THE EFFECTS OF VARIOUS GROWTH REGULATORS ON *HELIANTHUS* STEM TISSUE

by

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INTRODUCTION

Various growth-regulating compounds have been tested to determine their effect on intact and excised plant tissues. Colchicine is known to induce polyploidy in plants including Musa (Vakili, 1962) and Vitis (Gargiulo, 1960). Ferry and Ward (1959) have shown that 2,4-dichlorophenoxy-acetic acid (2,4-D), a plant herbicide at high concentrations, induces growth at low concentrations as shown by elongation and increase in fresh weight of sprayed stem sections in short periods of time. This growth is associated with increases in nucleic acid, protein, and ribonuclease content (Shannon, Hanson, and Wilson, 1964) and is not related to effects on respiration (Wedding and Black, 1964). Kinetin has been reported to stimulate cell division in carrot root tissues (Shantz, Mears, and Steward, 1958); however, there have been several reports, such as by Miller (1961), that the compound failed to cause division or did so only in combination with indoleacetic acid (IAA). Other effects of kinetin include initiation of shoot formation (Schrandolf and Reinert, 1959) and inhibition of roots in sunflower hypocotyl sections (De Ropp, 1956). Breaking of dormancy in several varieties of lettuce seeds by application of kinetin has been definitely established (Miller, 1956; Skinner et al., 1957; Haber and Tolbert, 1957). Glaziou (1957) reported an increase in respiration accompanying cell elongation in tobacco pith

cells. Also, kinetin increases mitotic activity in tobacco pith tissue when used in combination with IAA (Das, Patau, and Skoog, 1956). When applied to stem sections the compound seems to produce normal xylem growth, while auxins such as 2,4-D cause cambial activation and abnormal growth after seven days (Sorokin, Mathur, and Thimann, 1962).

A fourth substance, coconut milk, is not a chemically defined compound but the liquid endosperm of the coconut. Its composition is under study, but the mixture has been long tested for its growth-regulating properties. It is divided into active and neutral fractions, the latter consisting mainly of carbohydrates (Pollard, Shantz, and Steward, 1959) and the former containing several substances of a purine-like nature which are believed to be related to kinetin, plus many other substances which have not yet been fractionated but which have been shown to stimulate cell division (Shantz, Pollard, and Steward, 1959).

In view of these previous results, experiments were designed to provide information concerning the effects of various concentrations of the three substances colchicine, 2,4-D, and kinetin. Tissue effects were indicated by changes in fresh weight, dry weight, and specific stem tissues of Helianthus stem sections agitated in nutrient liquid medium. Results were determined first with each growth-regulating compound alone, then with each compound in combination with three per cent coconut milk.

MATERIALS AND METHODS

Test solutions of various concentrations of growth-regulating compounds, with Modified White's Medium and Modified White's Medium plus three per cent coconut milk as basal solutions, were used as culture media for the stem sections. Stock solutions for Modified White's Medium are:

A: $Ca(NO_3)_2 \cdot 4H_2O$ ..... 14 g
KNO_3 ..... 16 g
Make up to 500 ml with glass distilled water (GDW).

B: $MgSO_4 \cdot 7H_2O$ ..... 7 g
KCl ..... 6.5 g
Make up to 500 ml with GDW.

C: KH_2PO_4 ..... 2.5 g
Make up to 500 ml with GDW.

D: KI ..... 75 mg
$ZnSO_4 \cdot 7H_2O$ ..... 267 mg
H_3BO_3 ..... 160 mg
$MnSO_4 \cdot H_2O$ ..... 492 mg
Make up to 500 ml with GDW.

E: Glycine ..... 200 mg
Thiamine HCl ..... 10 mg
Nicotinic acid ..... 50 mg
Pyridoxine HCl ..... 10 mg
Make up to 500 ml with GDW.

