A Two Step Process for the Regeneration of *Arachis* spp. by Shoot Tip Culture of Greenhouse-Grown Plants

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ABSTRACT

Shoot-tips of peanut (*Arachis hypogaea L.*) cultivars Florunner and Pronto grown in the greenhouse, and the tips of *A. villosulicarpa* Hoehne from *in vitro* cultured plants were used for *in vitro* regeneration of peanut. The terminal and lateral buds excised from greenhouse grown plants were surface sterilized with 70% ethanol for 3 min followed by 0.525% sodium hypochlorite for 5 min. Shoot-tips (0.5-3 mm long) were isolated from the buds, transferred to a modified Murashige Skoog (MS) agar medium, and maintained at 26 C with a 16-h photoperiod. The modified MS medium contained MS mineral salts, B5 vitamins, and the hormones 1-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA). The best growth of meristems (0.5-1 mm) was induced with 5.0 µM NAA and 5.0 µM BA. Rooting was induced with 5.0 µM NAA. Both shoot growth and rooting occurred on medium that contained 5.0 µM NAA when large tips (1-3 mm) were cultured. Plantlets regenerated from the tips were successfully transferred to pots and grown to maturity.

Key words: Arachis, BA (6-benzyladenine), meristem, NAA (1-naphthaleneacetic acid), peanut, regeneration, tip culture, tissue culture.

Shoot-tip culture and meristem culture have been used with many legumes for obtaining virus-free plants, for clonal propagation of plants, and for the conservation of germplasm. Meristem culture has been used for elimination of pea seed-borne mosaic virus from over 100 breeding lines of *Pisum sativum* L. (10, 11, 12), and for obtaining virus-free *Trifolium* plants (7). Many legumes have been clonally propagated from either root or shoot meristems. The species include *Trigonella foenum graecum* L., *Vigna unguiculata* L.(4), *P. sativum, Cicer arietum* L., *Lens esculenta* L., *Phaseolus aureus* L., and *P. mungo* L. (3). Cultured meristems and the *in vitro* grown plantlets are being used for the exchange of and conservation of germplasm because they are generally pathogen free (4). Meristems or shoot-tips of about 20 species, including *Arachis hypogaea* L., have been stored in liquid nitrogen and subsequently cultured (19). Cryopreservation of meristems may permit the long-term storage of germplasm in a genetically stable and pathogen-free condition (19).

Success in obtaining plants from meristems or shoot-tips usually depends on the size of the starting material and the culture conditions. Shoot-tip explants (1-5 mm) are generally easier to excise and have a higher survival rate *in vitro* than meristem explants. The addition of NAA will induce complete plant formation from shoot pieces of several legumes (3). Although meristem and shoot-tip culture have been used in a variety of species (23), the application of these techniques to *A. hypogaea* has been limited. Kartha (12) and Russo (18) reported on the utilization of meristem and shoot-tip culture of peanut from plants grown *in vitro*. Additional work on tissue culture of *Arachis* has focused on regenerative responses of leaflet cultures (20), ovule and embryo cultures (22), and anther cultures (21). No reports have been published on tip culture of greenhouse grown peanut. Shoot tip cultures could be an important source of material as many germplasm collections are maintained in greenhouses or under non-aseptic conditions. This study was undertaken to investigate the requirements for culture and regeneration of plants from meristems and shoot-tips of two cultivars of peanut and *A. villosulicarpa* Hoehne. A preliminary report has been published (6).

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Materials and Methods

Seeds of the peanut cvs. Florunner (a runner type), and Pronto (a spanish type) were obtained from H. A. Melouk, USDA-ARS, Stillwater, OK and planted in the greenhouse. The shoots were collected three weeks later. After removing the leaves, the shoots were rinsed in tap water with Tween 20 (1%) for 5 min, 70% ethanol for 3 min, and 0.525% sodium hypochlorite for 5 min. This was followed by three rinses with sterile water. The buds were excised from the shoots under aseptic conditions. Meristem (0.5-1 mm) or shoot-tips (1-3 mm) were isolated from the buds and individually cultured in small test tubes (1.8 x 15 cm) containing 10 mL of nutrient medium. Shoot-tips and meristems of *A. villosulicarpa* were isolated from plants maintained as previously described *in vitro* (17).

The medium consisted of MS mineral salts (14), 3% sucrose, B5 vitamins (9), 0.8% agar, and BA and/or NAA were added in various concentrations. The pH of the medium was adjusted to 5.7 with KOH or HCl prior to adding the agar. The medium was then autoclaved at 120 C for 15 min. After autoclaving, filter sterilized ampicillin (25 mg/mL) was added to the medium to a final concentration of 80 mg/L.

