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High Efficiency Peanut Regeneration Using a Nonimbibed Immature Leaflet Culture Method

S.D. Utomo[1], A.K. Weissinger[2], and T.G. Isleib[2]*

ABSTRACT

Efficient plant regeneration is an essential part of gene transfer systems using recombinant DNA technology. Efficiency of regeneration from immature leaflets of peanut (*Arachis hypogaea* L.) was compared among several explant treatments in an effort to maximize recovery of plants from culture. In one experiment, explants were derived from leaflets of cv. NC 7 from dry mature seeds or from mature seeds which had been imbibed for 1 or 4 d. To avoid confounding treatment effects with variation among individual seeds, both nonimbibed and imbibed leaflets originated from a single seed. For each seed, four nonimbibed leaflets from a single leaf were excised, sterilized, rinsed, and plated on MS-based medium amended with 4 mg L^{-1} benzylaminopurine and 2 mg L^{-1} naphthaleneacetic acid. The embryonic axis with the other leaf (four remaining leaflets) and one cotyledon attached was then imbibed in water for 1 or 4 d. After 4 wk in culture, 53% of nonimbibed leaflets, 37% of leaflets imbibed for 1 d, and 6% of leaflets imbibed for 4 d produced shoots. Subsequently, regeneration efficiency was compared among immature leaflet cultures from nonimbibed seeds of four cultivars representing three botanical varieties from two subspecies. Shoot frequency after 4 wk in culture averaged 9% for Peruvian introduction A_2 (NC Ac 17090) representing subsp. *fastigiata* var. *peruviana* versus 53% for cv. NC 7 and Bolivian introduction B_2 (PI 274191), both representing subsp. *hypogaea* var. *hypogaea*. At 6 wk after plating, these frequencies increased to 28 and 61%, respectively. The response of Argentine introduction C_2 (PI 262000) representing subsp. *fastigiata* var. *vulgaris* was intermediate to vars. *hypogaea* and *fastigiata*. Shoot proliferation in var. *hypogaea* was significantly greater than in the other varieties, whereas it was significantly lower in var. *peruviana*. Regenerated plants developed normal flowers and pods in the greenhouse. The study indicated that *A. hypogaea* can be cultured efficiently from nonimbibed leaflets.

Key words: *Arachis hypogaea* L., tissue culture, imbibition.

Plant regeneration is an essential part of nearly all gene transfer systems using recombinant DNA technology. Somatic embryo culture from immature seeds (Hazra *et al.*, 1989; Ozias-Akins, 1989; Sellars *et al.*, 1990; and Ozias-Akins *et al.*, 1992), somatic embryo culture of axes from mature seeds (Baker *et al.*, 1995), and embryonic leaflet culture (Mroginski *et al.*, 1981; Pittman *et al.*, 1983; Seitz *et al.*, 1987; McKently *et al.*, 1991; Baker and Wetzstein, 1992; Cheng *et al.*, 1992; Clemente *et al.*, 1992; Eapen and George, 1993) have been employed to regenerate peanut (*Arachis hypogaea* L.) plants. Establishment of transgenic peanut plants from immature embryo culture is time-consuming and may be subject to somaclonal variation and reduced fertility. Delivering DNA into embryonic axes using microprojectile bombardment does not substantially impair regenerative ability, although the efficiency of DNA delivery to embryonic axes is lower than that to leaflets (Clemente *et al.*, 1992; Schnall and Weissinger, 1993).

If an acceptable frequency of regeneration can be obtained, embryonic leaflet culture could have advantageous applications in development of a transgenic peanut. The planar geometry of leaflets facilitates transformation using microprojectile bombardment. Leaflets could also be a suitable explant for transformation using *Agrobacterium tumefaciens*. Plants can be regenerated from embryonic leaflets in only 3 to 4 mo. Regeneration response of embryonic peanut leaflet is affected by the

[1]Dept. of Agronomy, College of Agric., Lampung Univ., Jl. S. Brodjonegoro 1 Bandar Lampung 35145, Indonesia.

[2]Dept. of Crop Science, N.C. State Univ., Box 7629, Raleigh, NC 27695-7629.

