# IN VITRO CULTURE OF PEANUT (ARACHIS SPP.)

## ANTHERS, COTYLEDONS, AND LEAFLETS

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TABLE OF CONTENTS

# CHAPTER I

## INTRODUCTION

The peanut (Arachis hypogaea L.) breeding program in Oklahoma has many long range objectives. These objectives include the development of superior germplasm lines which are resistant or tolerant to environmental and biological stresses. The ability to define and assemble the requisite genetic variability within the genetic resources is necessary to obtain these objectives. After the genetic material has been gathered, the plant breeder must recombine this genetic material and select from the gene pool those gene combinations which yield superior genotypes. Two major constraints limit the progress in plant improvement. First, the recurrent cycle of hybridization and selection under field conditions is time and resource consuming. Second, the range of genetic variability is usually restricted to the species of interest.

A great deal of progress in plant tissue culture has been made during the last few years. This progress has attracted considerable attention because technology has been developed which may mitigate these constraints. Genotype improvement by tissue culture approaches is an expectation founded on the premise that plants can be cultured under defined conditions; haploid cell lines or plants can be obtained; cell cultures can be induced to regenerate fertile plants; plant protoplasts can be fused with the consequent potential for somatic hybridization; and biochemical mutants can be isolated. This

dissertation is composed of a number of studies that have been conducted with emphasis on anther and explant culture. A list of the Arachis species used in tissue culture experiments is given in Table 1. Botanical nomenclature, taxonomic sections, series, species names, and parental numbers follow that of Gregory and Gregory (1) and Gregory et al. (2).

Chapter II examines the establishment of callus tissue culture from cotyledon fragments of A. hypogaea. Emphasis was placed on determining the response of cotyledon fragments to various rates and combinations of auxins and cytokinins in the culture media.

In the third chapter, androgenesis was investigated. Emphasis here was placed on studying the morphological and cytological response of anthers from several species of Arachis within seven taxonomic series.

The fourth chapter reports the results of in vitro culture of immature peanut leaflets. Representatives from five taxonomic sections of Arachis and their hybrids were investigated in these studies.

The fifth chapter is a study of morphogenesis in peanut leaflet cultures. Histological studies of the leaflet cultures were used to determine the areas of origin for developing meristematic areas.

Chapter VI presents a brief summary of the results obtained in Chapters II, III, IV, and V.

Chapters II through V are presented in a form acceptable for publication by the Crop Science Society of America (3). This format is also acceptable for Peanut Science and many other professional journals reporting agronomic research.

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3. American Society of Agronomy, Crop Science Society of America and Soil Science Society of America. 1976. Handbook and Style Manual for ASA, CSSA and SSSA Publications. Madison, WI.

# TABLE

TABLE 1

*Arachis* species used in tissue culture experiments[†]

| Section | Series | Name | OAES Accession No. | Parental No. |
|---|---|---|---|---|
| *Arachis* | $A_1$ annual diploid | *A. spegazzinii* nom. nud. | P-2364 | #8 |
| | | *A. stenocarpa* nom. nud. | | #42 |
| | $A_2$ perennial diploid | *A. chacoense* nom. nud. | P-1553 | #37 |
| | | *A. cardenasii* nom. nud. | P-1538 | #34 |
| | $A_3$ annual tetraploid | *A. monticola* Krap. et Rig. | P-1563 | #11 |
| | | *A. hypogaea hypogaea*[‡] | P-961 | |
| | | *A. hypogaea fastigata* | P-161 | |
| | | | P-936 | |
| | | | P-936 | |
| | | | P-2035 | |
| | | *A. hypogaea vulgaris* | P-1443 | |
| | | | P-2398 | |
| | | | P-2613 | |
| | | | P-3793 | |
| *Erectoides* | $E_2$ perennial diploid | *A. apressipila* nom. nud. | P-1537 | #52 |
| | | *A.* sp.[§] | P-3305 | |
| | | *A.* sp. | | |
| *Rhizomatosae* | $R_2$ perennial tetraploid | *A. hagenbeckii* Harms | P-2359 | #27 |
| *Caulorhizae* | C perennial diploid | *A. pintoi* nom. nud. | P-1556 | #96 |
| *Extranervosae* | Ex perennial diploid | *A. villosulicarpa* Hoehne | P-1565 | #9 |
| *Triseminalae* | T perennial diploid | *A. pusilla* Benth. | P-1557 | #98 |

TABLE 1 (Continued)

| Section | Series | Name | OAES Accession No. | Parental No. |
|---|---|---|---|---|
| Ambinervosae | AM annual diploid | A. pernambucensis nom. nud. | P-1558 | #100 |

† Botanical nomenclature and parental numbers follow Gregory and Gregory (1) 1979. P-numbers are accession numbers assigned by the Oklahoma Agricultural Experiment Station (OAES).

‡ Common names for cultivated A. hypogaea types are 'Chico' (P-2398), 'Colorado Manfredi' (P-2035), 'Comet' (P-1443), EC5 (P-3793), 'Jenkins Jumbo' (P-961), 'Tamnut-74' (P-2613), and 'Tennessee Red' (P-161).

§ Erectoides tetrafoliolata sp. are HLK555-556 and 30110.

# CHAPTER II

## INDUCTION OF CALLUS GROWTH FROM COTYLEDON FRAGMENTS OF PEANUTS (*Arachis hypogaea* L.)


### ABSTRACT

Establishment of callus tissue culture from cotyledon fragments of *Arachis hypogaea* L. is described. Explants from 'Tamnut-74' were grown on modified Murashige and Skoog's medium supplemented with indole-acetic acid (IAA) and benzylaminopurine (BAP). Callus growth from cotyledon fragments was favored when IAA and BAP were at levels of 0.125 to 0.25 mg/l respectively. When 'Chico' cotyledon explants were cultured on a combination Gamborg-Nitsch medium supplemented with naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine, callus growth was favored when the auxin and cytokinin levels were 1.0 to 2.0 mg/l.


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*Additional index words*: Explant, tissue culture.


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The ability to regenerate plants from cultured cells is necessary if genetic alterations, either spontaneous or induced, occurring at the cellular level are to be put into use by the plant breeder. Howland and Hart (9) reviewed the effects of UV and X-ray irradiation on cultured plant cells. Maliga (15) reviewed the isolation, characterization, and utilization of mutant cell lines in higher plants.

Plants have been obtained from cell cultured from several species since the early work of differentiating whole plants from tobacco (Nicotiana tabacum L.) and carrot (Daucus carota L.) cultures (30,31). Callus and cell cultures of various legumes have been reported, including alfalfa (Medicago sativa L.) (7, 27,28), sweetclover (Melilotus alba Desr.) (32), red clover (Trifolium pratense L.) (25) bush bean (Phaseolus vulgaris) (2,14), pea (Pisum sativum L.) (6), perennial clover (Trifolium repens L.) (24), lucerne (Stylosanthes hamata) (29), and winter pea (Lathyrus sativus) (21).

The development of effective tissue culture methods for Arachis continues to be a crucial problem. Joshi and Noggle (12), Joshi and Ball (11), Kumar (13), and Guy et al. (8) describe growth factors needed for the generation of callus from peanut tissue. Growth factors in which limited success was obtained when culturing ovules or anthers have been described by Martin (16) and Martin and Rabechault (17). Mroginski and Fernandez (18,19) and Mroginski et al. (20) described growth factors which resulted in peanut plantlets being formed from anthers and leaflets.

Studies in which seed fragments were used to produce plants include Nuchowicz's (23) work on embryonic axils, D'Cruz and Kale's (3) on excised embryos, Illingworth's (10) on deembryonated cotyledons,

Robbins and Whitwood's (26) on the effect of deep-cold treatment and callus production from excised cotyledons, and Braverman's (1) on aseptic culture of embryonic axis to improve phytosanitation.

The objective of the work reported here was to produce plantlets from cotyledon fragments from two Arachis hypogaea cultivars.

## MATERIALS AND METHODS

In the first study, cotyledons from 'Tamnut-74' with the seed coat and embryonic axis removed were surface sterilized by soaking in 70% ethanol with 0.01% Tween 80 for five minutes, 2.63% sodium hypochlorite with 0.01% Tween 80 for ten minutes and then rinsed with three changes of sterile glass distilled water. Cotyledons were cut into fragments approximately 3 $mm^3$ with a razor blade and aseptically placed on a modified Murashige and Skoog medium solidified with 0.8% agar. All sterile work was carried out in a laminar flow hood.

Murashige and Skoog's media as modified by Murashige and Nakano (22) was supplemented with indoleacetic acid (IAA) and benzylaminopurine (BAP) at the following concentrations of 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/l in all combinations. The pH of the medium was adjusted to 5.8 with HCl or KOH prior to adding agar. Disposable 20 ml scintillation vials were filled with ten ml of the medium prior to autoclaving for 15 minutes at 121°C and 15 psi. Each combination was replicated four times with one cotyledon fragment per vial.

Cultures were maintained in a growth chamber at 27/21°C on a 12/12 hour light/dark cycle. Grolux fluorescence tubes were used for lighting and produced approximately 500 lux at the top surface of the vials. After one week, cultures were examined for microbial contamination and discarded if contamination was present. Cultures were also examined for callus, shoots, and roots at irregular intervals during and at the end of a 45 day growth period.

Cotyledon fragments of 'Chico' were used for the second test. Explant preparation, sterilization, and growth chamber procedures were the same as described earlier. The medium contained Gamborg's macro

salts (5), Nitsch's micro salts as modified by Yeoman (33), Dulieu's vitamins (4), 6.0% agar, and 30 g/l mannitol. Naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and benzylaminopurine (BAP) were added to the medium at concentrations of 0.0, 0.025, 0.25, 0.5, 1.0, and 2.0 mg/l in all combinations. Each treatment was replicated five times with one cotyledon fragment per vial. During the experiment, cultures were examined for disease contamination and those with contamination were discarded. At the end of 55 days, the explants were evaluated for callus, shoot, and root production.

## RESULTS AND DISCUSSION

In the first study, involving Tamnut-74 cotyledons, callus formation was favored at IAA and BAP concentration between 0.125 and 1.0 mg/l (Fig. 1). The cultures exhibited callus formation in 27% of the cultures and appeared to be light-green to green indicating the presence of chlorophyll. The best production of callus appeared with IAA at concentrations between 0.125 and 0.5 mg/l and BAP at concentrations between 0.125 and 0.25 mg/l.

In the Chico experiment, all three growth regulators (BAP, NAA, and 2,4-D) had effects on callus initiation, as demonstrated in Fig. 2. Callus was produced in 18% of the various combinations. In all but seven cases in which callus was produced, BAP had a level of 1.0 to 2.0 mg/l. Callus tissue was produced with 2,4-D only at concentrations of 0.25, 0.5, 1.0 and 2.0 mg/l, but callus occurred at all concentrations of NAA. Callus color patterns for the cultures according to 2,4-D and NAA concentrations are shown in Fig. 2. Fig. 2 is divided into three zones to show general color trends. In zone 1, both white and yellow callus were produced. In zone 2, some cotyledons merely turned green while others produced callus. The cotyledon response of turning green without callus formation was the major occurrence in zone 3. Callus production appears to be regulated by the levels of auxin present and NAA appears to have less of an effect than 2,4-D. Reduction in NAA and 2,4-D concentrations results in a response shift from callus production to cotyledon greening.

In general, the results indicate that the transition of cotyledon tissue to callus is influenced by the levels of auxin and cytokinin present in the culture media.

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# LIST OF FIGURES

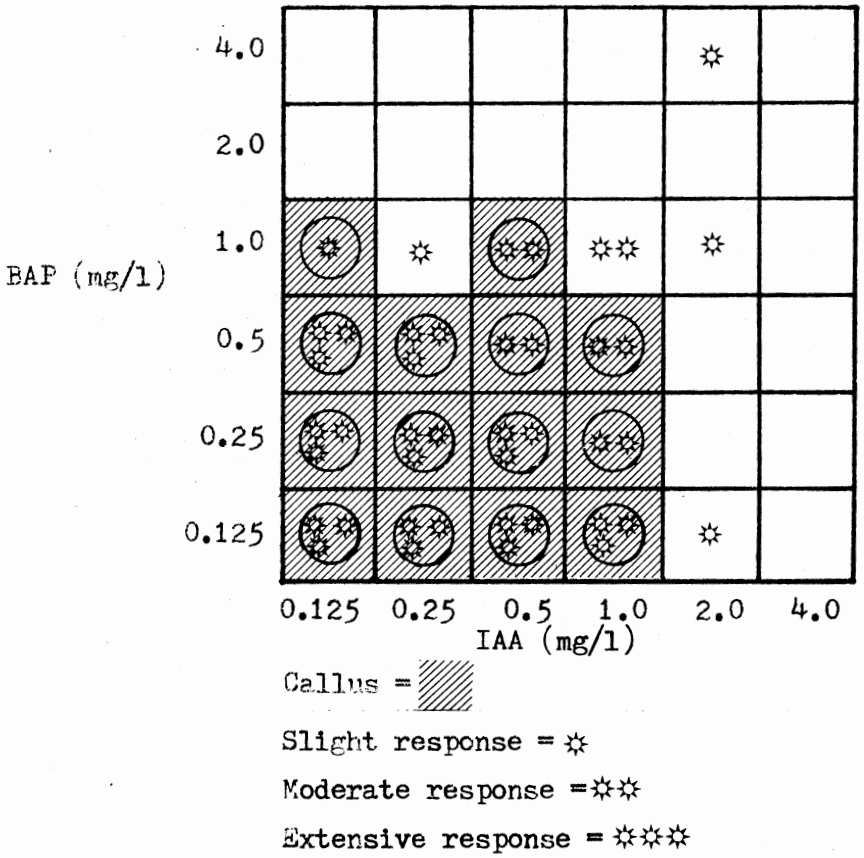


Fig. 1. Response of Tamnut-74 cotyledon fragments in culture to various combinations of IAA and BAP.

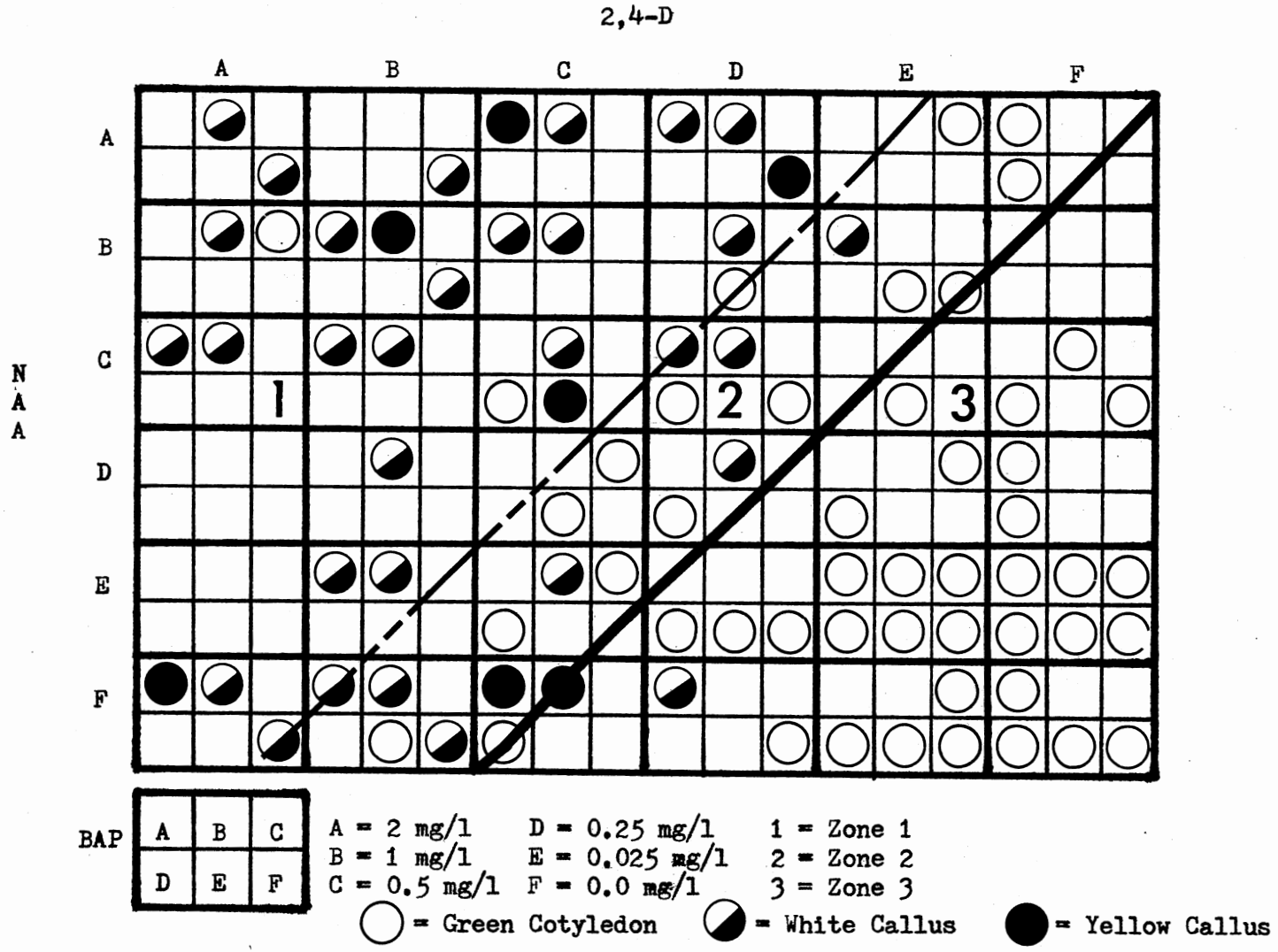


Fig. 2. Response of Chico cotyledon fragments in culture to combinations of 2,4-D, NAA, and BAP.

