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A protocol for repetitive somatic embryogenesis from mature peanut epicotyls

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Abstract The effects of 11 different auxins and one cytokinin-like compound were tested at four concentrations for their ability to induce primary and repetitive somatic embryos from mature, dry peanut (*Arachis hypogaea* L.) epicotyls of genotype AT120. Treatment with picloram and centrophenoxine at 83.0 and 124.4 μM resulted in the greatest number of embryos per explant and the highest percentage of explants responding. In a follow-up experiment, picloram, centrophenoxine, and dicamba were tested at 83.0 and 124.4 μM on four peanut genotypes (AT120, 59-4144, GK7, and VC1). Picloram and centrophenoxine induced similar numbers of globular-stage and total embryos from each genotype, while dicamba was less effective. Similar results were observed with percentage of responding axes. Genotypes AT120 and VC1 yielded more clusters of repetitive embryos than GK7 and 59-4144. After 5 months, embryos derived from repetitive embryogenic cultures were converted into mature plants.

Key words Picloram · Centrophenoxine · Auxin · *Arachis hypogaea* L. · Somatic embryogenesis

Abbreviations *2,4-D*: 2,4-dichlorophenoxyacetic acid · *2,4,5-T*: 2,4,5-trichlorophenoxyacetic acid · *BA*: N^{6}-benzyl adenine · *B5*: Vitamins according to Gamborg et al. (1968) · *CPPU*: *N*-(*c*-chloro-4-pyridyl)-*N′*-phenylurea · *Centrophenoxine (CX)*: (4-Chlorophenoxy) acetic acid 2-(dimethylamino) ethyl ester · *Dicamba*: 3,6-Dichloro-2-methoxybenzoic acid · *MS*: Murashige and Skoog (1962) · *NAA*: α-Naphthalene acetic acid · *Picloram* 4-Amino-3,5,6-trichloropicolinic acid · *PGR*: Plant growth regulator · *TIBA*: 2,3,5-Triidobenzoic acid

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Introduction

Gene transfer systems to improve peanut varieties require an efficient method for the production of transformable tissues and for the regeneration of transformed plants (Livingstone and Birch 1999). Repetitive somatic embryogenesis is the proliferation of somatic embryos from each other, and has been used for genetic transformation of various species (Merkle et al. 1990).

Transformation procedures typically result in transformation of individual cells within the explant tissue (Merkle et al. 1990). Whenever it is possible for somatic embryos to arise from single cells (Haccius 1978), repetitive embryogenesis may be used to first recover a somatic embryo from a transgenic cell, and then to increase the number of transgenic embryos available prior to plant recovery. Alternatively, when somatic embryos have multicellular origins (Williams and Maheswaran 1986), the original transgenic cell can at best give rise to a transgenic sector within a repetitive embryo. An additional cycle of embryogenesis is needed to obtain a fully transgenic embryo from the transgenic sector (Merkle et al. 1990). In essence, transformed embryos continue repetitive embryo production under selection, resulting in a reduction in chimeric events and in a greater number of embryos available for conversion into plants (Parrott et al. 1991). Hence, any improvement in protocols for repetitive somatic embryogenesis can potentially increase transformation efficiency.

Almost all of the literature on peanut somatic embryogenesis is specific to primary somatic embryos, and very little information is available on repetitive embryogenesis. Primary peanut somatic embryos have been induced from immature zygotic embryos (Hazra

et al. 1989; McKently 1995; Ozias-Akins et al. 1992), immature cotyledons (Durham and Parrott 1992; Ozias-Akins 1989; Ozias-Akins et al. 1992), leaflets from germinating seedlings (Baker and Wetzstein 1992), and dry, mature zygotic embryo axes (Baker et al. 1995; George and Eapen 1993). In addition, a variety of auxin types and concentrations have been compared for peanut somatic embryo induction (Eapen et al. 1993; Eapen and George 1993; McKently 1991). Systems utilizing immature zygotic embryos and cotyledons have been hampered by the difficulty of maintaining sufficient plant material at the correct developmental stage for initiation. Mature zygotic embryos from seed are a convenient source of explants, but the embryos produced from mature tissues are often differentiated and, thus, developmentally unsuitable for use in a repetitive system (Baker and Wetzstein 1995). Reports of repetitive embryogenesis in peanut have been limited to Baker and Wetzstein (1994, 1995), Durham and Parrott (1992), and Ozias-Akins et al. (1993). In each of these studies, repetitive embryos were larger than those used for transformation in other legumes, such as soybean (Hazel et al. 1998), and/or the frequency of repetitive embryogenesis was far lower than that reported in other legumes (e.g., Bailey et al. 1993).

