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# Detached Leaf Technique for the Establishment of Root Cultures of Peanut (*Arachis hypogaea* L.)

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**ABSTRACT**

Axenic cultures of peanut (*Arachis hypogaea* L.) roots can be initiated from *in vitro* cultured embryos and shoot meristems. Embryo axes produced more shoots than tissue taken from axillary meristems. For tissue derived from shoot cultures, virginia cultivars had a higher percentage of rooting explants than a spanish cultivar. Inoculation with *Agrobacterium rhizogenes* increased root yields. However, root propagation in liquid medium was unsuccessful. On the other hand, the use of detached leaves, incubated on a sand/mineral liquid medium, led to vigorous root production after inoculation with *A. rhizogenes*, regardless of the bacterial strain used. These roots could be propagated subsequently in liquid medium. The advantages of the detached leaf technique are discussed.

Key Words: Groundnut, root culture, *Agrobacte-rium rhizogenes*.

Root cultures are a useful tool for physiological studies because of the uniformity of clonal material. They have also been used to investigate root/microbe interactions under controlled conditions (Butcher, 1980) and have proved to be particularly suitable for the gnotobiotic culture of obligate parasites (Ingram, 1969; Zahka and Virányi, 1991) and vesicular-arbuscular mycorrhizae (Hepper and Mosse, 1980).

Mugnier (1988) obtained root cultures of peanut after inoculation with *Agrobacterium rhizogenes* (Riker *et al.*) Conn. He used surface-sterilized seeds as explant material. However, sterile peanut plants are often difficult to obtain from seeds because up to 100% of the cotyledons are systemically contaminated with fungi and bacteria (Pettit *et al.*, 1968). Tissue cultures derived from various plant organs have been successfully established for peanuts (Bajaj, 1984), but rooting occurs only infrequently (Moss *et al.*, 1988). This paper describes an efficient method for the initiation and maintenance of root cultures originating from leaf cultures of peanut inoculated with *A. rhizogenes*.

## Materials and Methods

***Plant Material.*** Two virginia types (cvs. VL GBON and 57-313) and one spanish type (cv. Malimba) were grown in pots (John Innes Compost N° 3) in a greenhouse. Up to 10 axillary buds (1-2 mm) or entire young leaves were taken from each plant as explant materials. All explants (10 axillary buds, five detached leaves, or five seeds) were surface-sterilized in 100 mL solution of 3% (v/v) NaClO containing two drops of Tween 80 for 15 min with agitation and then thoroughly rinsed with sterile distilled water. Embryos axes were isolated from surface-sterilized seeds and subjected to a second and identical sterilization treatment with five embryo axes in 100 mL fresh sterilant.

***Media and Growth Conditions.*** Embryos were cultured on a combination of Murashige-Skoog (MS) basal salts (Murashige and Skoog, 1962) and the growth substances (all from Sigma Chemical Co.) gibberellic acid (0.03 $\mu g\ mL^{-1}$), thiamine (0.1 $\mu g\ mL^{-1}$), riboflavin (0.01 $\mu g\ mL^{-1}$), and 2,4 dichlorophenoxyacetic acid (0.01 $\mu g\ mL^{-1}$) (Hale, 1969). Shoot meristems (*ca.* 0.5 mm) excised from axillary buds were placed onto MS medium containing indole-3-acetic acid (3 $\mu g\ mL^{-1}$), riboflavin (0.01 $\mu g\ mL^{-1}$) and 6-benzaminopurine (1 $\mu g\ mL^{-1}$). Both media contained 3% sucrose and 7 $g\ L^{-1}$ agar (Oxoid N° 3). Approximately 10 mL medium were placed in 30-mL McCartney bottles. Thirty embryos or shoot meristems (10 per cultivar) were placed singly into each bottle. The experiment was repeated three times.

Regenerated shoot cultures (*ca.* 1 cm) were transferred onto MS agar with the B vitamins thiamine (0.1 $\mu g\ mL^{-1}$) and riboflavin (0.01 $\mu g\ mL^{-1}$), but without growth regulators to induce rooting. Subsequently, plantlets were transferred to larger screw-cap jars, depending on the size of the plant.

