BIOLOGY

SYNTHETIC MEDIUM FOR GROWING SINGLE CELLS OF TOBACCO

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ABSTRACT.

1. Studies were done to find out a synthetic defined medium for growing single cells of tobacco. Two kinds of media were tried for these purposes. With several changes in components, the revised medium of LINSMAIER & SKOOG (6) seemed to be good for suspension and plating cultures of tobacco cells. The medium used by ERARLE & TORREY (3) for cells of Convolvulus also gave the desired results (table 1, plate 3).
2. Growth of both callus and single cell cultures occurred on comparable kinetin concentrations.
3. These studies (about the relationship between IAA and kinetin) gave no evidence of differentiation of the cell colonies within seven weeks after transferring the cells into the medium (plate 5).

INTRODUCTION.

MUIR et al. (7) first demonstrated that isolated single cells of higher plants can develop into cell groups and callus tissues by the use of the filter paper "nurse" culture method. TORREY (12) on the other hand used a microchamber technique for single cell growth. This was followed by microculture method (5) and more recently, single cells of higher plants have been more easily grown by a "plating" method (1, 2, 3).

The step from the "nurse" to the "plate" culture required the addition of complex nutrients such as coconut milk (1, 5, 7, 9, 10) or yeast extract (12, 13) to the media.

Because we know almost nothing about the active substance(s) of these two nutrients, experiments were done to find out a chemically defined synthetic medium for growing single cells of tobacco. For these purposes the microculture and the plating method were used.

MATERIAL AND METHOD.

a. Single cells.

Single cells of *Nicotiana tabacum* var. Wisconsin No. 38 were used as the main tissue. These cells were derived from callus which had been grown on 1% revised agar medium of LINSMAIER & SKOOG (6) with 200 γ/l

kinetin. From this medium the callus was successively transferred to the same medium which contained 30 γ/1kinetin respectively (media T_1 and T_2). Callus tissue pieces from medium T_2 were then transferred into 500 ml Erlenmeyer flasks. Each flask contained 100 ml of the synthetic medium which was agitated continuously at about 150 rpm on a horizontal-type rotary shaker. From this suspension culture single cells were obtained.

b. Medium.

Two kinds of media were used in these experiments. One of these contained basically the components of the revised medium of LINSMAIER & SKOOG (6), which was called medium M. The other was the synthetic medium used by EARLE & TORREY (3) (medium E).

Medium M contained the following components (4, 6):

I. Basal medium in mg/1: NH_4NO_3 1650; KNO_3 1900; H_3BO_3 6.2; KH_2PO_4 170; KI 0.83; $NaMoO_4.2\ H_2O$ 0.25; $CoCl_2.6\ H_2O$ 0.025; $CaCl_2.2\ H_2O$ 440; $MgSO_4.7\ H_2O$ 370; $MnSO_4.4\ H_2O$ 22.3; $ZnSO_4.7\ H_2O$ 8.6; $CuSO_4.5\ H_2O$ 0.025; Na_2Fe EDTA 35; sucrose 30.000.

II. Vitamins in mg/1: thiamine-HCl 0.40; *myo*-inositol 100.

III. Auxin in mg/1: 2,4-D 0.5 (instead of IAA) (4).

IV. *Cyto*-kinin in mg/1: kinetin 0.20.

V. To the above was added amino acid mixture Filner's (private communication to F. Skoog) containing the following *L*amino acids (mg/1): glumatic acid 51; aspartic acid 18; lysine-HCl 13; arginine 5; serine 10; threonine 8; cysteine 7; methionine 7; phenylalanine 16; tryptophane 4; histidine 11; alanine: 9 proline 16; glycine 5; valine 8; isoleucine 5; leucine 5.