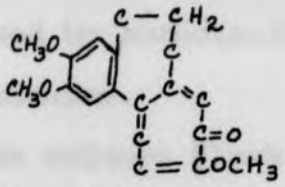
F: Dissolve 2.61 g of ethylene diamine tetraacetic acid in 27 ml of 1N NaOH after which 2.49 g of $FeSO_4 \cdot 7H_2O$ are added and dissolved by boiling, and then distilled water added up to a final volume of 500 ml.

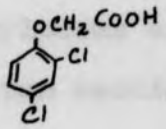
Modified White's Medium:

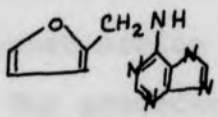
5 ml stock solutions A,B,C,D, and E
10 ml stock solution F
20 g sucrose
Make up to 1 liter with GDW;
adjust pH to 6.0-6.5.

Modified White's Coconut Milk Medium:

5 ml stock solutions A,B,C,D, and E
10 ml stock solution F
20 g sucrose
5 ml NH_4NO_3 solution (2 g NH_4NO_3 in 1 liter GDW)
30 ml coconut milk
Make up to 1 liter with GDW;
adjust pH to 6.0-6.5.

The growth-regulating compounds tested were colchicine, 2,4-D, and kinetin. Colchicine, which has the structural formula  , belongs to the group of plant alkaloids and is similar to nicotine.

2,4-D, which has the structural formula  , is classified as an auxin.

Kinetin, with the structural formula  , belongs to the family of compounds known as cytokinins (Skoog, Strong, and Miller, 1965). It was first isolated from herring sperm DNA (Miller et al., 1955).

Using Modified White's and Modified White's plus coconut milk as basal media, logarithmic concentrations of 10^{-3}M to 10^{-13}M of each growth-regulating compound were made from 10^{-1}M stock solutions of these compounds. To make the three stock solutions, 399 mg colchicine was dissolved in 10 ml of 95% ethanol; 221 mg 2,4-D was dissolved in 10 ml of 95% ethanol; and 215 mg kinetin was dissolved in 2 ml of dilute sodium hydroxide and added to 8 ml of 95% ethanol.

One control consisting of the basal medium was used for each series.

The resulting test solutions were dispensed into 125 ml erlenmeyer culture flasks with each flask containing 50 ml of solution. Duplicate series of six test solutions plus two controls for each basal medium and each test compound were used. The culture flasks containing the media were sterilized by autoclaving at 15 lbs pressure and 121° C for 15 minutes.

Each culture flask was then inoculated with four stem sections from the first internode above the cotyledons of six-week old Helianthus annuus plants. These stem sections had been surface sterilized with sodium hypochlorite solution by a procedure found to be effective in another series of experiments (Hendrick, unpublished data). The procedure included the following steps:

(1) "Wetting" the surface of an entire internode in a sterile petri dish with 50% ethanol.

(2) This was followed by soaking the internode for three minutes in a 1:4 solution of sodium hypochlorite:distilled water.

(3) The sodium hypochlorite solution was discarded and the sections were washed three times with sterile distilled water.

(4) The internodes were then cut into uniform 5 mm stem sections.

(5) The sections were finally transferred to the culture flasks.

The inoculated flasks were placed on a mechanical shaker

moving at ninety reciprocal motions per minute in constant light and maintained at a temperature of 20°C ± 2°.

At the same time the flasks were inoculated, representative stem sections were weighed and recorded as initial wet weights. These sections were then dried for 24 hrs at 38-40° C, reweighed, and these weights recorded as initial dry weights.

RESULTS AND DISCUSSION

At the end of four weeks the culture flasks were removed from the shaker and results were recorded. The wet and dry weights of the sections showed the greatest increase in the colchicine series at the 10^{-11}M concentration of colchicine in the plain medium (see Table 1) and at 10^{-9}M in the colchicine plus coconut milk series (see Table 2). The greatest inhibition of growth in both test media was found at a concentration of 10^{-3}M colchicine. The degree of inhibition, shown in the tables by the negative values for per cent deviation, was almost the same in the plain and coconut milk media. The final weights for the stem sections treated with coconut milk, however, were a great deal higher than those of sections cultured without coconut milk. Therefore, the coconut milk itself was responsible for a weight increase. Examination of the standard deviations within the experimental units shows a much smaller deviation (averaging ± 6.5 in the colchicine series as compared to ± 36.8 in the colchicine plus coconut milk series) in the stem sections cultured without coconut milk. This suggests somewhat random growth brought about by the coconut milk, while colchicine remained more consistent in its initiation of cell enlargement. The sections cultured in colchicine plus coconut milk weighed more than any other experimental group at the end of the test period. At the lower

