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Callus Induction and Plantlet Regeneration from Mature Cotyledonary Segments of Groundnut (*Arachis hypogaea* L.)

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We evaluated the efficiency of callus induction and plantlet regeneration from mature cotyledonary segments of groundnut cultivars VRI-2 and VRI-3. Callus cultures were induced from mature tissues using NAA and IAA in combination with KIN or BAP. Maximum induction was recorded with 3.0 mg/L IAA and 1.0 mg/L BAP. However, green, compact, and nodular calli were obtained in 2.5 mg/L of IAA or NAA combined with 1.0 mg/L of either BAP or KIN. Fresh and dry weights were highly influenced by auxin concentration. Compact and nodular calli were then transferred to shoot induction media. The highest mean number of shoots was observed in 3.0 mg/L BAP plus 0.5 mg/L IAA. Finally, the resulting plantlets were rooted with IBA and NAA.

Keywords: callus induction, cotyledonary segments, plantlet regeneration, root induction

The cultivated groundnut or peanut (*Arachis hypogaea* L.) is a valuable leguminous crop. Its seeds contain a higher percentage of protein and oil than do other legumes, and they are an excellent source of essential nutrients such as carbohydrates, trace elements and vitamins. This crop is also grown as forage and to checks soil erosion (Bajaj, 1984), and its leguminous properties mean that it can improve soil fertility during cultivation.

Progress in techniques for plant cell, tissue and organ culture makes it possible to introduce genetic variability and to more easily select for desirable traits. (Evans et al., 1984). An efficient plant transformation system is a prerequisite for genetic studies using *Agrobacterium tumefaciens* (Ozias-Akins et al., 1992). Regeneration from seed explants has been reported for a number of legume species, including the mung bean (*Vigna radiata*), and the pigeon pea (*Cajanus cajan*) (Mehta and Mohanram, 1980; Gulati and Jaiwal, 1990). However, because groundnut lacks such a system, improvements via tissue culture, selection, and genetic transformation have been slow (Eapen and George, 1994).

Although the cultivated groundnut is relatively recalcitrant in tissue culture (Cheng et al., 1992; Heatly and Smith, 1996), plantlet regeneration has been successful in both seed and seedling explants via organogenesis (Mroginski et al., 1981; Narasimhulu and Reddy, 1983; Pittaman et al., 1983; Atreya et al., 1984; Daimon and Mii, 1991; Vajranabhaiah et al., 1993; Venkatachalam et al., 1994, 1998). Likewise, groundnut plantlets have been directly regenerated using de-embryonated cotyledons (Sastri et al., 1981; Mhatre et al., 1985; Mckently et al., 1990). However, little research has been done toward improving callus induction and plantlet regeneration for commonly cultivated Indian cultivars of the groundnut. Therefore, our objective was to develop an efficient system for propagation from seed derived cotyledonary segments.

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METERIALS AND METHODS

Plant Material and Seed Sterilization

We obtained two commonly grown groundnut (*A. hypogaea* L.) cultivars, VRI-2 and VRI-3, from the Regional Research Station (RRS), Tamil Nadu Agricultural University, Virudhachalam, Tamil Nadu, India. Healthy and homogenous seeds were washed thoroughly in running tap water for 20 min to remove adhering particles, then with 1% (v/v) teepol for 15 min, and distilled water (dH2O) for 5 min. The seeds were then disinfected with 70% (v/v) ethannol for 30 s. followed by surface sterilization in 0.1 (w/v) HgCl2 for 10 min and five to seven rinses in sterile dH2O.

Collection of Cotyledonary Segments and Culture Media

We removed the entire embroyonal axis from each surface sterilized seed, then sliced, the proximal halves of the de-embryonated cotyledons into 0.5 × 0.2 cm pieces. This tissue was inoculated on an MS medium (Murashige and Skoog, 1962) that comprised B_5 Vitamins (Gamborg et al., 1968), 0.8% agar, 3% sucrose, and selected concentrations of NAA, KIN, IAA, and BAP ranging from 1.0 to 3.0 mg/L. The pH of the medium was adjusted to 5.8 before being autoclaving at 121°C for 15 min. Cultures were incubated under a 16 h/8-h light /dark photoperiod at 25 ± 20°C. Phillips cool white flutoscent tubes were used to obtain a light intensity of 80 μm Em^2s^{-1}. For the controls, we used sliced explants that were inoculated on MS media containing B_5 vitamins but no growth regulators.

