In Vitro Culture of *Arachis hypogaea* Peg Tips[1]

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ABSTRACT

Arachis hypogaea L. cv. NC 4 was used as a model plant system in an effort to develop an *in vitro* embryo rescue protocol which could have application to interspecific hybrid embryos, which often abort at very early growth stages. Embryo growth and development was studied in 1- to 4-day-old peg tips containing proembryos equivalent to a stage where many interspecific hybrid embryos abort. Three independent experiments were conducted to 1) determine the most favorable basal media, 2) evaluate the effects of auxins and cytokinins on growth, and 3) determine a favorable combination of auxins and cytokinins for *in vitro* peanut embryo growth. The results indicated that MS (Murashige and Skoog) medium with 3% sucrose was the most favorable basal medium among seven media and two sucrose concentrations analyzed. IAA (indole-3-acetic acid) at 1.5 mg L^{-1} in combination with a range of KN (kinetin) levels from 0.5 to 1.25 mg L^{-1} were the growth regulator combinations of choice. Proembryo growth reached the multicellular globular stage, but differentiation into heart-shaped embryos did not occur.

Key Words: Peanut, growth regulators, embryo culture, interspecific hybrids.

Peanut (*Arachis hypogaea* L.)—a major oil and protein-rich crop of the tropical and subtropical regions of the world, including the Southeastern U.S.A.—suffers severe yield losses from various diseases and insect pests. The wild *Arachis* species contain high levels of resistance to many of the diseases (Abdou *et al.*, 1974; Fitzner *et al.*, 1985; Subrahmanyam *et al.*, 1985) and insect pests (Amin, 1985; Stalker and Campbell, 1983) attacking the peanut. However, reproductive isolation barriers restrict the use of most *Arachis* species for the genetic improvement of *A. hypogaea*. Therefore, the utilization of these *Arachis* species requires that technology be developed to permit recovery of interspecific hybrids. Although *in vitro* ovule or embryo culture techniques can be used to rescue interspecific peanut hybrids from embryos cultured after they reached the heart-shaped stage (Sastri *et al.*, 1981; Thompson *et al.*, 1985; Stalker and Eweda, 1988), many hybrids contain only four- to eight- celled proembryos at the time they must be rescued (Halward and Stalker, 1987). These proembryos do not respond sufficiently to established ovule or embryo rescue techniques to obtain plants *in vitro*.

Williams and de Lautour (1980) used nurse cultures to support the nutritional and hormonal requirements of young interspecific hybrid embryos of three pasture legumes. The hybrid embryos were in the late globular to early torpedo stage at the time of culture, and they obtained interspecific hybrids. However, extremely immature globular embryos could not be successfully cultured with nurse endosperm. Peanut pegs (modified gynophores) containing fertilized ovules have been used as intact nurse tissue for proembryos in culture (Moss and Stalker, 1987; Moss *et al.*, 1988). Pattee *et al.* (1988) reported that 1-day-old peg tips of *A. hypogaea* cv. NC 6 containing one- or two-celled embryos, when cultured *in vitro* for 21 days, resulted in multinucleate globular embryo development. This continued growth of embryos after excision from the plant indicates a potential for obtaining hybrids from very young embryos, but differentiation between the globular and heart-shaped stage remains a critical step for plant regeneration.

The long-term goal of our research was to develop procedures to generate plants from proembryos found within interspecific hybrid pegs before abortion occurs. Because hybrid pegs which have entered the soil are difficult to obtain, proembryos contained within 1- to 4-day-old peg tips of *A. hypogaea* cv. NC 4 were used as test material as reported earlier by Moss *et al.* (1988). Our approach was to develop a workable system with *A. hypogaea* proembryos which may later be extended to culture hybrid pegs and recover interspecific hybrids. To achieve this, three specific objectives were defined: 1) establish the optimal basal medium for embryo culture, 2) evaluate the effects of auxins (NAA or IAA) and cytokinins (KN or BA) on 1- and 4-day-old peg tips of *A. hypogaea* grown *in vitro*, and 3) determine a favorable combination of an auxin and a cytokinin for embryo growth. Experiments involving objectives 1 and 2 were conducted independently based on the results of *in vitro* embryo growth responses reported earlier by Moss and Stalker (1987) and Moss *et al.* (1988) working with the proembryos of *A. hypogaea*. Objective 3 was undertaken based on the results from experiment 2.

