IN VITRO CULTURE OF EXCISED TISSUES OF COTTON

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IN VITRO CULTURE OF EXCISED TISSUES OF COTTON

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INTRODUCTION

Work with tissue cultures has progressed rapidly especially during the second quarter of this century. Excised plant parts have been successfully cultured *in vitro* and this technique is at present conveniently employed in many research studies.

White (22) summarizes the applications of cell culture technique into six categories:

> (1) cellular nutrition---the degree and manner of dependence of a single cell, type of cell, or tissue on the chemical properties of the external medium; (2) cellular metabolism---those aspects of cellular behavior which have an internal origin and control; (3) hormone relations---the behavior of cells toward the specific products, other than nutrients, of other cells, and the function of cells in producing such products; (4) morphogenesis---the production of integrated patterns of development by the interaction of cells or groups of cells; (5) pathology---the response of cells and tissues to agents of extra-cellular origin which, being neither nutrients, hormones, nor normal self-metabolites, are injurious to the cells in question; and (6) genetics---the behavior of individual cells as bearers of specific inherent characteristics.[1]

The economic importance of the cotton plant has prompted the author to use it as her material in this plant tissue culture research. To date, cotton has not yet been used extensively in *in vitro* culture studies. Robbins (15) in 1922 experimented with root and stem tips of cotton and found the root tips to grow remarkably well in a medium containing mineral salts and glucose.

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[1] Philip R. White, *The Cultivation of Animal and Plant Cells* (New York, 1954), p. 169.

The purpose of this study is to find out the behavior and capacity for growth of isolated tissues of the cotton plant, Gossypium hirsutum var. empire in vitro particularly with the use of indole-acetic acid, adenine, guanine and coconut milk. The investigation was earlier aimed at producing buds by excised tissues of cotton.

REVIEW OF LITERATURE

Haberlandt (8), at the beginning of the century, first formulated the concept of plant tissue culture. He isolated pieces of the tissue from tubers, stems, and leaves of higher plants and was successful in obtaining some cell division under certain conditions.

Stem tips and root tips of pea, corn, and cotton were first isolated and grown by Robbins (15) in 1922. The isolated root tips made considerable growth in the dark in solution cultures containing mineral salts and glucose. The excised shoot tips of corn and peas elongated considerably but developed only small leaves.

White (19), ten years later, grew isolated stem tips of Stellaria media and observed some tips to produce a limited differentiation. He also isolated root tips of tomato and showed that they are capable of independent existence in vitro for indefinite periods of time in certain artificial media, and also have the capacity to differentiate fresh leaf primordia.

In 1933, La Rue (9) studied the regeneration in certain mutilated seedlings which were isolated and grown on moist filter paper or on agar in Shive's nutrient solution. He observed the production of adventitious roots and shoots by the excised cotyledons and hypocotyls. These tissues were isolated when they were well out of their seed coats. Cotyledons of 41 species from 19 families regenerated roots in 4 to 37 days. Shoots were produced on cotyledons in 22 species in 6 to 58 days. Segments of cotyledons of 10 species were also found to

regenerate roots but they required a longer time to emerge. In 1936 he isolated segments of immature embryo parts of dandelion, wild lettuce, ox-eye daisy, and tomato (10). He successfully grew them into complete plants.

In 1939 Gautheret (7), working with the cambial tissue from carrot root, and White (20), experimenting on stem tissue of tobacco, reported that isolated plant parts could be grown for extended periods if they are repeatedly subcultured in fresh nutrients of suitable composition.

Bonner, Haagen-Smit and Went (3) maintained immature leaves excised from etiolated pea plants in a solution containing mineral salts and sucrose. The leaves were found capable of making a limited amount of growth. The addition of pea diffusate to the medium produced a greater growth, leading them to conclude that a non-specific leaf-growth hormone may exist which affects the leaf growth.

Loo (12) grew stem tips of Asparagus officinalis in sterile culture without the formation of roots. The results indicate that there are two substances involved: one is produced in the stem in the presence of light while the other is produced and transferred from the root system.

The development in vitro of stem tips and subjacent regions of Tropaeolum majus L. and of Lupinus albus L. in modified Pfeffer's culture fluid, was studied by Ball (1). He observed a decreasing capacity for growth and development in the tissues down the plant axis and that the shoot apex is the most capable of development.

Skoog (16) isolated tobacco callus cultures of the hybrid Nicotiana glauca x Nicotiana langsdorffii and observed the possibility of controlling growth and organ formation by manipulation of external factors including nutrient composition. Development of stems, leaves,

and roots have been obtained from these callus cultures. In 1953 Miller and Skoog (13) published their work on the chemical control of bud formation in tobacco stem segments cultured aseptically. Adenine enhanced bud formation in modified White's basal medium. Low concentrations of indole-3-acetic acid markedly reduced the amount of buds formed both in the presence and absence of adenine. Guanine was found to produce similar effects to that of adenine. They believe that both IAA and adenine affect organ formation and growth and plant tissues through their influence on the nucleic acid metabolism.

Caplin and Steward (6) isolated explants from carrot root employing coconut milk in addition to the basic nutrient medium. A significant effect on growth was noted. Nickell (14) observed a similar stimulation of growth with coconut milk in the virus tumor tissue of Rumex acetosa.

Root formation in isolated cotyledons of Brassica napus and Raphanus sativus has been studied by Carlson (4). Her report includes an anatomical study of root formation in the cotyledons. Shoots were not formed on the cotyledons in these cultures.

Caplin (5) studied the growth and morphology of tobacco tissue cultures in vitro. He stated that the increase in size of the cultures resulted from the production of knob-like protuberances which increase in number by cleavage and by the formation of new growing centers in the subsurface of older knobs.

Sterling (17) made a histological study of cultures of tobacco stem segments. He reported on the characteristic anatomical patterns of the stem cultures and callus formed which were affected by the constitution of the nutrient medium.

MATERIALS AND METHODS[1]

Cotyledon materials were secured from 2 to 4 weeks old seedlings of Gossypium hirsutum var. empire which were grown in the greenhouse. The cotyledons used included the petiole by cutting up to its base taking care that no macroscopically apparent meristematic bud tissue was included.

Internodal stem segments used were obtained from the second internode from the shoot apex of about 3 to 6 weeks old seedlings of Gossypium hirsutum var. empire which were also grown in the greenhouse. About 1 to 2 centimeters long segments were cut in sterile petri dishes with sterile blade. The apical portion of the segment was cut diagonally and the basipetal portion horizontally.

The stem segments and cotyledons were rinsed with distilled water, immersed momentarily in 60% alcohol and then sterilized with chlorox solution (1:5) for 2 to 5 minutes. They were then transferred to the corresponding containers. The cotyledons were placed in 250 ml. Erlenmeyer flasks, one piece to a flask. The internodal segments were cultured in 8-ounce prescription bottles. Two segments were put into each bottle. The culture containers were plugged with cotton and capped with aluminum foil. Replicates of 5 to 10 of the plant material per set of culture was maintained. All manipulations were carried out aseptically in an ordinary transfer room sterilized by steam.

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[1]The following abbreviations are used: IAA (indoleacetic acid), Ad. (adenine), C (control), and C.M.F. (coconut milk factor).

Nutrient medium: The basic medium employed was White's medium (21) of the following composition in mg./l of water: $Ca(NO_3)_2$ 200, KNO_3 80, $MgSO_4$ 36, KCl 65, NaH_2PO_4 16.5, KI 0.75, $MnSO_4$ 4.5, $Fe_2(SO_4)_3$ 2.5, $ZnSO_4$ 1.5, H_3BO_3 1.5, and sucrose 20,000. Ferric tartrate was employed, instead of $Fe_2(SO_4)_3$ in some of the experiments, the former dissolving more readily in water than the latter. Accessory nutrients used in addition to above were: glycine (3 mg./l), nicotinic acid (0.5 mg./l), thiamine (0.1 mg./l), and pyridoxine (0. 1 mg./l). The above mixture was taken as the control medium. Fifty milliliters of the nutrient solution was used per 8-ounce prescription bottle and 100 milliliters per 250 ml. flask.

The growth substance indoleacetic acid (IAA), and the purines, adenine (Ad.), and guanine were employed. Coconut milk obtained from a mature nut was also used. They were added to the basic medium before autoclaving the nutrient solutions. A liquid medium or semisolid medium of 0.75% agar concentration was employed. The nutrient media in the corresponding containers were sterilized in an autoclave for 15 minutes at 20 pounds pressure.

The cultures were kept at room temperature in diffuse light.

The first attempt to subculture the tissues resulted in bacterial contamination of most of the material. Hence, the plant tissues were allowed to grow as long as possible in the original medium.

For the histological study of the cultured material, the following procedure was employed. Stem segments and cotyledon were killed and fixed in Craf III. They were dehydrated by passing them through the dioxan series to butyl alcohol, and then embedded in paraffin. Sections,

12 microns in thickness, were cut in transverse planes. Safranin--differential staining method was used employing hemalum and tannic acid as mordants.

EXPERIMENTS AND RESULTS

Experiments with Cotyledons

Experiment 1: The effect of isolating cotyledons of 4 weeks old seedlings in liquid control media.

Results: The cotyledonary petiole base which was located above the surface of the medium produced callus on the 5th day and roots emerged from the calluses on the 7th day. Figures 5 to 8 show the extent of growth after 6 months.

Experiment 2: The effect of isolating shoot tips bearing one cotyledon each of 4 weeks old seedlings in liquid control media.

Results: Roots emerged on the 6th day of culture at the petiole bases. No callus was formed preliminary to root formation. The shoot tips developed to about an inch long producing 4 to 5 tiny leaves after 6 months (See Fig. 5).