Shoot Bud Regeneration and Elongation

After four weeks of culture, the compact, nodular calli were subcultured two to three times in a reduced auxin containing medium. Afterward, they were transferred to a shoot induction medium (SIM) containing either KIN or BAP (1.0 to 3.0 mg/L) Plus 0.5 mg/L auxin. After 60 d, the regenerated calli were transferred to fresh media that contained 5 mg/L of BAP or KIN in combination with 0.5 mg/L of IAA plus 0.25 mg/L and GA3 to promote shoot elongation.

Root Induction

The regenerated shoots were rooted on media containing IBA (ranging from 1.0 to 5.0 mg/L) and NAA (0.25 to 1.0 mg/L).

Statistical Analysis

Each experiment had a completely randomized design and was replicated three times. Appropriate standard deviations and means separations were calculated according to Duncan's Multiple Range Test.

RESULTS

Callus Induction

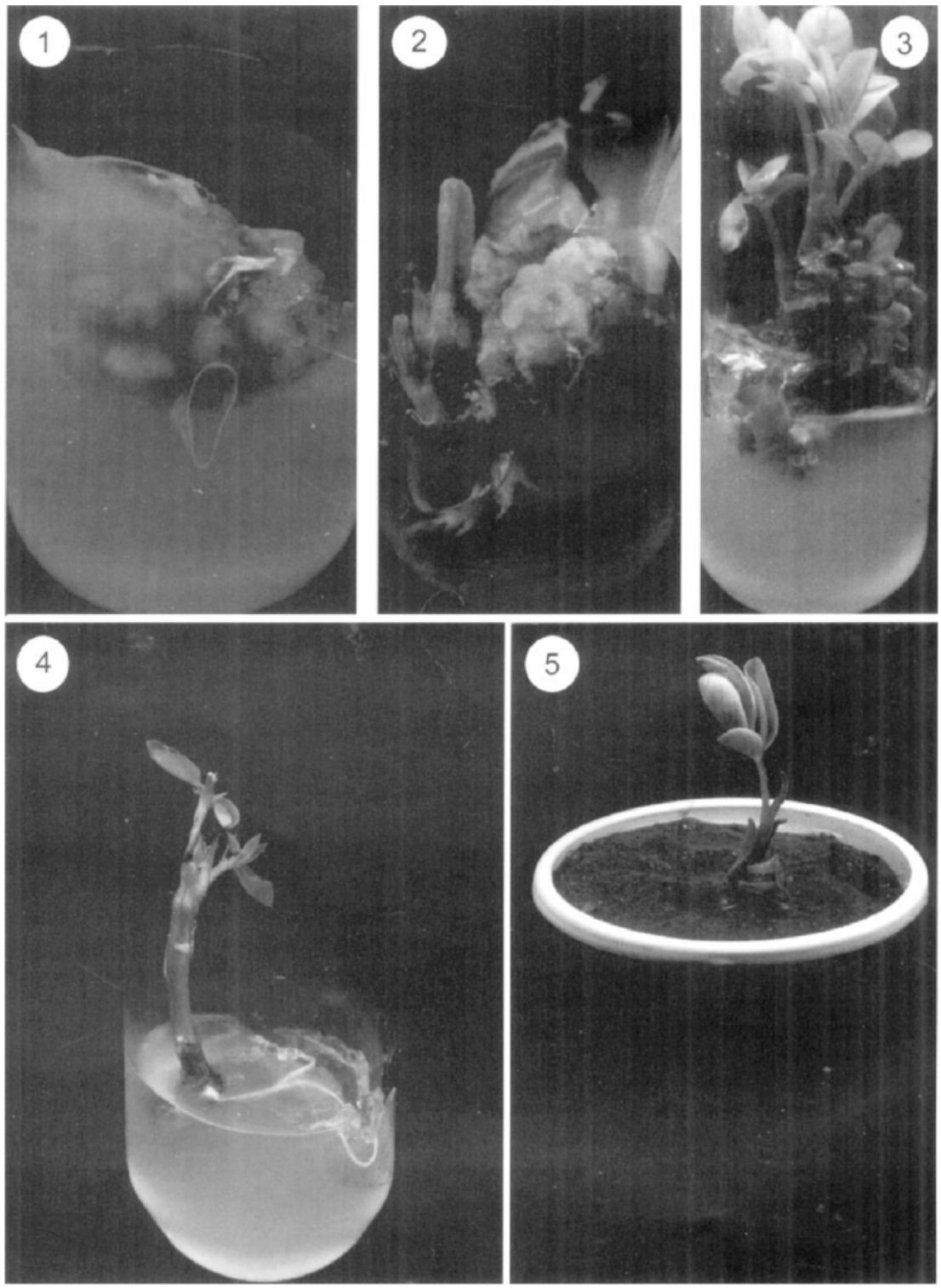
Cotyledonary segments on the MS media were subjected to different concentrations of NAA and IAA (1.0 to 3.0 mg/L) and KIN or BAP (1.0 mg/L) for callus induction. The segments began to enlarge with in 12 d of culture initiation (Table 1) Calli developed from the cut ends of the explants and turned either pale green in the IAA/BAP medium or slightly white in the NAA/KIN medium. Among the different auxin concentrations and combinations tested, 3.0 mg/L IAA with 1.0 mg/L BAP produced the highest percent callus formation (72.8% in VRI-2 and 66.8% in VRI-3 and 66.8% in VRI-3); results with NAA/KIN combinations were 68.6% and 63.0% respectively. However, more compact and nodular calli (Fig. 1) were obtained from 2.5 mg/L of IAA or NAA with 1.0 mg/L of KIN or BAP. The control explants never exhibited any callusing.