Materials and Methods

Plant Material

Arachis hypogaea cv. NC 4 was grown in the North Carolina State University Method Road greenhouses, Raleigh, NC during the summer of 1988. Self-pollinated flowers were tagged on the day of anthesis between 8 and 10 AM and subsequent flowers at the tagged node were removed before 8 AM on the day they opened. The entire tagged node was excised 1, 2, 3 or 4 days later before 10 AM of the respective day. The pegs were separated from the nodal tissues, bracts, stipules and other flower tissues in the laboratory. For culturing, pegs were sterilized in 600 mL of 20% Clorox (sodium hypochlorite 5.25% solution) with 3 mL Tween 80 for 15 min and washed three times for 15 min each in sterile distilled water. The peg tips were cut to a length of 1 mm to detach the peg meristem. All subsequent manipulations of the pegs were carried out aseptically in a laminar flow hood.

Tissue Culturing Procedures

Experiment 1. To identify a favorable basal medium for supporting peanut embryo growth, a factorial experimental design using two different sucrose concentrations (3.0 and 12.5%) with seven basal media was employed. The seven basal media were MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968), N6 (Chu, 1978), BO (Blaydes, 1966), SH (Schenk and Hildebrandt, 1972), Heller (Heller, 1953), and White (White,

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1963). The seven basal media differed mostly in the inorganic salt concentrations—Heller and White forming the low salt media; SH and BO the moderate salt media; and N6, MS, and B5 the high salt media. All media were adjusted to pH 5.7 and solidified with 7 g L^{-1} agar. Sterile plastic 60 x 15-mm disposable petri plates were filled with 9 mL of medium. Forty 3-day-old peg tips were cultured on each of the seven media combinations with 10 peg tips per replication. Each petri plate was sealed with parafilm to prevent desiccation and tissue contamination and then incubated in the dark at 25±1 C for 21 days. Ten peg tips were randomly selected from each media x sucrose combination for microscopic evaluation of embryo growth and classified as in Table 1.

Table 1. Description of embryo growth standards used in classifying the embryo growth and development in various experiments of the present study (after Pattee and Mohapatra, 1987).

Max. embryo growth stage	Description
D4	Linear 4-tiered, 4-celled proembryo
D5	Linearly packed 8-celled proembryo
1-0	Enlargement of 2 basal tiers of cells of the proembryo initiating suspensor formation
1-1	Rapid division of the apical tiers of proembryo with differentiation of proembryo and suspensor
1-2	Embryo becomes a spherical mass of small isodiametric cells with a distinguishable suspensor
1-3	Early globular embryo

Experiment 2. Concurrently with Experiment 1, a second test used MS (Murashige and Skoog, 1962) basal medium with 12.5% sucrose and 0.7% agar adjusted to pH 5.7 because a high osmoticum was believed at the time to be required for growth of very small embryos. The auxins, NAA (1-naphthalene-acetic acid) and IAA (indole-3-acetic acid), and cytokinins KN (kinetin) and BA (6-benzylaminopurine) were added in the concentrations outlined in Table 3. A total of 1167 peg tips were cultured *in vitro* using 1- and 4-day-old peg tips of *A. hypogaea* cv. NC 4 as described in Experiment 1. A split-plot design with age the main effect and the 25 media as subplots was used for the experiment. Ten pegs were randomly selected from each media x age combination for microscopic evaluation of embryo growth and classified as described in Table 1.

Experiment 3. Based on the results of Experiment 2 where large embryo growth occurred for both 1- and 4-day-old embryos, further comparisons of IAA at two levels (1.0 and 1.5 mg L^{-1}) and KN at five levels (0.5, 0.75, 1.0, 1.25, and 1.5 mg L^{-1}) were made using MS medium with 12.5% sucrose and 0.7% agar. Tissues were 1- and 2-day-old peg tips of *A. hypogaea* cv. NC 4 which were cultured as described in Experiment 1. Thirty peg tips with 10 per replication were used in the experiment. Peg tips were then histologically examined and the embryo growth classified as described in Experiment 1.