Experiment 3: The effects of IAA, IAA plus Ad., and Ad. on cotyledons of 4 weeks old seedlings. Four sets of liquid nutrient media were used:

a. Control medium

b. Control medium plus IAA (.02 mg./l)

c. Control medium plus IAA (.02 mg./l) and Ad. (40.mg./l)

d. Control medium plus adenine (40 mg./l)

Results: Callus started to form at the bases of the cotyledonary petioles on the 5th day after isolation of the cotyledons in the above media except in the nutrient medium containing adenine alone. On the 7th day roots emerged from all the petiole bases. On the 10th day 3 roots per cotyledon were formed in the control, 4 roots per cotyledon in both the media containing IAA, and IAA plus Ad., and 2 roots per cotyledon in the medium with Ad. After the 10th day the calluses increased in volume and secondary roots were formed. At the end of 5 weeks, photographs of the cultures were taken (See Figs. 1-4). After 6 months the longest root measured 20 centimeters. This root developed from the cotyledon cultured in the medium containing IAA (.02 mg./l).

Experiment 4: The effect of IAA (.02 mg./l) plus different concentrations of Ad. on cotyledons of 3 weeks old seedlings. Five sets of liquid nutrient media were used:

a. Control medium
b. Control medium plus IAA (.02 mg./l) and Ad. (40 mg./l)
c. Control medium plus IAA (.02 mg./l) and Ad. (60 mg./l)
d. Control medium plus IAA (.02 mg./l) and Ad. (80 mg./l)
e. Control medium plus IAA (.02 mg./l) and Ad. (100 mg./l)

Results: Results were not very satisfactory as most of the cotyledons were contaminated or did not produce any change at all. The cotyledons in all the different media started to form callus at the petiole base on the 5th day and roots started to emerge on the 6th day. On the 10th day the cotyledons in the media containing 40 to 60 mg./l of adenine produced 3 to 4 roots per cotyledon, while those in 100 mg./l

produced only 1 root. The cotyledon in the control medium produced 4 roots per cotyledon.

Experiment 5: The effect of various concentrations of coconut milk on cotyledons obtained from 2 weeks old seedlings. Three sets of liquid nutrient media were used:

a. Control medium

b. Control medium plus 1% coconut milk

c. Control medium plus 2.5% coconut milk

Results: The cotyledons in the medium containing 1% coconut milk produced roots from the cotyledonary petiole base as early as the 4th day of the experiment. Very little callus was formed. At the end of 10 days, approximately 10 roots were observed emerging radially from each petiole base. The cotyledons in the medium containing 2.5% coconut milk produced callus at its base on the 4th day and formed an average of 5 roots per cotyledon on the 10th day. The cotyledons in the control medium produced roots on the petiole base on the 4th day. In 10 days 3 to 4 roots with secondary rootlets per cotyledon were formed.

Experiment 6: The effect of low concentrations of IAA (.001 mg./l) plus different concentrations of guanine on cotyledons from 2 weeks old seedlings. Five sets of semi-solid media were used:

a. Control medium

b. Control medium plus guanine (5 mg./l)

c. Control medium plus guanine (20 mg./l)

d. Control medium plus guanine (40 mg./l)

e. Control medium plus guanine (60 mg./l)

Results: Callus started to form at the petiole base on the 5th day. Roots began to evolve on the 6th day from the calluses. On the 10th day 4 roots per cotyledon were produced in those cultured in the media containing 5 mg./l and 20 mg./l guanine, 5 roots in those cultured in 40 mg./l and 2 roots in the medium containing 60 mg./l guanine. The cotyledons in the control medium were all contaminated after the 5th day.

Experiments with Stem Segments

Experiment 1: The growth of isolated stem segments of 4 weeks old seedlings in a liquid control medium.

Results: The segments sank to the bottom of the container and they became dark brown after a few weeks.

Experiment 2: The effect of the removal of the epidermis and the cortex on the growth of isolated stem segments on a semi-solid control medium. (Epidermis and cortex were stripped off before sterilization of material.) Segments were obtained from 4 weeks old seedlings.

Results: The stem segments turned dark brown after a few days.

Experiment 3: The effect of IAA, IAA plus Ad., and Ad. on internodal stem segments of 6 weeks old seedlings. Four sets of liquid nutrient media were used:

a. Control medium
b. Control medium plus IAA (.02 mg./l)
c. Control medium plus IAA (.02 mg./l) and Ad. (40 mg./l)
d. Control medium plus Ad. (40 mg./l)

Results: Swelling of the stem segments started on the 2nd week of culture and callus began to form after a month at the basipetal ends (See Fig. 25). The culture was allowed to proceed for three months and then weights of the segments were taken. The data are shown in Table I.

TABLE I

Effect of IAA (.02 mg./l), IAA (.02 mg./l) Plus Ad. (40 mg./l) and Ad. (40 mg./l) on Internodal Stem Segments

	Wt. in mgs. of stem segments at beginning of experiment	Wt. in mgs. of stem segments after 3 months	Increase in wt. in mgs.	No. of segments	Average increase in mgs. per segment
a. Control	741	1094	353	5	70.6
b. IAA	720	1124	404	5	80.8
c. IAA plus Ad.	816	1215	496	5	99.2
d. Ad.	989	1485	399	5	79.8

Experiment 4: The effect of different concentrations of IAA on internodal stem segments of 4 weeks old seedlings. Five sets of semi-solid nutrient media were used:

a. Control medium

b. Control medium plus IAA (.001 mg./l)

c. Control medium plus IAA (.005 mg./l)

d. Control medium plus IAA (.01 mg./l)

e. Control medium plus IAA (.02 mg./l)

Results: Callus or swelling occurred mostly at the basipetal portion of the segments after a week of culture (See Fig. 26). The weights of the stem segments were recorded at the end of three months. The data are shown in Table II.

TABLE II

Effect of Different Concentrations of IAA on Internodal Stem Segments

	Wt. in mgs. of stem segments at beginning of experiment	Wt. in mgs. of stem segments after 3 months	Increase in wt. in mgs.	No. of segments	Average increase in mgs. per segment
a. Control	(Cultures were contaminated)				
b. .001 mg./l IAA	374.6	523	148.4	4	37.1
c. .005 mgs./l IAA	590	805	215	5	43
d. .01 mg./l IAA	593.4	881	287.6	7	42.5
e. .02 mgs./l IAA	670.5	927.3	246.8	6	42.8

Experiment 5: The effect of low concentration of IAA (.001 mg./l) plus different concentrations of Ad. on internodal stem segments of 4 weeks old seedlings. Seven sets of semi-solid nutrient media were used:

a. Control medium

b. Control medium plus IAA plus Ad. (1 mg./l)

c. Control medium plus IAA plus Ad. (5 mg./l)

d. Control medium plus IAA plus Ad. (10 mg./l)

e. Control medium plus IAA plus Ad. (20 mg./l)

f. Control medium plus IAA plus Ad. (40 mg./l)

g. Control medium plus IAA plus Ad. (60 mg./l)

Results: Swelling or callus formed chiefly at the basipetal region of the segments after a week of isolation (See Fig. 27). The weights of the excised tissues were taken after 3 months of isolation with the results noted in Table III.

Experiment 6: The effect of high concentration of IAA (.02 mg/l) plus different concentrations of Ad. on internodal stem segments of 5 weeks old seedlings. Six sets of semi-solid nutrient media were used:

a. Control medium

b. Control medium plus IAA plus Ad. (40 mg./l)

c. Control medium plus IAA plus Ad. (60 mg./l)

d. Control medium plus IAA plus Ad. (80 mg./l)

e. Control medium plus IAA plus Ad. (100 mg./l)

f. Control medium plus IAA plus Ad. (120 mg./l)

Results: No callus was observed but swelling occurred usually in regions behind the cut basipetal and apical ends (See Fig. 28). At the end of 3 months the weights of the tissue cultures were taken. The data are shown in Table IV.

TABLE III

Effect of Low Concentration of IAA Plus Different Concentrations of Ad. on Internodal Stem Segments

	Wt. in mgs. of stem segments at beginning of experiment	Wt. in mgs. of stem segments after 3 months	Increase in wt. in mgs.	No. of segments	Average increase in mgs. per segment
a. Control	379	456	77	6	12.3
b. IAA plus 1 mg./l Ad.	387	508	121	7	17.3
c. IAA plus 5 mgs./l Ad.	375	531	156	8	19.5
d. IAA plus 10 mgs./l Ad.	422	575	153	8	19.2
e. IAA plus 20 mgs./l Ad.	318	456	138	7	19.7
f. IAA plus 40 mgs./l Ad.	230	327	97	5	19.4
g. IAA plus 60 mgs./l Ad.	427	645	218	9	14.3

TABLE IV

Effect of High Concentration of IAA Plus Different Concentrations of Ad. on Internodal Stem Segments

	Wt. in mgs. of stem segments at beginning of experiment	Wt. in mgs. of stem segments after 3 months	Increase in wt. in mgs.	No. of segments	Average increase in mgs. per segment
a. Control	255	483.5	228.5	5	45.7
b. IAA plus 40 mgs./l Ad.	323	539	216	4	54
c. IAA plus 60 mgs./l Ad.	350	699.5	349.5	6	58.2
d. IAA plus 80 mgs./l Ad.	495	766.5	271.5	6	45.2
e. IAA plus 100 mgs./l Ad.	326	526	200	5	40
f. IAA plus 120 mgs./l Ad.	351	487	136	4	34

Experiment 7: The effect of different concentrations of coconut milk on internodal stem segments (1 centimeter long) of 3 weeks old seedlings. Six sets of semi-solid nutrient media were used:

a. Control medium

b. Control medium plus coconut milk (1%)

c. Control medium plus coconut milk (2.5%)

d. Control medium plus coconut milk (5%)

e. Control medium plus coconut milk (10%)

f. Control medium plus coconut milk (15%)

Results: Swelling occurred in regions behind the cut ends of the segments particularly at the basipetal end. No callus formation was observed (See Fig. 29). The weights were recorded after three months' time. The data are shown in Table V.

