In Vitro Reproductive Development of a Diploid Wild Species, *Arachis duranensis*[1]

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ABSTRACT

Embryo abortion at an early stage of reproductive development is a major impediment for introgressing germplasm from wild to cultivated species of *Arachis* by interspecific hybridization. Ovule and embryo culture techniques have been used to rescue aborting hybrid embryos, but increased efficiency and recovery of very young tissues are still needed. The objective of this study was to induce growth and differentiation of *A. duranensis* proembryos. Seven-, 10-, and 14-d-old peg tips were cultured on a modified basal medium containing MS and B_5 media combinations with 16 combination treatments using three growth regulators—1-naphthaleneacetic acid, gibberellic acid, and 6-benzylaminopurine—each at four levels. The results showed that seeds could be obtained *in vitro* by peg tip culture of four- to 16-celled proembryos. The favorable concentration ranges of growth regulators for pod formation and embryo development were 0.5-2.0 mg/L NAA, 0.05-0.5 mg/L GA_3, and 0.05-0.2 mg/L 6-BAP. Over all three selected ages of pegs, the three best combinations of growth regulators resulted in 4.8, 4.7, and 3.5% pod formation, respectively.

Key Words: Embryo, pod, peg, *in vitro* culture, tissue culture, peanut, *Arachis*, *Arachis duranensis*, wild species.

Embryo abortion is a major cause for failure of many interspecific hybrids between *Arachis hypogaea* L. and diploid *Arachis* species. One approach to rescue the hybrid embryos is *in vitro* culture of reproductive tissues before they abort. Bajaj *et al.* (1) reported the regeneration of hybrid plants from 30-d-old embryos between *A. hypogaea* and *A. villosa* Benth. Sastri *et al.* (10) obtained hybrid plants of *A. monticola* Krapov. and Rigoni x *A. glabrata* var. *glabrata* Benth. from heart-shaped or early cotyledonary embryos. Stalker and Eweda (11) recovered two hybrids from 30-d-old embryos of a cross between an *Arachis* hexaploid interspecific hybrid and a diploid species. These studies indicated the feasibility of rescuing differentiated embryos of interspecific hybrids. However, abortion of proembryos prior to development of heart-shaped embryos often occurs (4,5).

In attempts to develop techniques to rescue proembryos from selfed one- or two-celled proembryos of *A. hypogaea*, Pattee *et al.* (8) and Moss *et al.* (6) were able to obtain multicellular globular embryos. Ziv and Sager (12) achieved *in vitro* growth of selfed embryos of *A. hypogaea* and produced viable plants by a two-step process. Recently, proembryos of *A. hypogaea* were cultured in a one-step process from which mature plants were recovered by Feng (unpubl. data, 1994). The purpose of this investigation was to apply *in vitro* culture techniques to promote development of proembryos of a diploid wild species and induce differentiation to recover mature plants.

Materials and Methods

Plants of the wild diploid ($2n=2x=20$) species, *A. duranensis* Krapov. and W.C. Gregory (K 7988, PI 219823) were grown in a greenhouse at North Carolina State Univ., Raleigh, NC during the summers of 1992 and 1993 in 15 x 15-cm pots. Selfed flowers were marked daily with colored tags and aerial pegs were collected 7, 10 and 14 d later. Pegs were rinsed in running water for 5 min, surface-sterilized in 70% ethanol for 10 sec and 0.1% mercuric chloride for 8 min, and then washed three times for 10 min each with sterile distilled water. Peg tips were cut 10 mm from the apex, which left both the ovules and meristem within the specimen. Pegs were placed into 2.5 x 15-cm culture tubes by inserting 2 to 3 mm of the cut end vertically into the medium with the apex end upward. The basal medium contained the inorganic components of MS medium (7), the organic components of B_5 medium (3) plus 0.3 g/L casein hydrolysate and 60 g/L sucrose. All media were adjusted to pH 5.8 and solidified with Difco Bacto-agar at 6 g/L. Plant growth regulator treatments consisted of four levels as follows: 0.5, 1.0, 2.0, 4.0 mg/L 1-naphthaleneacetic acid (NAA); 0.05, 0.10, 0.50, 1.00 mg/L gibberellic acid (GA_3); and 0.05, 0.10, 0.20, 0.50 mg/L 6-benzylaminopurine (6-BAP). The 16 treatments were arranged so that each growth regulator was combined once with each level of the other two compounds (Table 1). A control with the basal medium without growth regulators was included. The cultures were placed in the light for 1 d and then kept in the dark at 27±1 C for 90 d in a growth chamber except for brief periodic observations. Three replications of 10 peg tips for each day and media combination were cultured, and observations were made after contaminated explants were discarded.