Data Collection

The peg tips were scored for macroscopic changes that had occurred during culture. The observed categories included 1) no change, 2) callus growth, and 3) protrusion of the ovule out of the cut end of the peg tip. Peg tips in the third category were eliminated from further analyses because of internal callusing in the protruding ovule (Pattee *et al.*, 1988). Ten peg tips without protruding ovules were randomly selected from each media x sucrose (Experiment 1), media x age (Experiment 2), and media x growth regulator (Experiment 3) combination for microscopic evaluation. The selected tissues were processed following the histological procedures described in the next section. The data collected involved the classification of embryo growth in microscopically observed tissues into various embryo growth categories developed for *A. hypogaea* by Pattee and Mohapatra (1987) (Table 1). All of the embryo sacs that contained multicellular embryos, indicating more growth than at the time of culture of peg tips, were scored. The percentage of growing peg tips was calculated as percent mean response.

Histological Procedures

Peg tips were fixed in FAA (5 pt formalin:5 pt glacial acetic acid:90 pt 70% ethanol) for 48 h and stored in 70% ethanol. Peg tips were dehydrated in an alcohol series, embedded in paraffin (Paraplast Plus, Fisher Chemical Company), microtomed at 10 μ and serial sections fixed on glass slides. The sections were deparaffinized with xylene and differentially stained using safranin-O/Fast Green/Orange G series, coverslips were affixed using Permount, and embryos microscopically observed. Syngamy was judged to have occurred by the dissolution or absence of starch grains within the embryo sac.

Data Analysis

Data analysis used the GLM procedure (SAS, 1985; 1986). The Waller-Duncan K-ratio t-test was performed to compare the means of the various media treatments on embryo growth and development in the cultured peg tips.

Results

Experiment 1

The initial objective was to determine if a preferred medium could be identified among a selected group of tissue culture media to culture 1- to 4-day-old peanut peg tips. Histological evaluation of the cultured peg tips for continued embryo development and maximum embryo developmental stage in Experiment 1 indicated a wide range in the percent developing embryos for a given culture medium (Table 2). Although the MS basal medium with 3% sucrose had a 50.2% growth response and was not significantly different from the White and BO media, it would appear to be the medium of choice. Maximum embryo growth achieved does not appear to be greatly affected by the growth medium. Multi-cellular embryos were produced on all media tested, with the BO medium yielding the largest embryos which reached stage 1-3. Increasing the percent sucrose in the MS and White media greatly reduced the percent growth response; increasing sucrose produced moderate percent growth reductions in BO and B5 media. Moderate growth increases in mean percent response due to sucrose increase were observed in Heller and SH media, while in the N6 medium a change from the least efficient to the most efficient embryo growth response was observed. Overall, increasing sucrose percentage in media was suppressive to embryo response, but had little affect on maximum developmental stage embryos obtained. The observed sucrose effect on percent growth response is in agreement with Williams and de Lautour (1980).

Table 2. Embryo growth and development in 3-day-old peg tips of *A. hypogaea* cv. NC 4 cultured on various basal media supplemented with two sucrose levels.

Medium	Mean response (%)	Max. embryo growth
	3% Sucrose	
MS	50.2	1-2
White	32.4	1-1
BO	32.1	1-3
B5	19.1	1-2
SH	7.3	1-1
Heller	5.9	1-1
N6	4.1	1-2
LSD (P = 0.05)	20.2	
	12.5% Sucrose	
N6	24.7	1-2
BO	14.1	1-0
Heller	13.2	1-3
SH	12.5	1-0
MS	6.6	1-1
White	3.1	1-0
B5	2.7	1-1
LSD (P = 0.05)	24.3	

Experiment 2

This experiment was designed to show the effects of auxins and cytokinins in the culture medium on the embryo growth and development.

