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A Protocol for High Frequency Plant Conversion from Somatic Embryos of Peanut (*Arachis hypogaea* L. cv. DRG-12)

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Abstract

A protocol was developed for somatic embryogenesis with 100% induction rate from immature zygotic embryo axes of peanut (*Arachis hypogaea* L. cv. DRG-12) cultured on MS medium containing 18.09 μM 2,4-D. The frequency of somatic embryogenesis (31.7%) as well as the number of somatic embryos induced per explant (6.6) decreased when the concentration of 2,4-D was increased to 72.4 μM. Morphologically abnormal somatic embryos were observed at a frequency of 43.3% on MS medium containing 72.4 μM 2,4-D. Somatic embryos isolated from 30-day-old cultures of immature zygotic embryo axes exhibited precocious germination with varied responses when placed on MS basal medium with 3% sucrose. Maximum shoot induction (80.0%) was observed from somatic embryos isolated from 60-day-old cultures of immature zygotic embryo axes when placed as a clump rather than individually on MS medium supplemented with 26.63 μM BA and 0.54 μM NAA. Shoots developed from somatic embryos rooted with higher frequency (93.3%) on Blaydes' medium containing 5.4 μM NAA.

Key words: Immature zygotic embryo axes, peanut, somatic embryos, plant conversion

Introduction

Regeneration through somatic embryogenesis (Ozias-Akins 1989; Sellars et al. 1990; Mckently 1995) and organogenesis (Atreya et al. 1984; Chengalrayan et al. 1995; Pestana et al. 1999; Venkatachalam et al. 1999a) has been reported in peanut. A variety of explants have been employed for initiating somatic embryogenesis, including leaflets (Baker and Wetzstein 1992; Gill and Saxena 1992; Venkatachalam et al. 1999b), immature cotyledons (Ozias-Akins et al. 1992; Baker and Wetzstein 1994; George and Eapen 1993), immature embryo axes (Hazra et al. 1989; Reddy and Reddy 1993), mature embryo axes (Mckently 1991; Baker et al. 1995), mature embryo derived leaflets (Chengalrayan et al. 1994 and 1997), hypocotyls (Venkatachalam et al. 1997) and epicotyls (Little et al. 2000). In general, young meristematic tissues such as immature zygotic embryos and developing leaves were more competent to form somatic embryos. Various growth regulators such as 2,4-D (Eapen and George 1993; Baker et al. 1995), NAA (Eapen and George 1993), picloram (Baker and Wetzstein 1994), dicamba (Eapen and George 1993), and cytokinins such as forchlorfenuron (Murthy and Saxena 1994) and TDZ (Saxena *et al.* 1992; Victor et al. 1999) have been employed for initiating somatic embryogenesis in peanut. In most of the reports, the plant recovery from somatic embryos ranged from 18-50% (Ozias-Akins 1989; Hazra et al. 1989; Reddy and Reddy 1993; Wetzstein and Baker 1993).

Few reports are available wherein the methods for obtaining high frequency plant conversion from somatic embryos. Little et al. (2000) reported that picloram at 83.0 μM resulted in the best conversion efficiency from mature somatic embryos of VC1 genotype. Chengalrayan et al. (1997) reported that incorporation of 22.7 μM TDZ triggered shoot differentiation in 92% of rooted somatic embryos after 8 weeks. Venkatachalam et al. (1997) achieved high frequency of embryo germination (VR1-2: 81.7% and TMV-7: 73.7%) on a medium containing 8.87 μM BA in combination with 1.34 μM NAA. Germinated somatic embryos developed into complete plants on

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MS basal medium within 2 weeks. Chengalrayan et al. (1998) showed a genotype-dependent variation in responses at each stage of somatic embryo development, including the phase of embryo conversion to plantlets. These studies have demonstrated that plant conversion of somatic embryos is influenced by several factors such as genotype, source of explant, media and growth regulators. The present study aimed at developing an efficient method for plant conversion from somatic embryos of peanut cv. DRG-12. The results obtained in the present study differ from previous reports wherein prolonged incubation of somatic embryos on induction medium favored high frequency shoot induction (80.0%) when placed as a clump (7-9) on MS medium containing 26.63 μM BA and 0.54 μM NAA. The shoots produced from somatic embryos rooted efficiently on Blaydes' medium with 5.4 μM NAA.