Table I

EFFECT OF COLCHICINE

CONCN	AVERAGE INITIAL WT WET	AVERAGE FINAL WT WET	SD WITHIN EXPT'L UNIT	SE EXPT'L VS. CONTROL	% DEVIATION	AVERAGE INITIAL WT DRY	AVERAGE FINAL WT DRY	% DEVIATION
control	18.0 mg	92.4 mg	±11.4			0.7 mg	8.8 mg	
10^{-3}	26.2	35.8	± 0.883	5.75	-61.3	1.6	3.7	-58.0
10^{-5}	26.2	86.6	± 4.05	6.09	- 6.3	1.6	9.5	7.95
10^{-7}	18.2	157.5	± 7.88	6.98	70.5	0.8	15.9	80.7
10^{-9}	11.0	151.5	± 8.31	7.09	63.9	0.6	19.2	118.0
10^{-11}	11.0	187.1	± 9.36	7.41	103.0	0.6	22.7	158.0
10^{-13}	18.0	61.0	± 3.22	5.96	34.0	0.7	5.9	32.9

Table 2

EFFECT OF COLCHICINE + 3% COCONUT MILK

CONCN	AVERAGE INITIAL WT WET	AVERAGE FINAL WT WET	SD WITHIN EXPT'L UNIT	SE EXPT'L VS. CONTROL	% DEVIATION	AVERAGE INITIAL WT DRY	AVERAGE FINAL WT DRY	% DEVIATION
control	47.8 mg	321 mg	± 19.5			4.9 mg	35.8 mg	
10^{-3}	43.4	190	± 7.91	12.2	-40.8	4.2	13.4	-62.6
10^{-5}	66.7	339	± 17.8	15.2	5.61	6.7	35.1	- 1.96
10^{-7}	43.4	628	± 29.7	20.5	95.6	4.2	53.9	50.6
10^{-9}	86.8	695	± 145	84.7	117	6.5	68.9	92.4
10^{-11}	86.8	545	± 26.2	18.9	71.4	6.5	57.9	61.7
10^{-13}	86.8	605	± 11.5	13.1	88.5	6.5	63.7	77.9

concentrations of colchicine the sections weighed approximately 600 mg each. Thus the high final weights of the sections seem to result from unopposed action of coconut milk in stimulating cell division.

Sections of stems treated in each test solution were fixed in a formalin-alcohol-acetic acid solution, then imbedded in paraffin, cut into 15 micron sections, and differentially stained with safranin and anilin blue. Microscopic examinations of these sections were made to determine the effect of the growth-regulators on the stem tissue and to correlate these effects with the increases in stem weight. When treated with 10^{-3}M colchicine the sections show inhibition of the cortical parenchyma. The number of cells in this region does not seem to be affected, i.e., normal cell division has not been altered, but the cells decrease in size, giving this region a compressed appearance. At the lower concentrations of colchicine a large region of cortical parenchyma is seen. The cells in this tissue mass have increased in size over corresponding cells in the control, but the number of cells has not increased.

In the colchicine plus coconut milk series proliferation of the cortical parenchyma and possibly an increase in vascular tissue was observed, but these are believed to result largely from the presence of coconut milk.