Peg elongation, peg tip swelling, pod formation, ovule growth, fresh weight of callus, and root number were scored at the end of 90 d. Pods were distinguished from tip swelling by a size of at least 3 x 2 mm (length x width) and netted veins on the pericarp surface.

Ovules or seeds were aseptically isolated from pods and subcultured on MS media containing 60 g/L sucrose, 0.3 g/L casein hydrolysate, 0.2 mg/L 6-BAP, and either 0.05 mg/L GA_3 or NAA. If seeds did not germinate within 1 mo, they were treated for 1 wk with a combination of 100 ppm ethylene and 5 mg/L each of GA_3 and 6-BAP and placed in soil until they germinated or died.

To histologically determine the stage of embryo development, swollen tips or ovules from enlarged pods were fixed in FAA (70% ethanol:glacial acetic acid:formalin, 18:1:1). Specimens were dehydrated in an alcohol series and paraffin embedded according to Berlyn and Miksche (2). The tissues were sectioned at 7 mm thickness, stained with safranin-fast green and prepared for observation under light microscopy. The experimental design for this study was a randomized complete block and the GLM procedure of SAS (9) was used for statistical analysis.

Results

Seven-d-old pegs of *A. duranensis* were 1 to 2 cm long and had three- to four-tier embryos (Fig. 1A and D) rapidly elongating. Ten-d-old pegs were 3 to 5 cm long and 14-d-old pegs generally were longer than 5 cm. Embryos of 10- (Fig. 1B and E) and 14-d-old pegs (Fig. 1C and F) consisted of four-tiers and eight to 16 cells. Basal embryos were more developed than apical ones in 7-d-old pegs, whereas in 10- and 14-d-old pegs, apical and basal embryos appeared to be at the same stage of development.

Between 2 and 3 d after the peg tips were placed in medium they started geotropic curvation and initiated elongation. Most peg tips, including 14-d-old pegs which

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Table 1. *In vitro* reproductive traits of *A. duranensis* peg tips excised 7, 10, and 14 d after pollination in different treatments of plant growth regulators for 90 d culture.