Materials and Methods

Seeds of peanut (*Arachis hypogaea* L. cv. DRG-12) were collected from Directorate of Oilseeds Research, Hyderabad. The cultivar DRG-12 is a high yielding Spanish bunch type and mature in 110-115 days in post-rainy season. Immature pods of peanut were collected from field-grown plants after 30-40 days of pollination. The plants grown in the field experienced an average day/night temperature of 24/30℃ approximately and relative humidity varied from 60-80%. The immature pods collected from field were surface sterilized with 70% ethanol for 1 min followed by 0.1% $HgCl_2$ for 20 min and rinsed three times in distilled water. Immature seeds were removed from the pods and the experiments were carried out under aseptic conditions. Immature seeds were sterilized with 70% ethanol for 1 min followed by 0.1% $HgCl_2$ for 20 min and rinsed three times in distilled water. Immature zygotic embryo axes were excised from the seeds and two explants were placed in sterile culture tubes (25 × 150 mm) containing MS (Murashige and Skoog 1962) medium with 3% sucrose, 0.85% agar (Agar powder, Extra pure, Hi-Media, India) and 2,4-D (9.05-72.40 μM) for induction of somatic embryogenesis. The observations on frequency of somatic embryogenesis and mean number of somatic embryos induced per explant were recorded after 30 days of culture.

Somatic embryos isolated from immature zygotic embryo axes after 30 days or 60 days of incubation on induction medium (MS medium with 18.09 μM 2,4-D) were placed on MS basal medium with 3% sucrose for germination and plant conversion. In another experiment, somatic embryos isolated from 60-day-old cultures of immature zygotic embryo axes were placed either individually or as a clump on MS medium supplemented with BA (17.75 μM-35.51 μM) and 0.54 μM NAA for inducing germination and plant conversion. The overall germination and shoot regeneration frequencies of the normal and abnormal somatic embryos were calculated after 30 days of culture on regeneration medium. Shoots produced from somatic embryos were placed on MS or Blaydes' (Blaydes 1966) medium with 3% sucrose, 0.85% agar and 5.4 μM NAA for stimulating root development. The frequency of root induction, duration for root induction and the number of roots that developed on the shoots were recorded after 30 days of culture. Somatic embryos that differentiated into plumule and radicle were taken into consideration for calculating the germination frequencies. The emergence of shoots with tetrafoliate leaves and developed roots were the criteria for evaluating the plant conversion of somatic embryos. For all the experiments on induction of somatic embryogenesis and plant regeneration, the cultures were maintained at 25 ± 2 ℃ under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 $\mu Em^{-2}s^{-1}$ provided by white fluorescent tubes. The initiation of somatic embryogenesis from immature zygotic embryo axes and germination responses of somatic embryos were observed and photographed under a Carlzeiss Macrozoom microsope.

Results and Discussion

The present investigation led to the development of simple and efficient procedure for induction of somatic embryogenesis and plant regeneration from immature zygotic embryo axes of peanut (Figure 1 a-d). The frequency of somatic embryogenesis, duration of induction and the average number of somatic embryos induced per explant varied with the concentration of 2,4-D used in the induction medium (Table 1). The frequency of somatic embryos was low (35.0%) in the presence of 9.05 μM 2,4-D with a longer duration of 15.3 days for induction and lesser number (6.4) of somatic embryos per explant. The best response of somatic embryogenesis (100%) was observed on MS medium supplemented with 18.09 μM 2,4-D with induction of 18.3 somatic embryos per explant after 30 days of culture. Somatic embryos were induced directly after 10.2 days of culture without any callus phase on immature zygotic embryo axes. Induction of somatic embryogenesis was found to be asynchronous and somatic embryos at different stages of development could be observed after 30 days of culture (Figure 1 a). There was gradual decrease in the frequency of somatic embryogenesis (88.3%-31.7%) as well as the number of somatic embryos induced per explant (11.1-6.6) with an increase in the level of 2,4-D from 27.15 to 72.4 μM.