A comprehensive study of different auxins over a range of concentrations, in which total numbers and morphology of embryos, secondary embryogenesis, genotypic effects, and conversion rates are evaluated concurrently, was needed to develop an efficient repetitive embryogenic system for peanut. The objective of this study was therefore to optimize the auxin type and concentration for the induction of somatic embryos and the establishment of repetitive cultures from mature epicotyls of four genotypes of peanut.

Materials and methods

Plant material

Embryonic axes were removed from mature seeds of peanut cultivars AT120, GK7, 59-4144, and VC-1 (AgraTech Seeds, Ashburn, Ga., USA) as described by Baker et al. (1995). The axes were surface-sterilized in 0.525% NaOCl (10% commercial bleach) for 5 min and rinsed three times in sterile water. The epicotyl of each axis was aseptically removed from the radicle.

Embryo induction on solid medium

The epicotyl portions of mature axes were placed, with the leaves protruding, on a basal medium composed of MS salts, B5 vitamins, 3% sucrose, and 0.8% agar (Agar-Agar, Sigma, St. Louis, Mo., USA; MSS3 medium) supplemented with one of several auxins as described below. The pH was adjusted to 5.8 with NaOH prior to autoclaving at 121 °C for 20 min. Stock solutions of each PGR were filter-sterilized and added to the cooled medium prior to pouring.

In the first experiment, 11 auxins (centrophenoxine, chlorogenic acid, *p*-chlorophenoxyacetic acid, *trans*-cinnamic acid, dicamba, NAA, 2,4-D, phenylacetic acid, picloram, 2,4,5-T, and TIBA) and one cytokinin-like compound (CPPU) were tested at 12.4, 41.5, 83.0, and 124.4 μM using cultivar AT120. For each PGR treatment, ten epicotyls were placed in a 100×15 mm Petri dish containing 25 ml of medium/plate. The experiment was replicated ten times. Plates were sealed with Nescofilm (Karlan Research Products Corp., Santa Rosa, Calif., USA) and incubated at 28 °C in the dark for 6 weeks, after which the number of embryos induced per explant and the percentage of responding explants were determined.

The auxins and concentrations that showed the best results were tested further. Picloram, dicamba, and centrophenoxine were tested at 83.0 and 124.4 μM on each of four peanut cultivars, AT120, GK7, 59–4144 and VC-1. Cultural conditions were as described above, and the experiment was replicated five times. After 6 weeks, the average number of globular-stage and total embryos per explant, and the percentage of responding explants were determined for each treatment/genotype combination. Clusters of globular-stage embryos 5 mm in diameter and containing five to ten globular-stage embryos were transferred to the same medium used for induction. After 4 weeks, each treatment was rated for the percentage of embryo clusters that produced repetitive globular-stage embryos. Such repetitive, globular-stage embryos were transferred every 4 weeks to fresh induction medium.

Embryo conversion

One hundred milligrams of repetitive embryos were collected from those treatments that continued to yield repetitive cultures after 5 months (fourth transfer) on induction medium. For each treatment and genotype, a maximum of three repetitive cultures (one culture from each of three replications) were used. Twenty-five embryo clusters 5 mm in diameter were placed onto each plate of MS medium with B5 vitamins, 6% maltose substituted for the sucrose, and 0.5% activated charcoal (Sigma) added after the pH was adjusted to 5.8 (MS0M6^{ac} medium, Bailey et al. 1993). After a 4-week incubation at 26 °C and a 23-h photoperiod under cool white fluorescent tubes at 10 μmol m^{-2} s^{-1}, embryo clusters were divided and transferred to MS0M6 medium (Finer and McMullen 1991) without activated charcoal and incubated another 4 weeks as before. The embryos were placed in empty Petri dishes, sealed, and desiccated for approximately 1 week (Durham and Parrott 1992) before transfer to modified germination medium consisting of FNL salts (Samoylov et al. 1998), B5 vitamins, 1 mM asparagine, 0.1 μM BA, 2.4% sucrose, and 0.8% agar (FNLB.1 medium). Plates were incubated under a 23-h photoperiod and a light intensity of 60 μmol m^{-2} s^{-1}. Elongated shoots were excised, transferred to 25×150 mm glass tubes containing 25 ml germination medium without BA (FNLO medium), and observed for root development. Tubes were capped with polypropylene closures (Kim-Kaps, Kimble Glass Inc., Vineland, N.J., USA). The percentage conversion rate of rooted plantlets obtained per number of embryos placed on germination medium was determined. Five of the rooted plantlets from each treatment were placed in potting mix (equal parts commercial potting mix and sand) and transferred to a greenhouse to examine fertility of the converted embryos.

