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In Vitro Culture of Immature Peanut (*Arachis* spp.) Leaves: Morphogenesis and Plantlet Regeneration[1,2]

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ABSTRACT

Green immature leaflets (2-5 mm in length from shoots of germinated seeds or greenhouse grown plants) from species representing seven taxonomic sections of the genus *Arachis (Ambinervosae, Arachis, Caulorhizae, Erectoides, Extranervosae, Rhizomatosae,* and *Triseminalae),* were cultured aseptically, *in vitro,* on a medium composed of Murashige and Skoog salts, Gamborg's B5 vitamins, 0.8% Difco agar, and supplemented with 1 mg/L each of naphthaleneacetic acid and N-6 benzyladenine. Histological examination of the cultures revealed that the meristematic areas originated from epidermal cells. Embryoids and meristematic shoots developed after lysis of the surrounding cells. All species of *Arachis* tested produced callus. Genotypic differences for the production of callus, shoots, and roots were observed with cultivated peanuts. Organogenesis occurred in the leaflet cultures, and plants were recovered from sections *Arachis* and *Extranervosae* of the genus *Arachis.*

Key Words: Leaf explants, organogenesis, tissue culture.

Recent advances in plant tissue culture techniques of nonlegumes (1, 6, 15, 17, 18) and legumes (3, 8, 10, 13, 14, 16, 19, 20, 21, 22, 23) have fostered interest in the use of these techniques for the improvement of cultivated peanuts.

Skoog and Miller (27), Skoog (26), and Saunders and Bingham (24) have demonstrated that organogenesis of explants can be induced by the controlled initiation of organ primordium through the manipulation of constituents in the culture media. Callus formation is usually considered as undesirable because the chromosome constitution of callus cells is unstable in many plants (4,5,25) and for propagation purposes the period of unorganized growth or callus should be minimized (7).

The development of tissue culture methods for *Arachis* which allow for effective plant improvement continues to be a crucial problem. The growth factors needed for the generation of callus from peanut plant tissue have been described by Joshi and Noggle (14), Joshi and Ball (13), Kumar (16), and Guy et al. (10). Martin (19) and Martin and Rabechault (20) describe growth factors for culturing peanut ovules or anthers in which limited success was obtained. Recent work by Mroginski and Fernandez (21,22) and Mroginski et al. (23) resulted in peanut plantlets being formed from anthers and leaflets. Plants used in their callus and plantlet regeneration studies were *A. hypogaea* types; but in anther cultures, Mroginski and Fernandez also included species from the section *Erectoides* (series *Procumbensae*), and section *Arachis* (series *Perennes*).

The objective of this study was to extend the observations of Mroginski et al. (23) to other *Arachis* species. Twenty-eight genotypes of *Arachis* which included cultivars, species, and hybrids were evaluated. Also, the cultures were examined microscopically to investigate the development of callus, shoots, and roots.

Materials and Methods

Cultivars, wild species, and hybrids of peanuts tested for culturing were from a collection maintained by Oklahoma State University and the United States Department of Agriculture (Tables 1 and 2).

Seeds of six commercial cultivars were employed in this study: Chico (spanish), Comet (spanish), Jenkins Jumbo (virginia), Colorado Manfredi (spanish-valencia), P-936 (spanish-valencia), and Tennessee Red (spanish-valencia). Seeds of each cultivar were placed in a transfer hood equipped with a germicidal ultraviolet light (UV) and exposed to UV light at a distance of 38 cm from the source for 48 hours except for Colorado Manfredi II which was sterilized chemically using ethanol and sodium hypochlorite (described below).

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[2]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by Oklahoma State University or the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

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Table 1. *Arachis* spp. used for leaflet cultures.

