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High frequency somatic embryogenesis in peanut (*Arachis hypogaea* L.) using mature, dry seed

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Abstract. Peanut (*Arachis hypogaea* L.) somatic embryos were produced from the embryo axes of mature, dry seeds of cultivar GK-7. Percent embryogenic explants ranged from 88-100% using 10-40 mg/l of 2,4-D in the induction medium. Neither 2,4-D concentration nor photoperiod during the induction period had a large effect on percent embryogenesis, mean number of embryos per explant, or embryo morphology. However, embryos obtained from cultures grown in the dark were easier to remove from the explant than those under a 16-h photoperiod. Somatic embryos developed on the epicotyl portion of the embryo axis, primarily on the young, expanding leaves. A survey of 14 genotypes indicated that genotype had a large influence on embryogenic capacity, with all genotypes being embryogenic to some extent. The ability to recover somatic embryos from axes of harvested, stored seeds represents significant advantages for the establishment of peanut embryogenic cultures, including the use of simple sterilization procedures and a constant source of explant tissue.

Abbreviations: B5: medium of Gamborg *et al.* (1968); 2,4-D: 2,4-dichlorophenoxyacetic acid; MS: Murashige and Skoog (1962) salts medium.

Introduction

A number of recent reports describe somatic embryogenesis in peanut (*Arachis hypogaea* L.) using a variety of different explants, including leaves (Baker and Wetzstein 1992), immature cotyledons (Ozias-Akins 1989, Ozias-Akins et al. 1992, Durham and Parrott 1992, Wetzstein and Baker 1993, Baker and Wetzstein 1994, Eapen et al. 1993, Baker et al. 1994, Baker and Wetzstein 1995), immature embryo axes (Hazra et al. 1989, Ozias-Akins et al. 1992, Ramdev Reddy and Reddy 1993, Eapen et al. 1993), embryo axes collected at harvest (McKently 1991), and whole immature embryos (Sellars et al. 1990).

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Although the use of explants from immature zygotic embryos for somatic embryogenesis and plant regeneration has become routine, several inherent difficulties are encountered in using such materials in peanut. These include the need to maintain appropriately aged parent material in the greenhouse or field, and the need to categorize embryos at specific developmental stages. In addition, high contamination rates are associated with the use of explants from a below-ground organ, the peanut peg. Finally, we have observed seasonal variation in peg development and response of greenhouse-grown plants, making the use of external pod characteristics to estimate embryo developmental stage difficult.

The development of protocols to establish embryogenic cultures using more readily available material would alleviate many of the problems associated with zygotic embryo explants. We describe a simple, high-frequency somatic embryogenic system which uses embryo axes from harvested, dry, stored seeds obtained from commercial sources.

Materials and Methods

Plant Materials. Seeds for all genotypes studied were obtained from Agratech Seeds, Ashburn, GA. The seeds were from pods which had been harvested at maturity, dried to 10.5% moisture, shelled and sized, then stored in a warehouse at ambient temperature, until they were brought into the laboratory. The embryonic axes were removed from the seeds in the laboratory, surface-sterilized for 5 min in 10% commercial bleach (0.5% NaOCl) with 2-3 drops per liter of Tween-20 detergent, and rinsed 3 times in sterile deionized water.

Medium. The basal medium consisted of Murashige and Skoog (1962) salts supplemented with B5 vitamins (Gamborg et al. 1968), 3% sucrose, and 0.4% Gel-gro (ICN Biochem., Irvine, CA) or 0.2% Gelrite (Merck and Co., Rathway, NJ). Growth regulators were used as described for the different experiments. Culture media were adjusted to pH 5.8 and autoclaved at 121°C and 105 kPa for 20 min.

Optimization Experiments. Seeds of cultivar GK7 were brought to the laboratory and stored at 4°C until used. Axes were placed horizontally in the medium, with 3 explants per 100 × 15 mm Petri dish containing 25 ml medium. After 30 d on induction medium, all explants were transferred to basal medium.

In experiment 1, whole embryo axes were placed on induction medium, consisting of basal medium supplemented with either 20 or 40 mg/l 2,4-D. Cultures were maintained in the dark, or under a 16-h photoperiod provided by cool white fluorescent lights at 130 μmol · m^{-2} · s^{-1}. In experiment 2, intact embryo axes or bisected embryo axes were used as explants. In the bisected explants, the apical portion (Fig. 1) contained the upper one-third of the axis, while the radicle portion comprised the lower two-thirds of the axis. Explants were placed on basal medium with 2,4-D at either 10, 20, 30 or 40 mg/l for induction. Cultures were maintained in the dark. All experiments were conducted at 26°C.