In both varieties, the amount of callus growth differed significantly according to the tested auxin concentration. The maximum fresh weights obtained from the

Table 1. Effect of NAA and IAA on callus induction and callus growth of mature cotyledonary segments of groundnut cultivars VRI-2 and VRI-3.

Hormones (mg/L)	Percentage of callus induction		Fresh weight/culture (gm)		Dry weight/culture (gm)	
	VRI-2	VRI-3	VRI-2	VRI-3	VRI-2	VRI-3
NAA + KIN						
1.0 + 1.0	31.0^{e}	28.4^{d}	1.15^{b}	0.958^{b}	0.1460^{bc}	0.1319^{bc}
1.5 + 1.0	41.0^{a}	36.4^{cd}	1.54^{b}	1.380^{b}	0.1612^{b}	0.1415^{bc}
2.0 + 1.0	54.6^{c}	41.2^{c}	2.16^{ab}	1.920^{ab}	0.1860^{ab}	0.1830^{ab}
2.5 + 1.0	57.8^{bc}	53.0^{b}	2.56^{ab}	2.070^{ab}	0.2090^{ab}	0.1923^{a}
3.0 + 1.0	68.6^{ab}	63.0^{ab}	2.84^{ab}	2.780^{ab}	0.2345^{a}	0.2138^{a}
IAA + BAP						
1.0 + 1.0	38.6^{de}	32.0^{cd}	1.91^{ab}	1.720^{b}	0.1203^{c}	0.1111^{c}
1.5 + 1.0	49.8^{cd}	41.6^{c}	2.07^{ab}	2.070^{ab}	0.1511^{bc}	0.1449^{bc}
2.0 + 1.0	59.0^{bc}	58.0^{ab}	2.62^{ab}	2.390^{ab}	0.1526^{bc}	0.1514^{b}
2.5 + 1.0	64.8^{b}	59.4^{ab}	3.13^{a}	2.690^{ab}	0.1699^{b}	0.1612^{ab}
3.0 + 1.0	72.8^{a}	66.8^{a}	3.52^{a}	3.150^{a}	0.1712^{ab}	0.1641^{ab}

Values with the same superscript are not significantly different at the 0.5% probability level according to Duncan's multiple ra nge test.



Figures 1-5. Plantlet regeneration from mature cotyledonary segments of groundnut. **1.** Green compact nodular calli derived from mature segments on MS + IAA (2.5 mg/L) + BAP (1.0 mg/L). **2.** Shoot regeneration from calli on MS + BAP (3.0 mg/L) + IAA (0.5 mg/L). **3.** Multiple shoot formation on MS + BAP (5.0 mg/L) + GA_3 (0.25 mg/L). **4.** Regenerated shoot in root induction medium containing MS + IBA (2.0 mg/L) + NAA (1.0 mg/L). **5.** Well-developed plantlet established in plastic cup containing sterile soil.

treatment of 3.0 mg/L IAA with 1.0 mg/L BAP, were 3.52 g for callus derived from VRI-2 and 3.15 g for VRI-3. Their respective dry weights were 0.171 g and 0.164 g. The most active callus production occurred in the proximal half of the cotyledon.

Plantlet Regeneration

The calli obtained from 2.5 mg/L IAA or NAA plus 1.0 mg/L BAP or KIN were transferred to a reduced auxin containing medium (1.5 mg/L auxin plus 1.0 mg/L cytokinin) After being subcultured, the calli were transferred to a SIM containing different concentrations of cytokinin plus 0.5 mg/L cytokanin). Shoot bud regeneration was observed within 15 d of culture initiation (Fig. 2). The maximum percentage of calli having shoot buds was observed from the treatment of 3.0 mg/L BAP plus 0.5 mg/L IAA (63.2% for VRI-2 and 57.4% for VRI-3). The second most successful combination was KIN plus 0.5 mg/L NAA (58.0% for VRI-2 and 53.0% for VRI-3). Further bud development produced multiple shoots (Fig. 3) with little callusing. The Highest mean number of shoots was obtained while using 3.0 mg/L BAP with 0.5 mg/L IAA (17.6 shoots from VRI-2 and 14.6 from VRI-3; Table 2).