# CHAPTER III

## CALLUS FORMATION AND SHOOT DIFFERENTIATION IN ANTHER FLOAT CULTURES OF *ARACHIS* SPP.


### ABSTRACT

White to yellowish-white anthers from five taxonomic sections of *Arachis* (*Arachis*, *Erectoides*, *Caulorhizae*, *Ambinervosae*, and *Rhizomatosae*) were cultured aseptically *in vitro* on media with agar and without agar supplemented with indoleacetic acid or naphthalene acetic acid and kinetin or benzyladenine. The media supplemented with indoleacetic acid or naphthalene acetic acid and kinetin stimulated callus production at the site where the filament and anther are attached. Shoots from callus occurred in anther float cultures of Murashige and Skoogs medium supplemented with naphthalene acetic acid and benzyladenine. Limited organogenesis occurred in *A. pintoi* cultures of section *Caulorhizae* but no plants were recovered.


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Additional index words: Tissue culture, explant, organogenesis.


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Research efforts aimed at androgenic embryoid formation by tissue culturing anthers or isolated pollen grains on artificial media have increased recently. Because of their potential as tools for the simplification and/or acceleration of breeding programs, haploids have attracted plant breeders. Reviews on this subject have been published by Melchers (25), Sunderland (36,37,38,30) and Reinert and Bajaj (33).

Magoon and Khanna (20) discussed haploids in general and various means of producing haploids other than by pollen and/or anther cultures. The various methods usually employed to induce haploidy are: 1) Delayed pollination, 2) Use of abortive pollen, 3) Distant hybridization, 4) High and low temperatures, 5) Radiation, and 6) Various chemicals. The low frequency of occurrence and the difficulties encountered in detecting the haploid individuals limits these approaches for incorporating haploids into a plant breeding program. Chromosomal elimination in barley (13,16,17) and semigamy in cotton (45,46) offer the breeders of these species an opportunity to incorporate haploids into their breeding programs because of the regularity at which the haploids occur.

In a review by Sink and Padmanabhan (35), immature pollen was found which could be induced to divide under optimum cultural conditions resulting in:

1) Direct development of embryoids (18,30,31,43,49). Haploid cells divided and developed in stages analogous to those of normal seed embryos.
2) Undifferentiated callus (2,4,6,9,12,34). Transfer to a regeneration medium resulted in callus differentiating to form roots and shoots which could result in ploidy variation.

Direct development of embryoids usually insures production of a haploid plant and is preferred to the callus pathway.

Sunderland (37,40) classified embryoid formation into three pathways. Haploidy is assured in pathway A by non-participation of the generative cell. Haploidy is also assured in pathway B, in which one of the two nuclei, though similar to its sister in size and staining response, undergoes limited division, and like the generative nucleus in pathway A, fails to participate in the final product. An extension of the A pathway is the C pathway which commences with a normal asymmetric mitosis. This route differs from the A route in that the generative cell is functional and participates in embryogenesis.

The nutrient requirements for pollen and anther culture vary widely from species to species. Sunderland (36), Nitsch and Norreel (29) and Reinert and Bajaj (33) have discussed the general nutrient and cultural requirements for pollen and anther culture for several species. Sunderland (37) found tobacco anther response usually lies between the tetrad and a stage just past the first pollen mitosis for haploid production. Sunderland and Roberts (42), with tobacco at 7-8°C for 12 days, and Malhotra and Maheshwari (21) with petunia at 6°C for 48 hours, have shown a cold treatment promotes the induction of embryogenesis and increases the number of pollen grains developing into embryoids. Sunderland and Roberts (41,42), Sunderland and Wildon (44), and Wernicke and Kohlenbach (47) increased the percentage of plantlet producing anthers of Nicotiana and Hyoscyamus by using a liquid medium.

Nuchowicz (32), working with peanuts, developed a medium in which fragments of embryos could be cultured. A medium for mesophyll cell culture of A. hypogaea was developed by Joshi and Noggle (15) and

Joshi and Ball (14). Vitamin requirements of peanut callus were studies by Kumar (19). Martin (22) using major salts of Murashige and Skoog, minor salts of Hildebrandt, Riker and Duggar as modified by Yeoman (48), and vitamins as recommended by Dulieu (5) was able to culture peanut ovules to produce plants. Martin et al. (24) reported that peanut flowers with pollen at the tetrad stage contain pollen grains which can be cultured to produce haploids. Later, Martin and Rabechault (23) produced callus from anthers that was composed of haploid, diploid and polyploid cells. Organogenesis occurred in their cultures but the plants were spindly and mostly albinos. Guy et al. (10) studied biochemical parameters of callus growth from three A. hypogaea cultivars ('Early Bunch', 'NC-Fla 14', and 'Florunner'). Mroginski and Fernandez (26) were able to produce callus with various ploidy levels from anthers of A. hypogaea ('Colorado Manfredi'), A. correntina and A. villosa. Later, Mroginski and Fernandez (27) cultured anthers of A. lignosa and A. sp. (HLK559) and obtained callus and plantlets which had the same chromosome number as the original material. Bajaj et al. (1) cultured anthers from A. hypogaea and A. glabrata which contained uni- and binucleated pollen. Although no chromosome counts were made, their study showed pollen underwent early segmentation, multinucleation, and multiplication, which led to the formation of embryoids which proliferated to form callus. Mroginski et al. (28) have been able to produce plantlets from leaflet cultures by modifying the auxin and cyotkinin rate of the medium used for anther culture.

The objective of the work reported here was to produce haploid plants by utilizing anthers from Arachis species representing five

*Arachis* sections and to determine their stages of development.

Preliminary experiments utilizing tobacco, where haploidy induction is well known, were conducted to develop expertise in general culture methods.

# MATERIALS AND METHODS

Seeds of Nicotiana tabacum cv. White Burley were germinated in a 10 cm pot, transplanted to larger pots and grown in a fiberglass greenhouse at temperatures ranging from 21 to 38°C. Procedures and methods as outlined by Sunderland and Roberts (42) were used when culturing anthers from tobacco. Only surface sterilized buds having anthers containing early bicellular pollen as described by Nitsch and Nitsch (30) were removed in a laminar flow hood by forceps and placed in Pyrex petri dishes (100 x 5 mm) containing 15 ml of culture medium. All dishes were sealed with Parafilm and incubated at 27°C in darkness for the first 14 days in a growth chamber. After 14 days, the cultures were maintained at 27/21°C on a 12/12 hour light/dark cycle. Grolux fluorescent tubes were used for lighting and produced approximately 500 lux when measured 24 cm from the tubes. Later, the culture methods were extended to peanuts.

Peanut species grown in a greenhouse or growth chamber which were utilized are listed in Table 1.

In the first study, flower buds of 'Chico', 'Jenkins Jumbo', and A. pernambucensis were given a pretreatment. Buds were placed between damp paper towels, wrapped in aluminum foil, placed in a sealed plastic bag, and stored at 6°C for 48 hours in a refrigerator. After pretreatment, the buds were surface sterilized in a laminar flow hood by soaking buds in 70% ethanol with 0.01% Tween 80 for two minutes, 2.63% sodium hypochlorite with 0.01% Tween 80 for five minutes, and rinsed with three changes of sterile glass distilled water.

The culture medium was composed of Gamborg's B5 macronutrients (7), Nitsch's micronutrients as modified by Yeoman (48), Dulieu's vitamins

(5), 2.0% sucrose and 0.8% agar. Naphthalene acetic acid (1.86 mg/l) and kinetin (0.0215 mg/l) supplemented the media. The pH was adjusted to 5.5 prior to adding agar. Disposable 20 ml scintillation vials with 10 ml of medium were autoclaved for 15 minutes at 121°C and 15 psi. One white or yellowish-white anther for each genotype was placed on solidified medium in a disposable 20 ml scintillation vial. Each genotype was replicated 96 times. Then, the vials containing the anthers were placed in the dark for 14 days followed by 12/12 hours day/night cycle at 27°C. Grolux fluorescent tubes were used as a light source which produced approximately 500 lux at the top of the vials.

Pollen viability for one species (A. hagenbeckii) was evaluated by placing the buds in cold storage at 6°C for various lengths of time. Heslop-Harrison and Heslop-Harrison's (11) method was used to evaluate pollen viability. Two buds were removed daily for evaluation. Fluorescence of pollen was observed under a Zeiss microscope equipped with an Epi-fluorescence condenser, mercury lamp, G 436 exciter, FT510 reflector and LP 515 barrier. The percentages of fluorescing and non-fluorescing grains were recorded for ten replications of one hundred grains each. The average percent of assummed fertile or sterile grains was then calculated.

In a second study, anthers from Jenkins Jumbo were placed on Murashige and Skoog's medium as modified by Sunderland and Roberts (41) with various combinations of indoleacetic acid (IAA) and kinetin (K). Buds were surface sterilized as described above. Murashige and Skoog's medium was supplemented with 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/l of IAA and K in all combinations. The pH was adjusted to 5.5 prior to adding agar. Ten ml of each combination of IAA and K were

put in 20 ml disposable scintillation vials with each combination consisting of four replications. Observations on growth and callus color were taken at the start and conclusion of the experiment.

In a third study, anthers were collected from plants previously growing in a fiberglass greenhouse or in a plant growth chamber maintained at 30/24°C on a 12/12 hour light/dark cycle. The anthers were surface sterilized as given in the procedure above. Anthers representing five sections of Arachis (Arachis (P-2364, P-1538, Chico and Jenkins Jumbo), Erectoides (30110 and HLK555-556), Caulorhizae (P-1556), Rhizomatosae (P-2359), and Ambinervosae (P-1558)) were used in this study. White or yellowish-white anthers were separated with forceps from the filament aseptically in a hood. Anthers were float cultured on Murashige and Skoog medium as modified by Sunderland and Roberts (41). Murashige and Skoog's major salts, minor salts, vitamins and sucrose were supplemented with naphthalene acetic acid (2 mg/l) and benzyladenine (0.5 mg/l) (27). The pH of the medium was adjusted to 5.6 with KOH or HCl prior to dispensing in Pyrex quadrant petri dishes. Three ml of medium were dispensed into each quadrant of the 100x15 mm petri dishes prior to autoclaving for 15 minutes at 121°C and 15 psi. Cultures were maintained at 27/21°C on a 12/12 hour light/dark cycle in a plant growth chamber. Grolux fluorescent tubes were used for lighting and produced approximately 500 lux when measured 24 cm from the tubes.

Later, anthers which had produced callus or shoots were sampled for histological studies. Callus and/or shoots were fixed in formalin-propiono-alcohol (90:5:5) for 24 hours and dehydrated through an ethanol series. Dehydrated tissues were embedded in Paraplast and

sectioned at 12 μ thickness on a rotary microtome. Sections were then mounted on glass slides and stained with safranin and fast green (3).

Photographs were made of the anther cultures at irregular intervals during the experiments.

# RESULTS AND DISCUSSION

Anthers from flowering tobacco plants were collected and cultured as outlined in the procedures and methods of Sunderland and Roberts (41). Fig. 1 shows an anther which had shed pollen into the medium after culturing for 40 days. Embryoids began to develop 10 days after culturing. Fig. 2 shows an embryoid with normal cotyledon and root development. Although no plantlets were transferred for further development, several embryoids were observed floating in the culture media indicating that the basic microtechnique and culture procedures had been mastered and could be extended to other species.

No difficulty was experienced in growing the Arachis species nor in collecting anthers and placing them in culture. Fig. 3 shows the stage of development of Jenkins Jumbo pollen when the anther colors were white to yellowish-white.

In the first study in which anthers from Chico, Jenkins Jumbo, and A. pernambucensis were given a pretreatment of storing for 48 hours at 6°C in a refrigerator followed by culturing in the dark for 14 days, only 11 of 288 anthers survived. Three anthers of Chico and eight anthers of Jenkins Jumbo produced callus at the site where the filament was attached to the anther (Fig. 4). All anthers from A. pernambucensis failed to produce any callus. Anthers which did not produce any callus decreased in size and turned dark.

A. hagenbeckii pollen showed a decrease in viability, when stored from zero to six days at 6°C. Fig. 5 shows an example of A. hagenbeckii pollen stained with fluorescein diacetate. The presumably viable pollen fluoresced brightly when viewed with ultraviolet light. Fig. 6 shows the decrease in peanut viability over a six day period when

stored at 6°C. Fig. 7 shows the effect of the cold pretreatment on pollen viability of peanut and tobacco. It is apparent that peanut pollen loses viability much faster than tobacco under these conditions. Pretreatment of peanut pollen at 6°C temperature seems questionable as a promoter of haploid production because viability is reduced at a fast rate.

In the second study, where anthers from Jenkins Jumbo were grown on Murashige and Skoog's medium supplemented with various rates of IAA and K in different combinations. All combinations of IAA by K produced callus except 0.1x1.0, 0.5x3.0, 1.0x0.1, 3.0x0.1, 3.0x1.0 and 5.0x1.0 (Fig. 8). Because callus was produced by the anther filament tissue rather than from the pollen, these culture media and methods are of little value for haploid production in Arachis spp.

In the third study, anthers were float cultured on Mroginski and Fernandez's (26) medium. The anthers of all species appeared normal but produced no callus even after culturing for one month. However, it was determined later that some of the anthers had sunk in the medium during the experiment. Results here indicate that meticulous care should be taken while observing and moving culture dishes with a liquid medium. Those anthers which had part of the filament still attached showed filament enlargement which probably resulted in the anther sinking and dying. In some cases, septum areas of the anther appeared to enlarge. This enlargement again caused the anther to sink and die. In the above experiments only one genotype, A. pintoi, produced shoots from the callus. White or yellowish-white anthers of this species appeared to enlarge and later changed colors. Callus-like growth produced by this species showed dark and light areas with shoots arising

from the former (Fig. 9). When the anther culture of A. pintoi showing shoots was transferred to a plantlet medium of Mroginski et al. (28), the culture started producing true callus (Fig. 10).

Figs. 11, 12, and 13 show histological sections of A. pintoi callus and plantlets. Leaves, leaflets, and meristematic areas can be seen in the photomicrographs. Fig. 11 shows a section of the dark callus-like growth from which the shoots originated. Chromosome counts could not be made from these materials nor could the origin of the shoots from the callus be discerned.

Although only one genotype, A. pintoi, produced shoots, float culturing peanut anthers might be effective if meticulous care were given to the regulation of the media depth. Additional experiments are needed to succeed in producing haploid peanuts.