TABLE V

Effect of Different Concentrations of Coconut Milk on Internodal Stem Segments

	Wt. in mgs. of stem segments at beginning of experiment	Wt in mgs. of stem segments after 3 months	Increase in wt. in mgs.	No. of segments	Average increase in mgs. per segment
a. Control	306	417	111	10	11.1
b. 1% Coconut Milk	107	149	42	3	14
c. 2.5% Coconut Milk	275	366	91	6	15.1
d. 5% Coconut Milk	131	239	108	4	27.2
e. 10% Coconut Milk	238	497	259	6	43.1
f. 15% Coconut Milk	232	438	206	6	34.3

Experiment 8: The effect of low concentration of IAA (.001 mg./l) plus different concentrations of guanine on internodal stem segments of 5 weeks old seedlings. Seven sets of semi-solid nutrient media were used:

a. Control medium
b. Control medium plus IAA plus guanine (1 mg./l)
c. Control medium plus IAA plus guanine (5 mg./l)
d. Control medium plus IAA plus guanine (10 mg./l)
e. Control medium plus IAA plus guanine (20 mg./l)
f. Control medium plus IAA plus guanine (40 mg./l)
g. Control medium plus IAA plus guanine (60 mg./l)

Results: Little callus was formed. The segments produced basipetal swelling or no change at all. The weights of the cultured tissues were taken on the 3rd month of culture. The data are illustrated in Table VI.

TABLE VI

Effect of Low Concentration of IAA Plus Different Concentrations of Guanine on Internodal Stem Segments

	Wt. in mgs. of stem segments at beginning of experiment	Wt. in mgs. of stem segments after 3 months	Increase in wt. in mgs.	No. of segments	Average increase in mgs. per segment
a. Control	126	172	46	4	11.5
b. IAA plus 1 mg./l Guanine	170	225	55	4	13.8
c. IAA plus 5 mg./l Guanine	81	124	43	3	14.3
d. IAA plus 10 mg./l Guanine	258.5	343	84.5	6	14.1
e. IAA plus 20 mg./l Guanine	254	349.5	95.5	6	15.9
f. IAA plus 40 mg./l Guanine	244	315	71	5	14.2
g. IAA plus 60 mg./l Guanine	174	229	55	5	11

DISCUSSION OF RESULTS AND OBSERVATIONS

Callus growth and root development were observed to occur only in parts of the cotyledon which were above the surface of the culture medium (See Figs. 1-8). Cotyledons whose petiole bases were located below the surface of the nutrient medium did not produce any change. Most of the cotyledons produced callus and roots at the bases of the petioles. In Fig. 2 callus is developed at the edge of the blade. This might have been due to wounding of the lamina. Callus formation usually preceded the appearance of roots. The largest callus produced after 5 weeks measured about 8 millimeters in diameter (See Fig.3). Figure 16 is a histological cross section of a portion of the callus formed after 6 months in media containing IAA (.02 mg./l). Division in various planes of the callus cells resulted in the production of disorderly protuberances. Scalariformly pitted tracheary elements occur in patches in irregular fashion throughout the callus. Dark lines of cells located almost to the edge of the callus appear to be dead cells which might have been crushed due to simultaneous division of peripheral cells and the enlargement and random division of the internal cells. The callus cells appear to develop into parenchymatous cells and xylem elements. Phloem elements were not very distinct in the sections.

The histological cross section of the petiole base of the cotyledon cultured in the media containing 1% coconut milk showed that the coconut milk growth factor stimulated largely cambial activity resulting in strands of meristematic cells that give rise to root primordia. (See Fig. 13).

The cross section of the petiole base of cotyledon isolated in medium containing IAA (.02 mg./l) shows less cambial activity but suggests that the roots were derived from them (See Fig. 15). Epidermal division and enlargement of collenchyma cells contributed to the increased bulk of the petiole base. The activity of the cambium and phloem elements might have initiated callus growth. (See Figs. 14-15).

Figure 16 shows a root derived from a group of callus cells. An isolated cotyledon has been observed to produce roots from the blade in regions adjacent to the lateral big veins (See Fig. 7). A few cotyledons produced roots along the petiole, while in others they evolved at the base of the laminae where the big veins originate (See Fig. 8).

The shoot tips bearing one cotyledon developed when they were located above the surface of the liquid medium. After six months they elongated to about an inch long producing 4 to 5 tiny leaves (See Fig. 5). Adequate aeration might possibly be needed for the growth and development of the excised cotton cotyledons. White (20) and Skoog (16) observed the contrary with tobacco callus cultures. The callus beneath the liquid or agar produced buds while those on the surface remained without buds. White considered difference in oxygen supply as the cause of differentiation into vegetative buds and roots. The stem tips of Lupinus were found by Ball (1) to require oxygen for their growth and development as they would grow only on the tops of agar or liquid medium. These findings suggest that various plant tissues exhibit different respiratory behaviors at varying levels of oxygen tension.

The cotyledonary blade because of its position in relation to the nutrient medium may be considered as the major region responsible for the absorption of food material in this culture. Figures 9 and 11 illustrate how the mechanism is accomplished. Figure 9 shows the callus

growth at the peripheral region of the blade. The cells protrude radially from the vascular area. In Figure 11, epidermal cell division of the ventral side of the laminae which was in contact with the agar medium might possibly serve for food absorption. It also shows the elongated and enlarged palisade cells. Roots were formed in certain cotyledonary blades in areas close to the big lateral veins and serve as organs for absorption (See Fig. 10).

Under the conditions in which the experiments were carried out, no buds were produced by the cotyledons in spite of the concentrations of Ad. and guanine employed. Therefore, growth was measured on the basis of roots developed up to the 10th day. After the 10th day the roots just continued to elongate and produce secondary roots. Generally, cotyledons capable of growth evolved roots at the petiole base. These roots were counted and recorded. Usually cotyledons obtained from younger seedlings, 2-3 weeks old, were observed to yield roots on the 6th day; those from 4 weeks old seedlings produced roots on the 6th or 7th day. This difference in dates of root emergence might be due to the greater amount of stored food and to the more embryonic stage of the younger cotyledons. It was only the cotyledons, 2 weeks old, cultured in media containing coconut milk which developed roots on the 4th day, much earlier than the cotyledons cultured in the media containing IAA and different concentrations of Ad. The controls for the cultures treated with IAA, Ad. or guanine started to produce roots usually on the same day that the cotyledons containing any or a combination of these chemical substances evolved roots. However, the number of roots produced on the 10th day showed that the IAA used (.02 mg./l) increased the number over that developed by the cotyledons in the control media. Experiments 3 and 4 demonstrate some inhibiting action of

Ad. especially at high concentrations on the root stimulating action of IAA. Guanine, at high concentrations, was also observed to produce the same results. The medium containing coconut milk at 1% concentration demonstrated its stimulating effect on root formation. Very little callus was formed. The number of roots produced on the 10th day was 2 1/2 times that yielded by the cotyledons in the control media, and twice as much as that developed in the media with 2.5% coconut milk.

Contamination of cultures hindered in securing accurate results from media containing coconut milk at high concentrations. The non-availability of coconut from the local markets prevented the use of a good representative number of replicates. From the meager results of the experiment, it can be suggested that the growth factor present in coconut milk at a certain concentration exhibits a more pronounced stimulation of root production than IAA at .02 mg./l concentration. Caplin and Steward (6) isolated carrot phloem fragments in media containing coconut milk and observed a more remarkable growth than that obtained with auxins. Wiggans (23), working with tissue segments from Red Core Chantenay carrot which are very sensitive to auxins, found that coconut milk is much more effective.

Observations on experiments 1 and 2 suggest that cotyledon or shoot tip bearing a cotyledon is capable of rooting and of producing callus in the basal medium of White containing the accessory organic substances. The presence of some chemical substance which was not found in the basal medium of White or in the cotyledon might be needed for the formation of buds.

The stem segments in the liquid control media sank to the bottom of the container and turned brown after a week (Experiment 1). The stem segments of cotton like the cotyledon possibly need an adequate supply

of oxygen in order that growth can occur. Hence, in the subsequent experiments conducted, a semi-solid medium was employed. The segments grown on agar media produced callus or mere swelling. Certain stem segments did not show any growth, even after several months of culture. This was the case observed in experiment 2 when the stem segments were stripped of their epidermis and cortex. The effect was assumed to be the result of the wound caused by handling and excising of the tissues. The longer segments (about 2 centimeters long) that remained healthy throughout the duration of the experiment produced callus at the basipetal end or swelling behind the cut ends (See Figs. 25-27). The shorter segments (about a centimeter long) rarely formed callus, but swelling was observed mostly between the cut ends of the segments. Such condition was noted in the stem segments in experiments 6-8 (See Figs. 28-30). Caplin (5) and Lee (11) observed the contrary and stated that smaller plant or parts always show a greater relative growth rate than larger plants or parts. The centimeter long segments probably were more mature morphologically than the two centimeter long segments employed. Went (18), however, pointed out the possibility of metabolites to diffuse across protoplasmic membranes of isolated tissues or organs. He stated that diffusion in macroscopical dimensions is a slow process but at microscopical dimensions, diffusion rates become greater, affecting the development of the cultures.

The cut ends of all the stem segments became brown after a few days. The callus that formed at the basipetal end tend to grow upward and away from the region of the segment that is in contact with the surface of the medium (See Fig. 17). The surface area of the excised stem touching the medium became irregular when observed after 3 months. Histological examination showed that the peripheral cells in this area

divided and enlarged, and possibly are responsible for absorption of nutrients from the medium, resembling the function of the cells of the cotyledonary blade in contact with the medium (See Figs. 11 and 17).