Name	Collection No.	PI No.	Ac. No. †	Section ‡
A. appressipila §	10002 GKP	262140	P-1537	E_2
A. chacoense §	10602 GKP	276235	P-1553	A_2
A. chacoense			P-1553L ¶	A_2
A. hagenbeckii Harms	10596 GK	276233	P-2359	R_2
A. hypogaea L.				
cv. 'Chico'		268661	P-2398	A_3
cv. 'Colorado Manfredi'			P-3796	A_3
cv. 'Comet'			P-1443	A_3
cv. 'Jenkins Jumbo'			P-961	A_3
cv. 'Tennessee Red'			P-161	A_3
cv. (name unknown)		262129	P-936	A_3
A. monticola Krap. et Rig.	7264 K	219824	P-1536	A_3
A. pernambucensis §	12946 GK	338453	P-1558	AM
A. pintoi §	12787 GK	338447	P-1556	C
A. pusilla Benth.	12922 GK	338449	P-1557	T
A. stenosperma §	410 HLK	338280	P-1847	A_1
A. sp.	555 HLKHe	338293	P-3305	E_2
A. spegazzinii §	10038 GKP	263133	P-2364	A_1
A. villosulicarpa Hoehne	484 HL	336984	P-1565	Ex

† Accession numbers assigned by the Oklahoma Agricultural Experiment Station.

‡ Classification follows that of Gregory and Gregory (9).

§ Undescribed species (nom. nud. Krap. et Greg.).

¶ P-1553L is a large-flowered selection from P-1553.

Table 2. *Arachis* hybrids used for leaflet cultures.†

Parents	Selection Numbers
Chico x P-1553W	M-193
Chico x (A . stenosperma x A. chacoense)F_1C_1	M-189, M-202, M-213
EC5 x (A. stenosperma x A. chacoense)F_1C_1	M-191, M-200, M-207
EM3 x (A. stenosperma x A. chacoense)F_1C_1	M-38, M-39, M-40
EM3 x US 98Y	

† P-1553W is a white-flowered selection from A. chacoense. EC5 and EM3 are breeding lines derived from a cross between Chico and Comet, both are spanish cultivars. US 98Y, a virginia botanical type, is a selection from the Bolivian accession 98 BPZ (P.I. 468295).

Intermittently the seeds were shaken so that all seed surfaces were exposed. Following UV sterilization, seeds were placed in sterile 20 mL disposable scintillation vials containing 5 mL of sterile water and allowed to germinate for a period up to 10 days. Green leaflets 2-5 mm long were selected from young uncontaminated seedlings for use as explants. All sterile operations were performed in a laminar flow hood.

Shoots from plants previously growing in the greenhouse or field plots representing seven sections of *Arachis (Ambinervosae, Arachis, Caulorhizae, Erectoides, Extranervosae, Rhizomatosae,* and *Triseminalae*) and interspecific hybrids were also used in this study. Shoots were surface sterilized ("standard chemical method") by soaking in 70% ethanol with 0.01% Tween 80 for 5 min, 2.63% sodium hypochlorite with 0.01% Tween 80 for 10 min, and then rinsed with three changes of sterile glass distilled water. Sterile green leaflets 2-5 mm long were removed from the shoots aseptically with forceps and placed on Murashige and Skoog's major and minor salts, B5 vitamins as modified by Gamborg (7), 0.8% agar (Difco Laboratories, Detroit, Michigan), and 3% sucrose. Naphthaleneacetic acid (NAA) and N-6 benzyladenine (BA) were added at the concentration of 1 mg/L each (21). The pH of the medium was adjusted to 5.8 with 0.1 N HCl or 0.1 N KOH prior to adding agar. Disposable 20 mL scintillation vials were filled with 8 mL of medium and autoclaved for 15 minutes at 121C and 103.5 KPa. Each treatment consisted of 15 replications with one leaflet per vial.

Cultures were maintained in a growth chamber at 27/21C on a 16/8 hour light/dark cycle. General Electric F20T12·CW fluorescent tubes were used which produced approximately 67 $\mu E\ m^{-2}\ sec^{-1}$ when measured 24 cm from the tubes with a Li-185 (Li-cor®) light meter with a Li-190s quatum sensor (400-700 mm range).

After one week, the cultures with fungal or bacterial contamination were discarded. During the experiment the cultures were examined and notes were recorded for callus, shoot, and root development. Cultures showing callus, shoots, and/or roots were sampled for histological studies by fixing in a solution of formalin-propionic-alcohol for 24 hours and then dehydrating through an ethanol series. Dehydrated tissues were embedded in Paraplast and serially sectioned at 12 μ. Sections were mounted on glass slides and stained with safranin and fast green (2). Photographs were taken during these observations. The experiments were terminated after 60 days.

