations of BAP (0.5–2.0 mg/l) in combination with 0.5 mg/l 2.4-D. About 80% of the globular and heart-shaped embryos of both the cultivars developed into torpedo and cotyledonary stages in this medium. The medium without BAP did not promote the growth of the embryos. Such embryo development was observed in all combinations with varying frequencies. The highest frequency of embryo maturation (VRI-2:82.7% and TMV-7:74.3%) was observed on a medium containing 2 mg/l BAP in combination with 0.5 mg/l 2,4-D in both the cultivars, which was highly significant (Table 2). Embryos were very loosely held on the surface of calli and matured synchronously. Maturation increased with the increase in the BAP concentration. After isolation of mature embryos from both cultivars, they were transferred to MS medium containing 2% (W/V) sucrose, different concentrations of BAP (0.5–2.0 mg/l) in combination with NAA (0.25 mg/l) and 0.3% (W/V) activated charcoal for germination. Germination of cotyledonary stage embryos varied with different concentrations of BAP. Germination of embryos was characterized by simultaneous production of root and shoot systems (Figure 1 *b*). Embryos induced from hypocotyls in the presence of 2,4-D or NAA and transferred to germination medium turned green and

developed by elongation of hypocotyl revealing the folded cotyledon. Later, cotyledons unfold and the shoot region developed further with the emergence of the first leaves. When MS medium was used with different concentrations of BAP (0.5–2.0 mg/l) in combination with NAA (0.25 mg/l), both shoot and root pole developed in all the combinations with varying frequencies. However, BAP (2.0 mg/l) and NAA (0.25 mg/l) combination was found to be best for highest frequency of embryo germination (VRI-2:81.7% and TMV-7:73.7%) in both the cultivars which was significant at 1% level (Table 3). Thus, BAP was necessary for germination of somatic embryos of the cultivars used in the present investigation. Germinated embryos produced good shoot and root systems when transferred to growth regulator free MS medium (Figure 1 *c*). Germinated embryos developed into complete plantlets on MS basal medium within 2 weeks. Regenerants were successfully transferred to the plastic cup (Figure 1 *d*) and subsequently transferred to the field. Properly hardened plants flowered normally and set viable seeds like that of seed grown plants (controls).

Table 2. Effect of BAP level in combination with 0.5 mg/l 2,4-D on embryo maturation from hypocotyl derived embryogenic calli of two groundnut cultivars

BAP concentration (mg/l)	No. of calli plated		No. of calli showing mature embryos		Embryo maturation response ± SD	
	VRI-2	TMV-7	VRI-2	TMV-7	VRI-2	TMV-7
0.0	32	26	0.0	0.0	0.0 ± 0.0	0.0 ± 0.0
0.5	34	29	8.6	6.1	25.3 ± 2.3[d]	21.2 ± 2.5[d]
1.0	38	32	17.1	13.2	45.2 ± 3.5[c]	41.5 ± 3.8[c]
1.5	42	35	25.7	19.3	61.2 ± 4.7[b]	55.4 ± 5.4[b]
2.0	45	41	37.2	30.4	82.7 ± 6.8[a]	74.3 ± 5.4[a]

Mean separation using Duncan's New Multiple Range Test, means within column with different letters are significant at 1% level.

Table 3. Effect of different concentrations of BAP in combination with 0.25 mg/l of NAA on germination of somatic embryos derived from hypocotyl explants into complete plantlets in two groundnut cultivars

BAP concentration (mg/l)	No. of mature embryos plated		No. of embryos germinated		Conversion per cent ± SD	
	VRI-2	TMV-7	VRI-2	TMV-7	VRI-2	TMV-7
0.0	52	54	0	0	0.0 ± 0.0	0.0 ± 0.0
0.5	58	61	13	11	22.4 ± 1.8[d]	18.0 ± 1.3[d]
1.0	65	68	27	25	41.5 ± 3.6[c]	36.7 ± 3.1[c]
1.5	74	75	46	44	62.1 ± 4.1[b]	58.6 ± 4.4[b]
2.0	82	80	67	59	81.7 ± 5.7[a]	73.7 ± 4.9[a]

Mean separation using Duncan's New Multiple Range Test, means within column with different letters are significant at 1% level.

Present studies have shown that it is possible to induce somatic embryogenesis and plant regeneration from hypocotyl explants of known commercial groundnut cultivars. Immature cotyledons[6,9], immature leaf[7,8] and immature embryos[11,16] have been successfully used for somatic embryogenesis in groundnut. Plantlet formation remained a problem in conversion of somatic embryos to plants, after transfer to basal medium, (ranged from 0 to 18%) in all the previous experiments[8–10]. In groundnut cultivars, 2,4-D was found to be the auxin of choice for the induction of somatic embryogenesis although NAA was also effective[10,17].