In the 2,4-D series some of the same general trends were found as in the colchicine series. When the stem sections

were removed from the culture flasks and weighed, certain obvious changes were observed. At a 10^{-3}M concentration of 2,4-D the diameter of the sections had decreased, while at the lower concentrations a definite increase in diameter was noted. The first observations were confirmed by actual weight increases of the sections. The greatest increase in fresh weight was found at a concentration of 10^{-7}M in 2,4-D alone (see Table 3) and at 10^{-11}M in the 2,4-D plus coconut milk series (see Table 4). The 359% increase over the control found in the average fresh weight of the sections treated with 10^{-7}M 2,4-D is much lower, only 67.8%, in the average dry weight of the same sections. This suggests a change in the osmotic regulation of the tissues caused by the 2,4-D. In the sections cultured in coconut milk media, no comparable change was observed. This indicates a regulating effect of coconut milk since the 359% increase found in sections treated with 10^{-7}M 2,4-D was reduced to 25.5% with the addition of coconut milk. Overall, the range of weights was 432% with 2,4-D alone, but only 116% with 2,4-D plus coconut milk. These results support a present hypothesis that coconut milk acts somewhat like kinetin in controlling abnormal growth (Sorokin et al., 1962). Further examination of the final weights of the stem sections showed a peak increase at the intermediate concentrations of 2,4-D in both the plain and coconut milk series with sharply falling values at both the highest and lowest concentrations of 2,4-D.

Table 3

EFFECT OF 2,4-D

CONCN	AVERAGE INITIAL WT WET	AVERAGE FINAL WT WET	SD WITHIN EXPT'L UNIT	SE EXPT'L VS. CONTROL	% DEVIATION	AVERAGE INITIAL WT DRY	AVERAGE FINAL WT DRY	% DEVIATION
control	23.0 mg	65.8 mg	± 2.54			0.8 mg	10.9 mg	
10^{-3}	23.0	17.6	± 1.82	1.58	-73.2	0.8	1.1	-89.9
10^{-5}	28.2	63.0	± 9.03	4.69	- 4.26	1.0	6.5	-40.4
10^{-7}	22.5	291.4	±21.3	10.7	359	1.2	18.3	67.8
10^{-9}	22.5	150.2	± 7.77	4.09	128	1.2	14.2	30.3
10^{-11} *								
10^{-13}	11.0	91.3	± 9.57	4.95	38.8	0.6	8.6	21.1

* Discarded because of contamination

Table 4

EFFECT OF 2,4-D +

CONCN	AVERAGE INITIAL WT WET	AVERAGE FINAL WT WET	SD WITHIN EXPT'L UNIT	SE EXPT'L VS. CONTROL
control	81.4 mg	420 mg	± 16.5	
10^{-3}	81.4	57	± 14.7	12.8
10^{-5}	38.4	114	± 16.1	13.3
10^{-7}	46.8	527	± 83.6	49.2
10^{-9} *				
10^{-11}	60.7	544	± 34.6	26.3
10^{-13}	47.8	320	± 12.6	12.0

* Discarded because of contamination

Table 3

T OF 2,4-D

SE EXPT'L VS. CONTROL	% DEVIATION	AVERAGE INITIAL WT DRY	AVERAGE FINAL WT DRY	% DEVIATION
		0.8 mg	10.9 mg	
1.58	- 73.2	0.8	1.1	- 89.9
4.69	- 4.26	1.0	6.5	- 40.4
10.7	359	1.2	18.3	67.8
4.09	128	1.2	14.2	30.3
4.95	38.8	0.6	8.6	21.1

Table 4

EFFECT OF 2,4-D + 3% COCONUT MILK

CONCN	AVERAGE INITIAL WT WET	AVERAGE FINAL WT WET	SD WITHIN EXPT'L UNIT	SE EXPT'L VS. CONTROL	% DEVIATION	AVERAGE INITIAL WT DRY	AVERAGE FINAL WT DRY	% DEVIATION
control	81.4 mg	420 mg	± 16.5			6.3 mg	27.2 mg	
10^{-3}	81.4	57	± 14.7	12.8	-86.4	6.3	5.8	-78.7
10^{-5}	38.4	114	± 16.1	13.3	-72.9	3.0	10.0	-76.0
10^{-7}	46.8	527	± 83.6	49.2	25.5	3.4	43.0	58.1
10^{-9} *								
10^{-11}	60.7	544	± 34.6	26.3	29.3	5.2	49.2	80.9
10^{-13}	47.8	320	± 12.6	12.0	-24.0	4.9	35.8	31.6