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# TABLE

# LIST OF FIGURES

TABLE 1

*Arachis* species used for anther culture.†

| Section | Series | Name | OAES Accession | Parental Number |
|---|---|---|---|---|
| *Arachis* | $A_1$ annual diploid | *A. spegazzinii* | P-2364 | #8 |
| | $A_2$ perennial diploid | *A. cardenasii* | P-1538 | #34 |
| | $A_3$ annual tetraploid | *A. hypogaea vulgaris* ‡ | P-3793 | |
| | | *A. hypogaea vulgaris* | P-2398 | |
| | | *A. hypogaea hypogaea* | P-961 | |
| *Erectoides* | $E_2$ perennial diploid | *A.* sp. § | | |
| | | *A.* sp. | P-3305 | |
| *Caulorhizae* | C perennial diploid | *A. pintoi* | P-1556 | #96 |
| *Ambinervosae* | AM annual diploid | *A. pernambucensis* | P-1558 | #100 |
| *Rhizomatosae* | $R_2$ perennial tetraploid | *A. hagenbeckii* | P-2359 | #27 |

† Botanical nomenclature and parental numbers follow Gregory and Gregory (8). P-numbers are accession numbers assigned by the Oklahoma Agricultural Experiment Station (OAES).

‡ Common names for cultivated *A. hypogaea* types are: EC-5 (P-3793), 'Jenkins Jumbo' (P-961), and 'Chico' (P-2398).

§ *Erectoides* section sp. are: HLK555-556 (P-3305) and 30110.

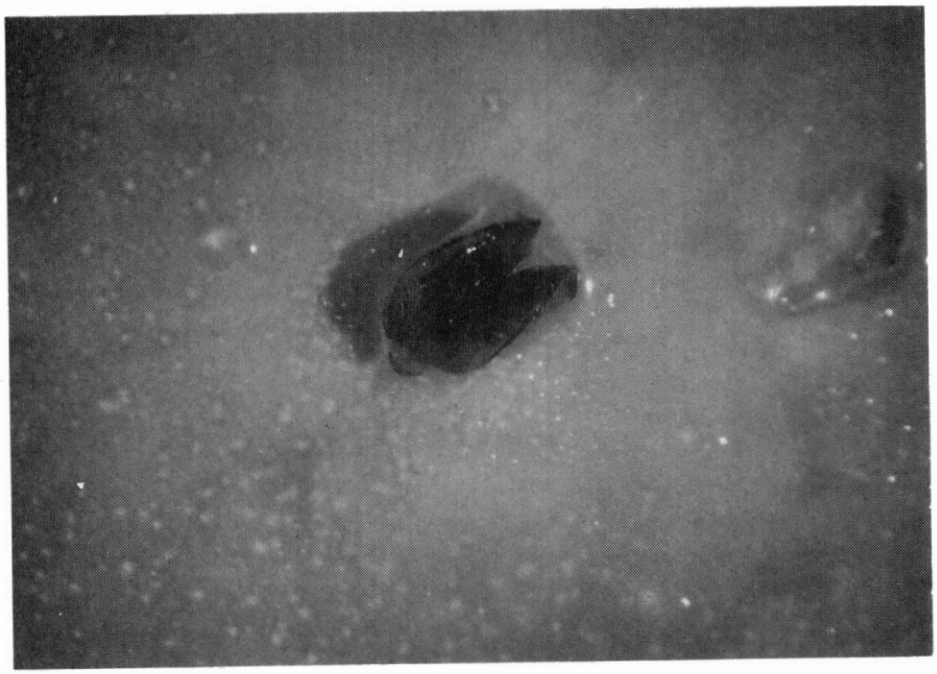


Fig. 1. Tobacco anther culture after 40 days. Pollen has shed into the medium. An embryoid is shown at extreme right.

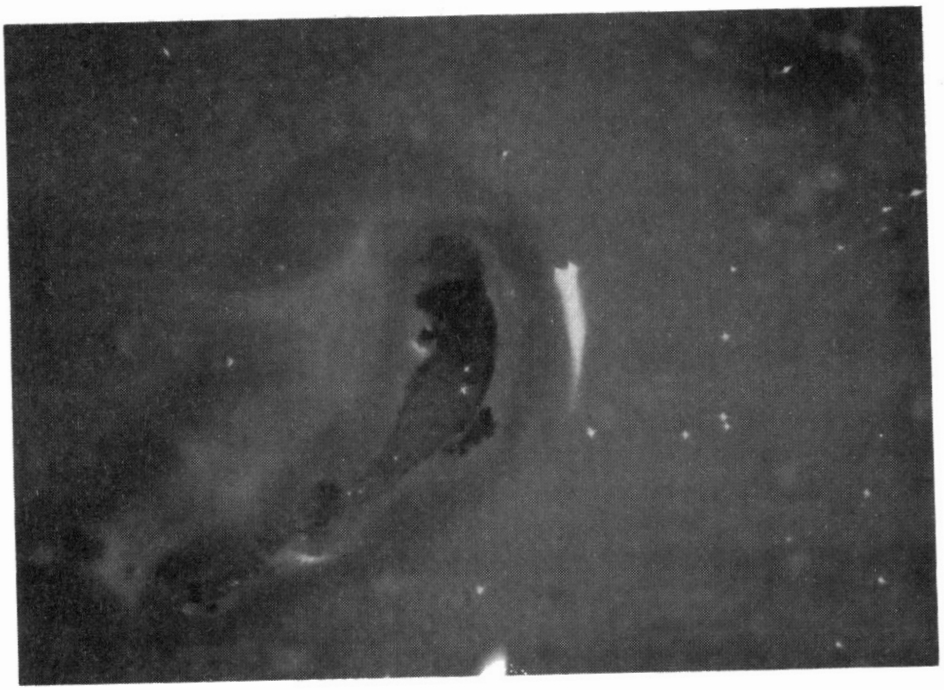


Fig. 2. Tobacco embryoid 40 days after culturing.

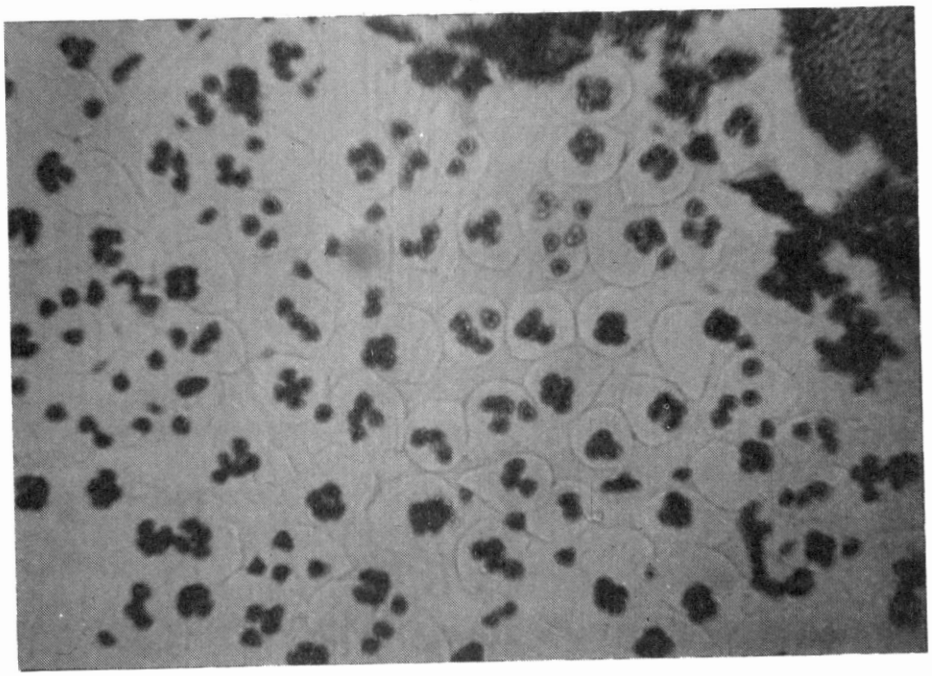


Fig. 3. Arachis hypogaea (Jenkins Jumbo) pollen at the late tetrad stage of development collected from anthers which were white or yellowish-white.

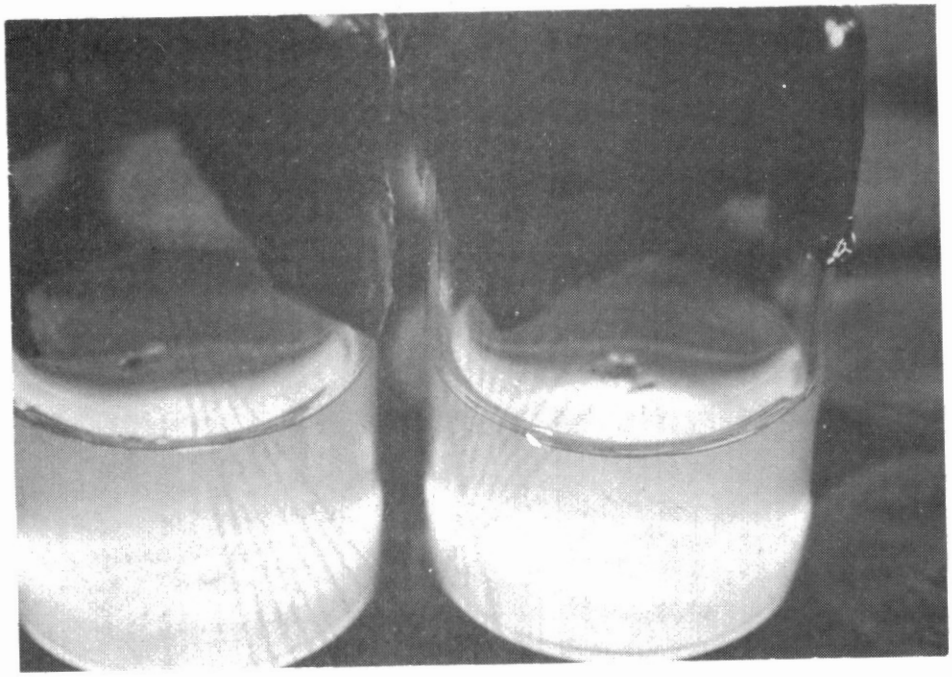


Fig. 4. An anther from Arachis hypogaea (Jenkins Jumbo) showing callus growing at the site where the filament was removed from the anther.

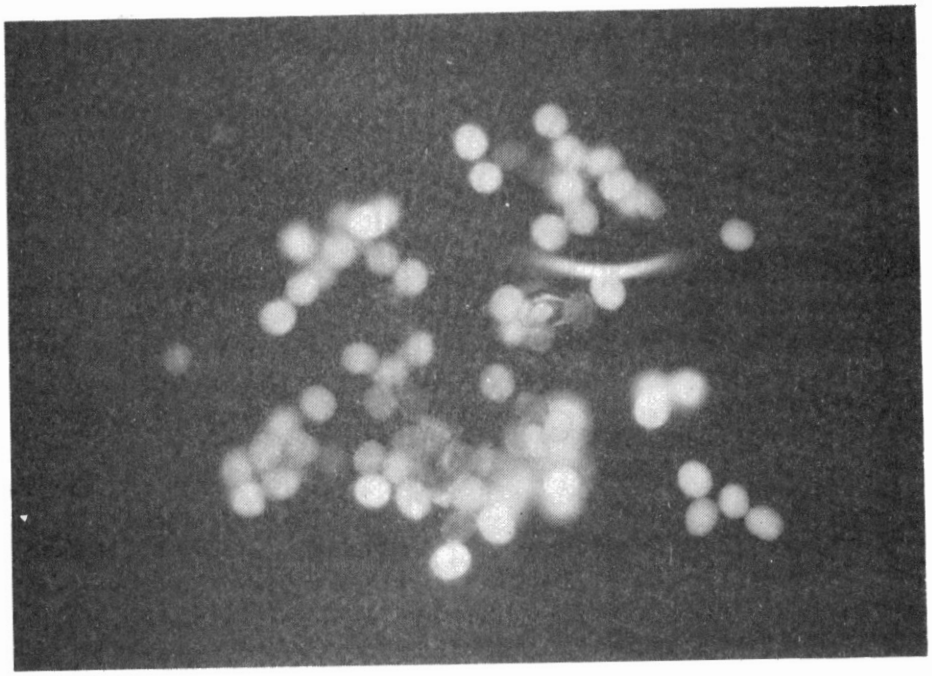


Fig. 5. Pollen grains from Arachis hagenbeckii stained with fluorescein diacetate. Bright grains are presumed to be viable.

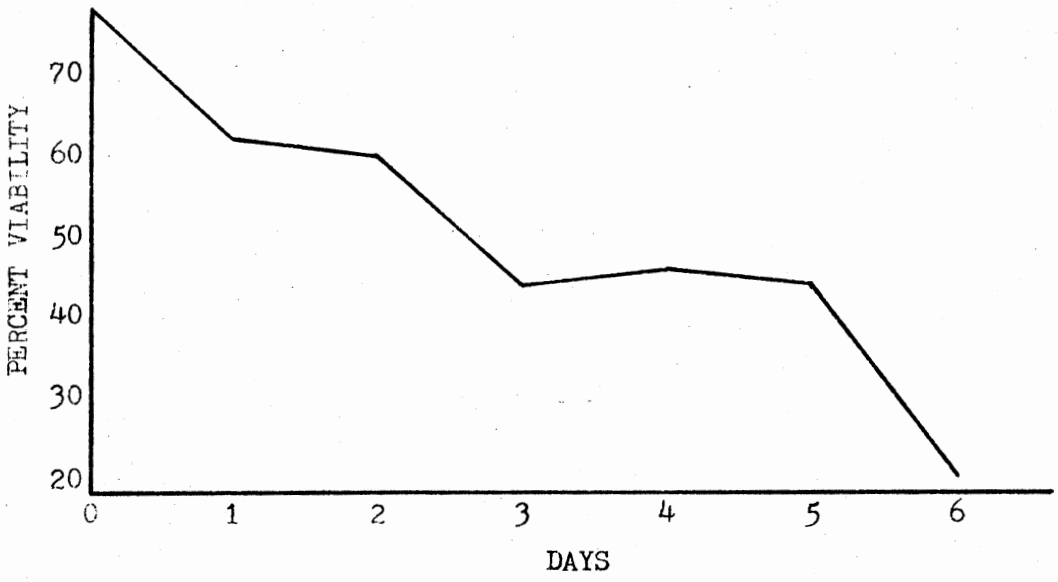



Fig. 6. Changes in pollen viability of A. hagenbeckii during pretreatment at 6 to 8°C.