Sixty meristems of each cultivated genotype were used for examining the effect of different combinations of regulators on regeneration of plants from meristems. Ten meristems of the sixty for each cultivar were placed on one of six media supplemented with BA and NAA in different concentrations. Results were recorded after 50 days.

In the initial experiments for regeneration of roots from shoots developed in culture, 75 shoots for each genotype were used. Three shoots of each were placed on one of 25 different media supplemented with different concentrations of BA or NAA. The results obtained on the 225 explants were recorded after 50 days.

For experiments in which growth of lateral and terminal buds form cultivars was compared, three terminal and three lateral buds for each cultivar were placed on one of 25 different media. A similar experiment was conducted using five shoot-tips of *A. villosulicarpa* on each of 5 media with different concentrations of NAA.

All cultures were maintained at a constant temperature of 26C with a 16-h photoperiod, and at a light intensity of 67 mE M^{-2} sec^{-1}. Plantlets were transferred to a pot containing a sandy loam soil and covered with transparent plastic for one week to maintain a high humidity.

Results

From the 120 meristems, large shoots (1-3 cm) from both cultivated genotypes were produced from meristems on the medium containing 5.0 µM NAA and 5.0 µM BA (Table 1). However, no roots formed on these shoots on this medium. Roots, but not shoots, were formed from meristems on a medium containing 10 µM NAA and 0.1 µM BA. No single medium induced both roots and shoots..

Because shoots regenerated from meristems failed to produce roots, they were transferred to other media with different concentrations of NAA and BA for the induction of

Table 1. Effects of different combinations of regulators on meristem culture of peanut[a].

Medium[b] BA (μM)	NAA (μM)	Results[c]
0.1	10	Callus and roots
1	10	Callus
5	10	Callus and multiple shoots
5	5	Multiple shoots
1	5	Multiple shoots
1	1	Single small shoot

[a] Meristems were from peanut cultivars "Florunner" and "Pronto". Ten meristems of each cultivar were used in each treatment.
[b] Medium contained MS mineral salts (14), B5 vitamins (9), 3% sucrose and 0.8% agar.
[c] Results were recorded after 50 days of culture.

roots (Table 2). The best root formation and shoot enlargement of the 225 explants occurred on the medium that contained only 5.0 μM NAA. Roots formed 20 days earlier for the shoots of Florunner and Pronto than for the shoots of *A. villosulicarpa*. In order to assure that induction of roots occurred on media supplemented only with NAA, another 30 explants were cultured on each of the four different concentrations of NAA. Again, the best growth was obtained in the 30 explants of each genotype cultured on the medium that contained 5.0 μM NAA. A greater percentage of cultivated genotypes rooted than *A. villosulicarpa*. An additional experiment was conducted with 150 shoots of each cultivar and 100 shoots of *A. villosulicarpa* on media supplemented with 5.0 μM NAA to examine the success of using only NAA for rooting shoots. All 150 cultured shoots of each cultivated genotype rooted, but only 80 out of 100 cultured shoots of *A. villosulicarpa* rooted.

Based on the results from the culture of meristems, different NAA and BA combinations were used to develop a medium for the development of shoots and roots directly from shoot-tips (1-3 mm). The best growth of terminal buds and lateral buds from each cultivar were occurred on medium containing 5.0 μM NAA (Table 3). There were minimal differences in the growth of terminal buds and lateral buds from either cultivar. In the similar experiment with terminal buds from *A. villosulicarpa*, the medium containing 5.0 μM of NAA was the most effective. There were growth differences between the genotypes. Within one month the shoot-tips of Florunner and Pronto had grown larger than the shoot-tips of *A. villosulicarpa*.

Table 2. Effects of different combinations of growth regulators on development and differentiation of shoots of different genotypes of peanut[a].

Medium[b] (μM)		Cultivar or Species		
BA	NAA	cv. Florunner	cv. Pronto	A. villosulicarpa
0	0	Small shoot	No response[c]	Small roots
0	2.5	Large roots	Long roots	Short roots
0	5.0	Large roots	Short roots	Callus, fine roots
0	7.5	Large roots	Short roots	Large root, callus
0	10	Fine roots	Callus	Small fine roots
0.05	0	No response	No response	Shoots, callus
0.05	2.5	No response	No response	Shoots, callus
0.05	5.0	No response	No response	Shoots, callus, roots
0.05	7.5	Callus	Little callus	Large roots, callus
0.05	10	Callus	Little callus	Callus
0.5	0	No response	Shoots	Shoots, callus
0.5	2.5	Small root	Shoots, callus	Small shoots, callus
0.5	5.0	Callus	Callus	Callus
0.5	7.5	Callus	Callus	Shoots, callus
0.5	10	Callus	Callus	Shoots, much callus
1.0	0	No response	Shoots	Shoots, callus
1.0	2.5	Callus	Shoots	Much callus
1.0	5.0	Callus	Shoots	Shoots, much callus
1.0	7.5	Callus	Callus	Much callus
1.0	10	Callus	Shoots, callus	Little callus
5.0	0	No response	No response	Shoots, much callus
5.0	2.5	Shoots	Shoots	Much callus
5.0	5.0	Callus	Callus	Much Callus
5.0	7.5	Callus	Callus	Callus
5.0	10	Shoots	Shoots, callus	Callus