Results and Discussion

The use of UV light for seed surface sterilization was very effective in minimizing contamination of leaflets. Losses due to bacteria or fungi averaged only 1% in leaflet cultures taken from germinating seeds, while leaflet cultures taken from shoot tips collected from the greenhouse and sterilized by the "standard chemical method" had an average contamination rate of 36%. All other entries were chemically sterilized by the "standard method". The results indicated that seeds surface sterilized with UV light and subsequently germinated can provide a good source of plant material for culture because bacteria or fungi on seedlings can be readily identified and discarded before culturing. Howland and Hart (12) reported that UV exposure of cells is effective in killing single cells or small aggregates. No mutations were observed in these studies, nor were any expected since the UV light could not penetrate the seed coat and cotyledons to reach the leaflet tissues. Material collected from the greenhouse or field-grown plants could not be easily identified as carrying bacteria or fungi until after culturing.

Genotypes differed significantly in their responses to the medium used by Mroginski et al. (22). A summary of the morphogenetic responses for genotypes used is presented in Table 3. *A. pernambucensis, A. spegazzinii, A. stenosperma,* EM3 x US 98Y, M-189, and M-213 produced only callus after six weeks. In addition to callus, roots were produced by Colorado Manfredi, Comet, Jenkins Jumbo, and *A.* sp. after three weeks. Shoots were

Table 3. Morphogenic responses of *Arachis* genotypes in culture.†

Genotype	Observation Period 1‡ % Callus	% Roots	% Shoots	Observation Period 2‡ % Callus	% Roots	% Shoots	Observation Period 3‡ % Callus	% Roots	% Shoots
Cultivated Peanuts									
Chico	47	0	0	53	0	40	71	0	29
Colorado Manfredi	57	0	0	14	0	79	14	57	7
Colorado Manfredi II	0	0	0	43	0	43	67	0	33
Comet	27	13	13	21	14	29	44	11	0
Jenkins Jumbo	53	13	7	7	20	27	50	36	36
P-936	21	14	21	8	8	33	50	0	40
Tennessee Red	53	7	7	20	0	73	50	0	50
Wild Species									
A. appressipila	0	0	0	71	0	14	29	0	14
A. chacoense	0	0	0	75	0	8	29	0	14
A. chacoense (P-1553L)	38	0	25	57	0	29	50	0	50
A. hagenbeckii	44	0	11	63	0	38	50	0	50
A. monticola	0	20	0	50	0	25	0	0	50
A. pernambucensis	25	0	0	25	0	0	25	0	0
A. pintoi	0	0	0	80	0	20	100	0	67
A. pusilla	20	0	0	60	0	0	33	0	33
A. sp. (P.I. 338293)	10	0	0	73	0	0	50	17	17
A. spegazzinii	0	0	0	67	0	0	90	0	0
A. stenosperma	9	0	0	90	0	0	100	0	0
A. villosulicarpa	7	0	0	58	0	33	13	0	88
Hybrids									
EM3 x US 98Y	22	0	0	100	0	0	100	0	0
M-38	28	0	0	72	0	43	72	0	57
M-39	17	0	0	100	0	67	100	0	50
M-40	22	0	0	86	0	57	100	0	50
M-189	20	0	0	80	0	0	100	0	0
M-191	8	0	0	74	0	8	100	0	18
M-193	0	0	0	100	0	11	100	0	25
M-200	23	0	0	76	0	8	92	0	15
M-202	80	0	0	75	0	25	75	0	25
M-207	40	0	10	100	0	50	90	0	10
M-213	0	0	0	50	0	0	50	0	0

†Percentages shown are based on noncontaminated vials (2-15 leaflets per genotype) showing callus, shoots, or roots at a given period of observation.

‡, ‡, and ¶ were at 5 to 10, 18 to 25, and 40 to 46 days respectively.

produced by all genotypes after three weeks except for those that produced only callus.