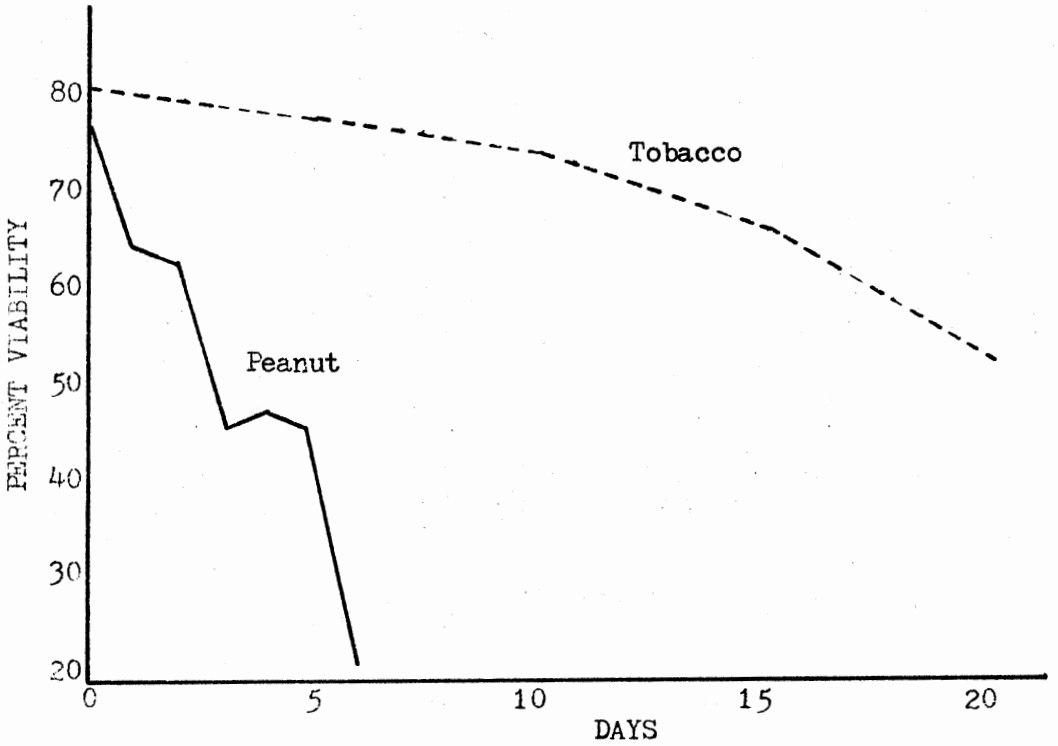
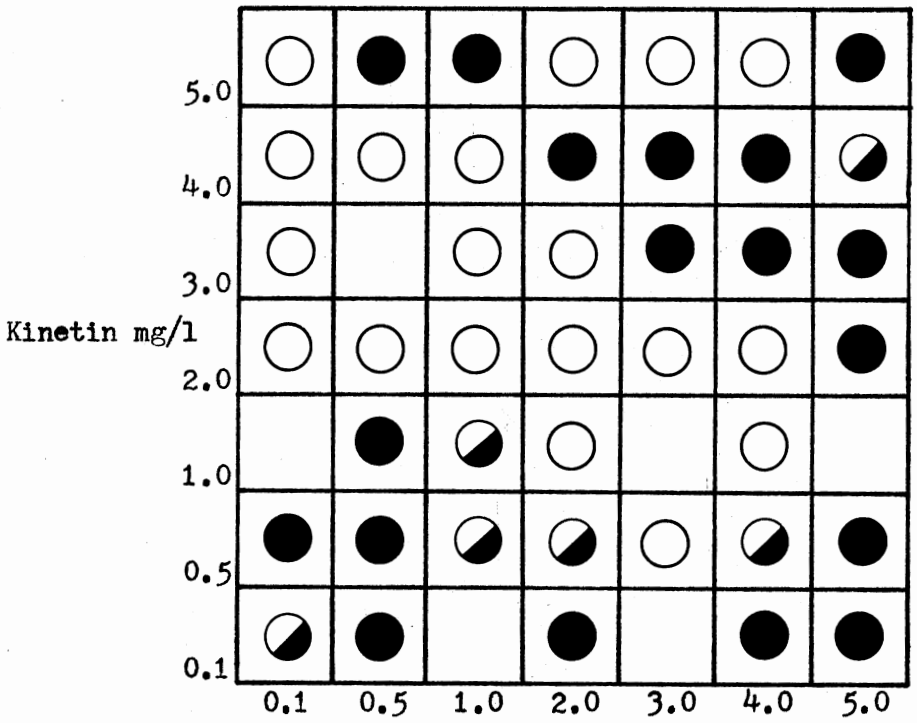



Fig. 7. Comparison of the viability of peanut pollen with Sunderland and Dunwell's (40) tobacco data.




Symbols used denote the following: ◑ = early callus production, ● = white callus, ○ = yellow callus.

Fig. 8. Responses of anthers from Jenkins Jumbo grown with various concentrations of indoleacetic acid and kinetin.

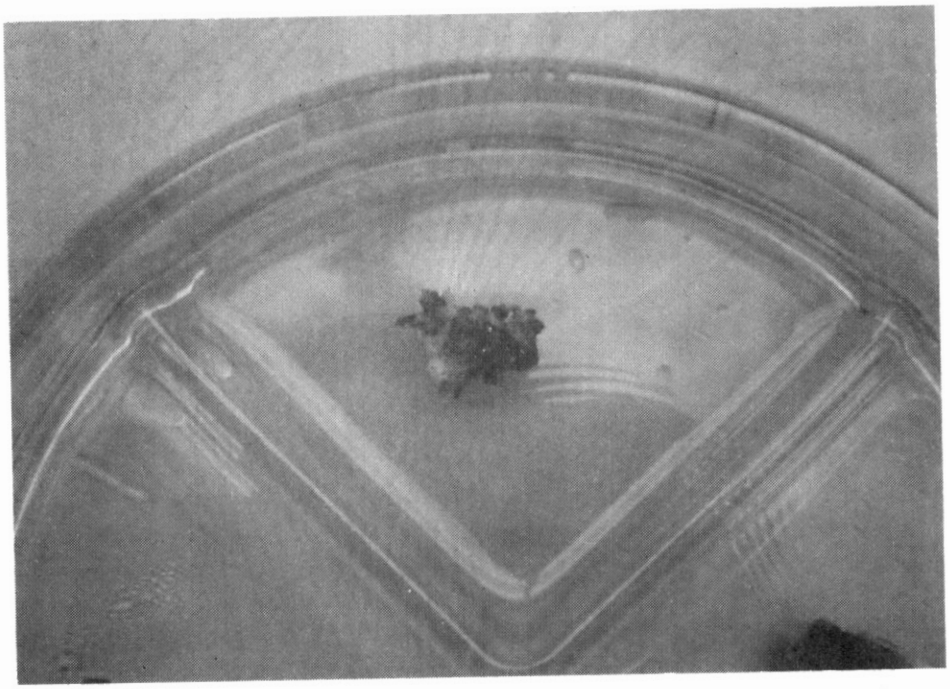


Fig. 9. Anther culture of Arachis pintoi. Shoots can be seen emerging from the dark callus.

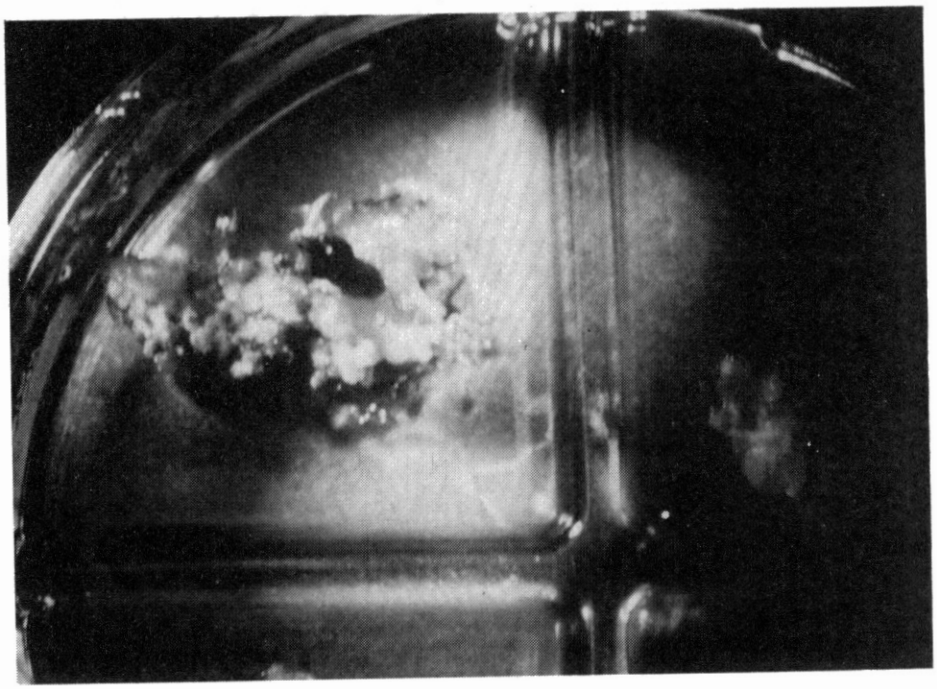


Fig. 10. An Arachis pintoi anther culture after being transferred several times.

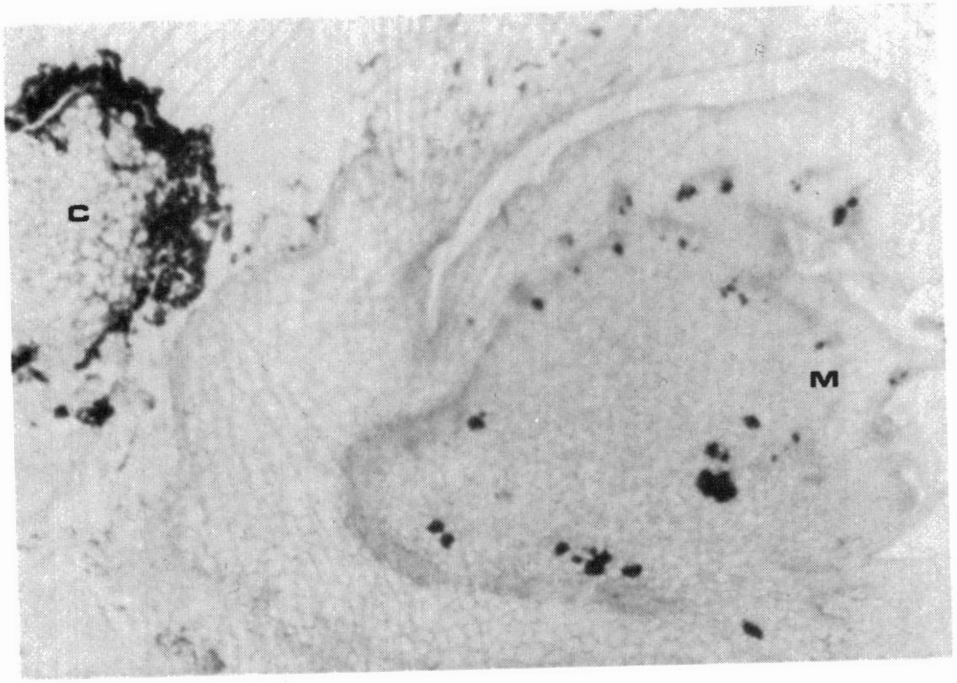



Fig. 11. A cross section of Arachis pintoi anther culture showing a shoot meristem (M) and dark callus (C).

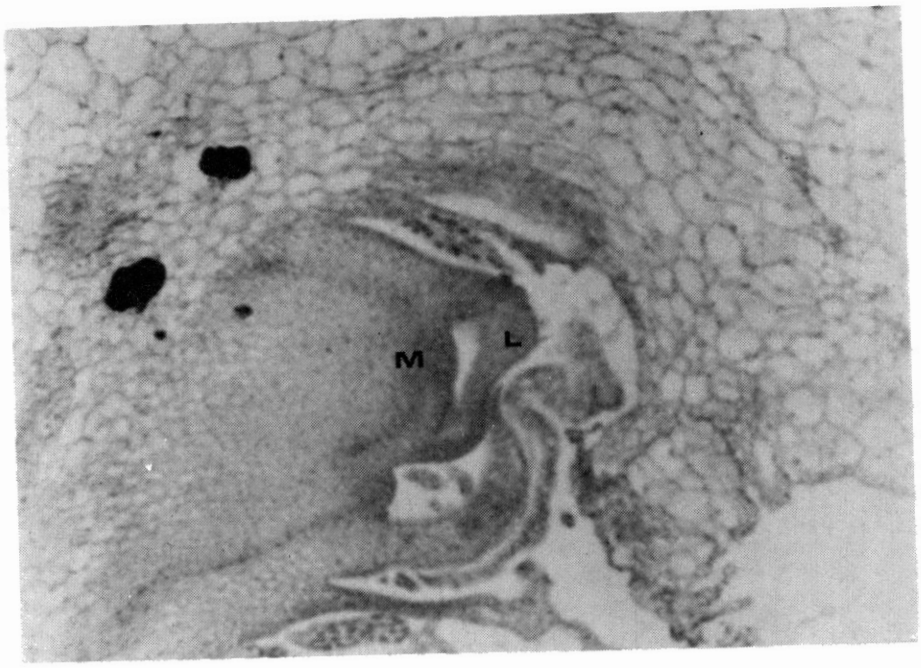



Fig. 12. A longitudinal section of Arachis pintoi anther culture showing the meristematic area (M) and leaves (L) of a shoot.

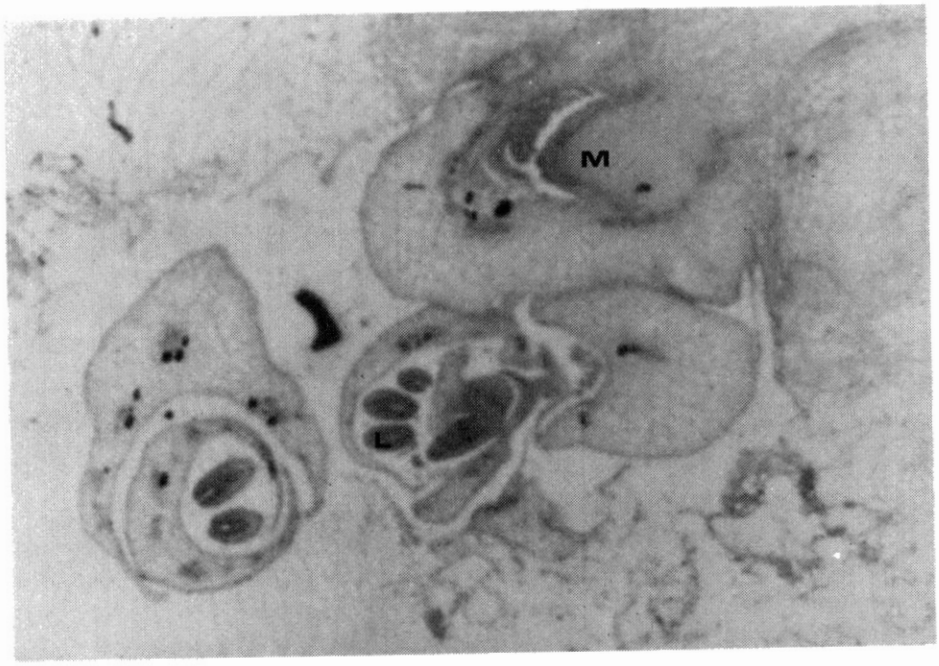



Fig. 13. A cross section of Arachis pintoi anther culture showing meristematic (M) areas and leaflets (L) of shoots.

CHAPTER IV

# *IN VITRO* CULTURE OF IMMATURE PEANUT (*ARACHIS* SPP.) LEAVES AND PLANTLET REGENERATION


## ABSTRACT

Leaflets (2-5 mm in length) from five taxonomic sections of *Arachis* (*Ambinervosae*, *Arachis*, *Caulorhizae*, *Erectoides* and *Rhizomatosae*) were cultured aseptically *in vitro* on Murashige and Skoog medium, Gamborg's B5 vitamins, 0.8% Difco Agar, and supplemented with 1 mg each of naphthalene acetic acid and benzyladenine. The medium was broadly supportive and suitable for leaflet culture of all species of *Arachis*. Growth response differences of the leaflets were greater between genotypes than within genotypes. Organogenesis occurred in leaflet cultures and plants were recovered from *Arachis* and *Extranervosae* sections of *Arachis*.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*Additional index words*: Leaf explants, organogenesis, tissue culture.


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Legumes play a key role in developing countries by augmenting the low protein content of the main starchy cereals and fruits. They are of special interest since they are high protein crops containing meal between 20 and 40% protein which complements cereals (2). Recent advances in plant tissue culture techniques of non-legumes (1,3,5,6,12, 15,18,19,28,31) and legumes (4,8,11,13,14,16,17,20,21,22,23,24,25,26, 27,29,30) during the past decade have fostered interest that these techniques can be utilized for Arachis improvement.

Green (9) divided the prospects for improving a given crop by tissue culture methods into four conditions. First, the species under investigation must have appropriate tissue culture methods developed which supports rapidly growing diploid and/or haploid cells or protoplasts from which plants can be regenerated. Second, the trait to be modified must be expressed in tissue culture and have an effective selection system which identifies the desired variant. Third, the selected variant cell lines must undergo heritable mutations and be capable of regenerating plants. Fourth, tissue culture methods must provide solutions to crop improvement problems not resolved by existing breeding or genetic methods.