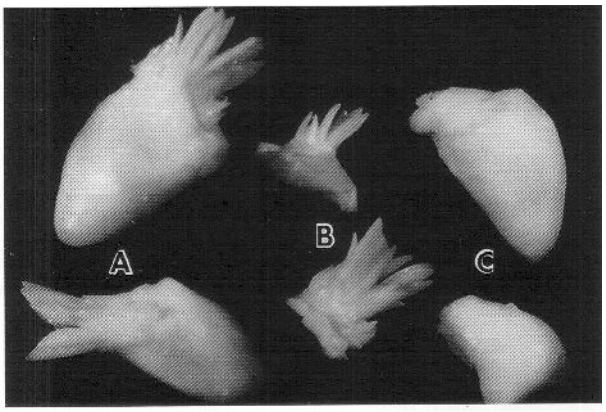

Fig. 1. Various explants used in these studies were isolated from mature dry seed of peanut. A. Whole embryo axis. B. epicotyl portion of axis. C. Radicle portion of axis.

After 8 weeks on basal medium, data were collected on percentage explants giving rise to embryos, and the number of embryos formed per explant. The morphology of somatic embryos was categorized using the system developed by Wetzstein and Baker (1993). Briefly, embryos were classified as follows: Class 1: single torpedo dicotyledonous embryos, Class 2: single torpedo multiple foliar embryos, Class 3: single tubular embryos, Class 4: embryos with broadened and distorted hypocotyls, Class 5: multiple fused embryos with individual embryo axes distinct, Class 6: multiple fused fan-shaped embryos, Class 7: precociously germinated embryos from any of the above classes. Data were analyzed by PC SAS (v. 6.03, SAS Institute Inc., Cary, NC 1990), using the PROC GLM procedure for a randomized complete block design with 10 replications, after transforming embryo count data using square root of (embryo count + 0.5). Mean separations were performed using Duncan's multiple range test.

For germination and conversion, individual somatic embryos were placed in GA7 boxes (Magenta Corp., Chicago, IL) containing 50 ml basal medium. Cultures were placed under a 16-h photoperiod with light and temperature conditions as previously described. Resulting plants were transferred into a 1:1 (v/v) mixture of perlite:vermiculite and acclimatized by gradually removing a plastic bag which covered the plant.

Genotype Survey. To test the response of various genotypes (listed in Table 3) for production of somatic embryos, axes from mature seed were placed on basal medium containing 30 mg/l 2,4-D. Twenty embryo axes were plated per 100 × 20 mm polystyrene Petri dish, and a total of 100 axes were plated per genotype. The cultures were maintained at 24 ± 1 °C, with a 23-h photoperiod, provided by cool-white fluorescent lights at 87 μmol·m^{-2}·s^{-1}. After 30 d of culture, plates were evaluated for the number of axes responding and the number of somatic embryos produced per axis. The 30 d period was chosen because this is the age at which somatic embryos would be used for the initiation of proliferative embryogenic cultures in liquid medium (Durham and Parrott 1992). Standard errors were computed using each Petri dish as a replicate. When contamination occurred, the entire dish was excluded from data collection.

Results and Discussion

Using axes of dried seed, an unlimited number of easily dissected explants are available. Only a simple sterilization using NaOCl is required. This is in contrast to our experience with immature pegs or cotyledon explants, in which contamination rates are invariably high. For greenhouse or field-collected fruit, others have prescribed the use of more environmentally toxic disinfectants such as mercuric chloride (Hazra et al. 1989, Ramdev Reddy and Reddy 1993, Eapen et al. 1993) or the need for multiple sterilizations during explant dissection (McKently 1991, Ozias-Akins 1989), often after extensive washing.