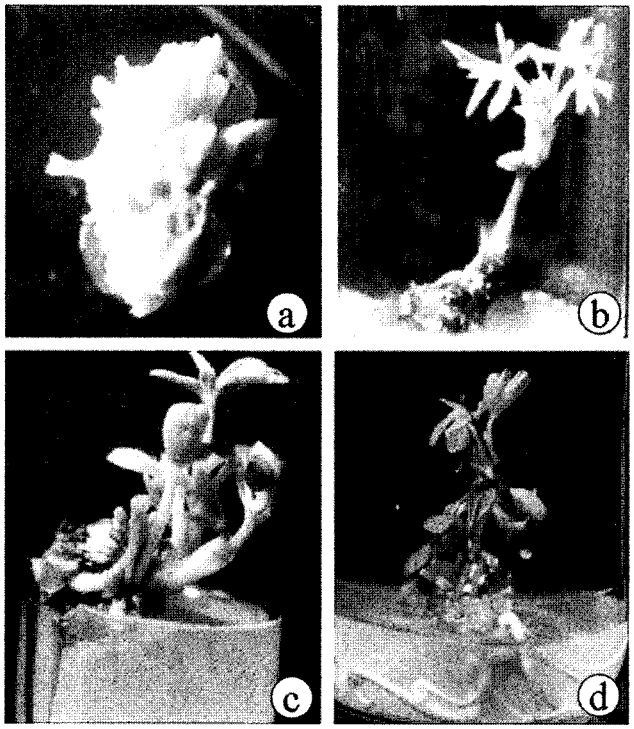

Figure 1. Regeneration of plants via somatic embryogenesis from immature zygotic embryo axes of DRG-12 cultivar of peanut: (a) Somatic embryos induced from immature zygotic embryo axes on MS medium with18.09 µM 2,4-D (b) Multiple shoot induction from abnormal somatic embryos on MS medium with 26.63 µM BA and 0.54 µM NAA (c) Shoot induction from clumps of somatic embryos derived from 60 day-old cultures on MS medium with 26.63 µM BA and 0.54 µM NAA (d) Root induction from shoots developed from somatic embryos on Blaydes' medium with 5.4 µM NAA.

Higher concentrations of 2,4-D notably increased the average duration for induction of somatic embryos from immature zygotic embryo axes.

Wetzstein and Baker (1993) observed that the 2,4-D level in the induction medium had little effect on embryo morphology in peanut. Chengalrayan et al. (1995) reported that somatic embryos formed from immature leaves of peanut demonstrated a high degree of morphological abnormality. In the present study, normal as well as divergent morphological types, such as long narrow torpedo and cotyledonary types, jar shaped with fused cotyledons, flattened embryo with tricotyledons and no distinct hypocotyls, embryos with elongated epicotyledonary region, tubular, bell shaped and embryo with multiple cotyledons (Figure 2 a-d) were induced at all the concentrations of 2,4-D tested. However, morphologically abnormal somatic embryos, induced on 72.4 µM 2,4-D, constituted 43.3% of the total somatic embryos compared to 25.8% on 18.09 µM 2,4-D. The distribution of different morphological classes of somatic embryos induced in immature zygotic embryo axes on medium with 18.09 µM 2,4-D varied with globular, heart, torpedo, cotyledonary and abnormal types occurring at a frequency of 7.8%, 6.5%, 23.2%, 36.7% and 25.8%, respectively. Similarly, in *Avalia cordata*, somatic embryos with bowling pin and jar-shaped cotyledons were frequently observed when the somatic embryos were formed on medium containing high concentrations of 2,4-D (Lee and Soh 1993). In contrast, higher levels of 2,4-D increased the production of normal somatic embryos in asparagus (Levi and Sink 1991) and soybean (Ranch *et al.* 1985). These differential observations may be governed by the species, genotype or nature of the explant that influences the endogenous levels of the auxins.