Data analyses

Analysis of variance was completed using the General Linear Models program of SAS version 6.10 (SAS Institute, Cary, N.C., USA). Replications were performed over time and were treated as blocks in the analysis. Percentage embryo data were transformed using the arc sine-square root transformation before analysis.

Results and discussion

The objective of the first experiment was to identify the most effective auxins and concentrations for the induc-

tion of somatic embryos, particularly globular-stage embryos, from the epicotyls of mature peanut axes. Six (phenylacetic acid, chlorogenic acid, *trans*-cinnamic acid, NAA, 2,4,5-T, and TIBA) of the 11 tested auxins did not induce any somatic embryos from mature peanut epicotyls. Treatment with phenylacetic acid, chlorogenic acid and *trans*-cinnamic acid at all levels, and NAA at 12.4 and 41.5 μM, permitted the elongation of the apical meristem of the axis into a shoot. The addition of NAA at 83.0 or 124.4 μM and 2,4,5-T at all levels resulted in the formation of callus, but not of somatic embryos. TIBA induced an elongation of the explant at 12.4 and 41.5 μM and death of the explants at higher levels. Five auxins (2,4-D, picloram, centrophenoxine, dicamba, and *p*-chlorophenoxyacetic acid) induced callus formation with the subsequent formation of somatic embryos from the explant tissue (Figs. 1A, C, E, 2). Centrophenoxine and picloram at higher concentrations were the most effective treatments for both percentage of responding explants (Fig. 2A) and total embryos per explant (Fig. 2B).

Substituted phenylurea compounds, such as CPPU (Murthy and Saxena 1994) and thiadiazuron (Gill and Saxena 1992; Murthy et al. 1995), have cytokinin-like activity and have been used to induce somatic embryos from peanut, but have not been examined in a repetitive system. In this study, treatment with CPPU resulted in an elongation of the embryo axis with high numbers of meristem-like structures developing from the base of the epicotyl without a callus phase. These structures had a tendency to develop into shoots instead of producing secondary globular embryos. CPPU was therefore not useful in a repetitively embryogenic system. Embryos produced by picloram, dicamba, and centrophenoxine at 83.0 and 124.4 μM were often at the globular stage of differentiation (Fig. 1A,E), while most embryos produced by 2,4-D and *p*-chlorophenoxyacetic acid were opaque, misshapen or fasciated, and in the torpedo or cotyledonary stage of development by 6 weeks post-induction. Previously, 2,4-D was used to achieve a repetitive somatic embryogenesis system in either liquid (Durham and Parrott 1992) or solid (Baker and Wetzstein 1995) medium, but very few of the embryos induced were in the repetitive globular stage useful for transformation.