Effect of auxins alone. The treatment containing 0.5 mg L^{-1} IAA alone induced embryo growth significantly more often than the treatment containing 0.5 mg L^{-1} NAA alone for both 1- and 4-day-old tissues (Table 3). In contrast, the treatment containing 1 mg L^{-1} IAA alone performed poorly in supporting embryo growth when compared to 1 mg L^{-1} NAA alone. Moreover, treatment 1.0 mg L^{-1} IAA induced significantly less embryo growth compared to either treatment 0.5 or 1.0 mg L^{-1} NAA. The NAA treatments produced large embryo growth in 4-day-old peg tips but induced embryo growth response significantly less often than 0.5 mg L^{-1} IAA. These observations indicated that there was a significantly detrimental effect on percentage of embryos which grew when the level of IAA was raised from 0.5 mg L^{-1} to 1 mg L^{-1}, while a similar increase in the level of NAA indicated a nonsignificant effect on embryo growth. However, none of the auxin combinations resulted in satisfactory numbers of developing embryos and growth to a large size.

Effect of cytokinins alone. The treatment comparisons included 0.25 mg L^{-1} KN, 0.5 mg L^{-1} KN, 2.5 mg L^{-1} BA, and 5 mg L^{-1} BA for both 1- and 4-day-old embryos (Table 3). The two KN levels induced embryo growth significantly more often than BA treatments. This indicated that media with KN were generally superior to media with BA, at least at the levels of each cytokinin tested in this experiment. The 0.25-mg L^{-1} KN treatment not only induced embryo growth significantly more often than treatments with BA, but also produced significantly larger embryo growth in 4-day-old peg tips. Use of 0.5 mg L^{-1} KN also showed a similar embryo growth response as the lower KN level but produced large embryo growth only in 1-day-old peg tips. Thus, using KN alone is believed to be more effective than BA, but a satisfactory percentage of embryos growing plus development to a large size for both 1- and 4-day-old tissues was not observed.

Table 3. Embryo growth observed for 1- and 4-day-old peg tips after 21 days averaged across replications in culture with different auxin and cytokinin levels in Murashige and Skoog (MS) basal medium with 12.5% sucrose.

Treatment[a]	# Ovules observed		Embryo growth (%)			Large embryo growth (%)	
	1 day	4 day	1 day	4 day	1+4 day	1 day	4 day
Control	20	16	30.0	75.0	50.0	0	0
0.5N	20	20	50.0	70.0	60.0	0	10.0
1.0N	19	20	47.4	90.0	69.2	0	10.0
0.5I	18	20	88.9**	80.0	84.2**	0	0
1.0I	21	20	33.3	5.0**	19.5**	0	0
0.25K	20	20	55.0	90.0	72.5*	0	40.0**
0.5N+0.25K	20	18	10.0	33.3*	21.1**	0	5.6
1.0N+0.25K	19	18	10.5	50.0	29.7	0	11.1
0.5I+0.25K	20	19	5.0*	63.2	33.3	0	15.8
1.0I+0.25K	20	12	65.0*	75.0	68.8	0	0
0.5K	20	16	80.0**	56.3	69.4	5.0	0
0.5N+0.5K	20	19	65.0*	73.7	69.2	0	5.3
1.0N+0.5K	19	16	47.4	43.8	45.7	0	6.3
0.5I+0.5K	20	19	40.0	78.9	58.9	0	0
1.0I+0.5K	20	20	65.0*	80.0	72.5*	10.0	15.0
2.5B	12	20	33.3	40.0*	37.5	0	5.0
0.5N+2.5B	14	18	7.1	66.7	40.6	0	16.7
1.0N+2.5B	20	19	0**	31.6**	15.4**	0	10.5
0.5I+2.5B	20	19	30.0	42.1*	35.9	0	0
1.0I+2.5B	20	20	60.0	75.0	67.5	0	20.0
5.0B	19	19	0**	5.3**	2.6**	0	0
0.5N+5.0B	20	17	35.0	41.2*	37.8	0	11.8
1.0N+5.0B	20	7	5.0*	71.4	22.2*	0	0
0.5I+5.0B	20	16	10.0	6.3**	8.3**	0	0
1.0I+5.0B	19	16	0**	50.0	22.9*	0	6.3
Total	480	444					

[a]N = NAA, I = IAA, K = KN, B = BA. Growth regulator concentrations given in mg L^{-1}.

*,**Results were significantly (P = 0.05 and 0.01, respectively) different from zero control.