Five detached leaves (*ca.* 2 cm) from single, regenerated shoot cultures or 10 replicate leaves (5-7 cm) from greenhouse-grown plants were placed into 100 mL sand and

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moistened with White's mineral solution (WMS) (White, 1943) in 250-mL conical flasks. A total of 10 leaves per cultivar were taken from greenhouse-grown plants. For shoot culture-derived leaves, the three cultivars were randomized so that equal numbers of leaves were taken from each cultivar. Due to the low number of successfully rooted material, the different cultivars are not distinguished in the results section. All plant cultures were incubated at 30 C with a 12-hr photoperiod. Overhead light with an intensity of about 20,000 lux was provided by 12 fluorescent tubes (40 W, Thorne, warmwhite) and four tungsten lamps (40 W, Philips).

After roots had reached a length of 1-2 cm, they were excised and transferred into 50 mL WMS containing 2% sucrose, yeast extract (0.2 g $L^{-1}$) and the vitamins thiamine (0.1 µg $mL^{-1}$), pyridoxine (0.1 µg $mL^{-1}$), and nicotinic acid (0.5 µg $mL^{-1}$) in 250-mL conical flasks. The flasks were incubated at 25 C on an orbital shaker (85 rpm) in the dark.

***Bacterial Inoculation.*** *Agrobacterium rhizogenes* (strains 4Y and 1610 obtained from Prof. M. Yeoman) was maintained on a medium containing 2% agar, 1% mannitol, and the mineral nutrients $K_2HPO_4 \cdot 3H_2O$ (655 mg $L^{-1}$), $MgSO_4 \cdot 7H_2O$ (200 mg $L^{-1}$), and NaCl (100 mg $L^{-1}$) at 25 C in the dark. The inoculum was suspended in 0.8% sterile saline and stab-inoculated at the base of the shoot cultures or the leaves. *Agrobacterium rhizogenes* was subsequently eliminated from root cultures in liquid medium by using 250 µg $mL^{-1}$ cefataxim (strain 1610), 50 µg $mL^{-1}$ kanamycin, or 250 µg $mL^{-1}$ carbenicillin (strain 4Y).

***Experimental Design and Statistical Analysis.*** The experiment was arranged in a completely randomized design with unequal replication. Treatments were four tissue origins (axillary meristems, embryo axes, leaves from greenhouse-grown plants, and leaves from shoot cultures); three cultivars (the two virginia types VL GBON and 57-313 and the spanish type Malimba); and three *Agrobacterium* inocula (none, strain 4Y, and strain 1610). Each treatment was replicated three times with a maximum of 10 explants per replicate. Data were collected on the number of successful shoot and root cultures.

The number of shoot and root cultures was expressed as the percentage of total explants, and the percentage data were analyzed using generalized linear models on Genstat 5 (Genstat 5 Committee of the Statistics Department of Rothamsted Experimental Station, 1993). Errors were assumed to be binomial. All factors (origin, cultivar, and inoculum) as well as their first- and second-order interactions were fitted sequentially into the regression model and tested. Where a term was not significant, it was excluded from the final model before predicting the fitted means and standard errors. Analysis of the shoot data was only based on material derived from axillary meristems and embryo axes because material derived from leaf cultures did not form shoots.

## Results

***Shoot Cultures.*** Approximately 50% of the shoot meristems regenerated into shoot cultures and were further transferred onto rooting medium after 8 wk. Up to 90% of cultured embryos regenerated into shoot cultures after 4 wk (Table 1). After 1 mo on rooting medium, shoot cultures branched and unrooted shoots were subsequently micropropagated at monthly intervals. The growth pattern of the parental cultivar (erect bunch for cv. Malimba, spreading bunch for the two virginia cultivars) was retained in micropropagated shoots for at least 10 subcultures in the absence of growth regulators. However, defoliation and callus formation occurred after more than 10 subcultures. Statistical analysis indicated that only the main effect for origin was significant. Material taken from embryo axes formed significantly ($P \leq 0.001$) more shoots (89%) than cultures derived from axillary meristems (50%).