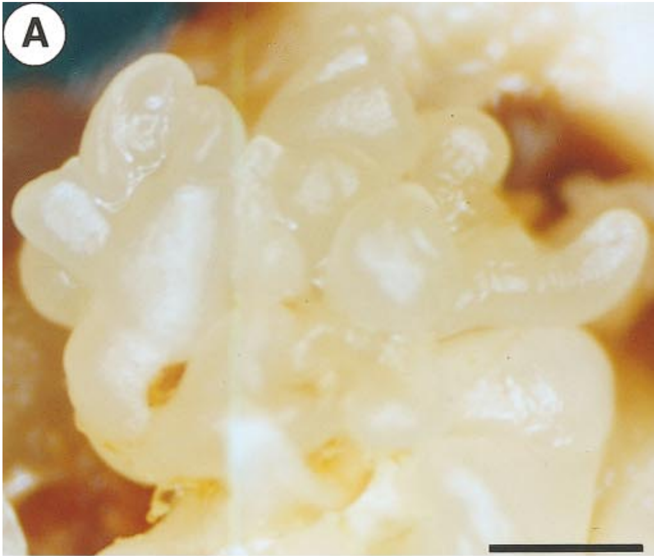
The objective of the second experiment was to increase the scope of the study by including different genotypes and to examine the effects of genotype, auxin, and concentration on the establishment and maintenance of repetitive globular-stage embryos. Treatment with picloram or centrophenoxine at either concentration resulted in a similar number of axes responding (78%–98%) for each genotype, while fewer responding explants were observed overall with dicamba (Fig. 3A). There was a highly significant effect of block, auxin, and the interaction of genotype and auxin, and a moderately significant effect of genotype, on the number of globular-stage embryos induced per responding explant (Table 1). For the total number of embryos induced per responding explant, all sources of variation were highly significant (Table 1). Picloram and centrophenoxine were more effective than dicamba for the induction of both globular-stage and total embryos per explant (Fig. 3B,C). The most effective treatment varied with genotype. Picloram at both concentrations was most effective for genotype GK7 and centrophenoxine at 83.0 μM was most effective for AT120, while little difference was seen between the picloram and centrophenoxine treatments for genotypes 59-4144 and VC1.

A highly significant genotype and auxin effect was observed on the number of transferred clusters of 6-week-old globular embryos that yielded secondary repetitive embryos 4 weeks following transfer to the same medium (Fig. 4). There was a weak interaction between genotype and auxin. Clusters of AT120 embryos produced the most repetitive embryos on centrophenoxine and picloram at 124.4 μM. Centrophenoxine and picloram at 83.0 and 124.4 μM and dicamba at 83.0 μM each induced a similar number of secondary embryos from VC1 clusters. Compared with the other two genotypes, 59-4144 and GK7 produced fewer secondary embryos using all auxin and concentration combinations, with no secondary embryos developing on dicamba. No secondary embryos were induced by dicamba at 124.4 μM for any genotype (Fig. 4).

Picloram-induced embryos tended to be the smallest and most uniform with more secondary embryos produced in each transferred cluster (Fig. 1B,D,F). After 4 months (third transfer), AT120 continued to produce the most repetitive embryo cultures, followed by VC1, 59-4144, and GK7 (Table 2). For all genotypes, once solid clusters of uniform, repetitive embryos were obtained, the number of repetitive clusters tended to

Table 1 Analysis of variance of globular-stage and total embryos/explant and of transferred clusters of embryos that remained repetitive after 4 weeks. [a] (*df* degrees of freedom, *MS* mean square) * Significant, ** Highly significant