* Discarded because of contamination

When a coconut milk control was compared to a plain White's Medium control, the sections treated with coconut milk showed a tremendous weight increase. At the various concentrations of 2,4-D this increase caused by coconut milk is repeatedly observed; however, it is found to be dependent on the concentration of 2,4-D in the medium. Although the stem sections treated with coconut milk weighed more at the end of the experiment than the ones cultured without coconut milk, the range of increase is significantly less than that observed in the 2,4-D series.

From microscopic study of the stem sections it was found that in the sections which had increased in weight over the controls, proliferation of the cortical parenchyma was observed. These cells appeared to be more numerous and were definitely larger in size than corresponding cells in the controls. Proliferation of cells in the vascular region was noted in the coconut milk-treated sections, both in the control and at the various concentrations of 2,4-D. This proliferation had been expected due to reports from Blakely and Steward (1964) on their work with carrot phloem cells treated with coconut milk. Proliferation of the phloem parenchyma cells was found in the sections treated with 10^{-5}M 2,4-D without coconut milk (see Fig. 1). Large masses of cells whose large, dark-staining nuclei made the entire tissue appear red at lower magnifications were found in the outer vascular region. This tissue did not appear to be

made up of mature phloem parenchyma cells, but rather of masses of dedifferentiated cells. Evidence of active cell division was found at a magnification of 1000x when various stages of mitotic figures were observed (see Fig. 2).

The same tissue masses were found in both the 10^{-5}M 2,4-D with or without coconut milk, but this particular effect was not noted at any other concentration. Thus the important factor seems to be 2,4-D and not the result of a synergistic effect with coconut milk.

Another tissue effect was observed with 2,4-D at 10^{-11}M plus coconut milk. The sections cultured in this medium produced adventitious roots which grew outward through the epidermis from the cortical region. Microscopic examination of sections of these adventitious growths revealed a definite root structure (see Fig. 3).

The stem sections treated with kinetin produced no adventitious roots, and generally showed an inhibition of normal weight increase. With kinetin alone the fresh weights at 10^{-11}M and 10^{-13}M showed a slight, but not significant increase (see Table 5). The per cent deviation of dry weights closely followed that of the fresh weights with the greatest inhibition of growth at 10^{-3}M kinetin and lower degrees of inhibition inversely proportional to the concentration of kinetin used.

The addition of coconut milk to the kinetin series greatly increased the final weights of the sections (see

Table 5

EFFECT OF KINETIN

CONCN	AVERAGE INITIAL WT WET	AVERAGE FINAL WT WET	SD WITHIN EXPT'L UNIT	SE EXPT'L VS. CONTROL	% DEVIATION	AVERAGE INITIAL WT DRY	AVERAGE FINAL WT DRY	% DEVIATION
control	13.0 mg	224.0 mg	± 24.4			0.75 mg	45.8 mg	
10^{-3}	13.0	66.8	± 2.80	12.2	-70.1	0.75	7.1	-84.5
10^{-5}	22.0	166.3	± 23.4	22.0	-25.8	1.0	21.5	-53.0
10^{-7}	11.8	57.7	± 32.4	20.2	-74.1	0.4	9.9	-78.4
10^{-9}	11.8	161.2	± 9.74	13.2	-28.0	0.4	18.6	-59.4
10^{-11}	24.8	247.2	± 4.42	12.4	10.4	1.8	22.6	-50.6
10^{-13}	24.8	235.0	± 18.4	15.3	4.91	1.8	24.7	-46.1

Table 6), and for the available concentrations an increase over the control was noted. However, the per cent increase was consistently small, especially when compared to the increases in the 2,4-D or colchicine series. Therefore, the increased weight of these sections is due primarily to the coconut milk and not the kinetin.