Effect of auxin-cytokinin interaction. For 1-day-old tissues, only three media combinations gave larger numbers of growing embryos than controls, including 1.0 mg L^{-1} IAA + 0.25 mg L^{-1} KN, 1.0 mg L^{-1} IAA + 0.5 mg L^{-1} KN, and 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} KN. The treatment with higher IAA+ KN levels was the only one having both auxins and cytokinins with a significant improvement in growth over the control (Table 3). Although not statistically significant, this growth combination also produced the greatest number of large embryos for both 1- and 4-day-old tissues. Overall, the cytokinin BA in combination with the auxins showed a detrimental effect on embryo growth. An exception was the treatment with 1.0 mg L^{-1} IAA + 2.5 mg L^{-1} BA which induced more consistent embryo growth response, as well as large embryo growth in 4-day-old peg tips, than the media combinations involving the two auxins and BA. It would appear from the above data that 1.0 mg L^{-1} IAA + 0.5 mg L^{-1} KN would be the medium of choice, as it not only induced embryo growth most often but also large embryo growth in both 1- and 4-day-old peg tips. However, if only older embryos were to be cultured (i. e., 4-day), then 0.25 mg L^{-1} KN without auxins appears to be superior.

Experiment 3

Based on the results of Experiment 2, a third experiment was conducted to verify and define an optimum combination of IAA and KN that supports proembryo growth from *A. hypogaea* peg tips. Because the highest levels of IAA (1.0 mg L^{-1}) and KN (0.5 mg L^{-1}) gave the most promising results in terms of percentage of tissues responding and large embryo growth (Table 3), this experiment tested two levels of IAA (1.0 and 1.5 mg L^{-1}) and a range of KN levels (0.5 to 1.5 mg L^{-1}). Further, 12.5% sucrose was used to be consistent with the previous experiment so comparison could be made for auxin/cytokinin levels without adding another variable. The data indicated that the different IAA and KN levels investigated had a non-significant effect on continued embryo development in both 1- and 2-day-old peg tips (Table 4). However, when the media containing the two IAA levels in combination with the five KN levels were analyzed as two separate groups, media 1.5 mg L^{-1} IAA (except in combination with 1.5 mg L^{-1} KN) produced more multicellular globular embryos as compared to media containing 1.0 mg L^{-1} IAA in combination with the five KN levels. The media containing 1.5 mg L^{-1} IAA with either 1.25 mg L^{-1} KN or 0.5 mg L^{-1} KN produced the largest embryos, which grew to stage 1-3. In contrast to the results of Experiment 2, the medium containing 1.0 mg L^{-1} IAA and 0.5 mg L^{-1} KN did not induce large embryo growth. The data also indicated a wide range in the percent developing embryos for both 1- and 2-day-old peg tips. The media containing 1.5 mg L^{-1} IAA in combination

with 1.0 mg L^{-1}KN induced the highest embryo growth response (23.3%) in 1-day-old peg tips; whereas, in 2-day-old peg tips, the media containing 1.5 mg L^{-1} IAA in combination with 1.25 mg L^{-1} KN induced a 48.4% embryo growth response. A lower IAA concentration of 1.0 mg L^{-1} in combination with 1.5, 0.75 and 0.5 mg L^{-1} KN induced 15.2, 12.1 and 11.5% embryo growth, respectively, in 1-day-old peg tips; whereas, in 2-day-old peg tips, the media containing 1.0 mg L^{-1} IAA in combination with 0.75, 1.0, 1.25 and 1.5 mg L^{-1} KN produced 33.2, 18.1, 15.9 and 16.7% embryo growth, respectively. Though no obvious trends were observed for inducing the growth of proembryos, the media with higher IAA levels appeared to favorably support the percent developing embryos as well as the maximum embryo growth in 1- and 2-day-old peg tips. In contrast, MS basal medium (control) showed only 1.7 and 8.3% embryo growth in 1- and 2-day-old peg tips but induced a stage 1-0 embryo.

Discussion

Early embryo abortion is a common occurrence in interspecific hybrids in the genus *Arachis* (Johansen and Smith, 1956). Reproductive development in peanut is unique because embryo development initiates immediately after fertilization, but growth is arrested for an extended period of time while the peg elongates. Pegs initiate growth the second day after fertilization, but by the fourth or fifth day the embryo stops dividing. Embryo development does not reinitiate until after the peg penetrates the soil and ceases to grow, which usually occurs 5-15 days later (Smith, 1956; Pattee and Mohapatra, 1987). Many of the problems encountered while culturing young peanut embryos are the result of this complex nature of reproductive ontogeny.