	Globular[a]			Total			Repetitive		
Source	*df*	MS	*P*>F	*df*	MS	*P*>*F*	*df*	MS	*P*>*F*
Block (rep)	4	50.22	0.0001**	4	171.74	0.0001**	4	0.06	0.0705
Genotype	3	11.11	0.0477*	3	110.31	0.0001**	3	0.48	0.0001**
Auxin	5	71.68	0.0001**	5	328.75	0.0001**	2	1.42	0.0001**
Genotype × Auxin	15	7.75	0.0317*	15	17.93	0.1198	6	0.07	0.0584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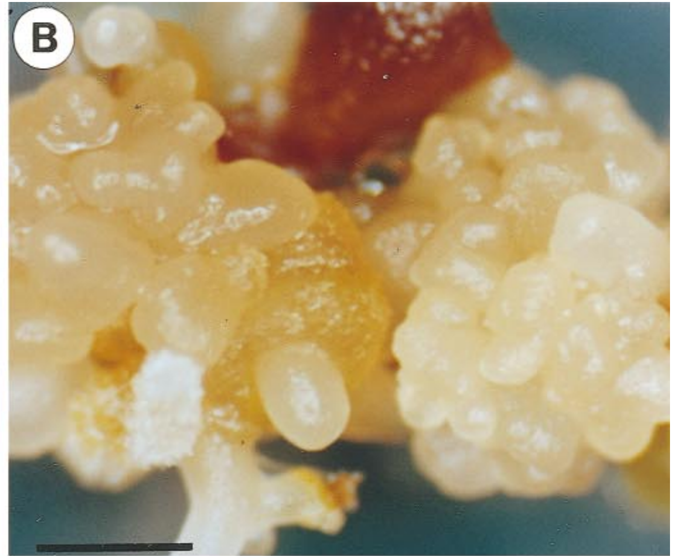
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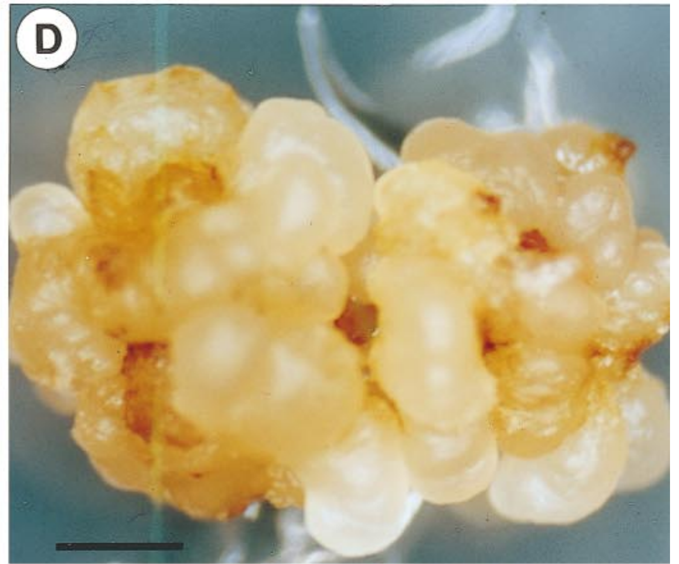
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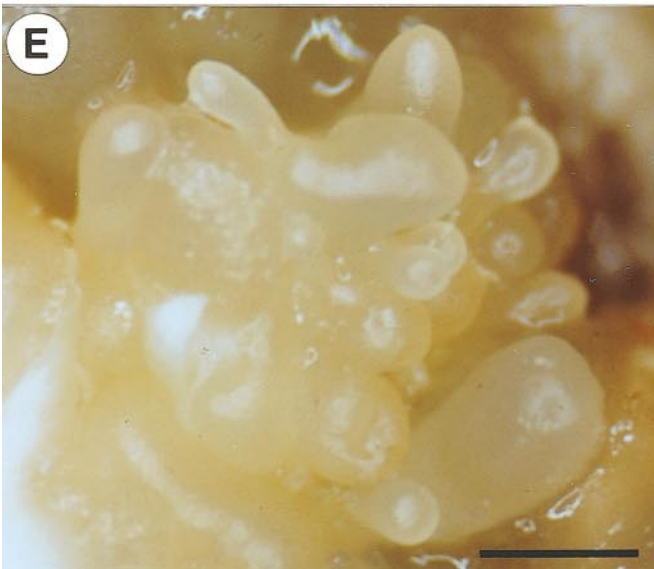
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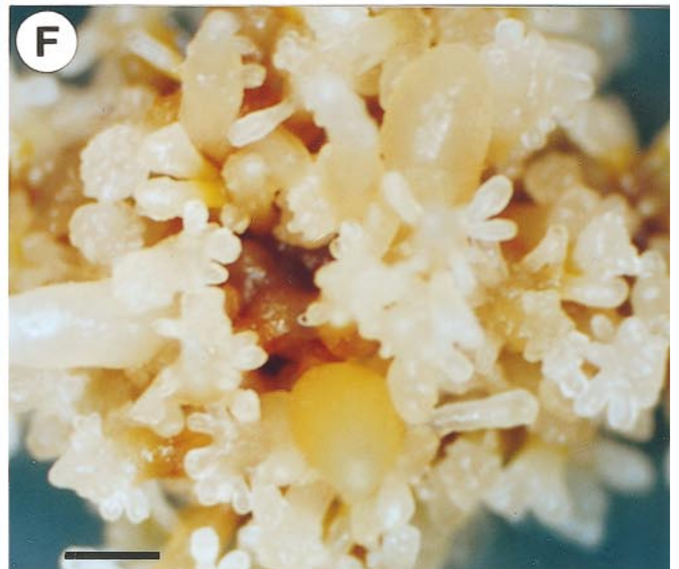
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Fig. 1 Repetitive somatic embryos induced from mature peanut axes on solid medium containing different auxins at various concentrations after 6 weeks (**A, C, E**) or 5 months (**B, D, F**). **A** Peanut cultivar GK7 on 83.0 μM centrophenoxine; **B** AT120 on 124.4 μM centrophenoxine; **C** 59-4144 on 12.4 μM dicamba; **D** AT120 on 83.0 μM dicamba; **E** VC1 on 83.0 μM picloram; and **F** VC1 on 124.4 μM picloram. *Scale bars* 1 mm