**Table 1. Number of explants used, their origin, successful establishment and rooting frequency with (+) and without (-) inoculation with *Agrobacterium rhizogenes* after 8 wk of incubation (pooled for three cultivars and three experiments).**

| Origin of explant | Inoculation with *A. rhizogenes* | Explants | Shoot cultures | Rooting cultures |
|---|---|---|---|---|
| | | | no. | |
| Axillary meristem | - | 88 | 45 | 21 |
| Embryo axes | - | 90 | 79 | 42 |
| Axillary meristem | + | 89 | 44 | 27 |
| Embryo axes | + | 90 | 82 | 52 |
| Greenhouse leaf | - | 30 | na[a] | 0 |
| Shoot culture leaf | - | 45 | na | 11 |
| Greenhouse leaf | + | 30 | na | 30 |
| Shoot culture leaf | + | 47 | na | 47 |

[a] na = not applicable.

***Root Cultures.*** Approximately 50% of shoot cultures produced roots after 2 mo (Table 1). However, when excised and transferred to WMS, root growth was poor and roots did not survive the second subculture. Rooted plantlets either died or stopped producing roots after their roots were pruned for the second time even though they were transferred onto fresh medium after every excision of roots.

After 4 wk, about 25% of shoot cultures inoculated with *A. rhizogenes* produced vigorously growing roots for up to 5 monthly subcultures. After 2 mo, over 50% had formed roots (Table 1). However, when transferred to liquid medium, the root growth was as poor as for non-inoculated roots.

Surface-sterilized leaves derived from greenhouse-grown plants did not produce any roots after culture for 2 mo. About 25% of leaves excised from sterile, regenerated shoot cultures rooted within a month. Following inoculation with *A. rhizogenes* all detached leaves rooted within 8 wk (Table 1; Fig. 1.). Root production was more vigorous than for shoot cultures and larger segments could be transferred into liquid medium. Rapid root growth continued and subcultures could be taken at monthly intervals. No tests were carried out to confirm the genetic transformation of inoculated roots. However, roots showed the typical phenotype of transformed roots with high growth rates, reduced apical dominance, and plagiotropism (Tepfer, 1984).

Statistical analysis showed that all main effects—origin, cultivar and inoculum and the origin x inoculum interaction–were highly significant ($P \leq 0.01$) in the generalized linear model. Because of the clear difference in response of the leaf and shoot culture material

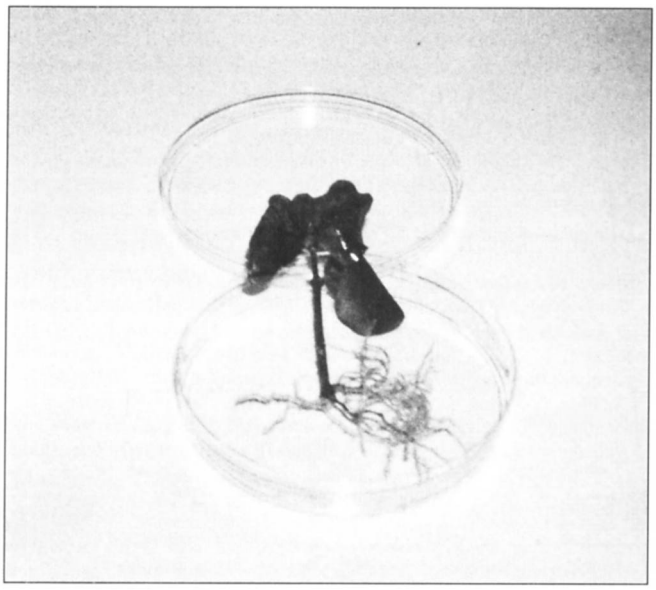

**Fig. 1. Adventitious roots are formed on detached peanut leaf (cv. VL GBON) after inoculation with *Agrobacterium rhizogenes*.**

(Table 2), these data were analyzed separately. In the case of leaf tissue from greenhouse-grown plants, explants did not root when leaves were not inoculated. However, following inoculation, success was 100% regardless of the strain of inoculum applied.

The analysis of rooted material derived from shoot cultures indicated that there were significant ($P \leq 0.05$) main effects due to origin and cultivar only. None of the interactions were significant. Explants originating from embryo axes were more successful at rooting (52%) than those originating from axillary meristems (27%). Among cultivars, the two virginia cultivars had higher success rates (48%) than the spanish cultivar (23%) ($P \leq 0.01$).