*Corresponding author.

developmental stages of explants (Mroginski *et al.*, 1981), auxin:cytokinin ratio (Seitz *et al.*, 1987; Clemente *et al.*, 1992) and plant genotypes (Mroginski *et al.*, 1981; Seitz *et al.*, 1987; Clemente *et al.*, 1992). Using MS-based medium amended with 1 mg L^{-1} each of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA), Mroginski *et al.* (1981) found the highest bud regeneration frequency (92.3%) on 2-5 mm leaflets imbibed in water. They also found 32.2% bud regeneration from nonimbibed leaflets indicating that imbibition is not an essential prerequisite for successful bud regeneration. Imbibition is required during germination to promote active growth of seed tissues. During germination, the cells of leaflet tissues enlarge until reaching maturity. Because of their low degree of maturity, nonimbibed leaflets may be potentially more responsive in culture than imbibed leaflets.

The objectives of this study were to examine the effect of imbibition period on regeneration response of embryonic leaflets and to compare the regeneration responses of diverse genotypes using a nonimbibed leaflet culture system.

Materials and Methods

Protocols for Leaflet Culture. Explants were derived from either dry mature seeds or mature seeds which had been imbibed in water for 1 or 4 d. To avoid confounding of treatment effects with variation among seeds, both nonimbibed and imbibed leaflets originated from a single seed. Each dry seed was split open. One cotyledon was discarded, and four nonimbibed leaflets from one leaf were excised for nonimbibed immature leaflet culture (NILC). The embryonic axis with the other leaf (four remaining leaflets) and the remaining cotyledon were imbibed for 1 or 4 d as described below.

For NILC, four leaflets from one leaf excised from a split dry seed were surface-sterilized with a 0.75% sodium hypochlorite solution for 4 min and rinsed in two changes of sterile distilled water. Four leaflets were plated on a shoot-induction medium in 60 x 15-mm disposable petri dishes containing 14-15 mL medium. The medium contained MS salts (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg *et al.*, 1968), 3.0% sucrose, 2 mg L^{-1} NAA, 4 mg L^{-1} BAP, and solidified with 0.8% agar (Clemente *et al.*, 1992). Leaflets were oriented vertically with the apex up and approximately half of the leaflet submerged in the medium. Petri dishes were sealed with Parafilm™ (American National Can, Greenwich, CT). Cultures were maintained in a growth chamber at 27-28 C and 16/8 hr light/dark cycle. General Electric F20T12 CW fluorescent tubes were used which produced approximately 66 $\mu E\ m^{-2}\ sec^{-1}$ when measured 15 cm from the tubes. At 3 d of incubation, explants were inspected and reoriented if necessary to keep 60-80% of the tissue submerged in medium. Cultures were transferred to fresh medium at 2-3 wk intervals. Due to space limitations, 60% of the cultures were transferred to approximately 15 mL of medium in 72-mm baby food jars and 40% to 60 x 15-mm plastic petri dishes for the first transfer. Explants which appeared to be developing shoots were transferred subsequently to baby food jars. All adventitious roots and callus tissue were removed from shoots at the time of transfer.

For imbibed immature leaflet culture (IILC), the embryonic axis with one attached leaf comprising four leaflets and one attached cotyledon were surface-sterilized as described for NILC. The axis then was transferred to a sterile Magenta box with water-saturated vermiculite and incubated at 27-28 C and 16/8 hr light/dark for either 1 or 4 d. After imbibition, axes were surface sterilized again for 2 min and rinsed twice with sterile distilled water. Four leaflets were excised, plated, and incubated as described for NILC.

Experiment 1: Cultural Response of NC 7 to Explant Imbibition. To measure the effects of imbibition treatments as well as seed-to-seed variation within mother plants, 20 mature seeds were harvested from each of five mother plants of NC 7, a large-seeded virginia-type cultivar developed by the North Carolina Agric. Res. Serv. (Wynne *et al.*, 1979). Two hundred experimental units were derived from the 100 seeds, each unit comprising a set of four leaflets. There were 100 units cultured by NILC, 50 by 1 d IILC, and 50 by 4 d IILC. The two sets of leaflets excised from the same seed were cultured simultaneously, one set by NILC and the other by either 1 d or 4 d IILC to provide paired comparisons of NILC with the other treatments. Experimental units from the same mother plant were placed on the same shelf in the incubator, confounding the effects of shelves and mother plants in an overall replicate effect. Individual seed effects were nested within mother plant/shelf effects. For imbibition treatments, the experimental design was an incomplete block with seeds as blocks and unequal replication of treatments.

Response variables measured or calculated for this experiment included:

(a) Frequency of responding explants at 4 wk after plating (WAP) as evidenced by development of both adventitious root and small (approx. 1 mm) swellings from which shoots may subsequently develop.