Root Induction

After elongating, the shoots were transferred to a root induction medium. Within 25 d roots developed from the cut ends (Fig. 4). Maximum root production was gained when using a combinations of 2.0 mg/L with 1.0 mg/L of NAA (data not shown) Concentrations of IBA > 3.0 mg/L lead to callus formation associated with this rooting. The rooted plantlets were transferred to plastic cups containing sterile soil (Fig. 5).

Table 2. Effect of KIN and BAP on shoot induction from mature cotyledons derived from callus cultures of groundnut cultivars VRI-2 and VRI-3, in combination with 0.5 mg/L of NAA and IAA.

Hormones (mg/L)	Calli with shoot buds (%)		Mean number of shoots/explant	
	VRI-2	VRI-3	VRI-2	VRI-3
KIN + NAA				
1.0 + 0.5	20.6^{d}	17.2^{d}	3.0^{d}	2.6^{d}
1.5 + 0.5	33.2^{c}	29.6^{cd}	3.4^{cd}	4.2^{c}
2.0 + 0.5	41.6^{bc}	34.8^{bc}	10.8^{b}	7.2^{bc}
3.0 + 0.5	58.0^{ab}	53.0^{ab}	14.4^{ab}	11.8^{ab}
BAP + IAA				
1.0 + 0.5	25.2^{cd}	22.0^{c}	4.0^{cd}	3.0^{cd}
1.5 + 0.5	39.0^{bc}	33.2^{bc}	6.0^{c}	5.0^{bc}
2.0 + 0.5	48.4^{b}	41.2^{b}	11.2^{ab}	8.8^{b}
2.5 + 0.5	60.0^{ab}	49.0^{ab}	13.8^{ab}	1.3^{e}
3.0 + 0.5	63.2^{a}	57.4^{a}	17.6^{a}	14.6^{a}

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

DISCUSSION

We have developed a procedure for repeatable and reproducible plantlet regeneration from matured seed - derived cotyledonary segment the groundnut. One advantage of this proposed system is experimental material would be available throughout the year, thereby accelerating the progress of groundnut breeding programs.

In our study, the proximal half of the cotyledon was the most active location of calli production in both the VRI-2 and VRI-3 cultivars. Illingworth (1968), had found that most of the occasional plantlet production was from the nodal region, where it also was most frequently the callus that differentiated into whole groundnut plants. Callus induction and groundnut plantlet rengenrartion also were reported by Bhattia et al., (1985) who cultured de-embryonated cotyledons in Petridishes lined with moist filter paper or on cotton wool in beakers or test tubes. A large number of cotyledons showed callus growth, which also regenerated in to plantlets.

Just as in our research, Sudhavani and Reddy (1996) were able to induce calli from de-empryonated chickpea cotyledons on a B_5 medium supplemented with different concentrations of auxin. We have found that increased auxin levels also produced a higher percentage of calli. In sesame (*Sesamum indicum*), similar observations were recorded when various seedling explant sources were used, such as hypocotyls, cotyledons, and the shoot apex (Goyal et al., 1995; Rao and Vaidyanath, 1997).

In the present study, we used a combination of BAP or KIN with auxin to regenerate shoot buds from cultured callus tissue. This had also been reported in groundnut by Mroginiski et al. (1981), Mckently et al. (1990), Cheng et al. (1992), Eapen and George (1994), and Venkatachalam et al. (1996). Barna and Wakhlu (1994), attributed the effect of BAP on shoot bud induction to the ability of plant tissues either to metabolize the natural hormone more readily than other, synthetic, growth regulators or to induce engogenous production of zeatin. The percentage of shoot bud differentiation and the mean number of shoots per culture increased at higher concentrations of cytokinim plus

auxin (see also planivel, 1998). This auxin and cytokinin ration is known to be critical for multiple shoot regeneration in the groundnut (Banerjee et al., 1988; Venkatachalam et al.,1998).

The regenerated shoots from our shoot elongation medium were then rooted using IBA and NAA, as had been achieved with other Arachis spp. (Moss et al., 1988). In contrast, Eapen and George (1994), Ganapathi and Nataraja (1993), and Venkatachalam et al. (1996) had used a medium plus NAA or IBA to induce rooting. The percentage of rooting success increased at higher auxin concentrations (see also Banergee et al., 1988; Barna and Wakhulu, 1994).

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