increase with each transfer. Genotypic differences for repetitive embryogenesis have been observed previously in peanut (Ozias-Akins et al. 1992) and in soybean (Bailey et al. 1993), but this is the first comparison of the effects of several auxins and concentrations on long-term (5 months) maintenance of repetitive embryogenesis in different genotypes.

In a previous study, picloram at 3 mg/l (12.4 μM) was used to develop repetitive embryogenic cultures on solid medium for use in a gene transformation system (Ozias-Akins et al. 1993). However, the somatic embryos were in various stages of differentiation. In this study, increasing the concentration of picloram to 20 or 30 mg/l (83.0 or 124.4 μM) maintained repetitive globular-stage embryos on solid medium for more than 5 months, and the use of these highly repetitive embryos from solid medium increased the efficiency for the initiation of liquid repetitive cultures suitable for use in a transformation system (data not shown). Such repetitive embryo cultures in liquid media have been

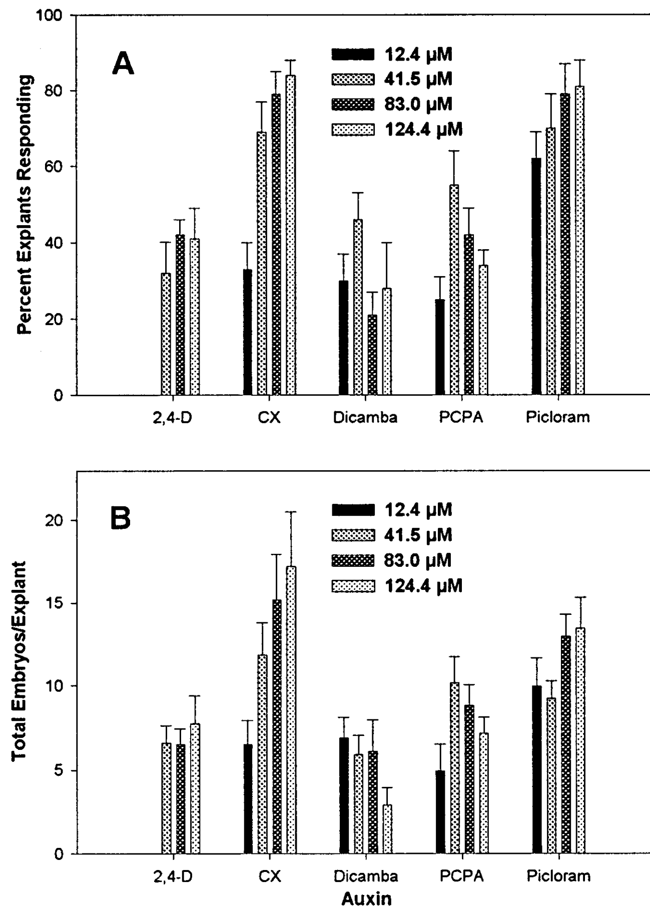

Fig. 2 Effect of auxin type and concentration on **A** percentage of cultivar AT120 explants responding and **B** number of total primary embryos per responding explant after 6 weeks on solid medium containing one of five auxins. *Error bars* Standard error