The medium E contained the following components (3):

I. Basal medium in mg/1; $Ca(NO_3)_2.4\ H_2O$ 242; $MgSO_4.7\ H_2O$ 42; KNO_3 85; KCl 61; KH_2PO_4 20; H_3BO_3 1.5; $ZnSO_4.7\ H_2O$ 1.5; $MnSO_4\ 4H_2O$ 4.5; $Na_2MoO_4.2\ H_2O$ 0.25; $CuSO_4.5\ H_2O$ 0.04; $FeCl_3.6\ H_2O$ 2.5; sucrose 40,000.

II. Vitamins in mg/1; thiamine-HCl 0.1; *myo*-inositol 100.

III. Amide in mg/1: *L*-glutamine 146.1.

IV. Purine in mg/1: adenine-sulfate 40.4.

V. Auxin in mg/1: 2,4-D 0.22.

V. Auxin in mg/1: 2,4-D 0,22.

VI. *Cyto*-kinin in mg/1: kinetin 0.22.

Sucrose, vitamins, auxin and kinetin were added to the media, and the pH was adjusted to pH 5.8 before autoclaving. At the time of sampling the

entire contents of a culture flask 14 days old were filtered aseptically as described by BERGMAN (1). The single cells or groups of cells were isolated from the filtrate by a plating method. A 1/2 - 1 ml aliquot was mixed with melted and cooled agar medium and the plated in Petri dishes. The dishes were then taped to prevent desiccation and contamination. These inoculated plates were kept at room temperature. By making the agar layer about 1 mm thick, the cells could easily be observed at low magnification (4 - 100 x) with a microscope. The influence of the media on the growth of the tobacco single cells were also made with the remaining cell suspension by the use of the "hanging drop" technique.

RESULTS AND DISCUSSION.

a. Suspension culture.

Preliminary experiments using the original revised medium of LINSMAIER & SKOOG (6) indicated, that this medium did not give the desired results. Generally speaking, single cells were not formed. Since others reported (1, 2, 12) the role of 2,4-D in suspension cultures, this component as much as 0,5 mg/l (4) was then added to the medium instead of IAA. High proportion of free cells were found, especially after adding the amino acid mixture. Division of these single cells occurred within 6 - 10 days after transferring the tissue from medium T_2 to the culture medium. This cell division continued for 4 days. Before cell enlargement took place, most of the cells died (plate 1). From plate 2 it was also clear, that the single cells of tobacco derived from the suspension culture were different in form and shape as compared with the regular shape of the control cells. Here again it was observed as reported by others (2, 7, 10, 11, 12) that cell division took place only when a higher number of cells were present in the drop. The adequacy of media M and E for growth was indicated by the increases in fresh weights of suspension cultures (table 1). Five weeks after transferring the callus tissue the increase in fresh weight was about 9 fold; the average number of cells per ml in both media was about 320 ± 80.

b. Plating culture.

The effectiveness of medium M and medium E for the plating method may be seen from plate 3 and fig. 1, which was expressed in terms of number of colonies per cm^2 (3). The total number colonies per cm^2 in both media goes up nearly in proportion to the concentration of kinetin within the specified range. Growth was seen to be proportional to the kinetin concentration in the range from 0 to 50 γ/l (table 2).

Table 1.

Mean fresh weight in gram.

Suspension culture	Time in weeks	
	0	5
Medium M	0.46 ± 0.06	3.10 ± 0.10
Medium E	0.46 ± 0.06	3.27 ± 0.16

Table 2.