A study of the cross section of the basipetal end of the excised stem cultured in the medium containing IAA (.02 mg./l) shows the knob-like or fan-shaped protuberances on the upper region of the tissue located away from the agar medium. The cambial cells appear to divide and enlarge by successive transverse divisions soon giving rise to an outgrowth of callus tissue which extends over and above the cut surface and finally grows independently into a large mass of cells. Aside from the cambium, the callus might have been initiated also by certain phloem elements (See Fig. 17). An enlarged section of a knob-like protrusion shows actively dividing cells found at and near the surface (See Fig. 18). This activity together with the enlargement of the inner lying cells presumably caused the crushing of the neighboring cells. Figure 19 shows a cross section of the whole callus area. Many fan-shaped outgrowths resulted from continuous division and proliferation of the callus cells. The cells divide in diverse planes resulting in an irregular mass of cells. Each knob is observed to contain one or more groups of scalariformly pitted tracheary elements and meristematic cells. Figure 20 is a magnified portion of a knob with three groups of such cells. Meristematic, elongated, sometimes binucleated cells appear to surround a group of xylem elements.

The above anatomical changes were similarly illustrated by the stem segments cultured in the control media. Segments cultured in media containing coconut milk, guanine and adenine mostly produced swelling. A cross section of the excised stem cultured in media with 1% coconut milk showed cambial activity, lignification of cells in the phloem area and

enlargement of the collenchyma cells (See Fig. 21). Breaking of the epidermal cells and elongation of the hypodermal cells in the lower area of the section illustrates the manner of food absorption in such tissues.

Under the conditions in which the experiments were conducted, none of the stem segments showed any root or bud initial formation. Hence, measurement of growth was based upon the increase in weights of the tissues after a period of 3 months. Callus growth was exhibited by all the segments cultured in the control media, IAA, IAA plus Ad. and Ad. in experiment 3 (See Fig. 25). The data presented in Table I show the amount of growth attained by the tissues. Addition of IAA caused an increase in weight over that in the control media. A combination of Ad. and IAA at the concentrations indicated produced an increase in weight above that caused by IAA alone while Ad. brought about results almost the same as the IAA.

Table II shows the effect of different concentrations of IAA on excised stems. Concentrations of .005 mg./l to .02 mg./l seem to have caused higher growth rate than the .001 mg./l concentration of IAA in the medium. However, comparison cannot be made with that of the control medium because cultures in the latter were contaminated. Concentrations of IAA higher than the .02 mg./l concentration used, may give more pronounced effect than the results obtained in these experiments.

Experiment 5 shows that the low concentration of IAA plus Ad. in concentrations of 5 mg. to 40 mg./l gave the maximum increase in weight (See Table III). Higher concentration of IAA combined with adenine, however, produced a more marked growth. With .02 mg./l IAA plus 40 mg.-60 mg./l of adenine, over 50 mg. increase per segment was noted (See

Table IV). Over 60 mg./l adenine in combination with IAA showed lower growth rate.

The stem segments cultured in media containing 5% to 15% concentration of coconut milk produced an increase of weight of two to three times that of the increase in weight of the segment in the control medium (See Table V).

Table VI shows the growth increment of stem segments in media containing low concentrations of IAA (.001 mg./l) in combination with different concentrations of guanine. These treatments were slightly effective in promoting section growth.

From the results of above experiments, it can be suggested that growth and organogeny primarily depend on the nutrients employed, the proper balance between growth stimulants as IAA and adenine or guanine, the repeated subculture of tissues for fresh nutrient supply, the size and age of plant tissues, and the oxygen gradient. The cultures presumably have been affected by the tightly applied cotton and aluminum foil covers of the containers. Boatner, Hall, Rollins, and Castillon (2) mentioned about the polyphenolic pigments in Gossypium. The pigments may have caused the excessive darkening of the cut ends of some of the stem segments and subsequently stalled regeneration in these areas.

Histological cross section of normal stem segment, cotyledonary petiole base and cotyledonary blade are included in the illustrations for comparison with the cultured tissues.

SUMMARY

1. This paper presents the results of a study on the culture *in vitro* of cotyledons and stem segments of *Gossypium hirsutum* var. *empire* in White's basal medium with and without addition of indoleacetic acid, adenine, guanine, and coconut milk.

2. Under the conditions in which the cultures were carried out only callus roots were developed from the cotyledons; and callus and/or swelling by the stem segments.

3. Addition of adenine and guanine which have been reported earlier by Miller and Skoog (13) to induce bud formation in tobacco stem segments did not produce buds in either tissues isolated.

4. Indoleacetic acid and coconut milk stimulated callus growth, root formation or swelling in the excised stem segments and cotyledons.

5. Shoot tips bearing one cotyledon cultured in liquid control medium developed to about an inch long in 6 months producing 4 to 5 tiny leaves.

6. Adenine and guanine exhibit some inhibiting effect on the root stimulating action of IAA at higher concentrations.

7. New meristematic responses occur more easily in younger tissues than in older more mature tissues.

8. In the growth responses reported in this paper, normal tissue differentiation occurred only in those cultures in which roots actually developed.

9. In cultures showing only cell enlargement or callus formation,

differentiation occurred not at all or differentiated vascular elements did not become normally oriented.

10. Some of the results indicate a relationship between growth in vitro and the size of the tissue isolate.

11. There are clear indications of a relationship of growth and differentiation to the oxygen gradient in the culture.

12. Photomicrographs have been included to show the histological changes resulting from the in vitro culture of the tissues.

13. The author aimed at inducing bud formation by the excised cotton tissues but results did not reveal any indication to this effect.

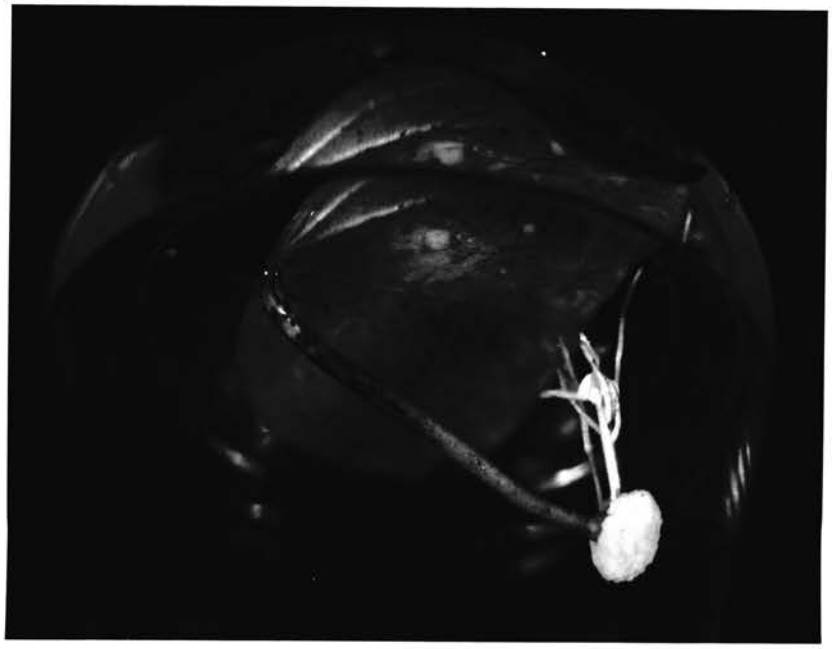

Fig. 1 Cotyledon in liquid control media after a period of 5 weeks.

Fig. 2 Cotyledon in liquid media containing Ad. (40 mgs./l) after a period of 5 weeks. (Cloudy medium is due to bacterial contamination.)

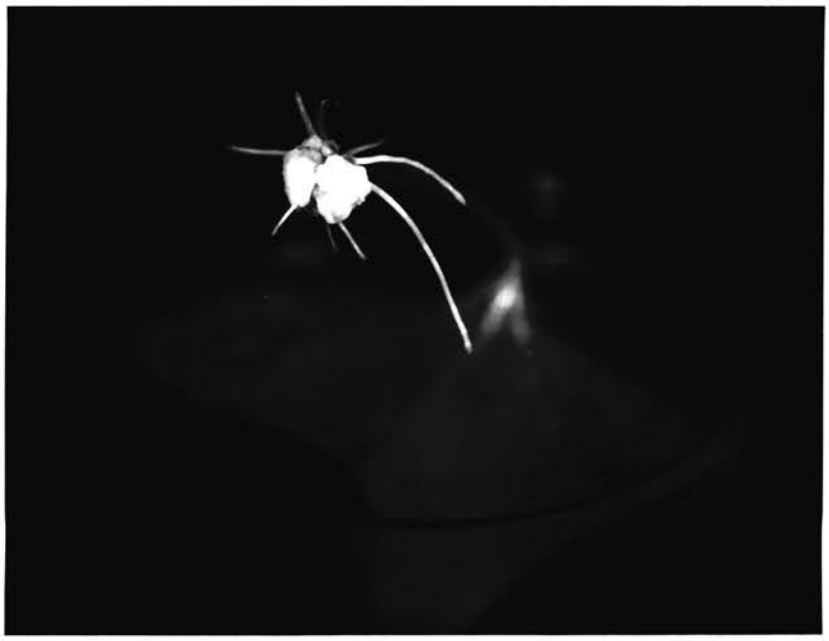

Fig. 3 Cotyledon in liquid media containing IAA (.02 mg./l) after a period of 5 weeks. (Cloudy medium is due to bacterial contamination)

Fig. 4 Cotyledon in liquid media containing IAA (.02 mg./l) and Ad. (40 mgs./l) after a period of 5 weeks.