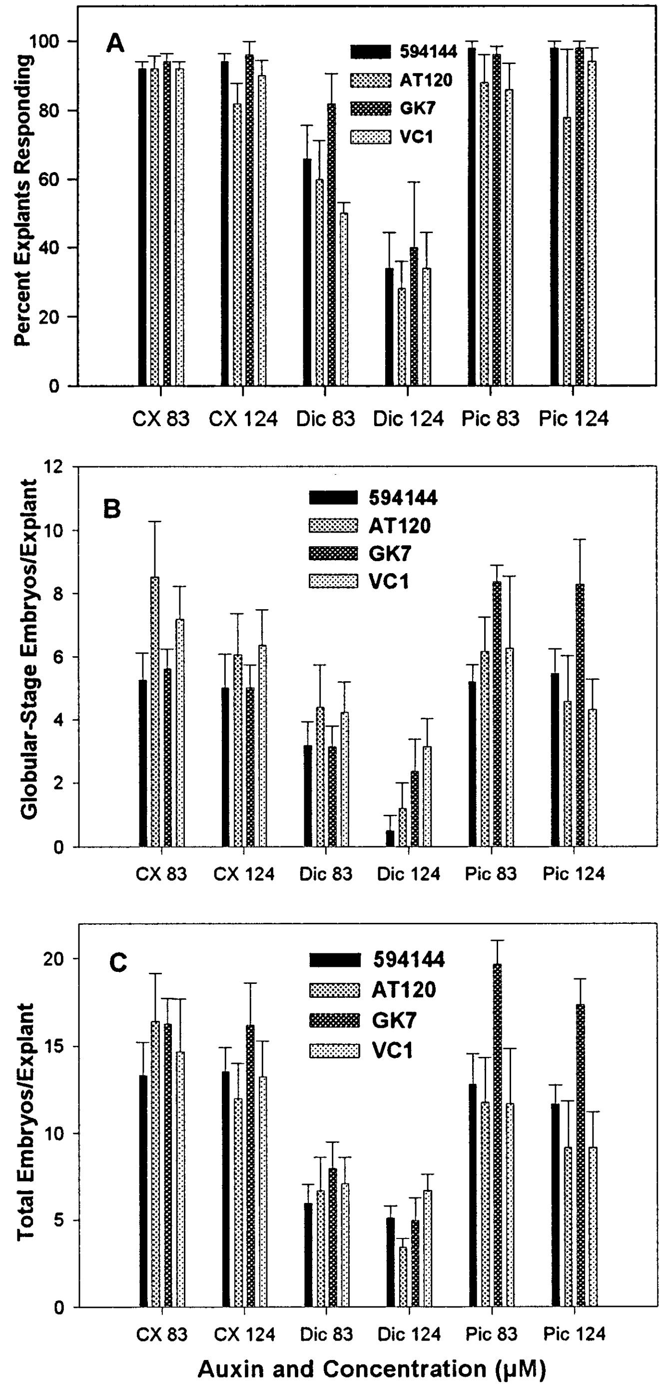

Fig. 3 Effect of auxin type and concentration on **A** percentage of explants that produce primary embryos, **B** the number of primary globular-stage and **C** total embryos/responding explant for four peanut cultivars after 6 weeks on solid medium. *Error bars* Standard error

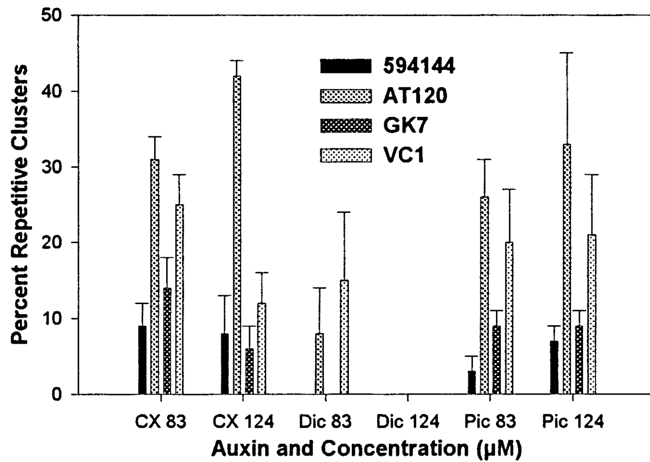

Fig. 4 Percentage of transferred 6-week-old primary embryo clusters exhibiting secondary repetitive globular-stage embryos 4 weeks following transfer to the same medium used for induction. Primary embryos were induced from four peanut cultivars on a medium containing one of three different auxins at one of two concentrations each. *Error bars* Standard error

Table 2 Number of treatment replications (out of five) that maintained repetitive embryos after 4 months in culture in relation to peanut genotype, auxin type, and auxin concentration

	Auxin treatment (μM)[a]			
	Picloram		Centrophenoxine	
Genotype	83.0	124.4	83.0	124.4
AT120	5	4	2	5
VC1	4	4	4	2
594144	1	3	1	3
GK7	1	3	1	0

[a] Only centrophenoxine- and picloram-induced embryos maintained repetitive embryogenesis after 4 months

useful for soybean (Finer and McMullen 1991; Stewart et al. 1996) and peanut transformation (Magbanua et al., submitted).