Examination of the cultures led to a number of interesting observations. Callus production generally started at the basal end of the leaflet. Shoot meristematic zones developed on the periphery of the callus (Figs. 1 and 2). Root formation appeared on the dorsal and ventral surfaces of the callus cultures of *A. villosulicarpa* (Fig. 2). Other cultures generally produced roots only from the upper most part of the callus. Densely stained areas of serial sections indicated the presence of meristematic growth centers.

Evidence that zones of preferential cell division arise from epidermal cells can be seen in Fig. 5. It is uncertain whether these zones are of single cell origin. A heart-shaped embryoid (Fig. 6) and shoot meristems (Figs. 7 and 8) show distinct separation zones within the callus mass. These zones appear to occur after the lysis of surrounding cells. The reason for lysis of the cells is unknown. The appearance of a shoot meristem in the case of *A. villosulicarpa* (Fig. 8) may be the result of meristemoid formation followed by separation from the explant. Shoot meristem (Fig. 7) and embryoid (Fig. 6) formation in *A. hypogaea* genotypes appeared after separation from the

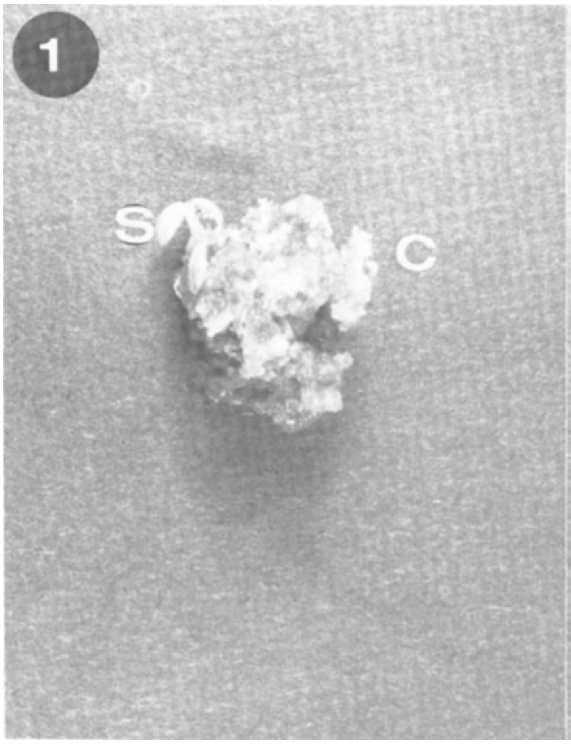

Fig. 1. *Arachis hypogaea* leaflet culture showing shoot (S) and callus (C) regeneration.

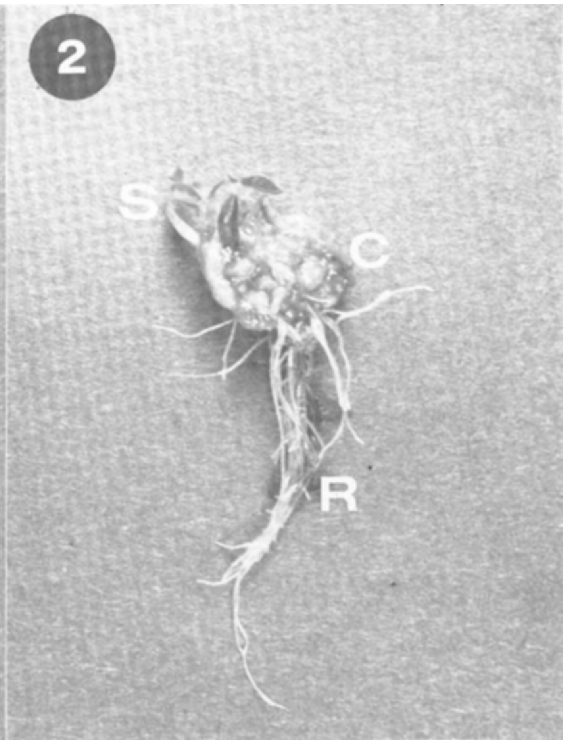

Fig. 2. *Arachis villosulicarpa* leaflet culture showing callus (C), shoot (S), and root (R) growth after 60 days in culture.