Trt.	Growth regulator NAA	GA_3	6-BAP	Peg tip age	Obs.	Peg length	Callus fresh wt	Roots/ peg	Tip swelling	Pod for-mation[b]
No.	--- mg/L ---			d	No.	cm	g/peg	Avg no.	%	%
1.	0.5	0.10	0.20	7	29	4.1	0.8	0.1	13.8	3.5
				10	27	4.3	0.7	0.0	14.8	3.7
				14	29	3.8	0.8	0.0	6.9	6.9
2.	2.0	1.00	0.05	7	30	3.7	1.7	1.9	11.0	3.3
				10	27	3.7	1.2	0.8	11.1	0.0
				14	29	3.6	1.3	0.3	10.3	0.0
3.	1.0	1.00	0.20	7	26	4.5	1.0	0.0	11.5	3.9
				10	23	3.8	1.0	0.0	4.4	0.0
				14	28	3.3	1.0	0.1	7.1	0.0
4.	4.0	0.10	0.05	7	24	3.4	2.5	5.2	4.2	0.0
				10	27	3.0	1.9	3.0	0.0	0.0
				14	29	3.4	2.5	2.2	3.5	0.0
5.	0.5	0.50	0.05	7	30	5.3	0.7	0.1	20.0	6.7
				10	27	4.2	0.5	0.0	14.8	3.7
				14	29	4.1	0.7	0.0	6.9	0.0
6.	2.0	0.05	0.20	7	28	4.0	2.1	0.4	3.6	3.6
				10	28	3.6	1.9	0.1	10.7	7.1
				14	28	3.3	2.2	0.4	28.6	3.6
7.	1.0	0.05	0.05	7	28	3.7	1.6	0.4	0.0	0.0
				10	29	4.2	1.4	0.0	0.0	0.0
				14	27	4.1	1.3	0.0	3.7	3.7
8.	4.0	0.50	0.20	7	29	3.6	1.7	0.4	3.5	3.5
				10	29	3.3	1.2	0.2	10.3	0.0
				14	29	3.4	2.0	0.2	17.2	0.0
9.	0.5	0.05	0.50	7	27	4.3	1.1	0.0	11.1	0.0
				10	26	3.7	1.0	0.0	11.5	3.9
				14	25	3.4	1.1	0.0	12.0	0.0
10.	2.0	0.50	0.10	7	29	3.7	1.4	0.1	10.3	0.0
				10	28	3.6	1.4	0.0	7.1	3.6
				14	28	3.5	1.5	0.1	14.3	0.0
11.	1.0	0.50	0.50	7	25	4.4	1.1	0.0	8.0	4.0
				10	24	3.5	1.2	0.0	4.2	4.2
				14	24	3.4	1.1	0.0	4.2	0.0
12.	4.0	0.05	0.10	7	27	2.9	2.5	3.3	7.4	0.0
				10	16	3.4	2.5	3.8	6.3	0.0
				14	26	3.2	2.1	1.4	3.9	3.9
13.	0.5	1.00	0.10	7	28	5.3	0.6	0.0	3.6	0.0
				10	19	4.8	0.7	0.0	0.0	0.0
				14	29	3.4	0.4	0.0	0.0	0.0
14.	2.0	0.10	0.50	7	23	3.9	2.0	0.0	4.4	0.0
				10	19	3.8	2.4	0.1	5.3	0.0
				14	29	3.3	2.1	0.0	10.3	0.0
15.	1.0	0.10	0.10	7	19	4.6	1.4	0.1	5.3	5.3
				10	27	4.2	1.3	0.0	7.4	3.7
				14	28	3.7	1.5	0.0	3.6	0.0
16.	4.0	1.00	0.50	7	16	4.8	2.5	0.0	0.0	0.0
				10	24	3.1	2.3	0.0	12.5	4.2
				14	27	3.2	2.0	0.0	11.1	0.0
17.	Control			7	22	4.8	0.0	0.0	0.0	0.0
				10	27	4.0	0.0	0.0	0.0	0.0
				14	29	3.0	0.0	0.3	0.0	0.0
Overall avg						3.8	1.4	0.5	7.7	1.8
LSD (P = 0.05)										
Between treatments						1.1	0.4	0.6	4.8	1.4
Between ages						0.5	0.3	0.2	3.2	1.1

[a]Observations were total numbers of peg tip explants for three replications after contaminated explants were discarded.

[b]Pod formation was distinguished from tip swelling by significant tissue enlargement to at least 3.0 x 2.0 mm (length x width) and having netted veins formed on the pericarp surface.

almost ceased elongation *in vivo*, rapidly elongated into the medium (Table 1). At 0.5 mg/L, NAA promoted peg elongation but, with increased levels, elongation was suppressed at all ages of pegs for all peg stages. GA_3 had significant positive effects on elongation at 7 d. 6-BAP had no significant effects at day 7 except for the 0.50-mg/L level. GA_3 and 6-BAP effects on elongation are confounded at 10- and 14-d ages because of peg shrinkage (Table 2).

In general, higher levels of NAA significantly ($P < 0.05$) enhanced callus production, whereas GA_3 had an opposite effect (Table 2). 6-BAP at all tested levels induced a large amount of callus (Table 2). When excessive callus was

Table 2. Effects of NAA, GA_3, and 6-BAP at different levels on *in vitro* reproductive traits of peg tips of *A. duranensis* after 90 d culture.