Cultures of repetitive embryos from genotypes AT120 and VC1 on centrophenoxine and picloram, and all four genotypes on picloram were tested after 5 months for conversion to rooted plantlets (Table 3). The remaining genotype/treatment combinations yielded no cultures with a sufficient amount of repetitive embryos (100 mg/repetition) to be tested for conversion. The combination of genotype VC1 and picloram at 83.0 μM resulted in the best conversion efficiency from mature embryos (59.5%) and also the highest average number of mature embryos/mg of tissue (Table 3). The VC1 somatic embryos on picloram were observed to be smaller and more numerous than the embryos from other treatments (Fig. 1F) which resulted in the high number of mature embryos/mg of tissue. The embryos from 59-4144 and GK7 were the most difficult to maintain in a repetitive state, and the poor conversion rates may be due in part to a deficiency of suitable embryo tissue. However, our conversion frequencies for somatic embryos are at or above the frequencies reported using other peanut somatic embryogenesis protocols (Durham and Parrott 1992; Ozias-Akins et al. 1992; Wetzstein and Baker 1993). In the greenhouse, the percentage of plants that flowered and set seed ranged from 20% to 60% of the total number placed in the greenhouse. The percentage of fertile plants may have been higher, but an infestation of thrips together with an outbreak of tomato spotted wilt virus resulted in severe stunting and death of many plants before their fertility could be determined. Fertility of the peanut plants obtained from somatic embryos induced using other protocols has not been adequately addressed in the literature, although Livingstone and Birch (1999) reported that 53% of transgenic plant lines obtained by particle bombardment of embryogenic callus were fertile.

This study is the most comprehensive evaluation of different auxins reported for the induction of primary and secondary embryos in peanut. It generally confirms previous studies which identified picloram (Eapen et al.

Table 3 Conversion efficiency of repetitive embryo cultures after 5 months on solid medium

Genotype	Auxin (μM)	No. of repetitive cultures converted[a]	Mean no. of mature embryos per culture[b]	% Conversion to plantlets	% Fertility[c]
GK7	Pic 124.4	1	46.0	21.3	20
VC1	Pic 83.0	2	559.5 ± 108	59.5	20
	CX 83.0	2	112.0 ± 32.5	22.9	20
AT120	Pic 124.4	3	114.73 ± 0.6	31.9	40
	CX 124.4	3	190.3 ± 50.5	44.2	20
594144	Pic 124.4	1	106.0	19.5	60

[a] Number of replications (with a maximum of three) from which repetitive embryos were used to test conversion. Only embryos from the treatments that yielded at least 100 mg of embryos were taken through the recovery process. One concentration was used for each auxin-genotype combination

[b] Values are means ± standard error. One hundred milligrams of repetitive somatic embryos from each culture was placed onto maturation medium and the total number of mature embryos was determined at the end of the shoot induction period (12 weeks)

[c] Percentage of plants (out of five) that flowered and set seed in the greenhouse. All plants suffered from severe thrip infestation in the greenhouse

1993; Eapen and George 1993; McKently 1991) and 2,4-D (Baker and Wetzstein 1994; Eapen et al. 1993; Eapen and George 1993) as particularly effective auxins. However, centrophenoxine, which was highly effective in our study, has not been previously identified as a useful auxin for peanut somatic embryogenesis. In contrast to previous studies (Baker and Wetzstein 1994; Eapen et al. 1993; Eapen and George 1993), the use of NAA proved ineffective. This may be due to our use of dry, mature axes as explants, rather than immature embryo explants.

This is also the first report that defines the culture conditions necessary to induce and sustain repetitive embryogenesis in peanut. Our results indicate that a high level (83.0 or 124.4 μM) of picloram or centrophenoxine in solid medium reliably induces highly repetitive globular-stage somatic embryos from mature peanut axes and maintains the long-term (5 or more months) continual production of embryos in the globular stage to serve as a convenient source of tissue for a gene transfer system.

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