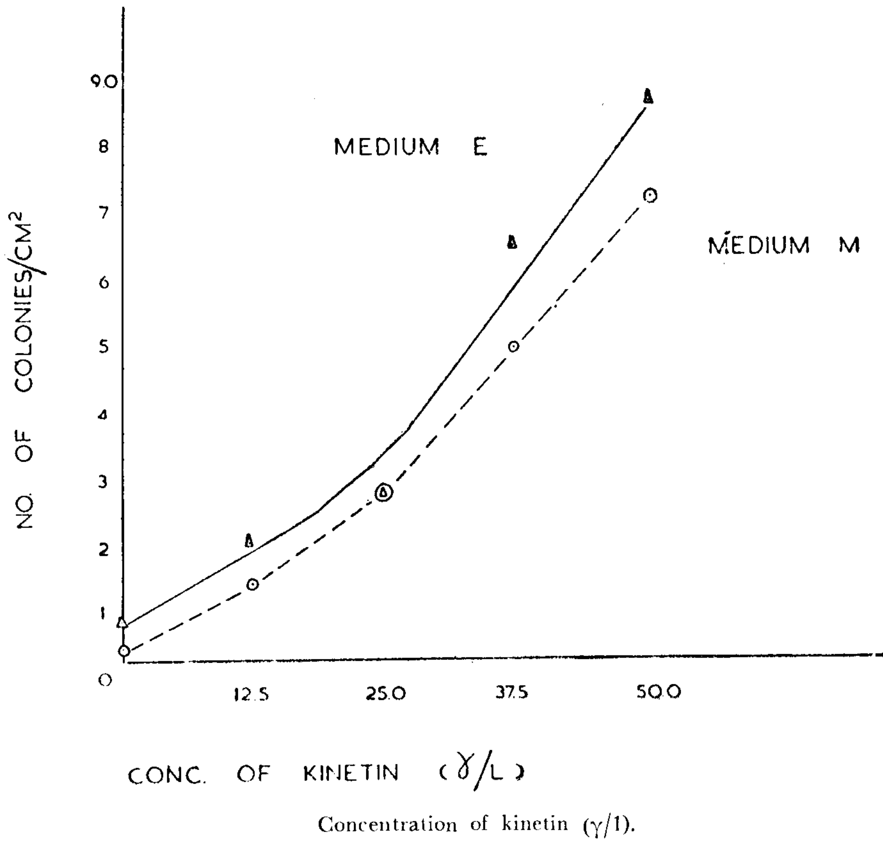
Mean number of colony per cm² in 5 ml agar medium.

Kinetin in γ/l	Medium	
	Medium M	Medium E
0	0.4	0.9
12.5	1.4	2.0
25.0	2.9	2.9
37.5	5.0	6.5
50.0	7.2	8.8

Table 3.

Percentage of cell colonies formed within 64 cm² Petri dish.

Concentration (mg/l)	Ave. area of colonies (cm²)	Ave. perc. of colonies	Concentration (mg/l)	Ave. area of colonies (cm²)	Ave. perc. of colonis
IAA : 2.0			Kinetin: 0.2		
Kinetin: 0	0	0	IAA : 0	1.16	1.8
0.05	0.94	1.5	0.5	3.48	5.4
0.1	5.76	9.0	1.0	5.48	8.6
0.2	10.22	16.00	2.0	11.28	17.6
0.3	40.38	63.1	3.0	2.48	3.9



Concentration of kinetin ($\gamma/1$).

Fig. 1. The relation between kinetin concentration and number of colonies per cm^2.

A kinetin concentration of 30 γ/l was used for maintaining the T_1 and T_2 callus cultures. Therefore, growth of both callus and single cell cultures occurred on comparable kinetin concentration.

c. The relation between IAA and kinetin in cell plating culture.

It was reported earlier from this laboratory (1957) that an auxin and a cytokinin appear to be essential for growth and organ formation of tobacco cultures.

Since medium M and medium E could be used for cultivating single cells of tobacco, the relationship between IAA and kinetin in single cell cultures was studied. This consisted of two experiments utilizing only medium M. In one experiment IAA was kept constant (2.0 mg/l) and kinetin was varied in the range from 0 to 0.3 mg/l. In the other the concentration of kinetin was kept constant at 0.2 mg/l and IAA varied in the range from 0 to 3.0 mg/l. The results of three experiments in each set were expressed in terms of percentage of colonies formed within the area of a Petri dish (appr. 64 cm^2).

From table 3 it was shown that in the first set of experiments, the average percentage of cell colonies formed increased proportionally to the concentration of kinetin. In the second set, the proportional increase to the concentration of IAA reached a maximum percentage of colonies at about 2.0 mg/l. This was probably due to the toxic effect of IAA.