(b) Estimates of the frequency of shoot induction a 4 and 6 WAP. Because shoot proliferation typically occurred continuously over a protracted period and shoot morphology varied, we sought to eliminate ambiguity in this measurement by defining "shoot" as an outgrowth bearing at least one tetrafoliolate leaf.

Experiment 2: Regeneration Responses of Three Diverse Botanical Types of Peanuts. Four homozygous peanut lines representing three botanical varieties from two subspecies were compared with respect to regeneration using NILC. NC 7, used as the standard genotype, exhibits the flowering and branching pattern characteristic of subsp. *hypogaea* var. *hypogaea* (virginia type) although it has some ancestry from subsp. *fastigiata* Waldron (Isleib and Wynne, 1992). Line B_2 (PI 274191) belongs to subsp. *hypogaea* var. *hypogaea* and was originally collected in Bolivia (Wynne, 1974). Line A_2 (NC Ac 17090) belongs to subsp. *fastigiata* var. *peruviana* (Peruvian valencia type) and was collected in Peru. Line C_2 (PI 262000) is a spanish-type line (subsp. *fastigiata* var. *vulgaris* Harz) collected from the Instituto Agronomico at Caacupé, Paraguay.

The experiment was arranged in a completely random design with 30 replications. Each experimental unit consisted of four leaflets excised from a single seed. Regenerated shoots were transferred every 2-3 wk until they developed at least four tetrafoliolate leaves, after which the shoots could be successfully transferred to one of two rooting media. One rooting medium consisted of full-strength MS salts supplemented with 3.0% sucrose, 1 mL L^{-1} B5 vitamins, 1 mg L^{-1} NAA, 0.04 mg L^{-1} kinetin, and 0.8%

agar (Narasimhulu and Reddy, 1983). Explants rooted slightly better when transferred to a half-strength rooting medium of MS salts with 1.5% sucrose, 0.5 mL L^{-1} B5 vitamins, 0.5 mg L^{-1} NAA, and 0.8% agar. Vigorous shoots with roots were transferred directly from rooting medium to a soil medium consisting of a 1:1 mixture of soil and sand in 5 x 5 x 5-cm plastic pots. When shoots appeared weak, then 1-2 wk after the appearance of roots, the tissues were transferred to hormone-free medium containing half-strength MS salts, 0.5 mL L^{-1} B5 vitamins, 1.5% sucrose, and 0.7% agar for 2-3 wk prior to transfer to soil medium. Pots were placed in a shaded mist system for 5-7 d. Plants were transferred to greenhouse flats containing Metromix™ (Grace Sierra, Milpitas, CA) and then placed on a shaded greenhouse bench for 4-7 d. Plants were moved subsequently to unshaded greenhouse benches and grown to maturity.

Additional response variables measured or calculated for each experimental unit in this experiment included those described for Experiment 1 as well as the following:

(a) Average number of shoots per explant at 6 WAP.

(b) Average number of leaves per shoot at 6 WAP. Experimental units that did not produce shoots were treated as missing for this trait.

(c) Maximum number of leaves per shoot at 6 WAP.

(d) Rooting frequency at 6 WAP as evidenced by the development of lateral roots.

(e) Frequency of regenerated plants capable of sustained growth in soil at 4 wk after transfer to soil medium as evidenced by the production of at least one new leaf.

(f) Frequency of flowering plants as evidenced by the production of at least one flower.

Statistical Analyses. Analyses of variance were performed using the general linear models (GLM) procedure in the SAS software package (SAS Institute, 1990). Square root transformation of data was applied when necessary to homogenize error variances. Linearity of response to number of days of imbibition in Experiment 1 was tested using polynomial regression. Because of the loss of experimental structure in Experiment 2 following transfer of shoots to rooting medium and subsequent transfer to Metromix, chi square analyses of contingency tables were used to compare the frequencies of rooted shoots and regenerated plants exhibiting sustained growth for the various treatments and genotypes.

Results and Discussion

Experiment 1. Imbibition significantly reduced the shoot response of NC 7. The frequency of responding explants at 4 WAP was significantly less for 4-d IILC than for NILC and 1-d IILC (Table 1). Shoot frequencies from NILC at 4 and 6 WAP were significantly higher than those from 1-d IILC while 1-d IILC was significantly higher than 4-d IILC. These results for IILC agree with those of previous studies. Clemente *et al.* (1992) found 6% shoot frequency using NC 7 explants imbibed 4 d. Seitz *et al.* (1987) reported 20% shoot frequency from NC 7 explants imbibed 3-4 d and cultured on MS-based medium amended with 1 mg L^{-1} BAP and 5 mg L^{-1} NAA. It should be noted that shoots were defined for these experiments as outgrowths bearing at least one tetrafoliolate leaf. In some cases, such structures do not develop functional meristems and development is arrested. Therefore, estimates of shoot proliferation frequencies may be biased and should be taken as upper limits of the response frequencies. Because the qualitative response was virtually identical among treatments, this bias would not affect comparisons among treatments within experiments.