Level	Peg tip age	Peg length	Callus fresh wt	Swelling	Pod formation	Roots/ peg
mg/L	d	cm	g/peg	%	%	Avg no.
			NAA			
0.5	7	4.8	0.8	12.3	1.8	0.0
	10	4.2	0.7	7.1	3.0	0.0
	14	3.7	0.8	6.3	1.8	0.0
1.0	7	4.2	1.3	6.1	3.1	0.1
	10	4.0	1.3	2.0	1.0	0.0
	14	3.6	1.3	4.7	0.9	0.0
2.0	7	3.8	1.8	7.3	1.8	0.6
	10	3.7	1.7	8.7	1.9	0.2
	14	3.4	1.8	15.8	0.9	0.2
4.0	7	3.5	2.3	4.2	1.0	2.4
	10	3.2	1.9	7.3	1.0	1.5
	14	3.3	2.1	9.0	0.9	1.0
			GA_3			
0.05	7	3.7	1.8	5.5	0.9	1.0
	10	3.8	1.6	7.1	4.0	0.7
	14	3.5	1.7	12.3	1.9	0.5
0.10	7	4.0	1.6	7.4	2.1	1.4
	10	3.9	1.5	3.0	1.0	0.8
	14	3.6	1.7	6.1	1.7	0.6
0.50	7	4.2	1.2	10.6	2.7	0.2
	10	3.6	1.1	7.4	0.9	0.1
	14	3.6	1.3	10.9	0.0	0.1
1.00	7	4.5	1.3	7.0	1.0	0.6
	10	3.8	1.3	7.5	1.1	0.2
	14	3.4	1.2	7.1	0.0	0.1
			6-BAP			
0.05	7	4.1	1.6	8.9	2.7	1.7
	10	3.8	1.3	4.5	0.9	1.0
	14	3.8	1.5	6.1	0.9	0.7
0.10	7	4.1	1.5	6.8	1.0	0.9
	10	4.0	1.4	3.3	2.2	0.7
	14	3.4	1.4	5.4	0.9	0.4
0.20	7	4.0	1.4	8.0	3.6	0.2
	10	3.7	1.2	8.5	2.8	0.1
	14	3.5	1.5	14.9	2.6	0.2
0.50	7	4.3	1.6	6.6	1.1	0.0
	10	3.5	1.7	8.6	2.2	0.0
	14	3.3	1.6	9.5	0.0	0.0
LSD (P = 0.05):						
Between ages		0.3	0.3	5.3	0.6	0.5
Between levels		0.1	0.2	4.6	0.4	0.3

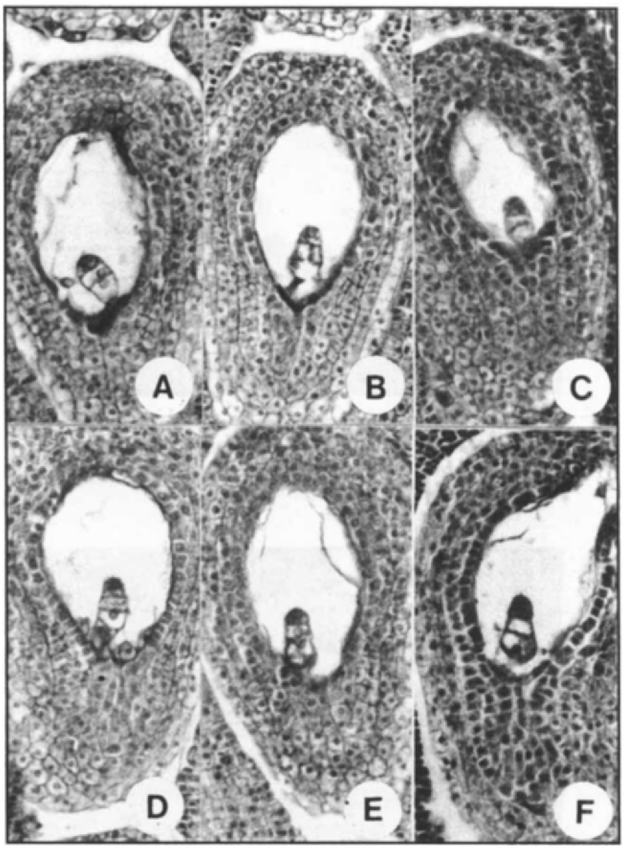

Fig. 1. Longitudinal sections of embryo sac and proembryo of *A. duranensis* at three collection stages: (A) 7-d-old apical embryo at three-tier stage; (B) 10-d-old apical embryo at four-tier stage; (C) 14-d-old apical embryo at four-tier stage; (D) 7-d-old basal embryo at four-tier stage; (E) 10-d-old basal embryo at four-tier stage; (F) 14-d-old basal embryo at four-tier stage.