Seven weeks after transferring the cells into the medium, no evidence of differentiation was observed in either set of experiments (plate 5). Further study about this relationship is of interest.

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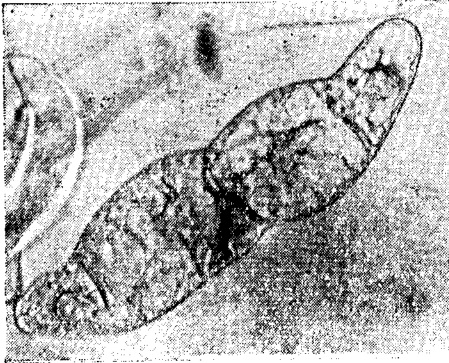
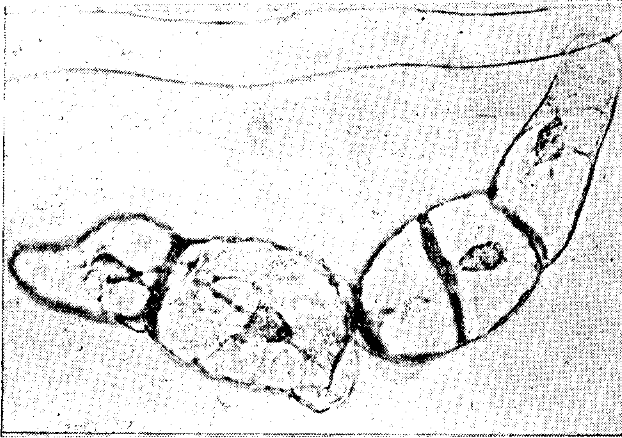
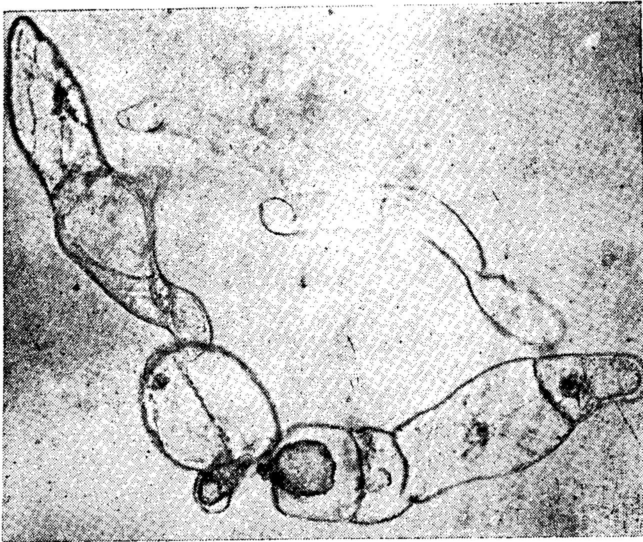
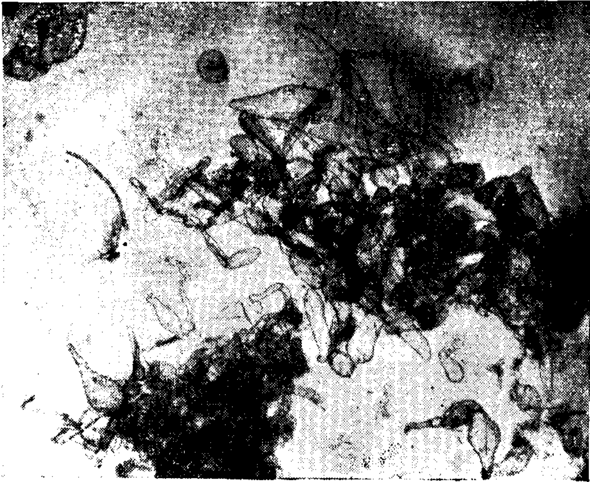


Plate 1. Cell division in suspension culture.