Somatic embryos isolated from 30-day-old cultures of immature zygotic embryo axes exhibited precocious germination with a frequency of 89.3% upon transfer to MS basal medium with 3% sucrose. They exhibited varied germination responses depending on the morphology of somatic em-

Table 1. Effect of 2,4-D on somatic embryogenesis from immature zygotic embryo axes of DRG-12 cultured on MS medium

Conc. (µM)	Somatic embryogenesis (%)	Mean number of somatic embryos/explant	Average duration for induction (days)
9.05	35.0 ± 2.89 ^{e}	6.4 ± 0.29 ^{e}	15.3 ± 0.20 ^{d}
18.09	100.0 ± 0.00 ^{a}	18.3 ± 0.20 ^{a}	10.2 ± 0.29 ^{f}
27.15	88.3 ± 1.67 ^{b}	11.1 ± 0.42 ^{b}	11.5 ± 0.23 ^{e}
36.2	75.0 ± 2.89 ^{c}	9.6 ± 0.29 ^{c}	11.7 ± 0.20 ^{e}
45.25	58.3 ± 4.41 ^{d}	8.6 ± 0.13 ^{d}	16.0 ± 0.51 ^{d}
54.3	50.0 ± 2.89 ^{d}	8.3 ± 0.38 ^{d}	23.8 ± 0.69 ^{c}
63.35	36.7 ± 1.67 ^{e}	7.2 ± 0.29 ^{e}	25.3 ± 0.17 ^{b}
72.4	31.7 ± 4.41 ^{e}	6.6 ± 0.29 ^{e}	26.6 ± 0.13 ^{a}

Data represent mean ± standard error of three replicates, with each replicate consisting of twenty explants. Means followed by same superscript alphabet in a column are not significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keul's multiple range test.

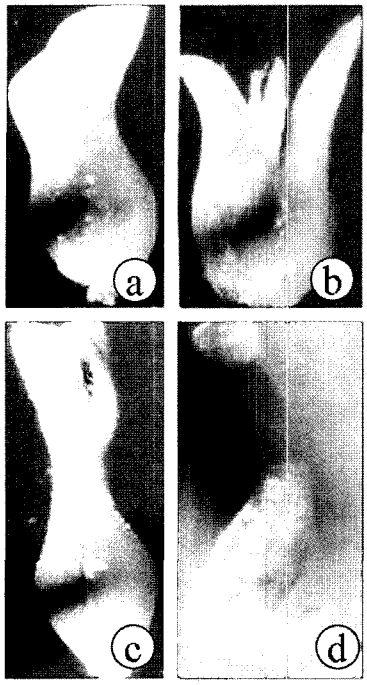

Figure 2. Abnormal somatic embryos developed from immature zygotic embryo axes cultured on MS medium with 72.4 μM of 2,4-D: (a) Jar shaped somatic embryo with fused cotyledons (b) Tricotyledonary flattened somatic embryo (c) Somatic embryo with elongated epicotyledonary region (d) Tubular somatic embryo with fused cotyledons.