The stem tissues treated with kinetin retained a normal orientation of tissues; neither the vascular bundles nor the cortical region appeared grossly affected. Some of the parenchyma cells were small and closely packed; however, most of the cells were normal in size. These small cells did not appear to be the products of initiated cell division in the parenchyma, thus upholding Miller's hypothesis (1961) that kinetin-induced cell division is questionable, and that when it occurs, it depends on varying experimental conditions.

In the kinetin plus coconut milk series proliferation of the cortical parenchyma was observed at 10^{-5}M kinetin. At 10^{-13}M kinetin the cortical parenchyma as well as the cambial cells had proliferated. However, an increase in cell size and number found in the xylem did not appear to be as extensive as that in the coconut milk control. Therefore, the inhibitory effect of kinetin on the stem tissues was reflected in its ability to reduce the effectiveness of coconut milk.

Table 6

EFFECT OF KINETIN + 3% COCONUT MILK

CONCN	AVERAGE INITIAL WT WET	AVERAGE FINAL WT WET	SD WITHIN EXPT'L UNIT	SE EXPT'L VS. CONTROL	% DEVIATION	AVERAGE INITIAL WT DRY	AVERAGE FINAL WT DRY	% DEVIATION
control	43.1 mg	337 mg	± 28.6			2.8 mg	40.4 mg	
10^{-3}*								
10^{-5}	41.6	568	± 142.0	83.6	68.6	5.2	57.4	42.1
10^{-7}	41.6	405	± 25.5	22.1	20.6	5.2	47.3	17.1
10^{-9}*								
10^{-11}*								
10^{-13}	33.3	420	± 39.8	28.3	24.6	3.0	43.0	6.44

* Discarded because of contamination

SUMMARY

With each of the growth regulators tested, the general trend of inhibition of growth at high concentrations of the compound with either a reduction in inhibition or an actual increase at the lower concentrations was observed. Colchicine favored enlargement of the cortical parenchyma cells, but did not initiate cell division. When used in combination with coconut milk, colchicine was found to produce the heaviest stem sections by the end of the experiment. This is believed due to colchicine's inability to inhibit the growth-initiating coconut milk.

The auxin 2,4-D was found to cause cell enlargement in the cortical parenchyma and, at a concentration of 10^{-5}M, to induce division in the phloem parenchyma. Active cell division in the dedifferentiated phloem tissue masses was shown by photographs of mitotic figures. The tremendous increase over the control observed in the fresh weights of the 10^{-7}M 2,4-D sections is believed due to a change in the osmotic regulation of the cells. In the 2,4-D plus coconut milk series, the coconut milk exerted a regulating effect on the 2,4-D and reduced the increase from 359% in 10^{-7}M 2,4-D alone to 67.8% in 10^{-7}M 2,4-D plus coconut milk. In addition to proliferation of the cortical parenchyma and vascular regions, 10^{-11}M 2,4-D with coconut milk induced adventitious root formation.

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Kinetin exerted a general inhibitory effect on weight increase. The compound did not appear to initiate either significant cell division or enlargement in any of the tissues. Increases observed in the kinetin plus coconut milk series resulted mainly from the coconut milk itself.

Each of these substances exhibited an influence on the growth and development of Helianthus stem tissue, and this influence was often affected by the presence of other growth regulators, in this case, coconut milk. These results again point to the interaction of several factors in the initiation and subsequent control of the cellular growth processes of stem tissue.