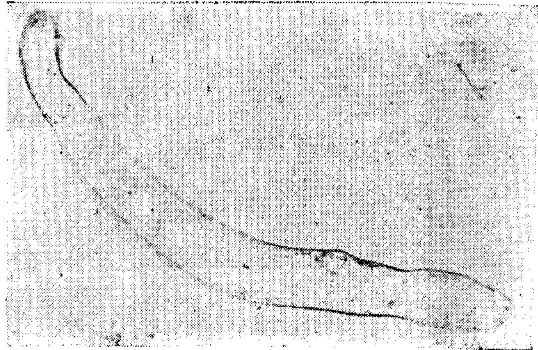


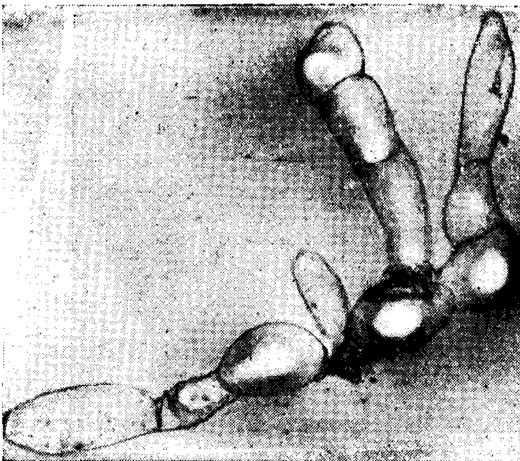


(a)

Plate 2. Different form and shape of single cells of tobacco derived from plateculture (a) and suspension culture (b) as compared with the regular shape of the control cells (c).

(b)





(c)

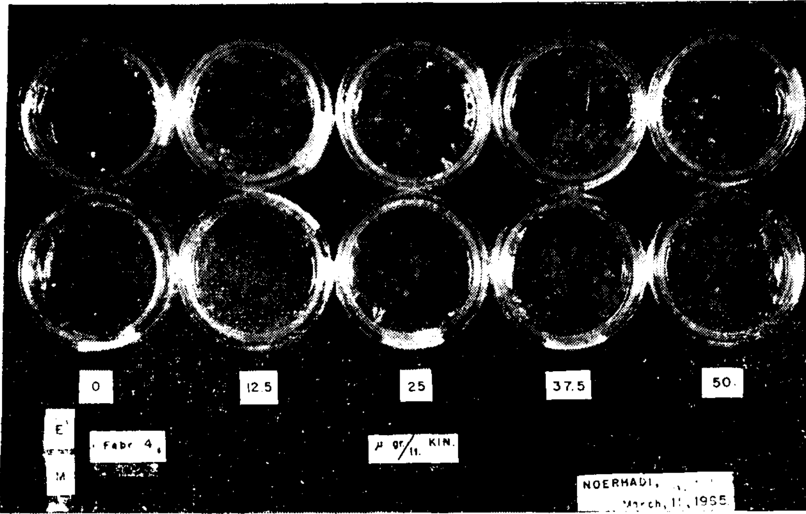
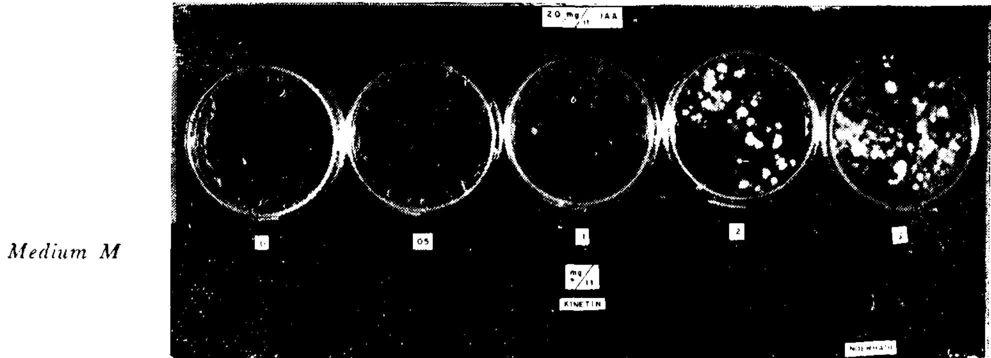
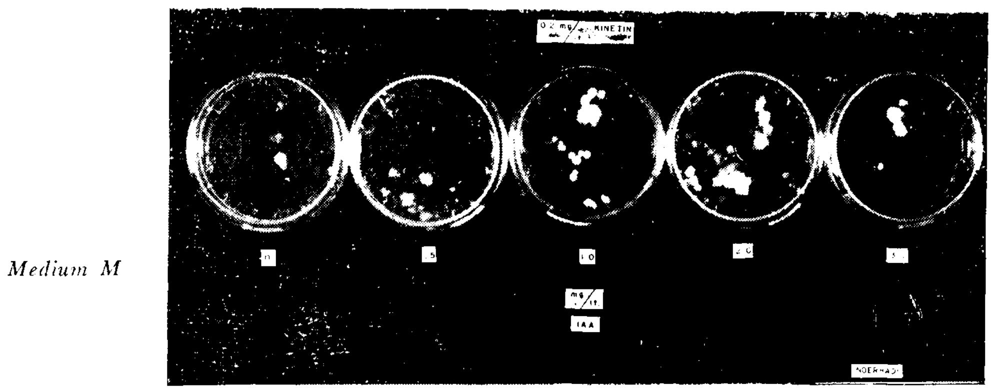