The development of tissue culture methods for Arachis which allows for effective plant improvement investigations 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. (11). Martin (20) and Martin and Rabechault (21) describe growth factors in which limited success was obtained when culturing ovules or anthers. Recent studies by Mroginski and Fernandez (22,23) and Mroginski et al. (24) have

resulted in plantlets being formed from anthers and leaflets. Plants used in the callus and plantlet regeneration studies were A. hypogaea types, but in anther cultures Mroginski and Fernandez also included species from Erectoides (Procumbensae) and Arachis (Perennes) sections.

This study used leaflets from Arachis sections Ambinervosae, Arachis, Caulorhizae, Erectoides, Extranervosae and Rhizomatosae.

# MATERIALS AND METHODS

The seeds of six commercial cultivars were employed in this study. Cultivars from each of the following botanical groups were used: 'Chico' (Spanish), 'Comet' (Spanish), 'Jenkins Jumbo' (Virginia), 'Colorado Manfredi' (Spanish-Valencia), P-936 (Spanish-Valencia) and 'Tennessee Red' (Spanish-Valencia). Petri dishes containing ten seeds of each cultivar were placed in a transfer hood equipped with a germicidal ultra-violet light. Seeds were exposed to UV light in an open petri dish at a distance of 38 cm from the source for 48 hours. Intermittently the seeds were moved in the petri dishes by shaking so that all seed surfaces were exposed. Then seeds were transferred to sterile 20 ml disposable scintillation vials which contained five ml of sterile water. Leaflets 2-5 mm were selected from young uncontaminated seedlings. All sterile operations were preformed in a laminar flow hood.

Shoots from plants previously growing in the greenhouse or field plots representing five sections of Arachis (Ambinervosae, Arachis, Caulorhizae, Erectoides, Extranervosae and Rhizomatosae) and crosses were also used in this study. Shoots were surface sterilized by soaking in 70% ethanol with 0.01% Tween 80 for five minutes, 2.63% sodium hypochlorite with 0.01% Tween 80 for ten minutes and then rinsed with three changes of sterile glass distilled water. Sterile leaflets 2-5 mm long were removed aseptically with forceps from the shoots and placed on Murashige and Skoog medium solidified with 0.8% Difco agar. Murashige and Skoog's major and minor salts as modified by Gamborg (7) were supplemented with B5 vitamins (7) and 3% sucrose. Naphthalene acetic acid (NAA) and benzyladenine (BA) were added at the

rate of one mg each (24). The pH of the medium was adjusted to 5.8 with HCl or KOH prior to adding agar. Disposable 20 ml scintillation vials were filled with 8 ml of the medium prior to autoclaving for 15 minutes at 121°C and 15 psi. Each treatment consisted of 15 replications with one leaflet per vial.

The cultures were maintained in a growth chamber at 27/21°C on a 16/8 hour light/dark cycle. General Electric F20T12•CW bulbs were used which produced approximately 67 microeinsteins $m^{-2}sec^{-1}$ when measured 24 cm from the tubes.

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. Photographs were also taken during these observations. The experiments were terminated after 46 days.

## RESULTS AND DISCUSSION

Two methods of surface sterilization have been used in the preparation of plant material for culture. The use of ultraviolet light for seed surface sterilization was very effective in these experiments. The amount of contamination averaged only 1% during culture, while shoot tips collected from the greenhouse sterilized by the standard method had an overall contamination rate of 36%. The results indicate that seeds surface sterilized with ultraviolet light and subsequently germinated can provide a good source of plant material for culture, because contaminated seeds can be readily identified and discarded before culturing. Material collected from the greenhouse or field-grown plants could not be easily identified as carrying a disease until after culturing.

The differences among the different genotypes in their responses to the medium used by Mroginski et al. (24) were notable. Table 1 shows a summary of the morphogenetic responses by genotypes used. Only five genotypes (A. pernambucensis, A. stenocarpa, A. spegazzinii, and two Chico x [C410 x #37] crosses) produced only callus after six weeks. Roots were produced by six genotypes after three weeks. These were Colorado Manfredi, Jenkins Jumbo, P-936, Comet, A. monticola, and HLK555-556 representing series $A_3$ and $E_2$. Shoots were produced by Colorado Manfredi, Jenkins Jumbo, P-936, Tennessee Red, Chico, Comet, A. apressipila, HLK555-556, A. pintoi, A. hagenbeckii, A. monticola, A. pusilla, A. villosulicarpa, A. chacoense, Chico x #37w, Chico x (C410 x #37), EC5 x (C410 x #37), #37L, EM3 x (C410 x #37) and EM3 x US 98yel after three weeks representing members from series $A_2$, $A_3$, C, $E_2$, Ex, $R_2$, T, $A_3xA_2$, $A_3x(A_1xA_2)$ and $A_3xA_3$.

The responses of A. hypogaea types are presented in Table 1. Photographs of representative cultures of selected genotypes taken at 40 days are presented in figs. 1-5. Although individual clones showed differences in responses, there were many overall similarities in the morphogenetic patterns obtained. For example, small white-yellowish friable calli were observed on all explants after two weeks of culturing for all genotypes. Bud regeneration generally occurred within two weeks in 25 genotypes but the number of leaflets which had produced buds as well as the number of buds per callus varied according to genotype. Root regeneration generally occurred within two weeks followed by reduced regeneration of roots after three weeks.

Fig. 6 shows graphs of the A. hypogaea data by genotype. Although individual genotypes showed some differences in culture responses for callus, shoot, and root production, the greatest differences within botanical types were noted in root formation. Callus and shoot production was very similar in all groups, including other species and crosses. Fig. 7 shows graphic responses of Colorado Manfredi, Comet, P-936 and A. villosulicarpa. 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 approximately 40 days in culture but only in those cultures which had explants removed from them during photography and replaced in the vial (fig. 8). Surviving shoots were transplanted to soil for Colorado Manfredi, 'M-213' (Chico x [C410 x #37]), 'M-38' (EM3 x [C410 x #37]), #37L, and A. villosulicarpa and are still growing. The plantlets produced in these cultures provide a demonstration of totipotency in leaflets of Arachis genotypes.

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## LIST OF TABLES



## LIST OF FIGURES

TABLE 1

Summary of data from *Arachis* leaflet cultures at 46 days.†

| Identification | Days of Culture | No. of Leaflets | % Forming Callus | % Forming Callus and Shoots | % Forming Callus and Roots | % Forming Shoots | % Forming Shoots and Roots | % Forming Roots | % Forming Callus, Shoots, and Roots |
|---|---|---|---|---|---|---|---|---|---|
| Colorado Manfredi‡ | 11 | 14 | 57 | | | | | | |
| ($A_3$) | 24 | 14 | 14 | 79 | | 7 | | | |
| | 46 | 14 | 14 | 57 | | 14 | | | 7 |
| Jenkins Jumbo | 11 | 15 | 33 | 7 | 13 | | | | |
| ($A_3$) | 24 | 15 | 7 | 27 | 20 | | 7 | 7 | 27 |
| | 46 | 14 | 50 | 7 | 7 | | | | 36 |
| P-936 | 11 | 14 | 21 | 21 | 14 | | | | |
| ($A_3$) | 24 | 12 | 8 | 33 | 8 | 25 | 8 | | 8 |
| | 46 | 10 | 50 | 40 | | 10 | | | |
| Tennessee Red | 11 | 15 | 53 | 7 | 7 | | | | |
| ($A_3$) | 24 | 15 | 20 | 73 | | | | | |
| | 46 | 14 | 50 | 50 | | | | | |
| Chico | 11 | 15 | 47 | | | | | | |
| ($A_3$) | 24 | 15 | 53 | 40 | | 7 | | | |
| | 46 | 14 | 71 | 29 | | | | | |
| Comet | 11 | 15 | 27 | | 7 | | | | 13 |
| ($A_3$) | 24 | 14 | 21 | 21 | | 29 | 14 | | |
| | 46 | 9 | 44 | 44 | 11 | | | | |
| Colorado Manfredi | 5 | 9 | | | | | | | |
| ($A_3$) | 24 | 7 | 14 | 43 | | | | | |
| | 40 | 3 | 67 | | | 33 | | | |

TABLE 1 (Continued)

| Identification | Days of Culture | No. of Leaflets | % Forming Callus | % Forming Callus and Shoots | % Forming Callus and Roots | % Forming Shoots | % Forming Shoots and Roots | % Forming Roots | % Forming Callus, Shoots, and Roots |
|---|---|---|---|---|---|---|---|---|---|
| A. apressipila (#52) | 8 | 7 | | | | | | | |
| ($E_2$) | 21 | 7 | 71 | 14 | | | | | |
| | 40 | 3 | 29 | | | 14 | | | |
| HLK555-556 | 8 | 10 | 10 | | | | | | |
| ($E_2$) | 21 | 11 | 73 | | | | | | |
| | 40 | 6 | 50 | 17 | | | | | 17 |
| A. pintoi (#96) | 8 | 5 | | | | | | | |
| (C) | 21 | 5 | 80 | 20 | | | | | |
| | 40 | 3 | 33 | 67 | | | | | |
| A. pernambucensis (#98) | 5 | 4 | 25 | | | | | | |
| (P) | 21 | 4 | 25 | | | | | | |
| | 40 | 4 | 25 | | | | | | |
| A. hagenbeckii (#27) | 9 | 9 | 44 | 11 | | | | | |
| ($R_2$) | 25 | 8 | 63 | 38 | | | | | |
| | 44 | 6 | 50 | 50 | | | | | |
| A. stenocarpa (#42) | 8 | 11 | 9 | | | | | | |
| ($A_1$) | 21 | 10 | 90 | | | | | | |
| | 40 | 2 | 100 | | | | | | |
| A. spegazzinii (#8) | 12 | 6 | | | | | | | |
| ($A_1$) | 22 | 6 | 67 | | | | | | |
| | 44 | 2 | 50 | | | | | | |
| A. monticola (P563) | 8 | 5 | | | | | | 20 | |
| ($A_3$) | 18 | 4 | 25 | 50 | | 25 | | | |
| | 40 | 4 | | | | 50 | | | |

TABLE 1 (Continued)

| Identification | Days of Culture | No. of Leaflets | % Forming Callus | % Forming Callus and Shoots | % Forming Callus and Roots | % Forming Shoots | % Forming Shoots and Roots | % Forming Roots | % Forming Callus, Shoots, and Roots |
|---|---|---|---|---|---|---|---|---|---|
| A. pusilla (P1557) | 9 | 5 | 20 | | | | | | |
| (T) | 22 | 5 | 60 | | | | | | |
| | 44 | 3 | | 33 | | | | | |
| A. villosulicarpa (P1565) | 8 | 14 | 7 | | | | | | |
| (Ex) | 18 | 12 | 58 | 8 | | 33 | | | |
| | 40 | 8 | 13 | | | 88 | | | |
| A. chacoense (#37L) | 9 | 12 | | | | | | | |
| ($A_2$) | 22 | 12 | 75 | 8 | | 8 | | | |
| | 44 | 8 | 75 | 38 | | | | | |
| A. chacoense (#37R) | 9 | 8 | 38 | | | 25 | | | |
| ($A_2$) | 22 | 7 | 57 | 29 | | | | | |
| | 44 | 6 | 33 | 50 | | | | | |
| Chico x #37w | 8 | 10 | | | | | | | |
| ($A_3 x A_2$) | 21 | 9 | 89 | 11 | | | | | |
| | 40 | 4 | 75 | 25 | | | | | |
| Chico x (C410 x #37) | 10 | 5 | 20 | 60 | | | | | |
| M-202 $[A_3 x (A_1 x A_2)]$ | 23 | 4 | 50 | 25 | | | | | |
| | 45 | 4 | 50 | 25 | | | | | |
| Chico x (C410 x #37) | 10 | 5 | 20 | | | | | | |
| M-189 $[A_3 x (A_1 x A_2)]$ | 23 | 5 | 80 | | | | | | |
| | 45 | 5 | 100 | | | | | | |
| Chico x (410 x #37) | 10 | 5 | | | | | | | |
| M-213 $[A_3 x (A_1 x A_2)]$ | 23 | 4 | 50 | | | | | | |
| | 45 | 2 | 50 | | | | | | |

TABLE 1 (Continued)

| Identification | Days of Culture | No. of Leaflets | % Forming Callus | % Forming Callus and Shoots | % Forming Callus and Roots | % Forming Shoots | % Forming Shoots and Roots | % Forming Roots | % Forming Callus, Shoots, and Roots |
|---|---|---|---|---|---|---|---|---|---|
| EC5 x (C410 x #37) | 10 | 10 | 30 | 10 | | | | | |
| M-207 $[A_3 x (A_1 x A_2)]$ | 23 | 10 | 60 | 40 | | 10 | | | |
| | 45 | 10 | 80 | 10 | | | | | |
| EC5 x (C410 x #37) | 10 | 13 | 23 | | | | | | |
| M-200 $[A_3 x (A_1 x A_2)]$ | 23 | 13 | 85 | 15 | | | | | |
| | 45 | 13 | 77 | 15 | | | | | |
| EC5 x (C410 x #37) | 10 | 12 | 8 | | | | | | |
| M-191 $[A_3 x (A_1 x A_2)]$ | 23 | 12 | 67 | 8 | | | | | |
| | 45 | 11 | 82 | 18 | | | | | |
| EM3 x (C410 x #37) | 10 | 9 | 22 | | | | | | |
| M-40 $[A_3 x (A_1 x A_2)]$ | 23 | 7 | 29 | 57 | | | | | |
| | 45 | 6 | 50 | 50 | | | | | |
| EM3 x (C410 x #37) | 10 | 6 | 17 | | | | | | |
| M-39 $[A_3 x (A_1 x A_2)]$ | 23 | 6 | 33 | 67 | | | | | |
| | 45 | 6 | 67 | 33 | | 17 | | | |
| EM3 x (C410 x #37) | 10 | 7 | 14 | 14 | | | | | |
| M-38 $[A_3 x (A_1 x A_2)]$ | 23 | 7 | 29 | 43 | | | | | |
| | 45 | 7 | 29 | 43 | | 14 | | | |
| EM3 x US98yel | 9 | 9 | 22 | | | | | | |
| $(A_3 x A_3)$ | 22 | 7 | 100 | | | | | | |
| | 44 | 7 | 86 | | | | | | |

† Percentages shown are based on non-contaminated vial showing callus, shoots, roots or a combination of each

‡ Classification follows that of Gregory and Gregory (10).

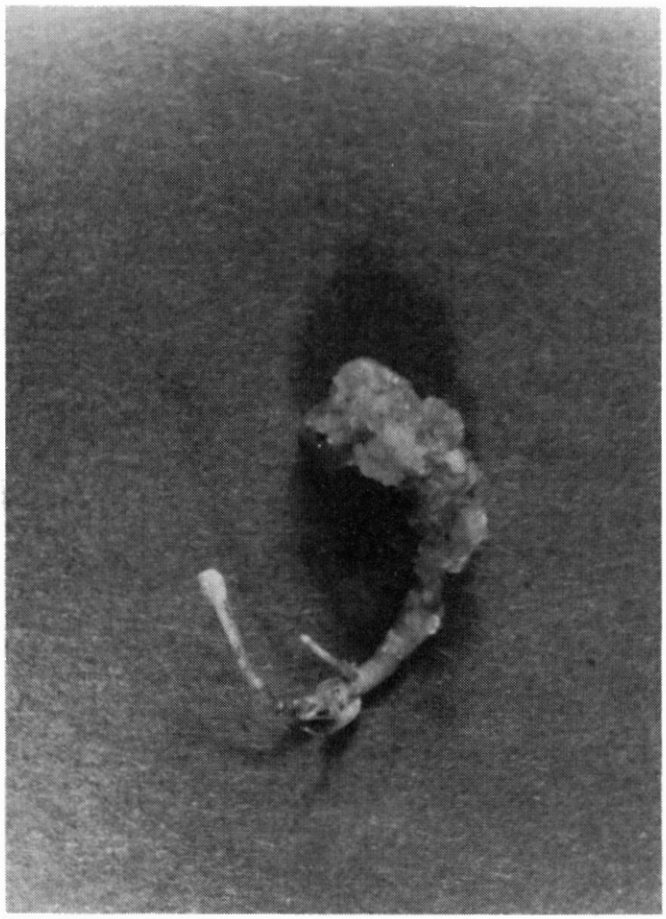

Fig. 1. Callus and root formation from a 40 day leaflet culture of Jenkins Jumbo.