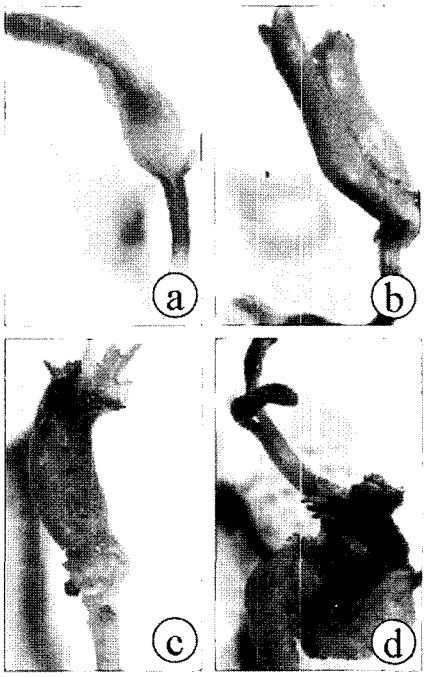

Figure 3. Germination responses from somatic embryos derived from 30 day-old cultures on MS basal medium with 3% sucrose: (a) Development of an elongated tubular plumule and distinct root (b) Development of broadened plumule and distinct root (c) Development of plumule with multiple leaflets and distinct root (d) Development of plumule with induction of bifoliate leaves and root.

bryos. The plumule and radicle elongated from tubular somatic embryos within one week of culture. The germination responses from cotyledonary somatic embryos included development of an elongated tubular plumule, broadened plumule with distinct root, induction of multiple leaflets, and enlargement of radicle and plumule region along with induction of bifoliate leaves (Figure 3 a-d). About 20% somatic embryos germinated with induction of spiny type trifoliate and tetrafoliate leaflets without further growth. Precocious germination and other abnormal tissue growth were prevented in soybean during growth and maturation *in vitro* using high concentrations of sucrose or mannitol in the medium or by addition of ABA to low osmotic medium (Obendorf and Wettlaufer 1984). In the present study, somatic embryos isolated from 60-day-old cultures of immature zygotic embryo axes failed to convert into plants when placed either individually or as a clump on MS basal medium. However, a combination of BAP with NAA promoted germination and shoot induction with varied frequencies from individual as well as clump of somatic embryos. Earlier studies showed that germination and conversion of somatic embryos occurred only in the presence of auxin (Eapen and George 1993) or a combination of different cytokinins (Chengalrayan et al. 1994 and 1997). In the present study, somatic embryos exhibited high frequencies of germination (85.8%-96.7%) irrespective of the concentration of BAP used in the medium (Table 2). Different germination responses were observed from morphologically abnormal somatic embryos when they were placed singly on MS medium with 26.63 μM BA and 0.54 μM NAA. These include differentiation of plumules with fused leaflets showing a cup-like appearance, broadened and distorted plumules showing abnormal leaflets without distinct petiole, broadened plumules with abnormal leaflets without distinct petiole and distinct root, elongated tubular plumules and broadened radicle without distinct root. Wetzstein and Baker (1993) found that conversion of peanut somatic embryos derived from cotyledon cultures was related to embryo morphology. Tubular embryos characteristically had the lowest conversion rates due to a poorly developed apical meristem compared to bipolar and broad-fasciated embryos, which converted at higher rates. In the present study, multiple shoot induction was observed with a frequency of 38.5% from abnormal somatic embryos after 60 days of culture on MS medium with 26.63 μM BA and 0.54 μM NAA (Figure 1 b).

Table 2. Effect of BA with 0.54 μM NAA on germination and shoot induction from somatic embryos of peanut

BAP (μM)	Germination (%)		Shoot induction (%)	
	Single somatic embryos	Clump of somatic embryos	Single somatic embryos	Clump of somatic embryos
17.75	87.5 ± 1.12 [b]	90.0 ± 1.29 [d]	23.3 ± 1.05 [d]	61.7 ± 1.67 [d]
22.19	90.0 ± 1.29 [ab]	92.5 ± 1.12 [bd]	28.3 ± 1.67 [c]	68.3 ± 2.11 [c]
26.63	92.5 ± 1.12 [a]	96.7 ± 1.05 [a]	39.2 ± 1.54 [a]	80.0 ± 1.03 [a]
31.07	91.7 ± 1.05 [ab]	91.7 ± 1.05 [cd]	36.7 ± 2.11 [ab]	74.2 ± 1.54 [b]
35.51	86.7 ± 1.67 [b]	85.8 ± 1.54 [e]	32.5 ± 1.12 [bc]	67.5 ± 1.12 [c]

Data represent mean ± standard error of three replicates, with each replicate consisting of 10 single somatic embryos or clumps of somatic embryos. Means followed by same superscript alphabet in a column are not significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keul's multiple range test.