[a] Each treatment consisted of three replicates. Each had the same result. Results were recorded after 50 days of culture.
[b] Medium contained MS mineral salts (14), B5 vitamins (9), 3% sucrose and 0.8% agar.
[c] No root, callus and shoot enlargement occurred.

Table 3. Comparison of bud source and different combinations of growth regulators on shoot tip culture of peanut[a].

Medium[b] (μM) BA	NAA	Terminal bud	Lateral bud
0	0	Small shoot	Small shoot
0	0.05	Large shoot	Large shoot
0	0.5	Large shoot	Small shoot
0	1.0	Large shoot, root	Large shoot, root
0	5.0	Large root, shoot	Shoot, root
0.05	0	Large shoot	Shoot, callus
0.05	0.05	Small shoot	Shoot, callus
0.05	0.5	Small shoot	Large shoot, callus
0.05	1.0	Large shoot, callus	Large shoot, little callus
0.05	5.0	Much callus	Root
0.5	0	Little callus, shoot	Large shoot
0.5	0.05	Large shoot, callus	Small shoot
0.5	0.5	Shoot, little callus	Shoot, callus
0.5	1.0	Large shoot, callus	Large shoot
0.5	5.0	Callus, one shoot	Small shoot
1.0	0	Large shoot, much callus	Large shoot
1.0	0.05	Shoot, little callus	Much callus
1.0	0.5	Large shoot, callus	Shoot, much callus
1.0	1.0	Large shoot, callus	Callus
1.0	5.0	Shoot, callus	Shoot, callus
5.0	0	Large shoot	Small shoot
5.0	0.05	Shoots	Large shoot, callus
5.0	0.5	Small shoots, callus	Small shoot
5.0	1.0	Small shoots, callus	Callus
5.0	5.0	Small shoots, callus	Callus, shoot

[a] Each treatment has three replicates from both peanut cultivars "Florunner" and "Pronto". The results for each replicate of each cultivar were similar. Results were recorded after 50 days of culture.
[b] Medium contained MS mineral salts (14), B5 vitamin (9), 3% sucrose and 0.8% agar.

Discussion

A previous study (12) found that a medium containing 0.1 μM BA and 10 μM NAA was good for meristem culture of peanut. However, in this study no whole plantlets were formed when meristems from greenhouse grown cultivated peanuts and *in vitro* cultured *A. villosulicarpa* were transferred to a medium supplemented with 0.1 μM BA and 10 μM NAA.

In this study plant regeneration from peanut meristems from two cultivated and wild genotype was best achieved in two steps. The sequential placement of tissue explants on media of different composition has been used for ovule and embryo culture of *Arachis* interspecific hybrids (22) and regeneration from anther callus in *A. paraguarienies* (21). In this study, the first step of shoot growth was induced on medium supplemented with 5.0 μM NAA and 5.0 μM BA. The second step, root formation, was then induced on a medium supplemented with 5.0 μM NAA. Rooting of larger shoot tips from the three genotypes required a medium supplemented with only 5.0 μM NAA. Other researchers (2, 5, 15, 16, 17) found a medium supplemented with NAA alone stimulates root production of explants from peanut. This is true of both cultivated (2, 5, 15, 16) and wild (5, 16, 17) genotypes. This is the first report of growth of shoot-tips from greenhouse grown medium which is supplemented with only NAA.

There are several advantages to using shoot-tip culture instead of meristem culture to propagate peanut. First, shoot tip culture is less laborious than meristem culture. No subculture is required when larger tips are cultured *in vitro*. Secondly, plants regenerate more rapidly. Once root

production is stimulated in cultured shoot-tips, shoot enlargement and growth rapidly follow. This response has also been observed in other species (3). Finally, shoot-tips survive in tissue culture better than meristems.

Peanut is an important source of protein for food and livestock feed production, and peanut production can suffer from diseases. Some of these diseases, e.g. peanut mottle virus (PMV), peanut stripe virus (PStV), and *Sclerotinia*, may be seed transmitted and thus spread by germplasm exchange (1, 8, 13, 24). Shoot-tip culture has been reported in other crop species to be useful in virus elimination, clonal propagation and the conservation of germplasm. The application of this technique to peanut may contribute to the control of virus disease and improvement of peanuts.

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