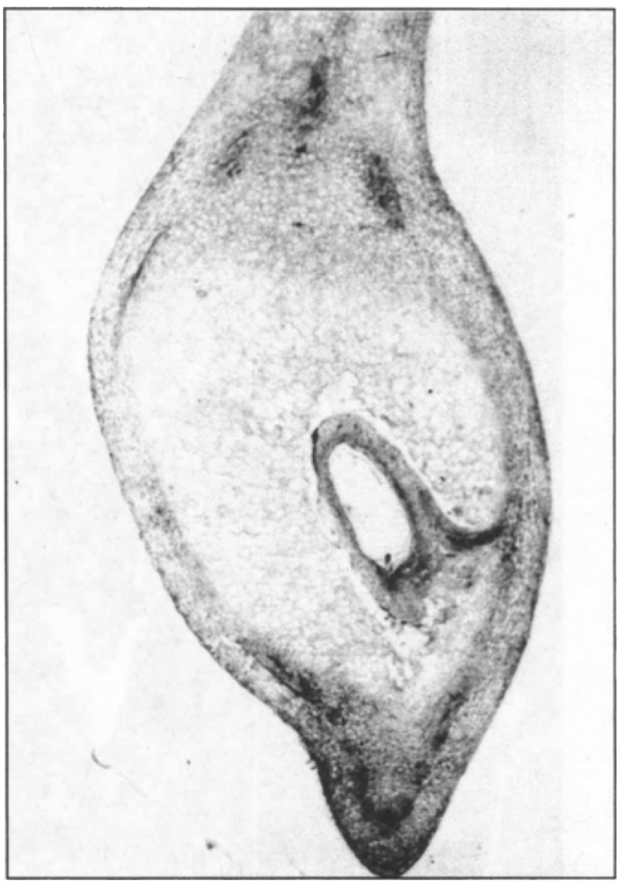

Fig. 2. An *in vitro*-cultured peg tip, showing the enlarged ovule with a globular embryo and cell expansion of pericarp.

present, a corresponding suppression of peg elongation and pod formation was observed. Roots appeared to originate either from callus or the cut end of peg tip explants. NAA induced, while GA_3 and 6-BAP inhibited, root formation with increased concentrations (Tables 1 and 2).

Overall, 7.7% (103/1340) of the explants swelled across all treatments (Table 1). Swelling occurred at the basal area of the ovary and was the result of both pericarp expansion and ovule growth (Fig. 2). No significant difference in swelling was observed among the three ages, and no significant relationship between peg swelling and growth regulator concentration was observed. After 90 d of culture, most basal embryos had grown to the globular stage, whereas apical embryos remained at the 8- to 16-cell stage. Eleven ovules isolated from swollen tips and subcultured turned brown and then died within a month; whereas three ovules turned green and produced callus, but later died. Histological observation indicated that these embryos remained at the globular stage.

All pods developed from the basal locule of the ovary. Nine, 9, and 6 pods were recovered from 7-, 10-, and 14-d-old peg tips, respectively. They had an average size of 7.2 mm long x 3.9 mm wide (Fig. 3A-C). Secondary meristematic activity was initiated between the basal and apical ovule of most pods which also resulted in elongating peg tissues beyond the basal ovule (Fig. 3B). Although no significant overall relationship between pod formation and levels of growth regulators was found, the most suitable levels of GA_3 for pod formation ranged from 0.05 to 0.5 mg/L, of 6-BAP from 0.05 to 0.2 mg/L, and of NAA from 0.5 to 2 mg/L (Table 2). However, pod formation varied with different growth regulator treatments (Table 1). The highest rates of pod formation occurred in treatments 1, 5, and 6 across all three ages, where an average of 4.7, 3.5, and 4.8% pods with lengths x widths of 8.8 x 3.8, 8.7 x 5.2, and 5.3 x 2.9 mm, respectively, were observed.