**Table 2. Effect of origin and inoculum on percentage of successfully rooted explants in peanut (values are back-transformed means; the transformed data are given in parentheses).**

| Origin of explant | No inoculation | Strain 4Y | Strain 1610 |
|---|---|---|---|
| | ---------- % ---------- | | |
| Axillary meristem | 24 (-1.24) | 31 (-0.87) | 29 (-0.96) |
| Embryo axes | 47 (-0.16) | 53 (0.13) | 62 (0.53) |
| Greenhouse leaf | 0 (-12.19) | 100 (9.85) | 100 (9.85) |
| Shoot culture leaf | 24 (-1.22) | 100 (9.53) | 100 (9.53) |

## Discussion

Shoot and root induction was quicker and more efficient from cultured embryos than from shoot meristems. However, a seed must be sacrificed in order to obtain one embryo, whereas 10 or more meristems can be excised from a single plant.

When roots were excised from nontransformed plantlets, the plantlets exhausted themselves quickly and had to be discarded after the second subculture. Inoculation with *A. rhizogenes*, which has been reported to prolong plant longevity in tobacco (Ackermann, 1976), also increased the success rate of rooting and extended the period over which roots could be obtained from peanut plantlets.

During transformation with *A. rhizogenes*, a root-inducing piece of DNA from the bacterial Ri-plasmid is inserted into the plant genome. This stable insertion changes the developmental fate of the cell, and roots are initiated in the wound tissue (Tepfer, 1984). Because the root-inducing genes are integrated into the plant genome, root production persists even after *A. rhizogenes* has been eliminated from cultures by antibiotics.

Although *A. rhizogenes* increased overall root yields, no sustainable root culture could be established in liquid medium even with inoculated plants. Mugnier (1988) successfully obtained root cultures of peanut from surface-sterilized seeds after inoculation of sterile seedlings with *A. rhizogenes*. In experiments preceding the present study, however, surface sterilization of seeds proved unsuitable for routine use due to the high proportion of systemically contaminated seeds. Also, hot water treatments were discontinued due to their low effectiveness accompanied by severe reduction in seed germeability (data not shown). This study indicates that shoot cultures of peanut are not an efficient starting material to produce sufficient root cultures for use in replicated experiments.

In contrast to shoot cultures, detached leaves incubated in a sand/mineral liquid medium yielded vigorously growing roots after inoculation with *A. rhizogenes*. These roots could be maintained and propagated in liquid medium. High rooting percentages on solid/liquid interface have also been reported by Moss *et al.* (1988) who induced rooting on filter paper strips in a MS-derived liquid medium. It is still not clear what causes the differences in survival between *A. rhizogenes*-treated roots from meristems or regenerated plantlets, which were incubated on a MS-derived medium first, and roots from detached leaves, which were produced in WMS and sand. Possibly roots initiated in MS medium could not adapt to WMS after transfer, whereas leaves were incubated in WMS from the beginning of *in vitro* culture.

Detached leaf culture was originally described by Subrahmanyam *et al.* (1990) as a bioassay for obligate leaf pathogens. In nonsterile conditions, rooting occurs after 1 mo (data not shown). No root induction was observed after 2 mo for surface-sterilized leaves. Meristematic activity is believed to originate from adaxial epidermal cells near the midrib of the leaf (McKently *et al.*, 1991). Epidermal cells could have been damaged during the sterilization process. This interpretation is in agreement with the observation that some leaves rooted if they were derived from sterile, regenerated plantlets and were used without surface sterilization. Whether the root yield can be increased without transformation merits further study. In that respect, a more in depth comparison of different cultivars would also be useful, as the virginia cultivars had a higher percentage of rooting explants than the spanish cultivar tested.

Detached leaf culture, combined with *A. rhizogenes* inoculation, was not only an efficient method for root

production; it also has other practical advantages. Due to the shorter incubation period required, root production is more synchronized. Adventitious roots formed at the leaf base are very uniform and well suited for experiments which require an isogenetic background of plant material. The incubation medium (WMS) does not contain sucrose and is thus less prone to contamination. The technique may, therefore, be particularly suitable for laboratories with limited facilities for sterile work.

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