The only prior report of embryogenesis from dry, mature peanut axes (George and Eapen 1993) made no attempts to optimize embryogenic parameters for this particular explant. Otherwise, all other previous studies have used immature axes or axes from freshly harvested seed as explants, which presents the host of difficulties discussed previously. Hazra et al. (1989) reported embryo axes 3-6 mm long were a particularly embryogenic stage, while Ramdev Reddy and Reddy (1993) induced somatic embryos from embryo axes (2-5 mm long) taken from 30- to 40-d-old immature pods. Embryo axes 3 mm long had more somatic embryos per explant than axes which were 1 or 5 mm long. McKently (1991) obtained embryogenic cultures using embryo axes collected from greenhouse-grown plants at time of harvest. Ozias-Akins et al. (1992) evaluated genotype effects on somatic embryogenesis from cotyledons and axes of different developmental stages. Maturity of the embryo explant did not show a significant effect on response, although there was a trend toward reduced embryo formation on older material. Our results showed that mature seed embryos were highly responsive in culture.

Optimization experiments

Experiment 1. The percentage of axes with somatic embryos was 89-100% in all photoperiods and growth regulator treatments (Table 1). Most embryos formed on the epicotyl portion of the axis, particularly on the expanding, young leaves. At 4 weeks after initiation when cultures were transferred to basal medium, leaf blades exhibited extensive tissue proliferation and an increase in thickness. By 3 d on basal medium, raised embryogenic regions were evident (Fig. 2). In contrast, the radicle area rarely formed somatic

Table 1. Quantitative and qualitative effects of photoperiod and 2,4-D concentration during induction on peanut epicotyl explants, 8 weeks after induction.

Photo-period	2,4-D (mg/l)	No. explants	Percentage embryogenic cultures[a]	Mean no. embryos per explant	Percentage embryos in morphological classes				
					No. embryos	Class 1,2	Class 3	Class 4,5,6	Class 7
0-h	20	28	96 a	7.6 a	212	1	2	79	18
	40	28	89 a	6.4 a	167	4	2	87	7
16-h	20	23	100 a	5.7 a	131	0	0	76	24
	40	26	96 a	4.6 a	120	0	0	77	23

[a]For a given photoperiod, means within a column followed by the same letter are not significantly different at $p \leq 0.1$.

embryos, and either formed a root or non-embryogenic callus. Somatic embryos were densely arranged on the explants after 4 weeks on basal medium (Fig. 3). There were no statistical differences in mean number of embryos produced per explant between different auxin concentrations for induction within photoperiod treatments. Few somatic embryos were single, bipolar morphological types (Classes 1,2,3) (Table 1). Over 75% of the embryos had broadened hypocotyls or were fasciated/fused (Classes 4,5,6).

Photoperiod had little effect on percentage embryogenesis or mean number of embryos per explant. However, a greater incidence of precocious germination and proliferation of non-embryogenic callus was observed in cultures grown under a 16-h photoperiod. These embryos were less succulent and more difficult to separate from the explant than those from dark-grown cultures. In light-grown cultures, a fluffy, white, non-embryogenic callus was prevalent, which eventually covered the somatic embryos. Similar callus proliferation was absent in cultures grown in the dark. Baker et al. (1994) observed similar morphological characteristics in peanut immature cotyledon somatic embryogenic cultures grown in light versus dark conditions. Others have described aberrant embryo development under some light conditions (Lazzeri et al. 1987, Michler and Lineberger 1987, Ammirato 1974).

Experiment 2. The concentration of 2,4-D during induction had little effect on the percentage of embryogenic cultures or on mean embryo numbers, regardless of explant type or light regime. Using whole embryo axes, from 89-100% of the explants were embryogenic with the 2,4-D levels used during induction (Table 2), although the mean number of embryos was lower when 10 mg/l 2,4-D were used for induction. In our other studies with axes, frequency of embryogenesis and mean number of somatic embryos per explant were not significantly lower until a level of 60 mg/l was used during induction (data not shown).

During culture, the axis typically enlarged and produced some callus. As observed in Experiment 1, somatic embryos formed primarily at the epicotyl portion of the axis. Embryos were prevalent on the expanding immature leaves. In treatments in which the axis was cut and the epicotyl portion used as the explant, embryogenesis was over 88% (Table 2). Percentage embryogenesis decreased slightly when 40