ILLUSTRATIONS

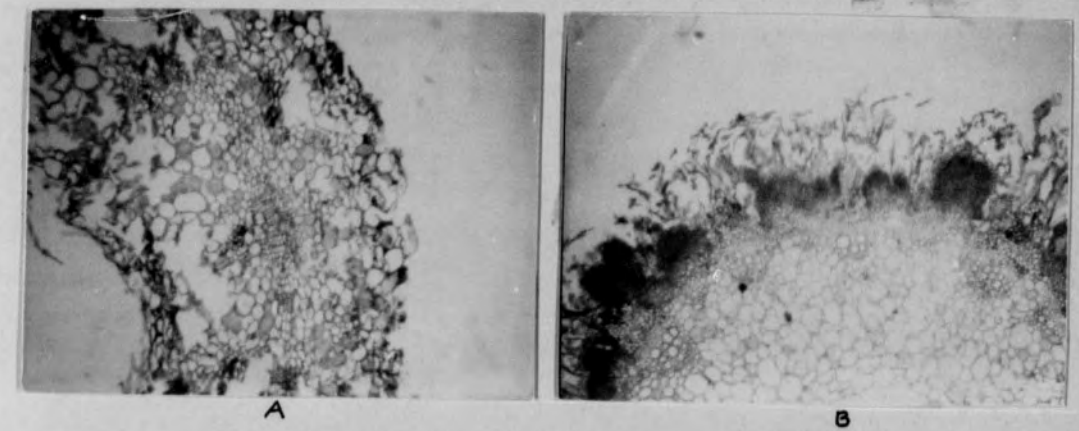

Fig. 1

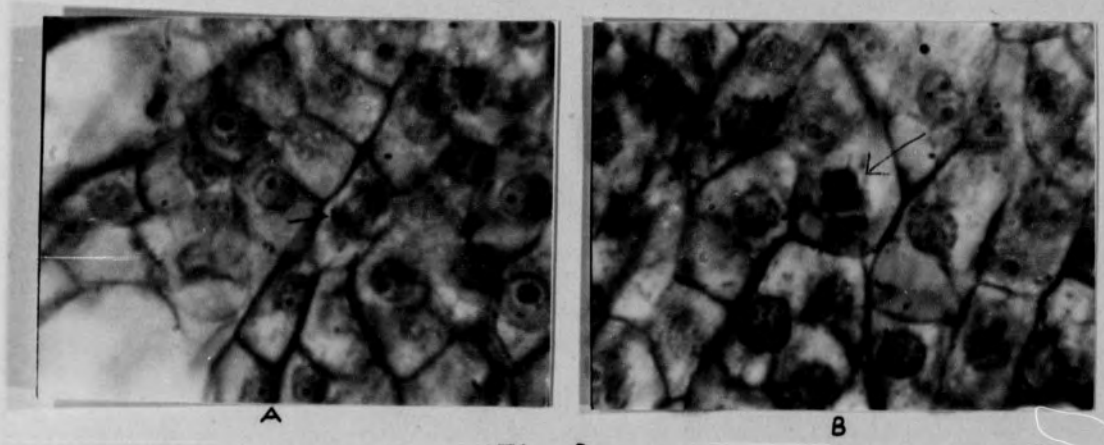

Fig. 2

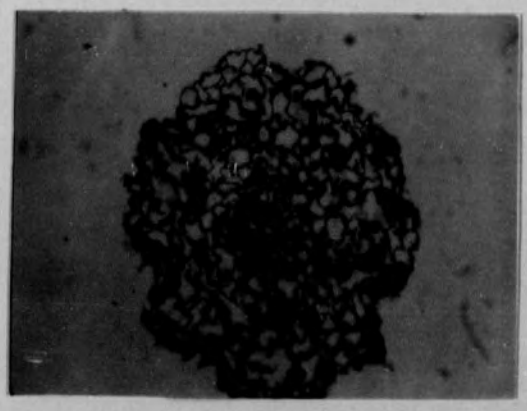

Fig. 3

LEGEND

Fig. 1. 35x magnification of a control section (A) compared with a section (B) cultured in 10^{-5}M 2,4-D.

Fig. 2. 930x magnification of mitotic figures in the proliferated phloem parenchyma of stems treated with 10^{-5}M 2,4-D. One cell (A) is in anaphase, another (B) is in telophase.

Fig. 3. 53x magnification of a cross section of an adventitious root produced on a stem cultured in 10^{-11}M 2,4-D plus coconut milk.

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