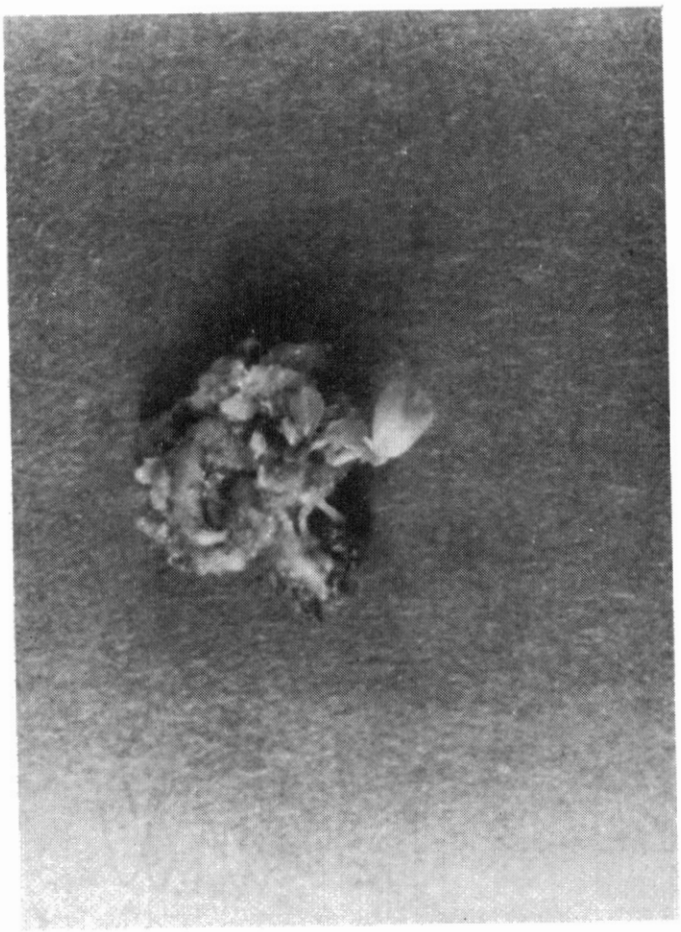

Fig. 2. Callus, shoot, and root formation from a 40 day leaflet culture of Chico.

Fig. 3. Callus and shoot formation from a 40 day leaflet culture of EM3 x (C410 x #37).

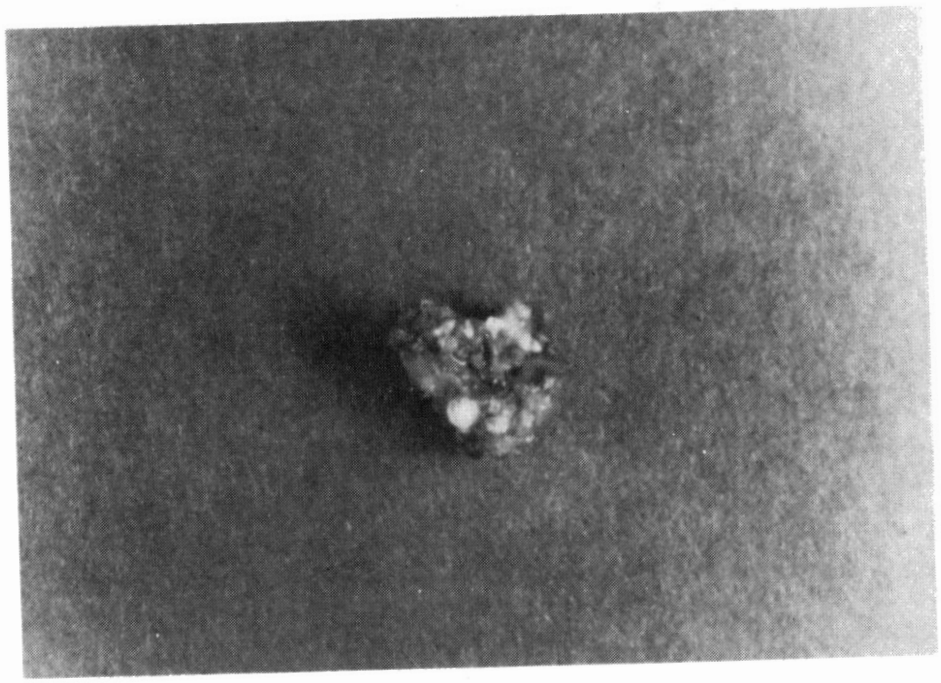

Fig. 4. Callus and shoot formation from a 40 day leaflet culture of EM3 x US98yel.

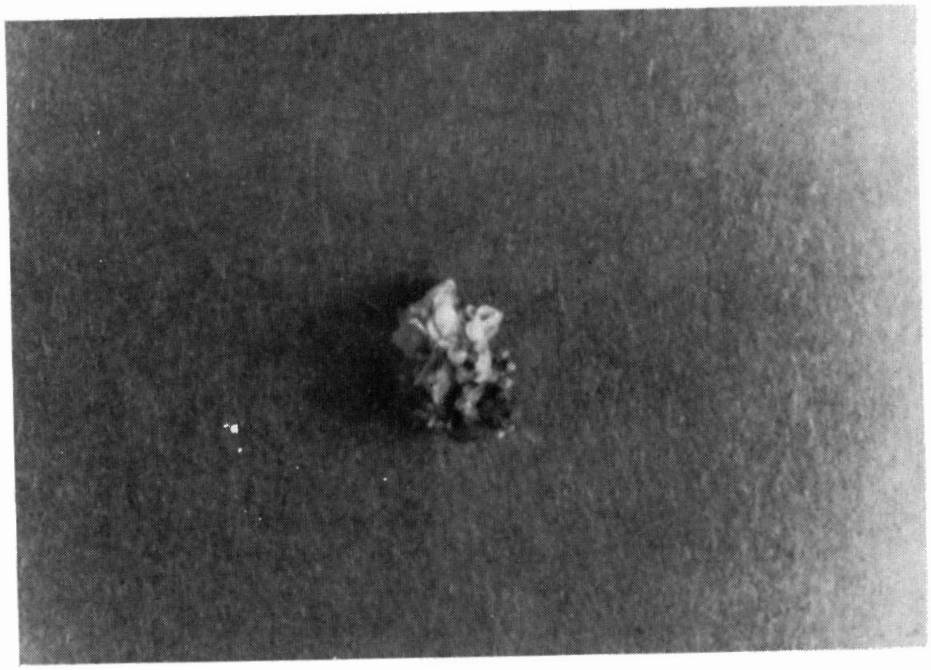

Fig. 5. Callus and shoot formation from a 40 day leaflet culture of EM3 x US98yel.

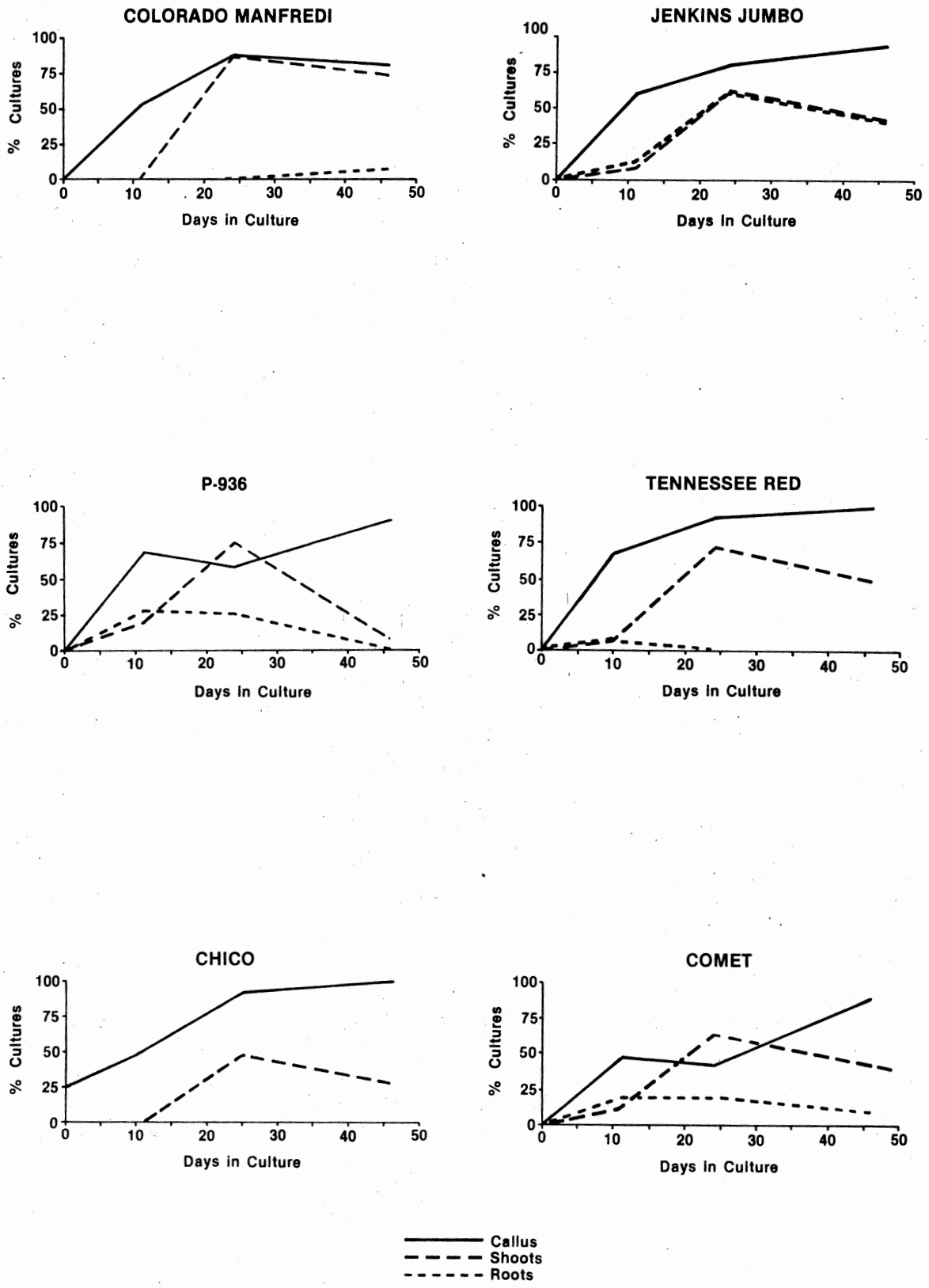


Fig. 6. Response of A. hypogaea genotypes for callus, shoot, and root growth.

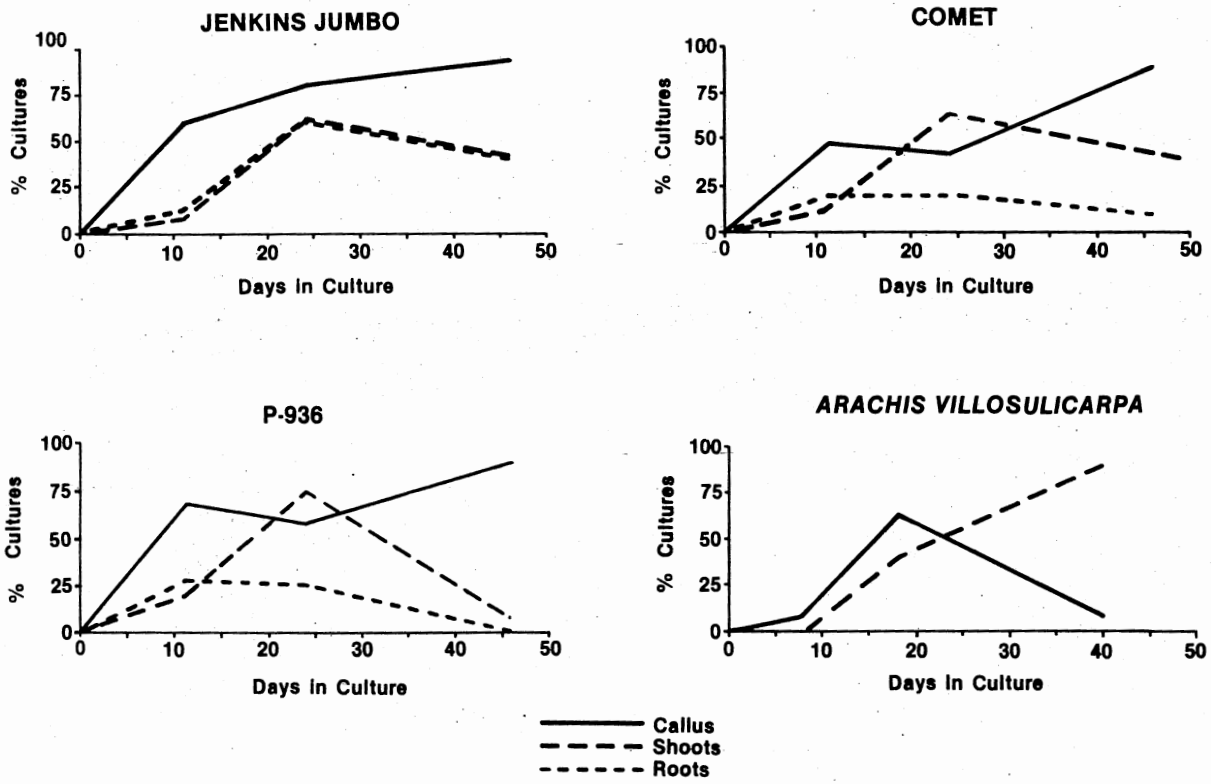


Fig. 7. Comparison of A. hypogaea types with A. villosulicarpa.

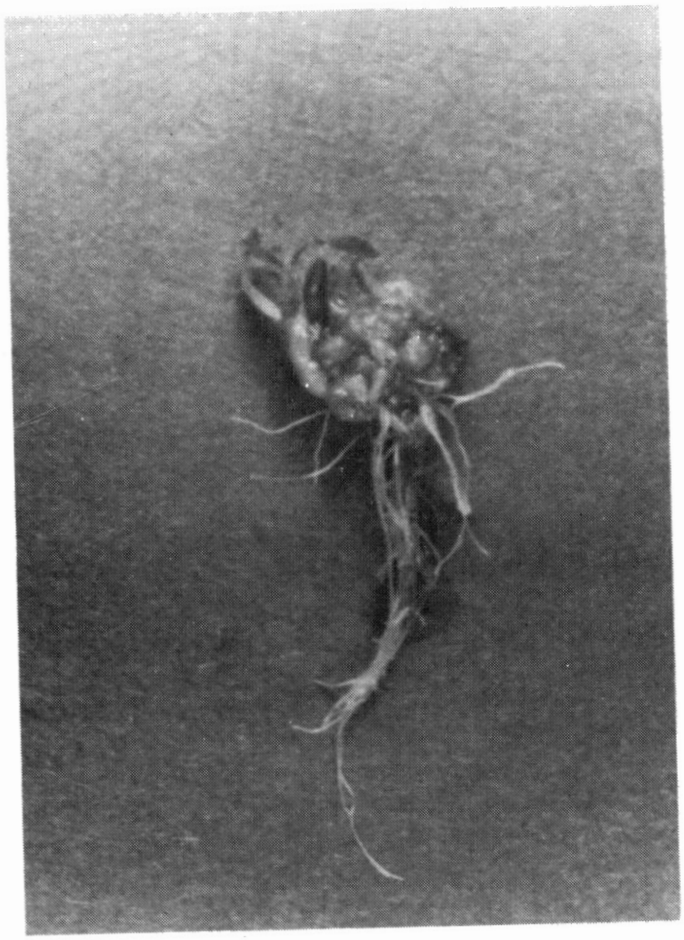

Fig. 8. Callus, shoot, and root formation on a 60 day leaflet culture of A. villosulicarpa.

CHAPTER V

# MORPHOGENESIS IN PEANUT (*ARACHIS* SPP.) LEAFLET CULTURES

## ABSTRACT

Peanut (*Arachis* spp.) leaflets in culture were induced to produce callus, shoots, and roots after growing on Murashige and Skoog's major and minor salts, Gamborg's B5 vitamins, and 2 mg/l each naphthalene acetic acid and benzyladenine for 50 days. Histological examination of the cultures revealed that the meristematic areas originated from epidermal cells. Meristematic shoots and embryoids developed after lysis of the surrounding cells.