Table 3. Root induction from shoots of somatic embryos on MS and Blaydes' medium with 5.4 μM NAA

Media and Growth regulators	Root induction (%)	Average duration of response (days)	Mean number of roots induced per shoot
MS + 5.4 μM NAA	80.0 ± 2.89 [a]	11.0 ± 0.22 [a]	3.80 ± 0.06 [a]
Blaydes' + 5.4 μM NAA	93.3 ± 1.67 [b]	9.6 ± 0.12 [b]	10.93 ± 0.35 [b]

Data represent mean ± standard error of three replicates, with each replicate consisting of twenty shoots. Means followed by same superscript alphabet in a column are not significantly different ($P < 0.05$) according to one-way ANOVA followed by Newman-Keul's multiple range test.

Multiple shoots induced from abnormal somatic embryos elongated when cultured on MS medium containing 8.88 μM BA and 0.54 μM NAA. The induction of multiple shoots may be due to the existence of multiple meristems within the somatic embryos. Chengalrayan et al. (1997) also reported multiple shoot formation in somatic embryos in the presence of 8.9 μM BA and 14 μM KN. Joshi et al. (2003) obtained multiple shoot differentiation in the plumule of the somatic embryos in the presence of 22.7 μM TDZ.

An interesting observation in the present study is that transfer of somatic embryos as a clump (7-9), consisting of normal as well as abnormal types, facilitated shoot induction with high frequencies rather than individual separation and culture (Table 2). Shoots were induced with a frequency of 61.7% and 68.3% on MS medium containing 17.75 μM and 22.19 μM BA, whereas BA at 31.07 μM and 35.51 μM in the presence of NAA triggered shoot induction with a frequency of 74.2% and 67.5%, respectively. High frequency shoot induction (80.0%) was observed from somatic embryos placed as a clump on medium supplemented with 26.63 μM BA and 0.54 μM NAA (Figure 1 c). In contrast, somatic embryos placed singly differentiated into shoots at low frequencies (23.3%-39.2%). Similarly, Durham and Parrott (1992) achieved embryo development, germination, and conversion by placing embryo clumps of peanut onto hormone-free, solid medium. The high frequency shoot induction observed from clumps of somatic embryos may be due to the beneficial effect of some unknown factor that need to be further investigated.

In the present study, roots were induced from shoots derived from somatic embryos on MS or Blaydes' medium with 5.4 μM NAA (Table 3). High frequency of rooting (93.3%) was observed from shoots on Blaydes' medium with 5.4 μM NAA and the root induction was observed within 9.6 days of culture (Figure 1 d). Shoots cultured on MS medium with 5.4 μM NAA rooted at a frequency of 80.0 % with induction of few roots (3.8) and longer duration (11.0 days) for root induction. An average of 74.6% of somatic embryos obtained from immature zygotic embryo axes converted into plants. Efficient plant conversion of somatic embryos isolated from 60-day-old cultures might be due to maturation induced with depletion of nutrients upon prolonged culture. Similarly, Chengalrayan et al. (1997) reported that incubation of the cultures of somatic embryos for 8 weeks instead of 4 weeks increased the frequency of plantlet formation in all the cytokinin concentrations tested.

In the present investigation, a simple method for somatic embryo induction (100%) and efficient plant conversion (74.6%) was developed in DRG-12 cultivar of peanut. Somatic embryos isolated from 60-day-old cultures of immature zygotic embryo axes developed into shoots with higher frequency (80.0%) when placed as clump (7-9) on medium containing 26.63 μM BA and 0.54 μM NAA. The shoots developed from somatic embryos rooted at a higher frequency (93.3%)

on Blaydes' medium with 5.4 μM NAA. This protocol will be useful for fundamental and applied research in DRG-12 cultivar in particular and peanut in general.

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