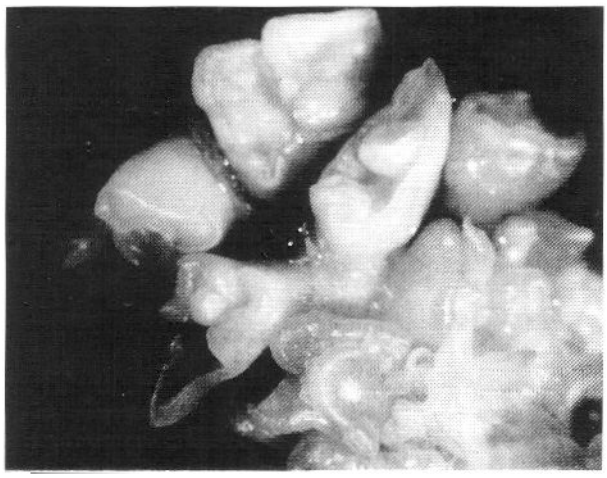

Fig. 2. Epicotyl portion of a peanut mature seed embryo axis explant after 3 d on basal medium following induction. Leaves have expanded and increased in thickness. Embryogenic regions are evident on leaflet blades.

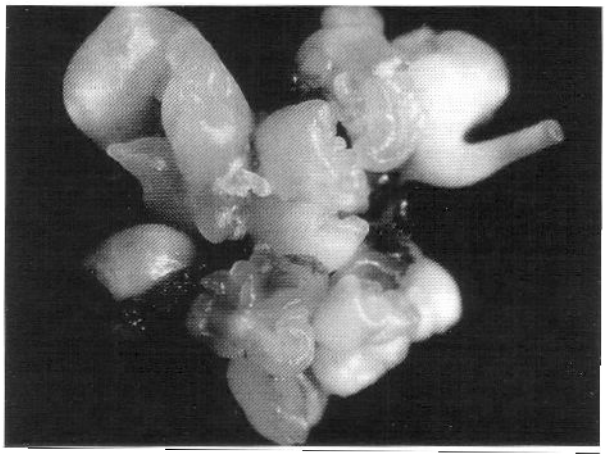

Fig. 3. Somatic embryogenic culture of peanut obtained from an epicotyl portion explant induced on medium with 30 mg/l 2,4-D, then transferred to a basal secondary medium for 30 d.

mg/l 2,4-D was used during induction. There were no differences in mean numbers of embryos produced due to induction auxin level. Callus proliferation of non-embryogenic tissues was also less prevalent. Radicle explants, in contrast to the other explant types, exhibited very low levels of embryogenesis (Table 2) and generally browned, callused over and/or produced roots.

The distribution of embryos in morphological classes was not affected by the range of concentrations of 2,4-D tested during induction. Although a few embryos were typically bipolar, the majority were fused, fasciated types. Such embryos often have multiple meristematic regions and convert into plants exhibiting multiple shoots. 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. Bipolar and broad-fasciated embryos converted at higher rates. Two percent or less of the somatic embryos were the typical single, torpedo-shaped forms found in zygotic development (Table 2). Most embryos were broadened in morphology, with distorted or fasciated hypocotyls. Tubular embryos made up 0 - 5% of the embryos.

The 2,4-D concentration or explant type (whole axis or epicotyl portion) did not affect germination frequency (data not shown), while morphology did, as discussed previously (Wetzstein and Baker 1993). About 30 - 35% of somatic embryos transferred to basal medium in GA7 boxes for germination exhibited shoot and root growth (data not shown), and of these, most converted into plants. We consistently have achieved conversion rates ranging from 15% across all classes to 40% for Classes 1 and 2, as reported previously (Durham and Parrott 1992, Wetzstein and Baker 1993). Once somatic embryos have been obtained, plants from them have been established in a greenhouse within 2 months. Regenerated plants exhibited the morphological characteristics of the cultivar. Using this method, over 150 plants have been hardened and transferred to soil (Fig. 4). Some plants were allowed to set seed, with the resulting R_1 progeny being uniform and appearing true to GK7 cultivar type in the field.

Genotype Survey

Because liquid embryogenic cultures can produce virtually unlimited numbers of somatic embryos (Durham and Parrott 1992), we chose to evaluate the genotypic study at 4 weeks, the stage at which embryos would be used to establish embryogenic liquid cultures. Consequently, the number of embryos per explant was lower than it would have been after 8 weeks, the time period used for the first two experiments.

Genotype effects were apparent for the production of somatic embryos from mature embryonic axes of

Table 2. Quantitative and qualitative effects of peanut explant type and 2,4-D concentration during induction on peanut epicotyl explants at 8 weeks after induction. Cultures were induced and maintained in the dark.