When Figs. 3 or 4 are combined, zones of preferential cell division can be seen arising near the midrib area of leaflet. Preferential zones of cell division later became massive and covered the entire leaflet surface (Fig. 4). The genotypes varied in the amount and kinds of meristems produced (Figs. 1 and 2). *A. villosulicarpa* produced extensive roots in addition to callus and shoots, whereas Chico produced only callus and shoots.

Although individual clones responded differently, there were many overall similarities in the morphogenetic patterns obtained. For example, small white-yellowish friable calli were observed for all genotypes after two weeks of culturing. Bud regeneration generally occurred within three weeks in 73% of the genotypes, but the number of leaflets which had produced buds, as well as the number of buds per callus, varied according to genotype (Table 3). Root regeneration generally occurred within two weeks, followed by reduced regeneration of roots after three weeks. Root regeneration decreased or ceased because callus production obscured or inhibited further root regeneration.

callus mass.

The pattern for organogenesis in the different *Arachis* genotypes appears to be in the "indirect pattern" of Hick's (11). The indirect organogenesis pattern is: primary explant ⧫ callus ⧫ meristemoid ⧫ organ system. "Indirect organogenesis" is a sequence which includes a callus stage.

In Fig. 9, graphs of the *A. hypogaea* data by genotype are shown. Although individual genotypes showed some differences in culture responses for callus, shoot, and root production, the greatest differences within botanical types (Chico vs Comet, spanish types; and Colorado Manfredi vs P-936, spanish-valencia types) were noted in root formation. Cultures should be recultured after 25 days because shoot production peaks between 20 and 30 days after culturing. Callus and shoot production were very similar in all groups, including other species and hybrids. Graphic responses of *A. villosulicarpa* are also shown in Fig. 9. Callus production was low, while shoot production was high for *A. villosulicarpa*. It is interesting that in three *A. villosulicarpa* cultures root production started after ap-