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Additional index words: Explants, tissue culture.

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Skoog and Miller (18), Skoog (17) and Saunders and Bingham (13) have shown organogenesis of explants can be brought about by the controlled initiation of organ primordium through the interaction and manipulation of constituents in the culture media. Hicks (9) presented two schemes for organogenesis. One sequence was described as showing "direct organogenesis" which occurred in either of two patterns. The direct organogenesis patterns are:

primary explant ⟶ organ

or

primary explant ⟶ meristemoid ⟶ organ system

"Indirect organogenesis" is a sequence which produces a callus stage. The indirect organogenesis pattern is:

primary explant ⟶ callus ⟶ meristemoid ⟶ organ system

Callus formation is considered as undesirable for two reasons: First, the chromosome constitution of callus cells is unstable in many plants (4,5,16). Second, for propagation purposes the period of unorganized growth or callus should be minimized (19).

Organogenesis of shoots and roots has been described for many years (2,8,13,14). Some workers (14,21) have described zones of preferential cell division which took place under both organ-forming and non-organ-forming conditions. Thorpe and Murashige (21) characterized the cells of meristemoids as being small, isodiametric, thin walled, lacking apparent vacuoles and having densely staining nuclei and cytoplasm. Konar and Nataraja (10) showed that accessory embryos in Buttercups (Ranunculus) originated from epidermal cells. Later studies by Konar et al. (11) and Thomas et al. (20) showed that embryoids can arise from single cells but they were uncertain as to

whether all are of single-cell origin.

Chlyah (3) and Tran Thanh Van et al. (23) found an antagonistic correlation existing among tissues of an organ fragment. In Torenia fournieri, the epidermis, normally capable of bud formation, does not proliferate if grown alone. The opposite is observed in subepidermal parenchyma tissue. Also, cell division centers are formed according to a gradient, increasing in a basal direction which is the direction of auxin transport and accumulation. Tran Thanh Van (22) later characterized epidermal cells or subepidermal cells as capable of organ formation although they are high differentiated. They are adequate models to describe morphogenesis at the very first stage.

In this study, organogenesis and/or embryogenesis from leaflets was attempted by culturing 32 genotypes of Arachis on a medium recommended by Mroginski et al.(12).

## MATERIALS AND METHODS

Seeds from six commercial cultivars and shoots from plants representing five taxonomic sections of Arachis and hybrids among them were used in this study (Table 1). Seeds were surface sterilized for 48 hours with a UV germicidal lamp as described in Chapter IV. After sterilization, the seeds were germinated in 20 ml disposable scintillation vials containing sterile distilled water. Seedlings from vials showing no contamination were used for culturing. Leaflets were cultured without any other sterilization. Shoots from selected plants in the greenhouse or from the field were surface sterilized by soaking in 70% ethanol with 0.01% Tween 80 for five minutes, 2.63% sodium hypochlorite with 0.01% Tween 80 for 10 minutes, and then were rinsed through three changes of sterile glass distilled water. Leaflets 2-5 mm in length were cultured on modified Murashige and Skoog's medium by placing them on the surface of the medium.

Murashige and Skoog's as modified by Gamborg (6) major and minor salts were supplemented with B5 vitamins (6) and 3% sucrose. Napthalene acetic acid and benzyladenine were added at a concentration of 1 mg each per liter (12). The pH of the medium was adjusted to 5.8 prior to adding agar. Disposable 20 ml scintillation vials were filled with 8 ml of medium prior to autoclaving for 15 minutes at 121°C and 15 psi and placed in a growth chamber. Each treatment, arranged at random, consisted of 15 replications with one leaflet per vial. The cultures were maintained at 27/21°C on a 16/8 hour light/dark cycle. General Electric F20T12•CW bulbs were used for lighting and produced approximately 67 microeinsteins $m^{-2}sec^{-1}$ when measured 24 cm from the tubes.

The leaflet cultures were grown for 46 days. Cultures showing callus, shoots and/or roots were sampled for histological studies, fixed in formalin-propionic-alcohol for 24 hours, and then dehydrated 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 (1).

## RESULTS AND DISCUSSION

Table 1 shows the responses of the various genotypes. Callus and shoot growth was usually evident within one week after the leaflets were placed on the medium. All genotypes produced callus, while 78% produced shoots and 19% produced roots. A. pernambucensis, A. stenocarpa, A. spegazzinii, and two Chico x (C410 x #37) crosses 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 outer periphery of the callus (Figs. 1 and 2). Root formation appeared above and below the culture medium for 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.

A typical peanut leaf cross section is shown in Fig. 3. When Fig. 3 is compared with Fig. 4, 5, and 6, several interesting observations can be made. Zones of preferential cell division can be seen arising near the midrib area of leaf. Preferential zones of cell division later became massive and covered the entire leaflet surface (Figs. 5 and 6). Figs. 1 and 2 show genotypic response variation in the amount and kinds of meristems produced. A. villosulicarpa produced extensive roots in addition to callus and shoots whereas Chico produced only callus and shoots.

Evidence that zones of preferential cell division arise from epidermal cells can be seen in Fig. 7. It is uncertain whether these zones are of single cell origin. Shoot meristems (Figs. 9 and 10) and

a heart-shaped embryoid (Fig. 8) show distinct separation zones within the callus mass. These zones appear to occur after the lysis of surrounding cells. The appearance of a shoot meristem in the case of A. villosulicarpa (Fig. 10) may be the result of meristematic growth followed by separation from the callus mass. Shoot meristem (Fig. 9) and embryoid (Fig. 8) formation in A. hypogaea genotypes appearred after separation from the callus mass.

The scheme for organogenesis in the different Arachis genotypes used here appears to be the indirect pattern according to Hick's (8) classification.

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## LIST OF TABLES



## LIST OF FIGURES

TABLE 1

Responses of *Arachis* genotypes to leaf culture in Murashige and Skoog's modified medium

| Genotype | Response | | |
|---|---|---|---|
| | Callus | Shoots | Roots |
| A. Colorado Manfredi† ($A_3$) | X | X | |
| B. Jenkins Jumbo ($A_3$) | X | X | X |
| C. P-936 ($A_3$) | X | X | X |
| D. Tennessee Red ($A_3$) | X | | X |
| E. Chico ($A_3$) | X | X | |
| F. Comet ($A_3$) | X | X | X |
| G. Colorado Manfredi ($A_3$) | X | X | |
| H. *A. appressipila* (#52) ($A_3$) | X | X | |
| I. HLK555-556 ($E_2$) | X | X | X |
| J. *A. pintoi* (#96) (C) | X | X | |
| K. *A. pernambucensis* (#90) (P) | X | | |
| L. *A. hagenbeckii* (#27) ($R_2$) | X | X | |
| M. *A. stenocarpa* (#42) ($R_2$) | X | | |
| N. *A. spegazzinii* (#8) ($A_1$) | X | | |
| O. *A. monticola* (#11) (P1563) ($A_3$) | X | X | X |
| P. *A. pusilla* (#98) (P1557) (T) | X | X | |
| Q. *A. villosulicarpa* (#9) (P1565) (Ex) | X | X | |
| R. *A. chacoense* (#37L0 ($A_2$) | X | X | |

TABLE 1 (Continued)

| Genotype | Response | | |
|---|---|---|---|
| | Callus | Shoots | Roots |
| S. A. chacoense (#37R) ($A_2$) | X | X | |
| T. Chico x #37w ($A_3 x A_2$) | X | X | |
| U. Chico x Comet ($A_3 x A_3$) | X | X | |
| V. Chico x (C410 x #37) M-202 $[A_3 x (A_1 x A_2)]$ | X | X | |
| W. Chico x (C410 x #37) M-189 $[A_3 x (A_1 x A_2)]$ | X | | |
| X. Chico x (C410 x #37) M-213 $[A_3 x (A_1 x A_2)]$ | X | | |
| Y. EC5 x (C410 x #37) M-207 $[A_3 x (A_1 x A_2)]$ | X | X | |
| Z. EC5 x (C410 x #37) M-200 $[A_3 x (A_1 x A_2)]$ | X | X | |
| AA. EC5 x (C410 x #37) M-191 $[A_3 x (A_1 x A_2)]$ | X | X | |
| BB. EC3 x (C410 x #37) M-40 $[A_3 x (A_1 x A_2)]$ | X | X | |
| CC. EC3 x (C410 x #37) M-39 $[A_3 x (A_1 x A_2)]$ | X | X | |
| DD. EM3 x (C410 x #37) M-38 $[A_3 x (A_1 x A_2)]$ | X | X | |
| EE. EM3 x US98yel ($A_3 x A_3$) | X | X | |

† Classification follows that of Gregory and Gregory (7).

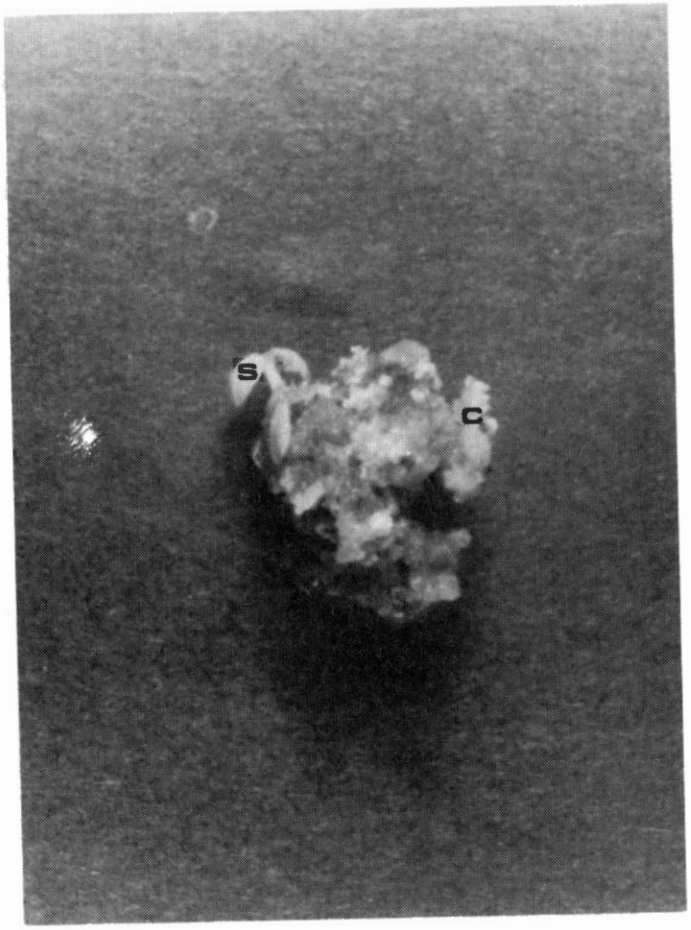


Fig. 1. Arachis hypogaea Chico leaflet culture showing shoot (S) and callus (C) regeneration.

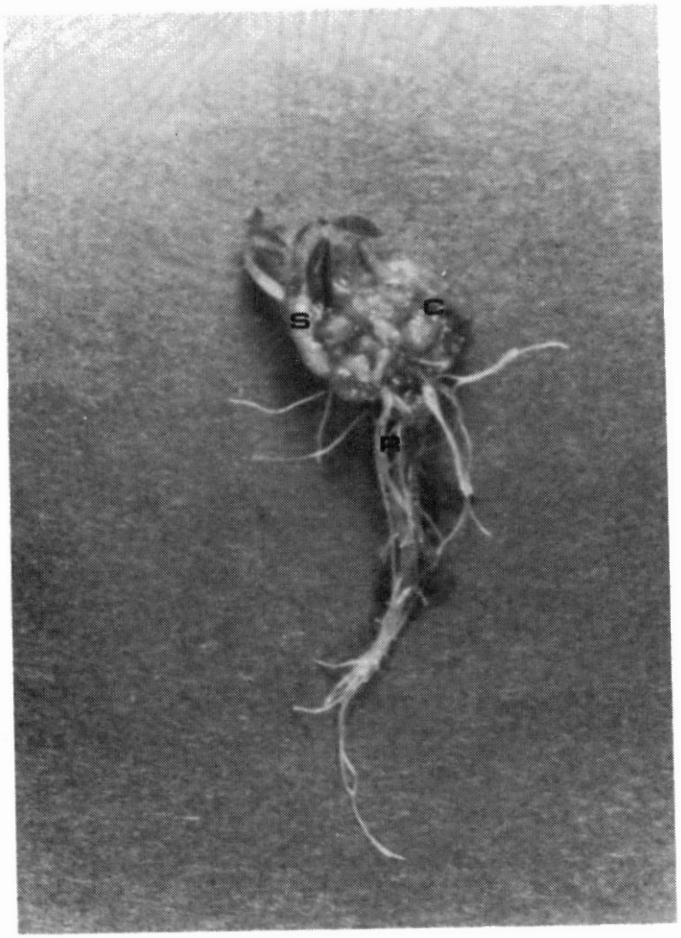


Fig. 2. Arachis villosulicarpa leaflet culture showing callus (C), shoot (S), and root (R) growth after 60 days in culture.

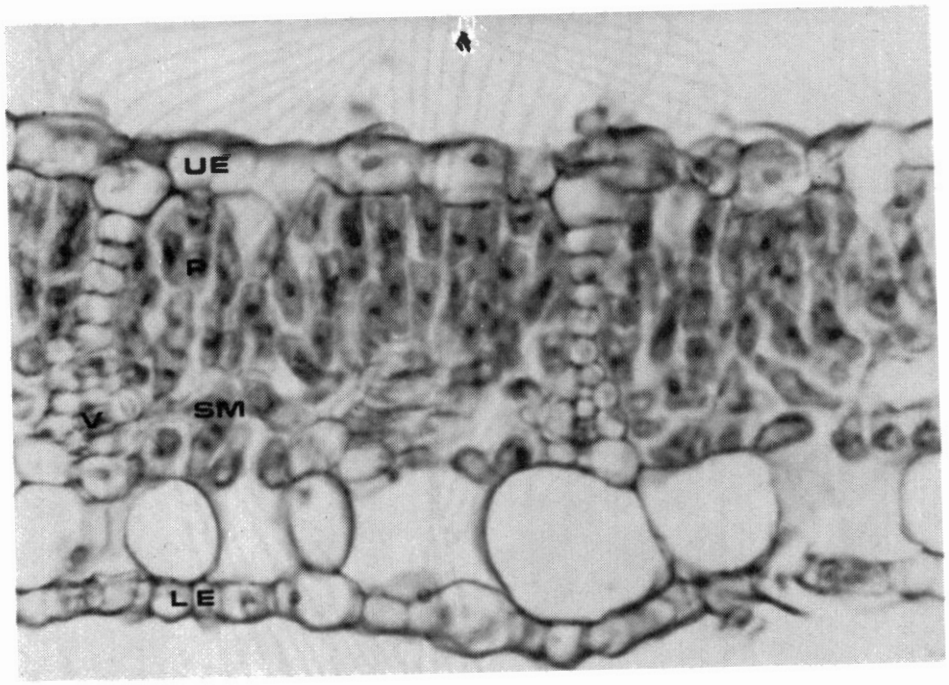


Fig. 3. Cross section of an Arachis leaflet showing upper epidermis (UE), palisade cells (P), spongy mesophyll (SM), lower epidermis (LE) and vascular bundle with support cells (V).