Table 1. Frequencies of responding explants at 4 wk after plating (WAP) and explants with shoots at 4 and 6 WAP for NC 7 explants imbibed for 0, 1, or 4 d.[a]

Imbibition period	Frequency of Responding explants at 4 WAP	Shoot production at 4 WAP	Shoot production at 6 WAP
d	%	%	%
0	94 a	53 a	56 a
1	92 a	37 b	38 b
4	46 b	6 c	6 c

[a]Means within a column followed by the same letter are not significantly different (P<0.05) by t-test.

Polynomial regression of the three response variables on days of imbibition indicated that all responses were linear functions of time of imbibition with no significant curvature. Slopes were negative for all three characters and indicated that for each day of imbibition there would be a corresponding reduction of 11.4±1.4% in frequency of responding explants at 4 WAP, 12.4±1.2% in frequency of shoots at 4 WAP, and 13.1±1.2% in frequency of shoots at 6 WAP. Only 6±2% of leaflets imbibed 4 d produced shoots by 6 WAP.

No significant variation among mother plants of NC 7 was observed for any of the three response variables. Seed-to-seed variation was significant ($P \leq 0.10$) for shoot proliferation frequency at 4 and 6 WAP. This variation, verging upon significance, may be the result of slight differences in the maturities of the seeds. Peanut exhibits highly indeterminate flowering and fruiting. Even though efforts were made to select fully mature seeds from the five mother plants, it is highly unlikely that all seeds harvested simultaneously from a single plant had identical maturity.

Differences in pattern of development of shoots regenerated from leaflets at different periods of imbibition were not apparent. Leaflet explants responded by developing adventitious roots at the basal end of midrib submerged in the medium (first observed as early as 7 d after plating) as well as small (approx. 1 mm) swellings associated with the development of shoots (observed as early as 10 d after plating). These swellings appeared on the adaxial surface opposite the adventitious roots. Tetrafoliolate leaves were observed as early as 4 WAP. Following removal of adventitious roots and calli and transfer of shoots to fresh shoot induction medium, shoots with 4-5 tetrafoliolate leaves developed as early as 8 WAP. These shoots were ready for transfer to rooting medium. Lateral roots were observed by 10 WAP.

Experiment 2. The frequencies of responding explants of the four genotypes were greater than 90% with

no significant variation among them (Table 2). However, the frequency of responding explants was a poor indicator of the shoot frequencies at 4 and 6 WAP. NC 7 and B_2 exhibited significantly higher shoot frequencies than A_2 and C_2. A_2 had the lowest shoot frequency, although it did not fall significantly below C_2 until 6 WAP.

The two fastigiate lines, A_2 and C_2, were slower to develop shoots than were NC 7 and B_2. Shoot frequency increased from 56% at 4 WAP to 66% at 6 WAP for NC 7, 50 to 55% for B_2, 9 to 28% for A_2, and 21 to 41% for C_2. These results suggest that explants should not be discarded until at least 6 WAP, especially explants from subsp. *fastigiata*. Overall shoot frequencies of subsp. *hypogaea* genotypes NC 7 and B_2 were higher than those of subsp. *fastigiata* genotypes A_2 and C_2. Contrary results were reported by Clemente *et al.* (1992). Using 4-d IILC plated on similar medium, they found the shoot frequency of fastigiate lines Tamnut 74 and UPL Pn-4 to be higher than that of NC 7. Livingstone and Birch (1995) used MS basal medium + 1 mg L^{-1} NAA + 3 mg L^{-1} BAP and found that 50% of nonimbibed leaflets of cv. Robut (subsp. *hypogaea*) and 66% of those of cv. Gajah (subsp. *fastigiata*) produced buds or shoots at 6 WAP. Seitz *et al.* (1987) found no significant variation among shoot frequencies in lines of subsps. *hypogaea* and *fastigiata*. Although the shoot frequency of A_2 was lower than those of the other lines tested in Experiment 2, all responses were much higher than those reported by Seitz *et al.* (1987) and higher than the highest shoot response reported by Clemente *et al.* (1992) for subsps. *fastigiata* var. *fastigiata*.