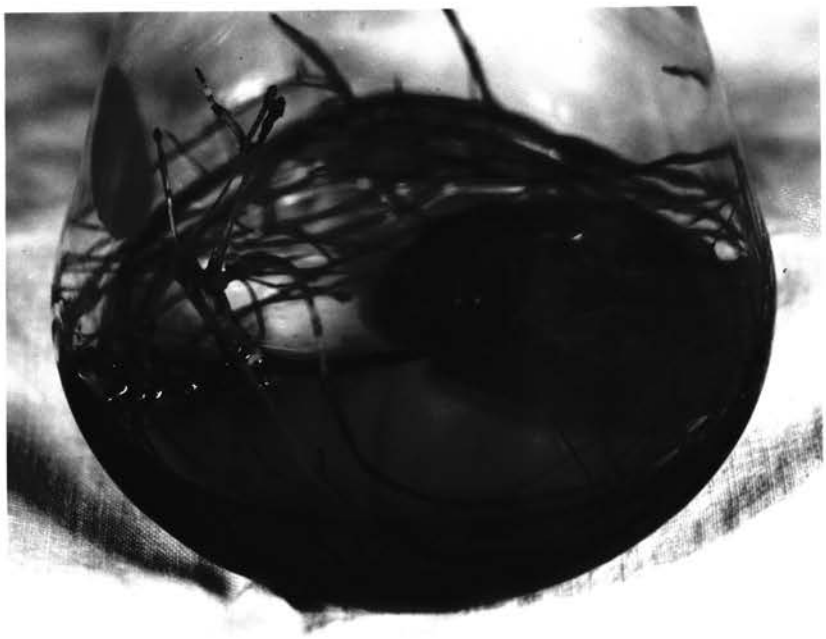

Fig. 5 Cotyledon with shoot tip in liquid control media after a period of 6 months.

Fig. 6 Cotyledon in liquid control media after a period of 6 months.

Fig. 7 Cotyledon in liquid control media after a period of 6 months showing 2 roots emerging from lateral veins of blade.

Fig. 8 Cotyledon isolated in liquid control media for a period of 6 months showing roots emerging from base of blade where big veins originate.

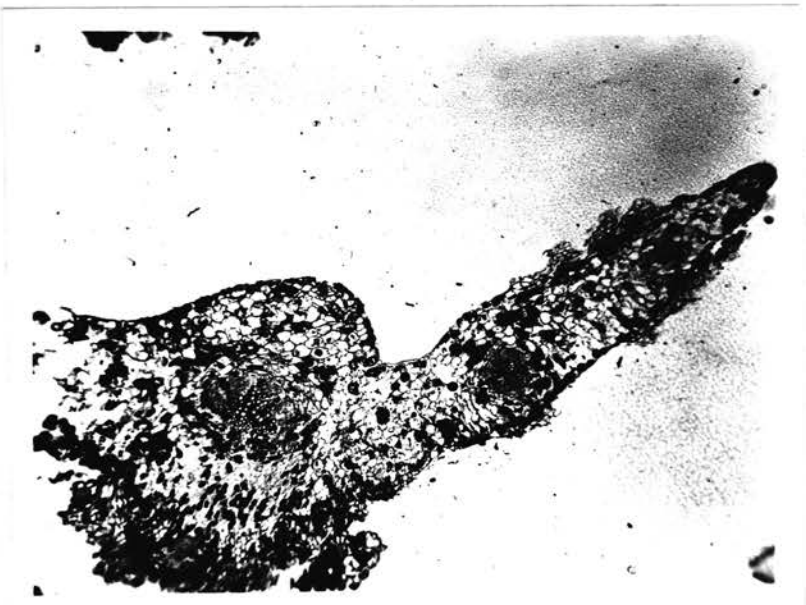

Fig. 9 Histological cross section of portion of cotyledonary blade after 6 months in liquid control medium. X 30

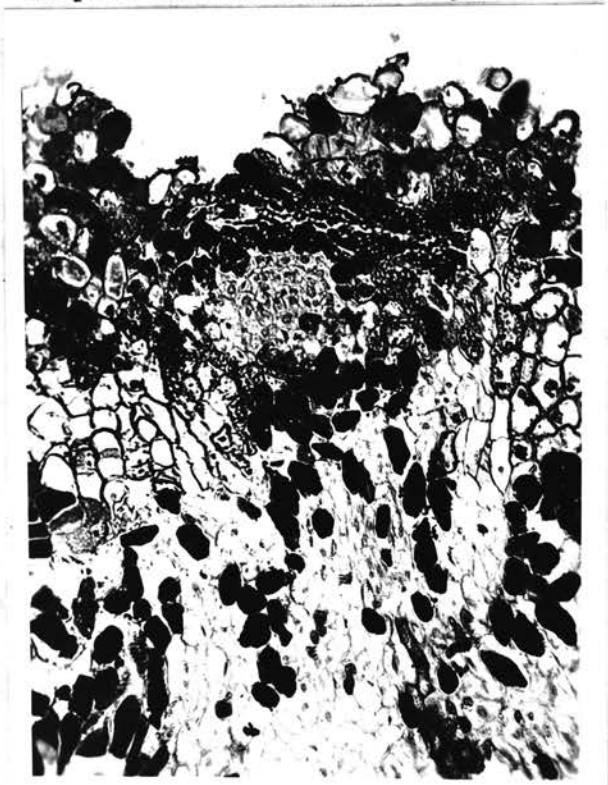

Fig. 10 An enlarged section of callus from Fig. 9 showing root primordium developing from group of cells above strand of xylem elements. X 80

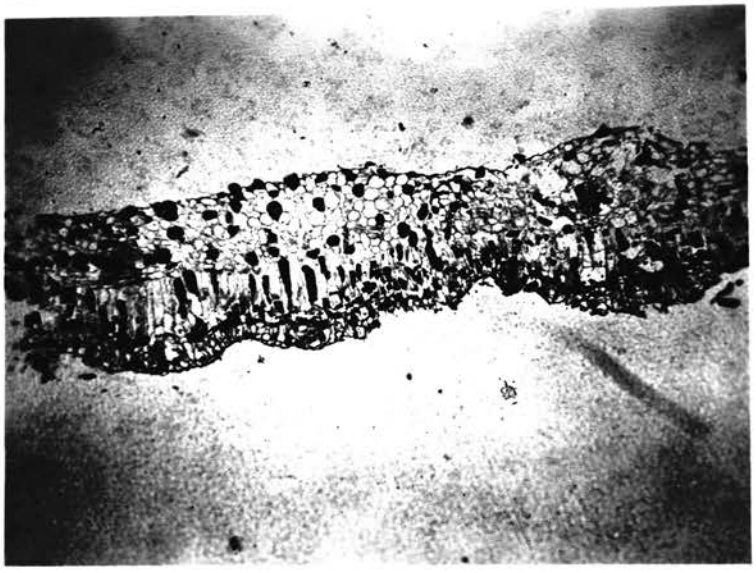

Fig. 11 Histological cross section of portion of cotyledonary blade after 3 months of culture in semi-solid medium containing 1% Coconut milk. X 30

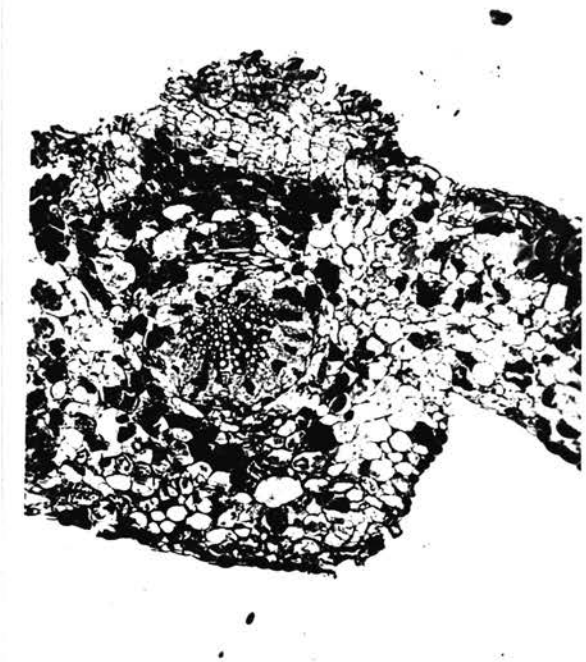

Fig. 12 Histological cross section of portion of cotyledonary blade cultured in semi-solid medium containing IAA (.02 mg./l) plus Ad. (40 mgs./l) for a period of 3 months showing callus growth opposite big vein. X 30

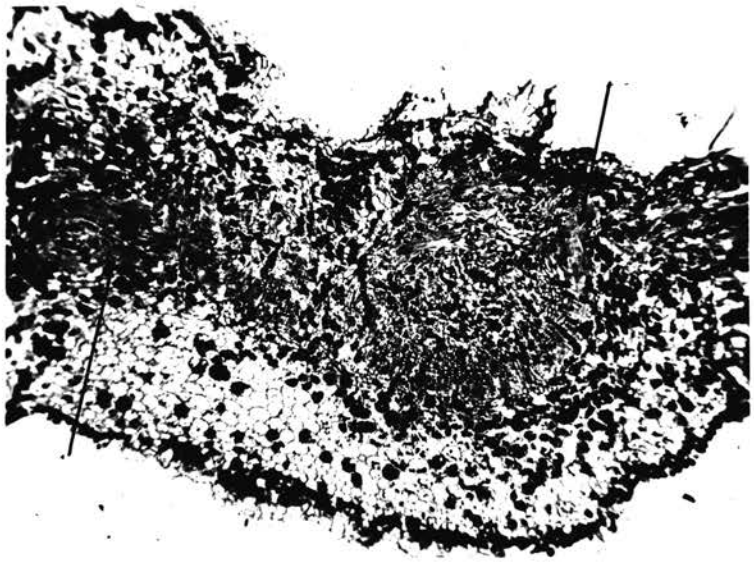

Fig. 13 Histological cross section of cotyledonary petiole base cultured in semi-solid medium containing 1% coconut milk for a period of 3 months showing callus growth and root formation. (Arrows point to strands of meristematic cells.) X 30