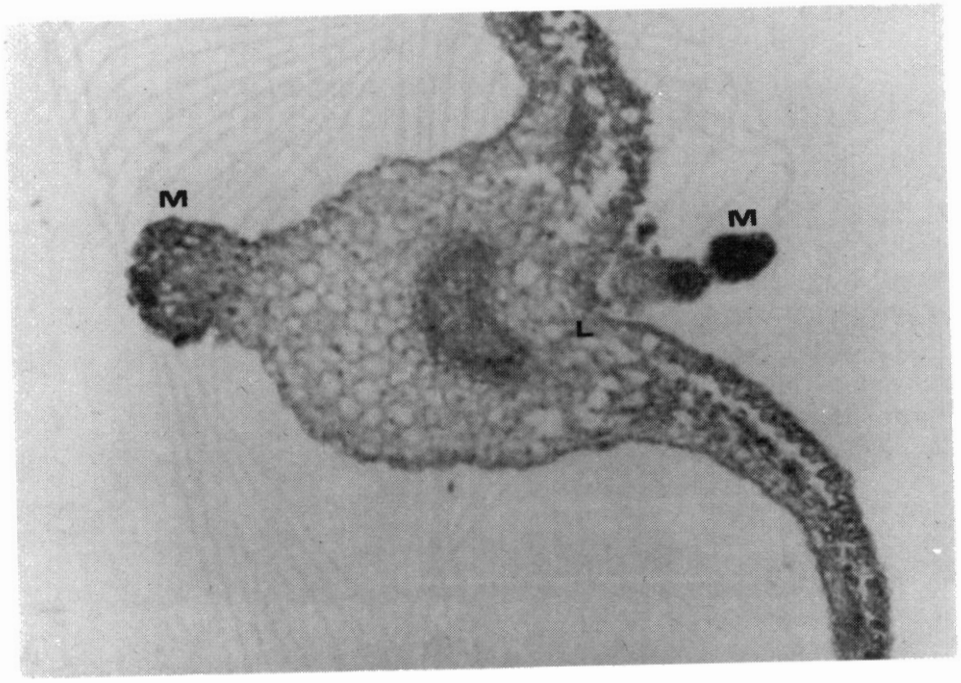


Fig. 4. Basal cross section of a leaflet (L) of an Arachis hybrid, EM3 x (C410 x #37), showing two meristemoid (M) areas.

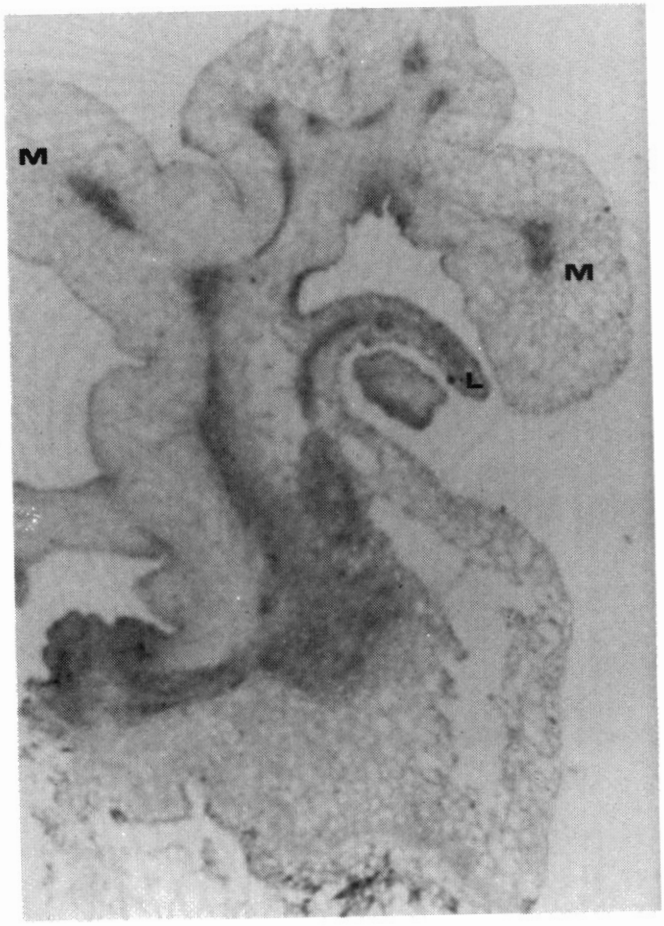


Fig. 5. Cross section of a P-936 leaflet showing part of the leaflet (L) and meristemoids (M).

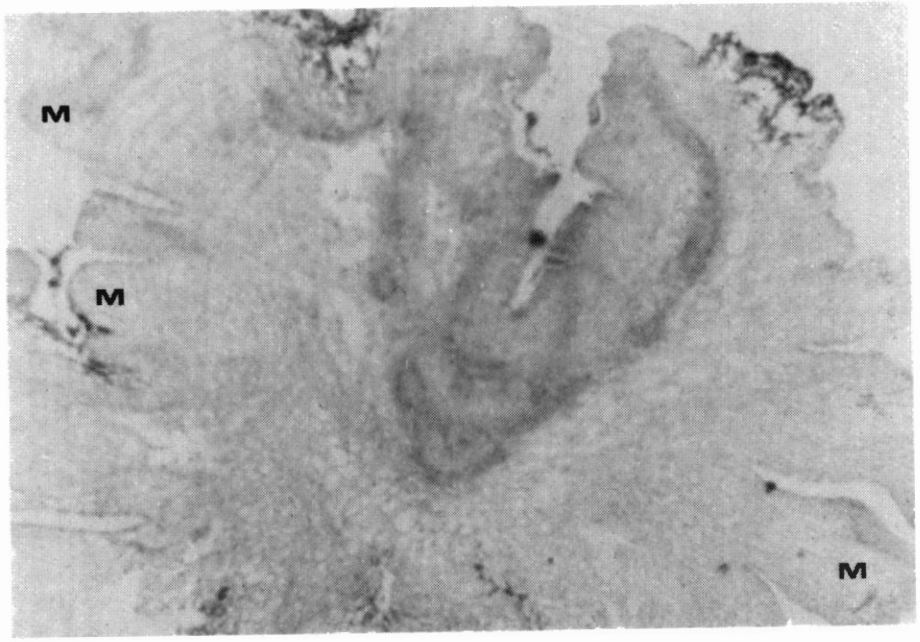


Fig. 6. Leaflet cross section of a hybrid Chico x Comet showing meristemoids (M).

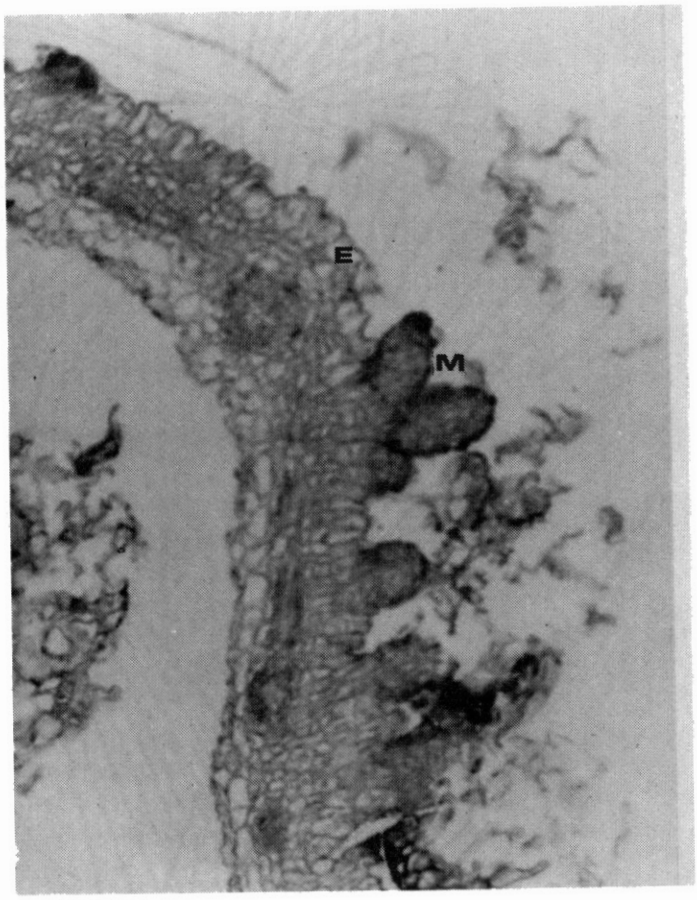


Fig. 7. Cross section of a Tennessee Red leaflet illustrating meristemoids (M) coming from epidermal cells (E).

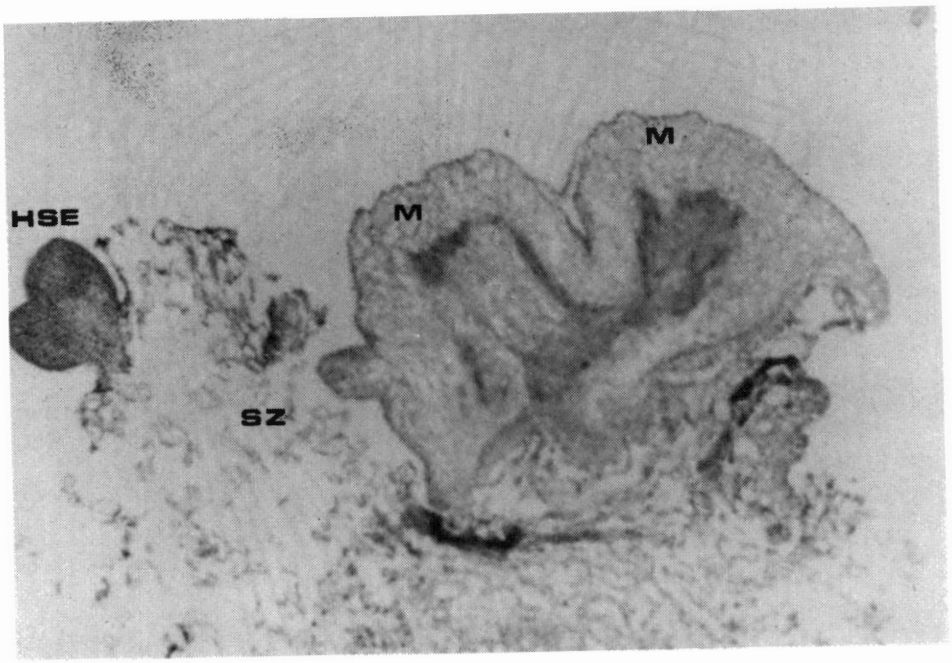


Fig. 8. Leaflet cross section of hybrid Chico x Comet showing a young heart-shaped embryo (HSE), separation zone (SZ) and meristemoid area (M).

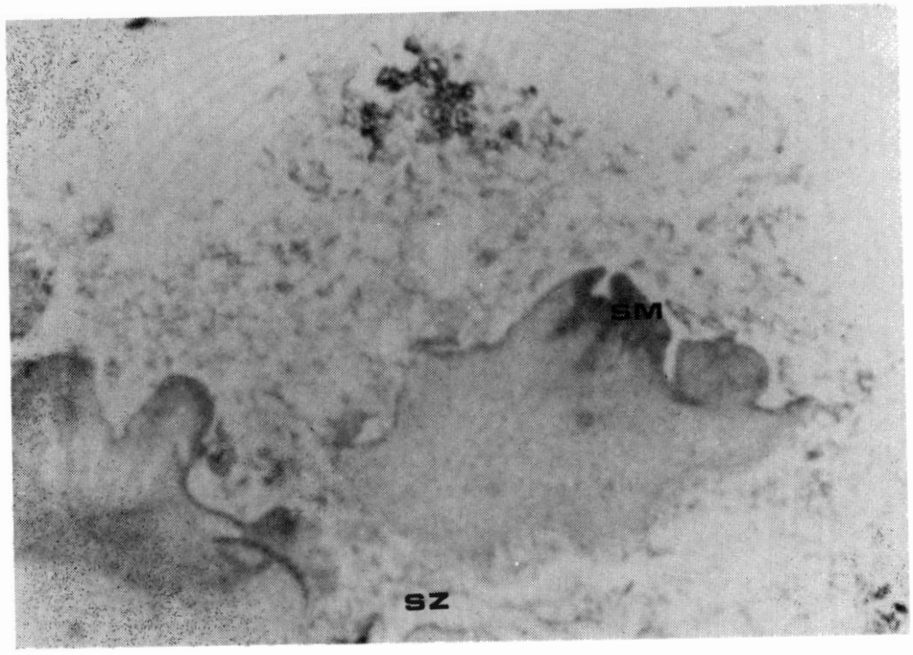


Fig. 9. Tennessee Red section showing a separation zone (SZ) and shoot meristematic area (SM).

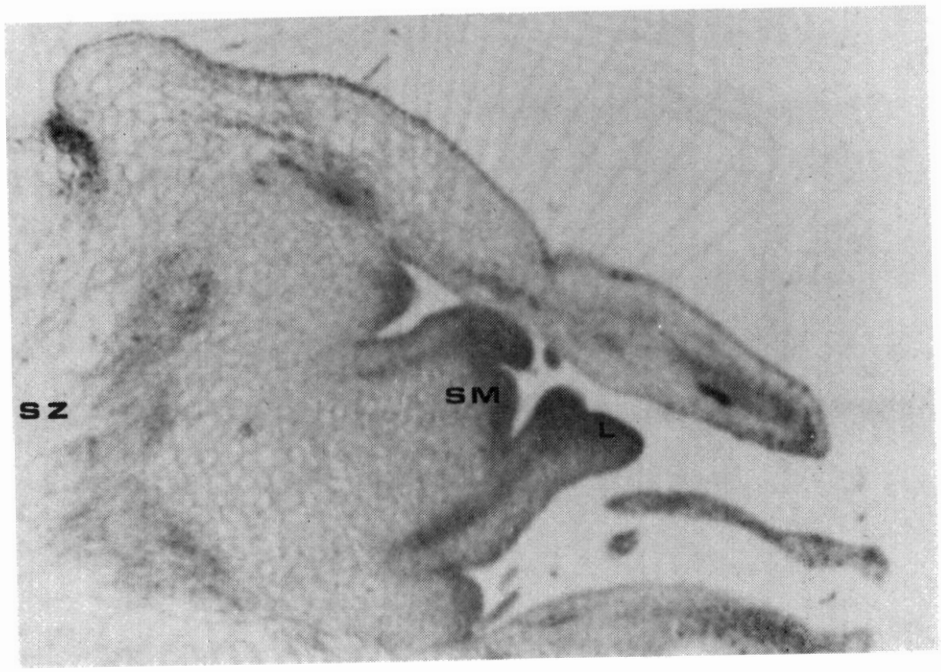


Fig. 10. Arachis villosulicarpa shoot meristem (SM) with embryonic leaves (L) and a separation zone (SZ).

# CHAPTER VI

## SUMMARY

Several *in vitro* culture studies involving peanut anthers, leaflets and cotyledon material were conducted to develop some useful tissue culture techniques specifically for peanuts which would aid in germplasm development and propagation.

In a study of callus induction from 'Tamnut-74' cotyledons, it was determined that fragments responded to IAA and BAP at concentrations between 0.125 and 0.5 mg/l. 'Chico' cotyledon fragments produced callus when BAP was between 1 to 2 mg/l, 2,4-D between 0.25 to 2 mg/l and NAA between 0.025 to 2 mg/l.

In the anther culture study, it was found that pollen from anthers of *Arachis hagenbeckii* which had a cold treatment lost viability very rapidly. Pollen with two or three days of cold treatment was only about 50% viable. At the end of six days, pollen viability was reduced to about 20%. These results indicate that more critical cold treatment periods for peanuts should be examined. Anthers which had part of the filament intact or which were damaged tended to produce callus at the site of filament connection or at the site of damage. Anthers cultured on a solid medium with kinetin tended to produce callus easily. In float cultures, only one genotype (*A. pintoi*) produced shoots and leaves. No chromosome counts were made to determine the ploidy level in these tissues. In most cases, anthers

that were float cultured started to produce callus, but they would sink and die. Future float culture experiments with anthers should give consideration to depths of media and meticulous care in handling to prevent sinking of the anthers.

The use of immature peanut leaflets in culture proved to be a very effective means of propagation. The results here show evidence that Arachis spp. can be propagated in vitro by using immature leaflets cultured on a medium with appropriate levels of naphthalene acetic acid and benzyladenine according to genotypic needs. Seeds which were sterilized by UV light prior to germinating were found preferred to material collected from the field or greenhouse.

Histological examinations of the leaflet cultures showed that meristematic areas originate from epidermal cells. It is uncertain that the zones of preferential cell division are of single cell origin.

In the studies that were conducted, some tissue culture techniques were developed and some new approaches are discussed which should aid in peanut germplasm development and propagation.

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