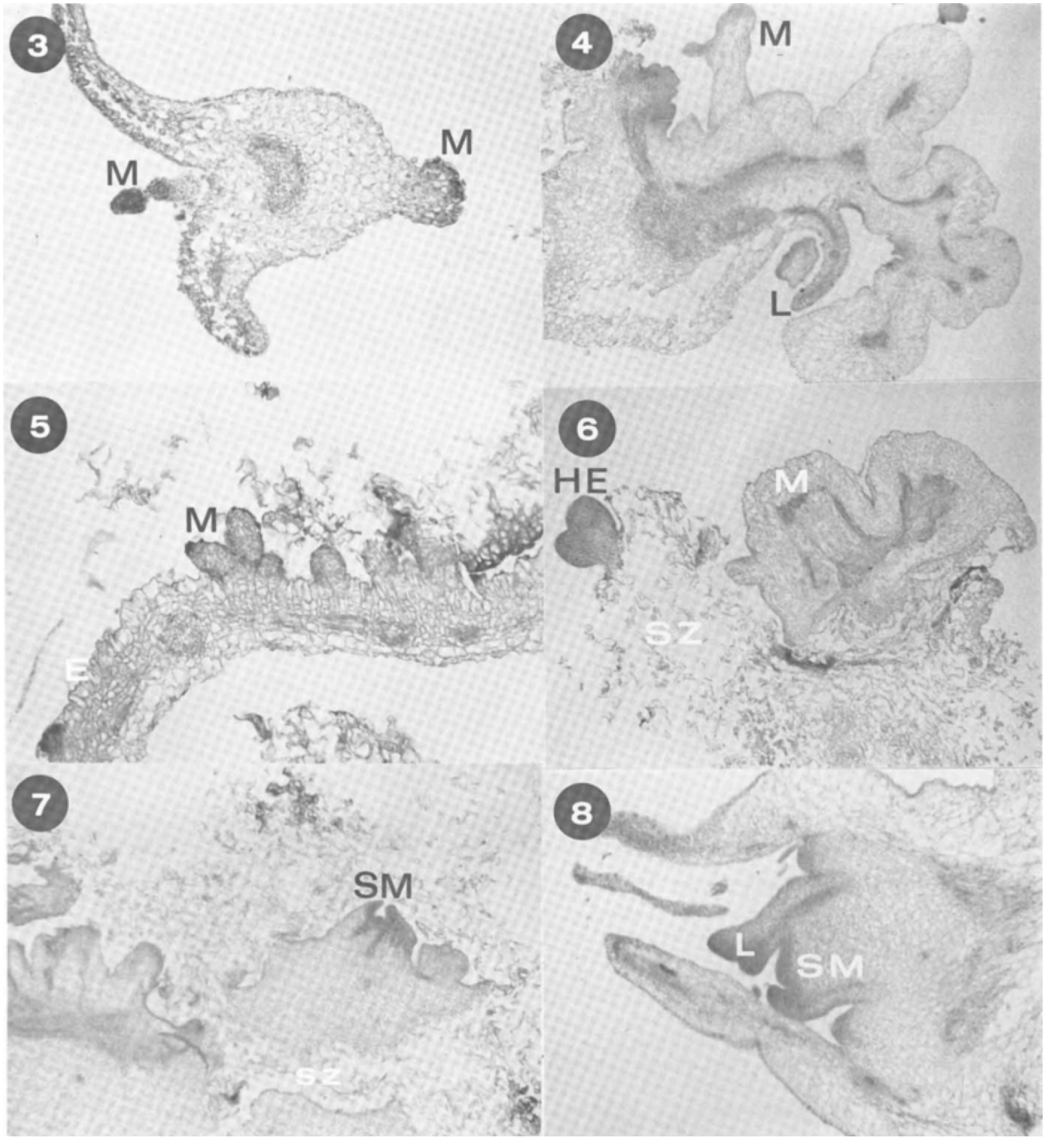

Fig. 3. Basal cross section of a leaflet of an *Arachis* hybrid, EM3 x (*A. stenosperma* x *A. chacoense*)F_1C_1, showing two meristemoid areas (M).

Fig. 4. Cross section of a P-936 leaflet showing part of the leaflet (L) and meristemoids (M).

Fig. 5. Cross section of a Tennessee Red leaflet illustrating meristemoids (M) arising from epidermal cells (E).

Fig. 6. Leaflet cross section of hybrid Chico x Comet showing a heart-shaped embryo (HE), separation zones (SZ), and meristemoid area (M).

Fig. 7. Tennessee Red section showing a separation zone (SZ) and shoot meristematic area (SM).

Fig. 8. *Arachis villosulicarpa* shoot meristem (SM) with embryonic leaves (L).

proximately 40 days in culture, which was after the cultures had been plotted for graphs and then removed for photographing and then replaced back in the vials (Fig. 2). Surviving shoots of Colorado Manfredi, M-213 (Chico x [*A. stenosperma* x *A. chacoense*]F_1C_1), M-38 (EM3 x [*A. stenosperma* x *A. chacoense*]F_1C_1), *A. chacoense* (L.), and *A. villosulicarpa,* were transplanted in soil and are still growing. The plantlets produced in these cultures

provide a demonstration of totipotency in leaflets of *Arachis* genotypes.

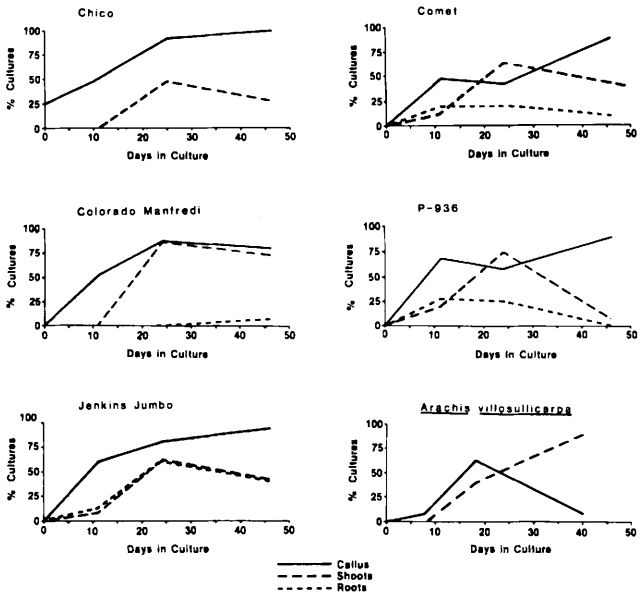

Fig. 9. Responses of *Arachis* genotype for callus.

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