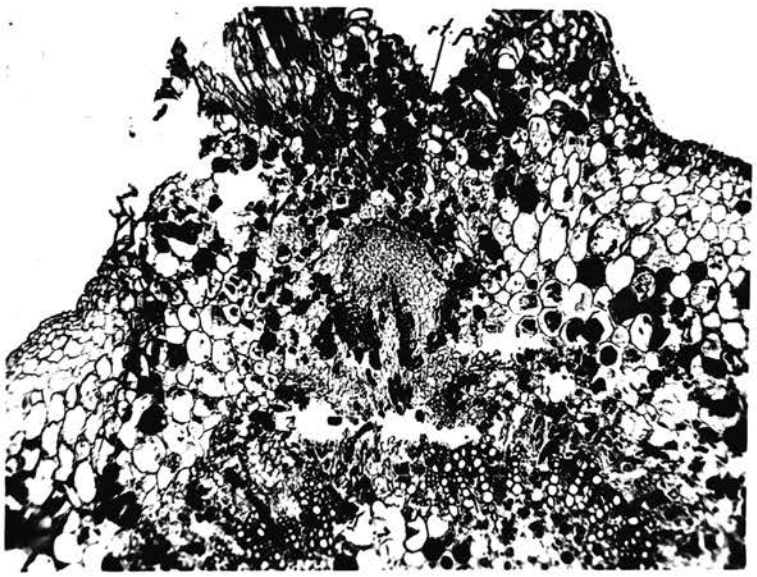

Fig. 14 An enlarged portion of cotyledonary petiole base showing root primordium (rt.p.) after 3 months of culture in liquid medium containing IAA (.02 mg./l). (Arrow indicates root primordium.) X 50

Fig. 15 Histological cross section of cotyledonary petiole base showing callus growth and root primordium (rt.p.) cultured in liquid medium containing IAA (.02 mg./l) after a period of 6 months. X 30

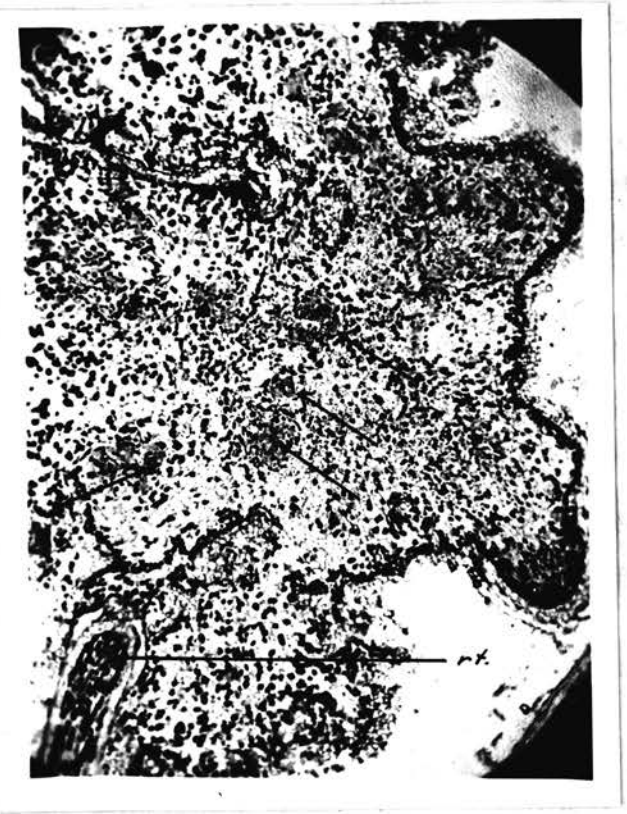

Fig. 16 Histological cross section of callus formed on cotyledonary petiole base after 5 months of culture in liquid medium containing IAA (.02 mg./l). Longitudinal section of developing root is seen at lower left-hand corner (rt.). (Arrows point to some groups of scalariformly pitted tracheary elements.) X 30

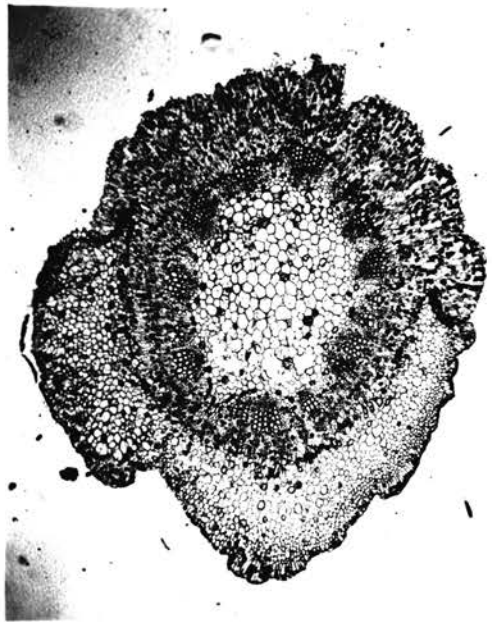

Fig. 17 Histological cross section of stem segment cultured in nutrient medium with IAA (.02 mg./l) for a period of 3 months. X 20

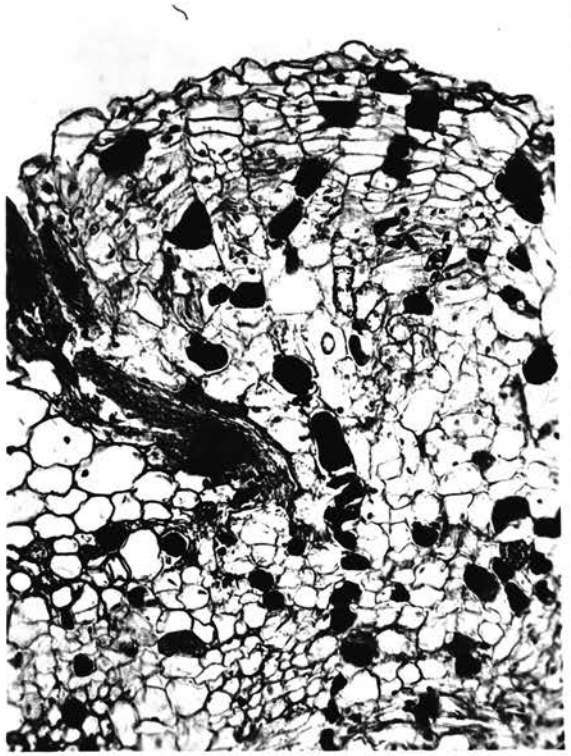

Fig. 18 An enlarged knob-like protuberance from Fig. 17 showing actively dividing cells at and near the surface. X 80

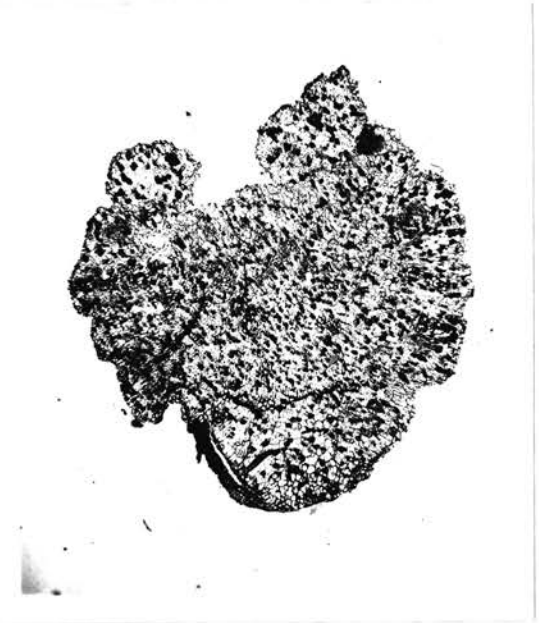

Fig. 19 Histological cross section of callus formed at the basipetal end of stem segment cultured in IAA (.02 mg./l) for a period of 3 months. X 15

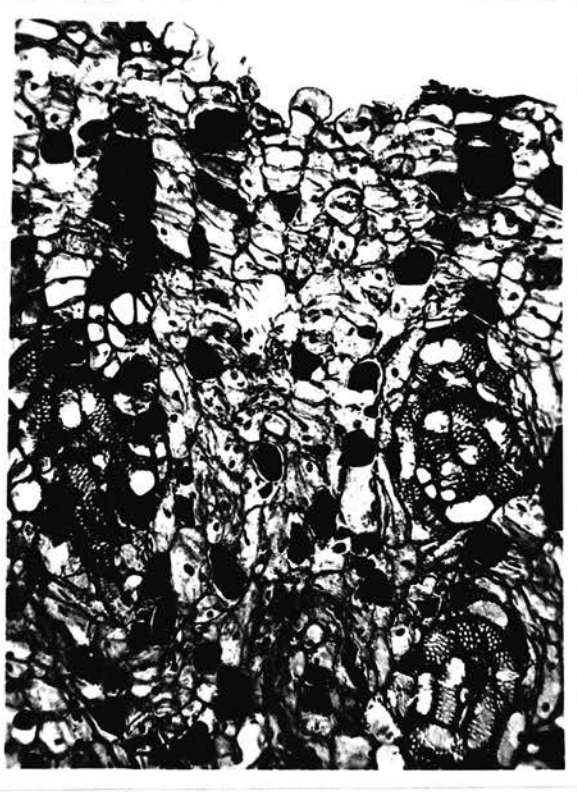

Fig. 20 An enlarged portion of callus from Fig. 19 showing patches of scalariformly pitted tracheary elements. X 80