To avoid the developmental stage where the embryo remains quiescent for an extended period of time and abortion may occur because of failure to reinitiate growth, peg tips with enclosed ovules and embryos (ranging from 1 to 20 days) were cultured for both *A. hypogaea* and interspecific hybrids (Moss and Stalker, 1987; Moss *et al.*, 1988; Pattee *et al.*, 1988). Pattee *et al.* (1988) found that some embryos in 1-day-old peg tips grew to the globular stage. They also indicated that, once the sequence of event is initiated which slows embryo growth, it is not easily reversed *in vitro*. The continued embryo growth in 1-to 4-day-old peg tips to the multicellular stage in the different experiments of this study is a promising indication of the application of peg tip culture to rescue early stage proembryos which, otherwise, would abort. This finding is in concurrence with the earlier observations of Moss and Stalker (1987) and Pattee *et al.* (1988).

Table 4. Embryo growth and development averaged across replications for 1- and 2-day-old peg tips of *A. hypogaea* cv. NC 4 cultured on Murashige and Skoog (MS) basal medium supplemented with 12.5% sucrose and various levels of IAA and KN.

IAA and KN (mg L^{-1})	Mean response (%) Day 1	Day 2	Maximum embryo growth
1.50 IAA + 1.50 KN	7.7	0	D4
1.50 IAA + 1.25 KN	19.1	48.4	1-3
1.50 IAA + 1.00 KN	23.3	19.1	1-2
1.50 IAA + 0.75 KN	9.8	28.9	1-2
1.50 IAA + 0.50 KN	14.5	21.5	1-3
1.00 IAA + 1.50 KN	15.2	16.7	1-0
1.00 IAA + 1.25 KN	2.6	15.9	1-0
1.00 IAA + 1.00 KN	9.7	18.1	1-0
1.00 IAA + 0.75 KN	12.1	33.2	1-0
1.00 IAA + 0.50 KN	11.5	8.3	D5
MS basal (control)	1.7	8.3	1-0
LSD (p = 0.05)	19.3	31.6	--

Although the different basal media tested in this study showed no significant differences in terms of maximum embryo growth stages achieved, the MS medium with 3% sucrose resulted in the highest mean response (50.2%). Therefore, it can be concluded that this combination of basal medium appears to be more favorably supporting embryo growth in *A. hypogaea* than the other six media combinations. The assumption is that interspecific hybrids, which may have both endosperm and nuclear deficiencies, will respond in a similar way to basal media and growth regulators as *A. hypogaea* which has normal endosperm. However, the optimal sucrose level of 3% for cultivated peanut may be too low for interspecific hybrids where endosperm deficiencies are expected. The observations further suggest that the young peg tips of *A. hypogaea* may already contain many or all of the growth hormones required for embryo growth. However, a critical step in future work will be inducing growing embryos to differentiate. Observations using different levels of growth regulators supported the previous results which indicated that the greatest percentage response for embryo growth *in vitro* occurred when growth regulators were added to media. The observations indicated that a higher auxin (IAA) level of 1.5 mg L^{-1} in combination with the various cytokinin (KN) levels, except for the highest KN level (1.5 mg L^{-1}) studied, induced a higher percentage of embryo growth and large embryo growth in 1- and 2-day-old peg tips than when lower IAA concentrations were tested. The results of these experiments can be used to redefine the favorable medium that appears to support proembryo growth and development as basal MS plus 3% sucrose and a hormonal combination of 1.5 mg L^{-1} IAA with a range of KN levels varying from 0.5 to 1.25 mg L^{-1}. However, additional experiments will be needed to verify the combination of low sucrose with the suggested best growth regulators. Moreover, most of the embryos did not develop past the 32-celled stage, thereby not growing as much as they would have naturally on the plant. This suggests that a change of media may be required before 21 days in culture. Further research will be required to obtain differentiation of the multicellular globular embryos to plantlets.

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