Explant	2,4-D (mg/l)	No. explants	Percentage embryogenic cultures[a]	Mean no. embryos per explant	Percentage Embryos in morphological classes				
					No. embryos	Class 1,2	Class 3	Class 4,5,6	Class 7
Whole axis	10	18	100 a	6.6 b	119	0	3	88	9
	20	18	89 a	9.2 a	166	0	2	92	6
	30	24	91 a	9.6 a	230	2	1	93	4
	40	22	91 a	8.6 a	189	1	5	86	8
Epicotyl portion	10	26	96 a	9.1 a	235	1	1	86	12
	20	26	100 a	11.3 a	295	0	1	88	11
	30	26	100 a	11.5 a	300	0	0	89	11
	40	26	88 b	10.1 a	260	0	0	90	10
Radicle portion	10	19	5 a	7.0 a	-[b]	-	-	-	-
	20	23	4 a	2.0 a	-	-	-	-	-
	30	21	5 a	7.0 a	-	-	-	-	-
	40	21	0 a	0 a	-	-	-	-	-

[a] For a given explant, means within a column followed by the same letter are not significantly different at $p \leq 0.1$.
[b] Not done because embryos from radicle portion explants were too few for evaluation

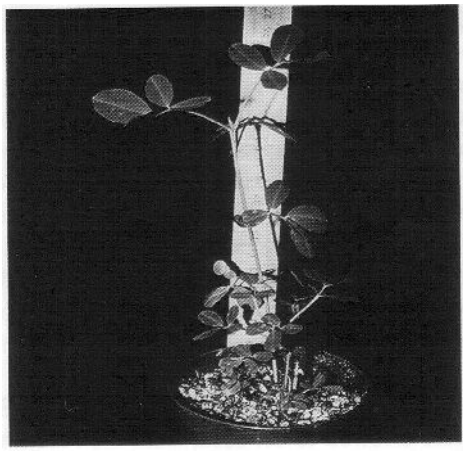

Fig. 4. Peanut plant regenerated from a somatic embryo derived from an epicotyl axis explant.

peanut (Table 3). The response ranged from 5.2 embryos/axis for genotype AT108 to 1.9 embryos/axis for genotype B18-63. The percentage of axes responding also varied from 10-47%, but this response was not correlated (r^2=0.12) to the number of embryos produced per axis.

Genotype remains the factor with the greatest influence on frequency of somatic embryogenesis from mature axes. Genotypic effects had previously been reported for embryogenesis from other explants of peanut (Sellars et al. 1990, Ozias-Akins et al. 1992, George and Eapen 1993). Nevertheless, in no genotype was the frequency of embryogenesis low enough to be a limiting factor.

Conclusions

This study shows that embryo axes from mature seed are a convenient, accessible, and efficient explant for somatic embryogenesis in peanut, that can be applied to a wide range of genotypes. Advantages of this system are ease in obtaining explant material, lack of need to maintain greenhouse or field plantings, low rate of contamination (0-5%), high frequency of embryogenic explants, and year-round availability of seed. Using only the epicotyl portions as explants, instead of the whole embryo axes, reduced the proliferation of non-embryogenic callus which often originated from the radicle portion of the axis. An advantage observed in epicotyl explants was the greater proportion of explant tissue which was embryogenic. Isolating the epicotyl region from the axis also introduced a wound site, which may be of advantage in *Agrobacterium*-mediated transformation applications.

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Table 3. Genotype effects on peanut somatic embryo production from mature embryonic axes, 30 d after being placed on induction medium.

Genotype	Percentage of axes responding	Average no. somatic embryos per responding axis ± SE
Florunner	10	2.67 ± 0.33
Arkansas Valencia	16	3.44 ± 1.16
F435AT[a]	18	3.67 ± 0.87
GK7	18	3.45 ± 0.52
NC7	18	4.05 ± 0.74
B18-63[b]	19	1.88 ± 0.52
GK3	25	2.43 ± 0.43
Georgia Runner	26	2.54 ± 0.65
27-6772	27	2.83 ± 0.36
GK19R-1	28	2.76 ± 0.46
GK17	29	2.78 ± 0.46
VC-1	31	4.72 ± 0.66
AT108	33	5.21 ± 1.19
1252[c]	47	3.93 ± 0.44

[a] Contains high oleic acid content
[b] Near isoline of F435AT, lacking high oleic acid
[c] BC_3 of high oleic acid trait into Sunrunner

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