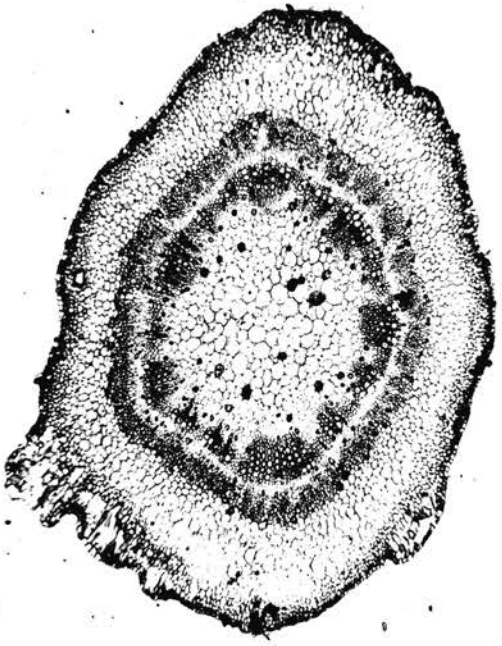

Fig. 21 Histological cross section of stem segment cultured in media containing 1% coconut milk for a period of 3 months. X 20

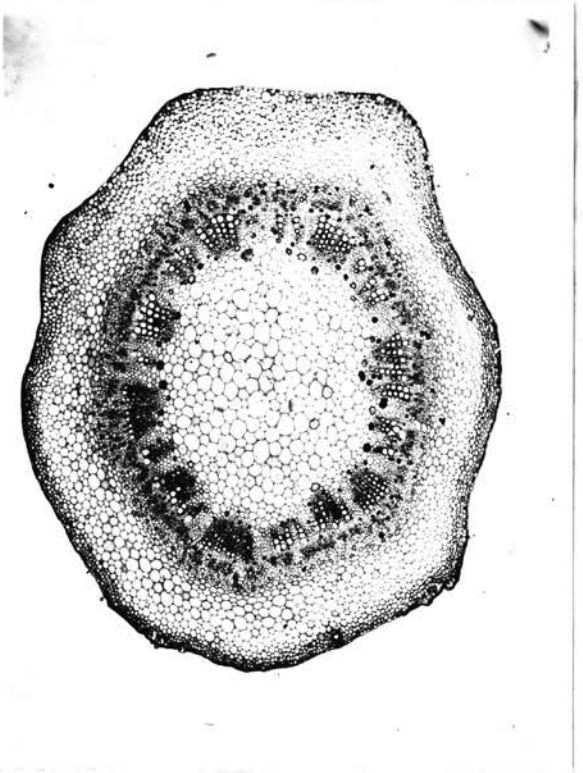

Fig. 22 Histological cross section of normal stem (2nd internodal stem segment). X 20

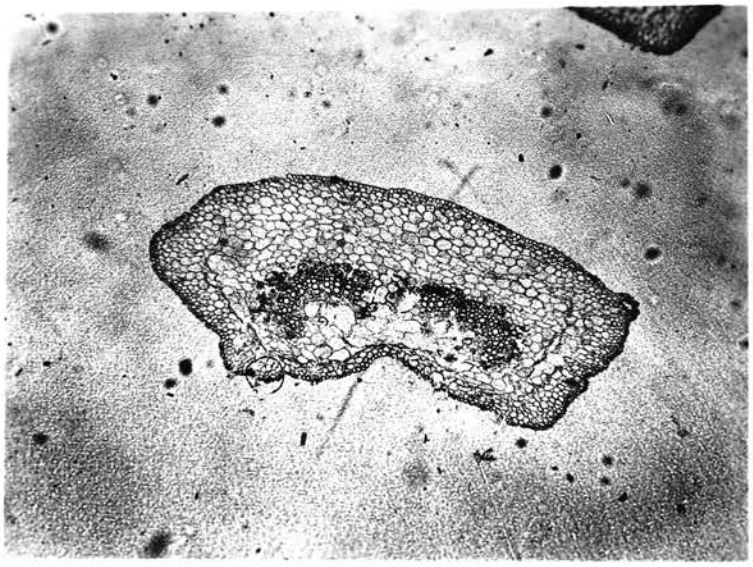

Fig. 23 Histological cross section of normal cotyledonary petiole base. X 30

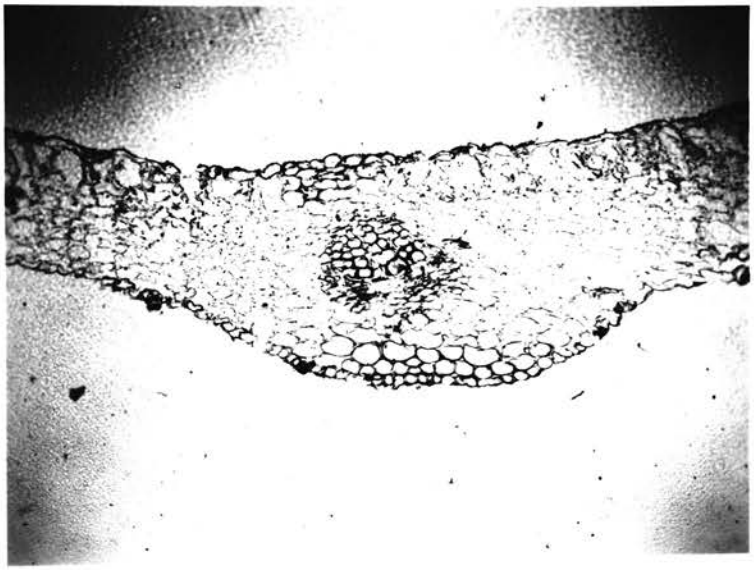

Fig. 24 Histological cross section of portion of normal cotyledonary blade showing a vascular area. X 80

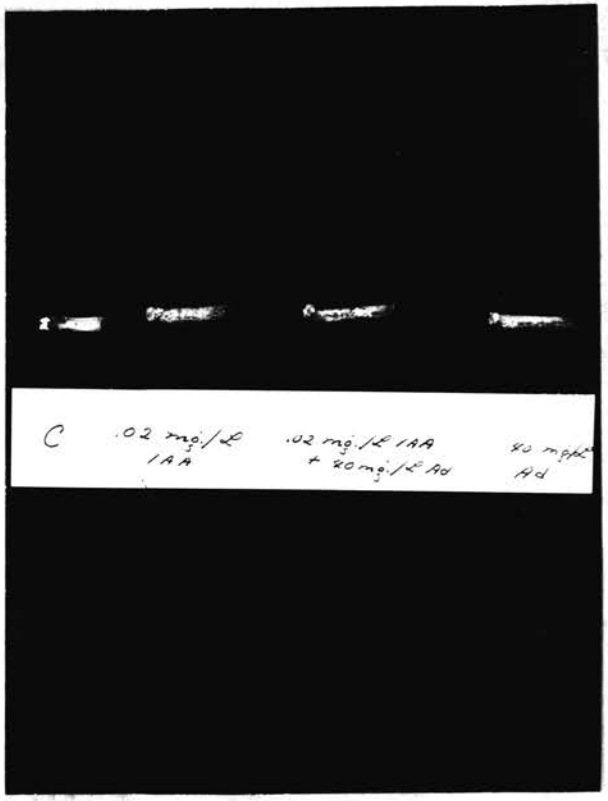

Fig. 25 Representative samples of stem segments cultured on semi-solid media containing growth substances: IAA (.02 mg./l), IAA (.02 mg./l) plus Ad. (40 mgs./l), Ad. (40 mgs./l) after a period of 3 months.

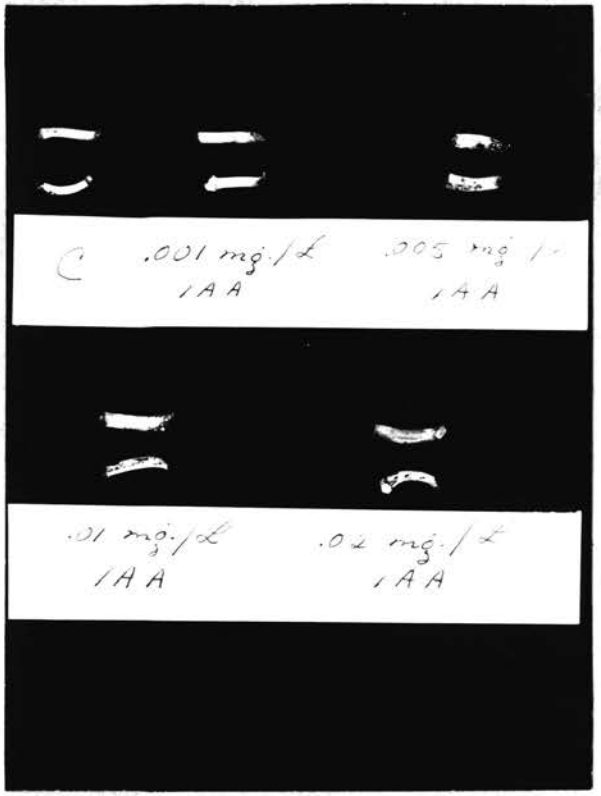

Fig. 26 Representative samples of stem segments cultured on semi-solid media containing different concentrations of IAA after a period of 3 months.