Fourteen large ovules (average size 2.9 mm long x 1.5 mm wide) and six seeds (average size 7.3 mm long x 4.1 mm wide) were recovered from the 24 pods (Fig. 3). For 7-, 10-, and 14-d-old peg tips, the numbers of large ovules were 7, 5, and 2, respectively, and seeds were 2, 1, and 3. Treatments with the highest rate of pod formation also had more ovule growth and embryo development. Seeds (one from treatment 1, one from 2, two from 5, one from 6, and one from 9) were distinguished by having a cotyledonary embryo (Fig. 3D) covered with a thin seed coat. Seven ovules and all six seeds were aseptically isolated from the pods and subcultured on the MS medium containing 0.2 mg/L 6-BAP and 0.05 mg/L GA_3. None of these ovules or seeds germinated. Anatomical observation of the other

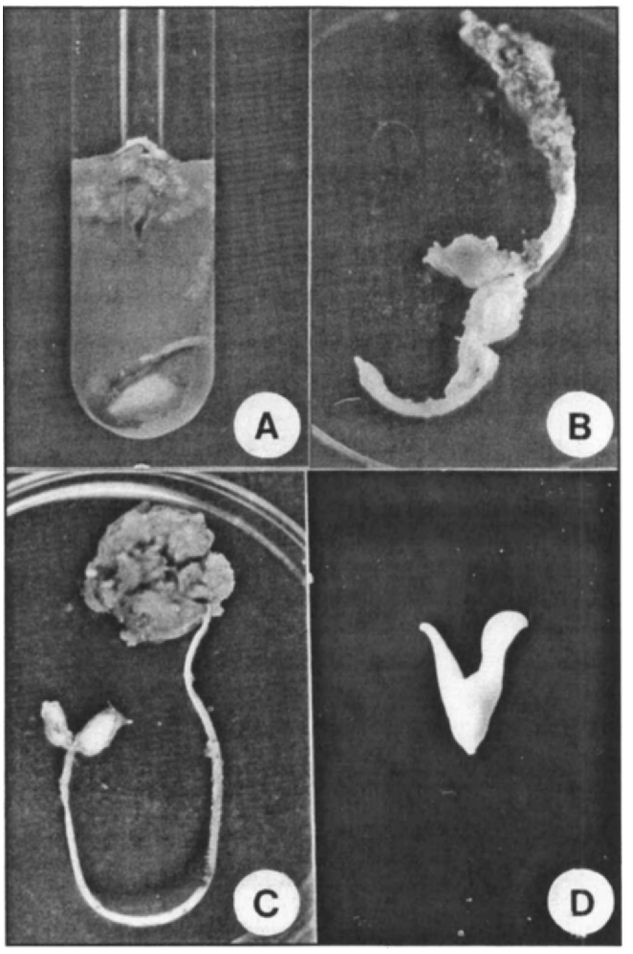

Fig. 3. *In vitro* peanut pods, seeds, and a cotyledonary embryo recovered from peg tips of *A. duranensis* after 90 d culture. (A) A pod in the medium with 0.5 mg/L NAA, 0.5 mg/L GA_3, and 0.05 mg/L 6-BAP; (B) a seed inside the pod which occurred at the middle of the peg and with an elongated tip at the apex end; (C) a pod containing a seed cultured on the medium with 2.0 mg/L NAA, 0.05 mg/L GA_3, and 0.2 mg/L 6-BAP showing the pod formation at the end of the peg tip; (D) an *in vitro*-raised embryo at late cotyledonary stage recovered from a 7-d-old proembryo.

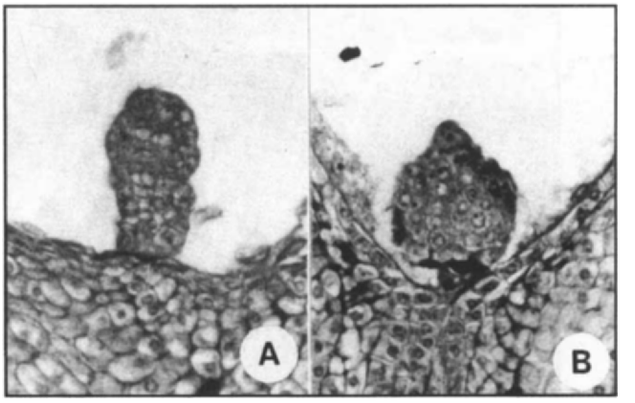

Fig. 4. Globular embryos recovered from proembryos of *A. duranensis* by *in vitro* peg tip culture. (A) A globular embryo which had a poorly differentiated suspensor; (B) a globular embryo without a suspensor.