Plate 4. The effect of kinetinin the presence of IAA on the growth of single cells of tobacco.





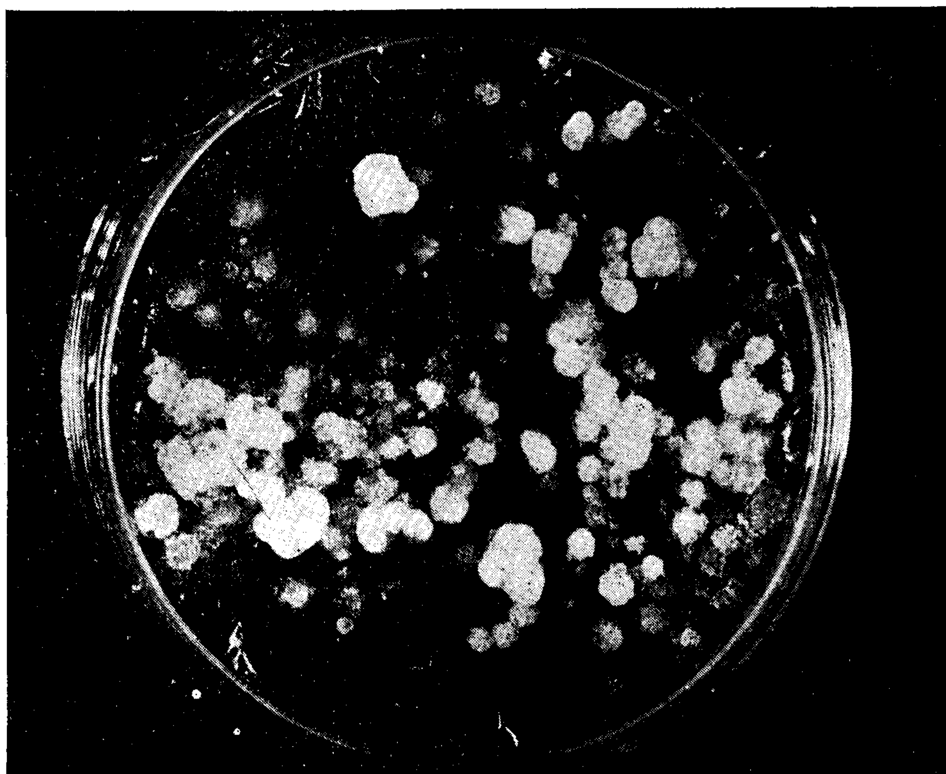


Plate 5. Cell colonies on agar plate medium M, nine weeks after transferring the cells into the medium.

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