In media supplemented with 2,4-D (1.0–30 mg/l), clusters of somatic embryos developed from the cotyledon part of the adaxial side of the hypocotyl. This was accompanied by callusing. In media containing lower concentrations of NAA (1, 5 mg/l), callusing was observed from the cotyledon part of the hypocotyl without any sign of embryogenesis. However, at higher concentrations (10–30 mg/l), clusters of yellowish green somatic embryos differentiated from the cotyledonary nodal region of hypocotyl. 2,4-D and NAA[8,10,11,14] were widely used for somatic embryo induction in groundnut. Addition of cytokinin (BAP or KIN) to the medium promoted growth, development, maturation and conversion into plants of somatic embryos in culture. Cytokinins are known to enhance shoot morphogenesis. However, when cytokinins were used along with auxin, it showed positive effect on somatic embryogenesis in groundnut[13,16]. Embryogenesis was induced with a frequency of 40.5% with 11 somatic embryos/explant on MS medium containing 20 mg/l NAA and 0.5 mg/l BAP. However, higher concentrations (25 to 30 mg/l) of 2,4-D or NAA in combination with 0.5 mg/l BAP decreased the embryo induction frequency as well as number of somatic embryos/explant. Reddy and Reddy[16] and McKently[18] similarly observed somatic embryos on 2,4-D and BAP containing medium. The effectiveness of an auxin type appears to be tissue and species specific.

In the present study, frequency of embryogenesis and number of embryos per explant decreased with increasing concentrations of auxin level beyond 20 mg/l. McKently[18] found in embryo axis cultures, that as auxin concentrations increased (1–10 mg/l), the probability of obtaining a normal-shaped groundnut somatic embryo decreased. Higher concentrations of auxin not only decreased the embryos/explant but also delayed the embryogenesis[16]. Present studies have shown that conversion of somatic embryos into plants was dependent on the type and concentration of auxin used in the somatic embryo induction medium. The best plant conversion frequency (81.7%) was obtained when 2,4-D was used for somatic embryo induction from hypocotyl. Among the various cultivars studied in India[6,8,10,13] so far and in the present study, somatic embryogenesis seems to be better suited for genetic manipulation stud-

ies, because of high frequency of plant conversion, etc. The frequency of somatic embryogenesis in the present study was found to be the result of interaction between genotype and the type and concentration of auxin used. Parrott *et al.*[19] found that the genotype had a significant effect on the ability of immature soybean cotyledons to undergo the auxin-stimulated somatic embryogenesis.

In conclusion, an efficient protocol was developed for high frequency somatic embryogenesis using a simple medium. The present protocol has a distinct advantage over the earlier published protocols, the explant is hypocotyl by germinating seeds, which is available throughout the year.

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Influence of metalaxyl on *Glomus fasciculatum* associated with wheat (*Triticum aestivum* L.)

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Soil incorporation of metalaxyl [methyl *N*-(2-methoxyacetyl)-*N*-(2,6,xylyl)-DL-alaninate] significantly enhanced root colonization of the vesicular–arbuscular (VA) mycorrhizal fungi *Glomus fasciculatum* associated with wheat. The stimulatory response of VA mycorrhizal fungi to low concentration of metalaxyl resulted in increased plant biomass production, nutrient uptake and grain yield of wheat. However, higher concentrations of metalaxyl, particularly 2.5 ppm of metalaxyl affected the mycorrhizal infection and seed yield of wheat. Addition of urban compost to an extent ameliorated the toxic effect of fungicide on VA mycorrhizal colonization, plant growth and yield of wheat when compared to unamended soil.

THE role of agro-chemicals on vesicular–arbuscular mycorrhizal development and efficiency requires better understanding because mycorrhizal fungi are necessary components of most plant systems, and have significant effect on plant growth, physiology and nutrition. Several workers[1–3] have reported the adverse effect of systemic and nonsystemic fungicides to VA mycorrhizae. Recently, considerable attention has been given to a systemic fungicide metalaxyl [methyl *N*-(2-methoxyacetyl)-

Table 1. Effect of metalaxyl on per cent mycorrhizal colonization of wheat

Treatments		Days after planting			
	Fungicide dose (ppm)	40	80	120	Mean
Without compost	Control	18.00	45.00	64.00	42.33
	0.5	21.00	52.00	69.00	47.33
	1.0	26.00	38.00	76.00	46.67
	2.5	12.00	25.00	43.00	26.67
	Mean	19.25	40.00	63.00	40.75
With compost	Control	29.00	58.00	73.00	53.33
	0.5	28.00	49.00	78.00	51.67
	1.0	16.00	63.00	70.00	49.67
	2.5	22.00	31.00	42.00	31.67
	Mean	23.75	50.25	65.75	46.58
		SEm±		LSD (0.05)	
	Stages	2.4688		6.8431	
	Compost	2.0158		5.5874	
	Treatments	2.8507		7.9018	