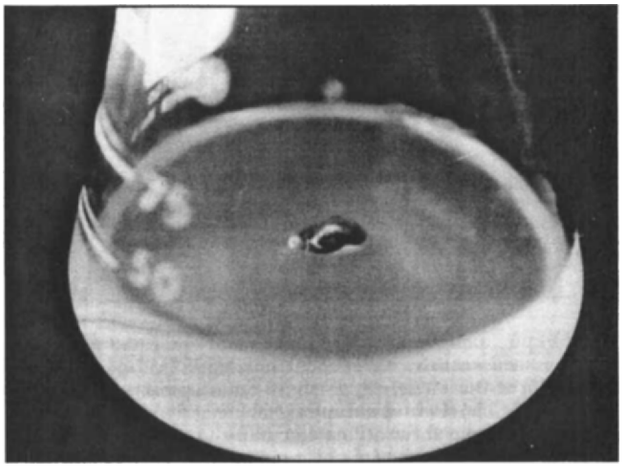

Fig. 5. An *in vitro*-recovered seed germinated with only radicle protrusion and no further growth in the MS medium with 0.2 mg/L 6-BAP and 0.05 mg/L NAA after released-dormancy treatment with growth regulators.

seven ovules indicated that they contained globular embryos with either a poorly differentiated suspensor (Fig. 4A) or no suspensor (Fig. 4B).

After removing seeds from the media and treating them with ethylene, GA_3, and NAA for a week, two seeds initiated germination with a protruding radicle. No further growth was observed, however, even after transfer to pots containing vermiculite supplied with 1/2 strength MS liquid solution. Four other nongerminated seeds were recultured on the MS medium with 0.2 mg/L 6-BAP and 0.05 mg/L NAA. Two produced radicles within 3 wk (Fig. 5); however, no further growth was observed after 2 mo of culture. The seed coat was removed from the other two seeds, but they did not grow during almost 4 mo of culture.

Discussion

Recovery of interspecific peanut hybrids by *in vitro* culture of immature embryos at the heart-shaped or cotyledonary stage has been possible (1,10,11), but experiments with proembryos have had less success (6,8). Culture of very young reproductive tissues in peanut is believed to be necessary because many potential hybrids abort within a few days after fertilization. Although the entire reproductive developmental process from a proembryo to a mature plant by *in vitro* peg tip culture of *A. hypogaea* has been successful (Feng, unpubl. data, 1994), culturing wild species is more difficult. This implies that interspecific hybridization will be easier if *A. hypogaea* is used as a female parent; however, if reciprocal crosses are desired, then a need exists for developing techniques to rescue female tissues of wild *Arachis* species.

The percentage of *in vitro* pod formation (1.8%) in *A. duranensis* was low. The results are believed to be largely due to excessive amounts of callus on explants which may have suppressed pod formation and seed set. The acceptable ranges of growth regulators for *in vitro* pod formation and embryo development were 0.5 to 2.0 mg/L NAA, 0.1 to 1.0

mg/L GA_3, and 0.05 to 0.2 mg/L 6-BAP for both the cultivated (4) and the wild species used in this experiment. The three best treatments in this study were 1, 5, and 6.

After 90 d in culture, the pegs and pods contained only proembryos, globular, or late cotyledonary embryos or seeds, whereas no heart-shaped and early cotyledonary embryos were observed. The failure to find intermediate stages indicates that growth and development will continue to maturity when an embryo differentiates to the heart-shaped stage. Because of the low frequency of pods, a critical step in reproductive development of peanut appears to be differentiation from the globular to heart-shaped embryo. It is proposed that differentiation failures may be related to less-than-optimal hormone concentrations or poorly developed suspensors associated with many globular embryos.

Because the *in vitro*-developed seeds were intensely dormant, they did not germinate into plants even after treatment with growth regulators. However, this is the first report of seed production with mature embryos from *in vitro*-cultured proembryos of wild peanut species. Development of techniques to break dormancy will be needed before applying this *in vitro* culture protocol to recover interspecific hybrids.

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