A_2 and C_2 developed fewer shoots per explant than NC 7 and B_2. NC 7 and B_2 both developed more than one shoot per explant, while C_2 developed only one per two explants, and A_2 fewer than one per 10 explants. If this result is true for other lines from vars. *vulgaris* and *peruviana*, then it will be necessary to culture more explants of fastigiate types to obtain the same number of regenerated plants. At 6 WAP, the *hypogaea* lines also showed a significantly higher growth rate of shoots than the fastigiate lines based on the mean and maximum numbers of tetrafoliolate leaves per shoot. Within subsp. *hypogaea*, B_2 showed more rapid growth than NC 7. There was no significant difference between A_2 and C_2.

Except for the frequency of responding explants, subsp. *hypogaea* genotypes showed higher responses to NILC than subsp. *fastigiata* genotypes. However, because the samples of genotypes from the three botanical varieties in this study were small, it is premature to generalize from these results to other genotypes within varieties or subspecies. It is possible that the individual genotype rather than the subspecies or botanical variety is the primary factor determining response in culture.

Rooting responses were similar for full-strength and half-strength rooting media (Table 3) as well as for all four peanut lines. Likewise, there was no significant variation among the four lines with respect to capability for sustained growth. All plants capable of sustained growth produced normal flowers and pods although their flowering date was delayed 2-4 wk compared to control plants regenerated from seeds.

In conclusion, the two experiments indicate that culture of nonimbibed immature leaflet is an efficient method for regeneration of peanuts, particularly NC 7 and B_2 which are two representatives of the alternate-branching class of peanut associated botanically with *A. hypogaea* subsp. *hypogaea* var. *hypogaea*. Imbibition of NC 7 seeds prior to excision of embryonic axes caused significant reduction in shoot development in culture. Although the nonimbibed leaflet culture method was less efficient for A_2 and C_2 (two representatives of subsp. *fastigiata*) than for the representatives of subsp. *hypogaea*, the method was sufficiently successful to be useful in the regeneration of the fastigiate cultivars.

Table 2. Shoot growth response of four genetically diverse peanut lines under nonimbibed immature leaflet culture.[a]

Line	Frequency of Responding explants at 4 WAP	Frequency of Shoot production 4 WAP	Frequency of Shoot production 6 WAP	Average number of shoots per explant 4 WAP	Average number of shoots per explant 6 WAP	Number of leaves per shoot Maximum	Number of leaves per shoot Mean
	%						
NC 7	94 a	56 a	67 a	0.72 a	1.23 a	2.63 a	1.52 b
B_2	98 a	50 ab	54 ab	0.67 ab	1.14 a	2.97 a	2.35 a
A_2	96 a	9 c	28 c	0.10 c	0.07 b	0.76 b	1.05 c
C_2	97 a	20 b	41 bc	0.21 bc	0.57 b	1.17 b	1.08 c
subsp. *hypogaea*	96	53[b]	60[b]	0.70[b]	1.19[b]	2.80[b]	1.94[b]
subsp. *fastigiata*	96	15	34	0.16	0.45	0.97	1.06

[a]Means followed by the same letter are not significantly different (P<0.05) by t-test.

[b]Difference between means for subsp. *hypogaea* and subsp. *fastigiata* significant (P<0.01) by t-test.

Table 3. Rooting response of four genetically diverse peanut lines under nonimbibed immature leaflet culture.

Line	Rooting medium: Full-strength: Trans-ferred	Full-strength: Rooted	Full-strength: Freq.	Half-strength: Trans-ferred	Half-strength: Rooted	Half-strength: Freq.	Plants exhibiting sustained growth: Trans-ferred	Grow-ing	Freq.
	%			%			%		
NC 7	20	18	90	18	17	93	18	15	83
B_2	14	9	64	75	63	84	14	10	71
A_2	0	0	--	38	36	95	3	1	33
C_2	0	0	--	11	10	91	3	1	33
ssp. *hypogaea*	34	27	79	93	80	86	32	25	78
ssp. *fastigiata*	0	0	--	49	46	94	6	2	33
Total	34	27	79	142	126	89	38	27	71

Chi-square tests:

Among lines	1.943 with 1 df (0.10<P<0.20)	2.292 with 3 df (0.50<P<0.70)	2.153 with 3 df (0.50<P<0.70)
Ssp. *hypogaea* vs. ssp. *fastigiata*		1.273 with 1 df (0.20<P<0.30)	2.991 with 1 df (0.05<P<0.10)
Full-strength vs. half-strength medium		1.358 with 1 df (0.20<P<0.30)	

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