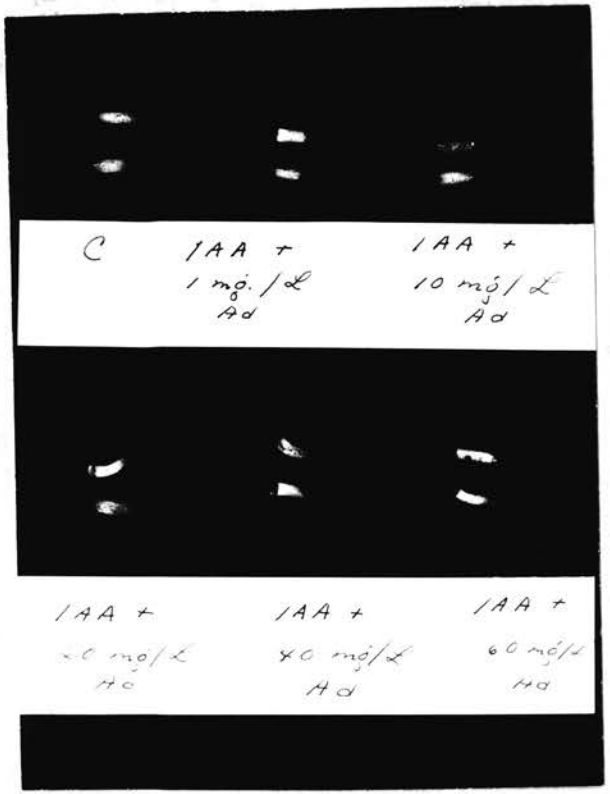

Fig. 27 Representative samples of stem segments cultured on semi-solid media containing low concentration of IAA (.001 mg./l) and different concentrations of Ad. after a period of 3 months.

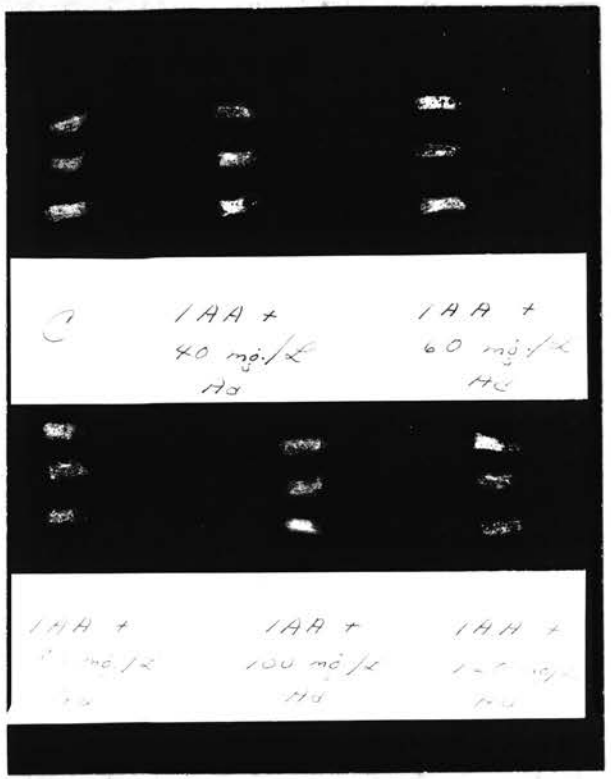

Fig. 28 Representative samples of stem segments cultured on semi-solid media containing high concentration of IAA (.02 mg./l) and different concentrations of Ad. after a period of 3 months.

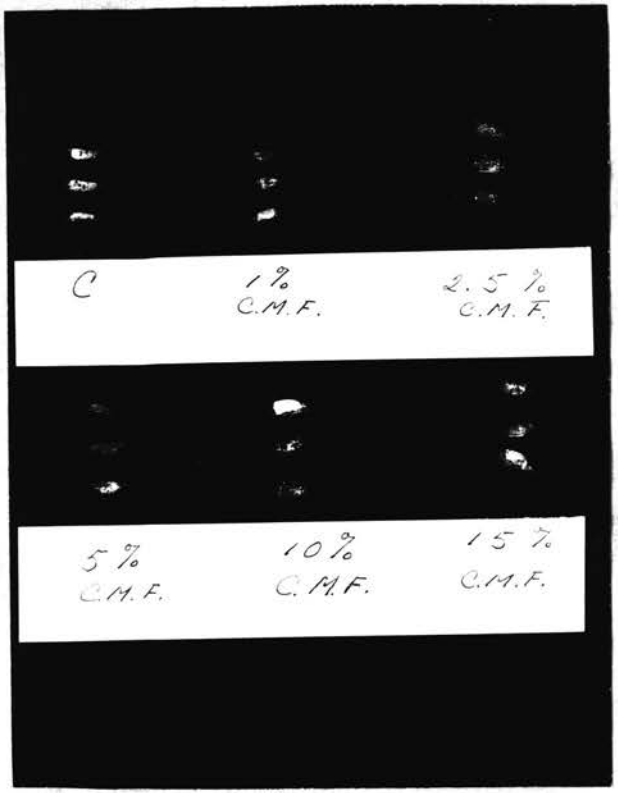

Fig. 29 Representative samples of stem segments cultured on semi-solid media containing different concentrations of coconut milk (C.M.F.) after a period of 3 months.

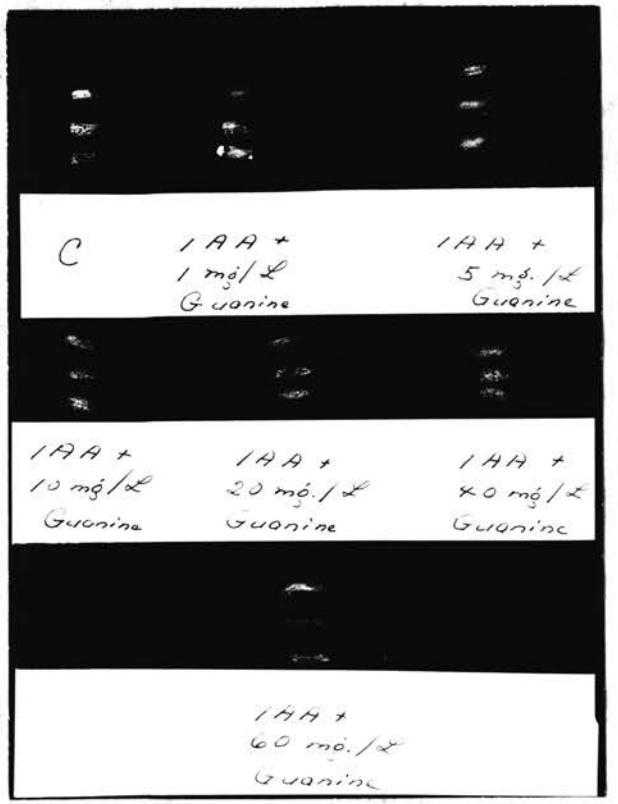

Fig. 30 Representative samples of stem segments cultured on semi-solid media containing low concentrations of IAA (.001 mg./l) and different concentrations of guanine.

LITERATURE CITED

1. Ball, E. 1946. Development in sterile culture of stem tips and subjacent regions of Tropaeolum majus L. and of Lupinus albus L. Am. J. Botany, 33: 301-318

2. Boatner, C. H., Catherine M. Hall, Mary M. Rollins, and Leah E. Castillon. 1918. Chemistry and histology of glands of cotton. Jour. Agr. Research, 13 (8): 419-436.

3. Bonner, D. M., A. J. Haagen-Smit, and F. W. Went. 1939. Leaf growth hormones. I A bio-assay and source for leaf growth factors. Bot. Gaz., 101: 128-144.

4. Carlson, Margery C. 1953. Root formation in isolated cotyledons of Brassica napus and Raphanus sativus. Am. J. Botany, 40 (4): 233-238.

5. Caplin, S. M. 1947. Growth and morphology of tobacco tissue cultures in vitro. Bot. Gaz., 108: 379-393.

6. Caplin, S. M. and F. C. Steward. 1948. Effect of coconut milk on the growth of explants from carrot root. Science, 108: 655-657.

7. Gautheret, R. J. 1939. Sur la possibilite de realiser la culture indefinie des tissus de tubercules de carotte. C.r. Acad. Sci. Paris, 208: 118-120.

8. Haberlandt, G. 1902. Kulturversuche mit isolierten pflanzenzellen. Sitzungsber. Akad. Wiss. Wien, math. naturw. Kl., 111B: 69-92 Quoted from Caplin (5) and Robbins (15).

9. La Rue, C. D. 1933. Regeneration in mutilated seedlings. Proc. National Acad. Sci., 19: 53-63.

10. ———. 1936. Tissue cultures of spermatophytes. Proc. National Acad. Sci, 22: 201-209.

11. Lee, A. E. 1950. The growth in culture of intact seedlings and isolated seedling organs. Am. J. Botany, 37 (4): 312-318.

12. Loo, S. W. 1945. Cultivation of excised stem tip of asparagus in vitro. Am. J. Botany, 32: 13-17.

13. Miller, C. O. and F. Skoog. 1953. Chemical control of bud formations in stem segments. Am. J. Botany, 40: 768-773.

14. Nickell, L. G. 1950. Effect of coconut milk on the growth in vitro of plant virus tumor tissue. Bot. Gaz., 112: 225-228.

15. Robbins, W. J. 1922. Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz., 73: 376-390.

16. Skoog, F. 1944. Growth and organ formation in tobacco tissue cultures. Am. J. Botany, 31: 19-24.

17. Sterling, C. 1950. Histogenesis in tobacco stem segments cultured in vitro. Am. J. Botany, 37 (6): 464-470.

18. Went, F. W. 1954. Physical factors affecting growth in plants. "Dynamics of Growth Processes," pp. 130-147. Princeton, N. J.: Princeton University Press.

19. White, P. R. 1933. Plant tissue cultures. Results of preliminary experiments on the culturing of isolated stem-tips of Stellaria media. Protoplasma, 19: 97-116.

20. ------. 1939. Potentially unlimited growth of excised plant callus in an artificial nutrient. Am. J. Botany, 26: 59-64.

21. ------. 1943. A handbook of plant tissue culture. Lancaster, Pa.: Jaques Cattell.

22. ------. 1954. The cultivation of animal and plant cells. New York: The Ronald Press Company.

23. Wiggans, S. C. 1954. Growth and organ formation in callus tissues derived from Daucus carota. Am. J. Botany, 41: 321-326.

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Date of Final Examination: July, 1955

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