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INVESTIGATIONS ON THE GROWTH AND DIFFERENTIATION OF TOBACCO TISSUE CULTURES IN VITRO

BY

P. BOYSEN JENSEN

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1. A Method to Estimate the Increase in Tissue Cultures.

(a) Starting of the tissue cultures. According to WHITE (1939) sterile tissue cultures of *Nicotiana glauca* × *Langsdorffii* can be obtained from young stems of the hybrid by breaking them 4–5 cm. from the tips and removing cones of tissue from the exposed, aseptic surface with a sterile scalpel.

Besides this method I have also used callus produced by seedlings to start the cultures. Seeds of the hybrid were wrapped in moist filter paper and placed in a pulp of crushed tomatoes for 48 hours. Afterwards they could be sterilized in the usual manner with calcium hypochlorite. The sterile seeds were placed in Freudenreich flasks on a semisolid nutrient containing 1 per cent. agar and the usual salts. When the seedlings had developed, the tip was severed from the basal part with a pair of scissors and the callus produced on the cut surface was used for setting up the cultures.

It seems that cultures prepared after the last method (Strain 18 in Table 2) grow a little faster than those prepared after the first one.

(b) Culture technique and nutrients. The cultures were maintained in 100 ml. Erlenmeyer flasks of Duran-, Jena-, or Pyrex glass.

The water used for preparation of the nutrients was tap water distilled first over alkaline potassium permanganate (per l. 7 ml. 0.2 per cent. $KMnO_4$ + 2.5 ml. 10% KOH), then over barium hydroxide (per l. 2.5 ml. 0.8 per cent. $Ba(OH)_2$, and finally without any addition (WHITE 1932).

The following stock solutions were prepared:

I b 0.95 g $Ca(NO_3)_2$
1.7 g $MgSO_4$
0.95 g Na_2SO_4

0.38 g KNO_3
0.31 g KCl
0.08 g KH_2PO_4
500 ml. triple distilled water

II 0.037 g KJ
0.125 g $Fe_2(SO_4)_3$
0.220 g $MnSO_4$
0.075 g $ZnSO_4$
0.080 g H_3BO_3
500 ml. triple distilled water

III a_1 0.03 g glycine
0.005 g thiamin
100 ml. triple distilled water

III a_2 0.03 g asparagine
0.005 g nicotinic acid
100 ml. triple distilled water

III a_3 0.03 g glycine
0.03 g asparagine
0.005 g nicotinic acid
200 ml. triple distilled water

III a_4 0.03 g glycine
0.03 g asparagine
0.005 g thiamin
200 ml. triple distilled water

III a_5 0.03 g glycine
0.005 g thiamin
0.005 g nicotinic acid
200 ml. triple distilled water

III a_6 0.03 g asparagine
0.005 g thiamin
0.005 g nicotinic acid
200 ml. triple distilled water

IIIa_7 0.005 g thiamin
0.005 g nicotinic acid
200 ml. triple distilled water.

The nutrients were made by mixing 100 ml. Ib + 10 ml. II + 10 ml. of one or two of the stock solutions $IIIa_1$—$IIIa_7$ + 20 g sucrose + 800 ml. triple distilled water.

Each culture flask was charged with 8—10 ml. of the nutrient and plugged with non-absorbent cotton, covered with filter paper. Transfers were made at intervals of 10 days, in some periods during the war only once monthly.

The cultures were maintained at room temperature in weak light.

(c) Estimation of the increase. At intervals of about a month the increase of the cultures was estimated in the following way. The tissue fragment from a culture was placed in a small, dry sterilized glass box (fig. 1, diameter 3.0 cm., height 2.5 cm.), and the box with the fragment was weighed; afterwards the fragment was transferred to a culture flask with fresh nutrient, and the box was weighed anew. The difference between the weight of the fragment, estimated in this manner, and the weight a month ago is the increase in mg. in the said month.

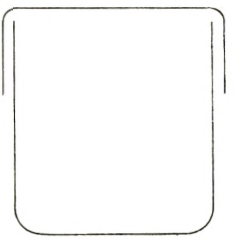

Fig. 1.

The fragments are very seldom contaminated by this procedure.

The method permits consecutive measurements on the same fragment.

(d) Calculations. Blackman (1919) has found that the equation $W_t = W_0 e^{rt}$, which implies that the increase is proportional to the weight in every moment, is applicable for the growth of an annual plant. According to Caplin (1947) this formula is also particularly applicable for describing the growth of tobacco tissue cultures. Still, as the cultures mainly or exclusively grow on the surface, it is more probable that the increase is proportional to the surface and not to the weight of the fragment, and I have therefore preferred to estimate the increase in mg. per day per sq. cm. surface. It is assumed that the fragment is spherical and that the specific gravity is 1.

The calculations are carried out in the following way. Through the method described above the weight of a fragment is estimated. The corresponding surface is taken from the curve in fig. 2,

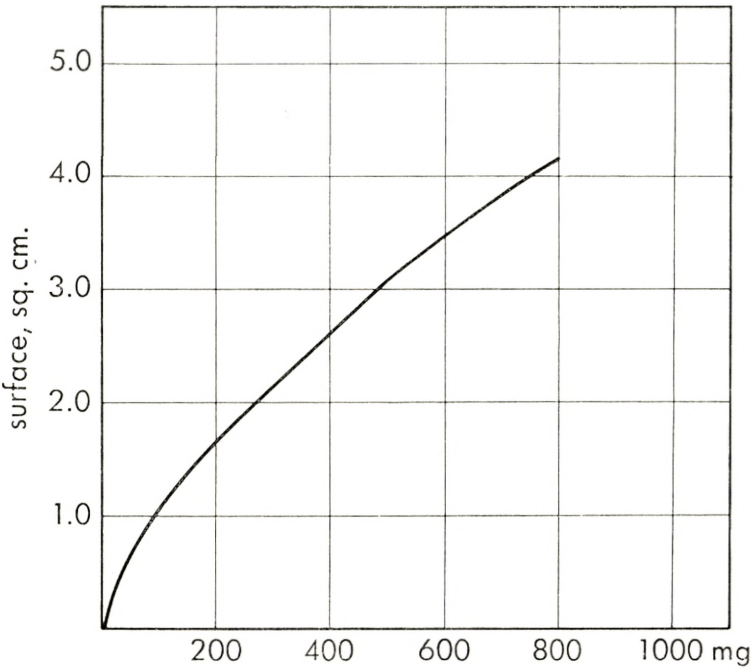

Fig. 2.

in which the surface of a sphere is rendered as a function of its weight in mg. The increase during a period of a month is divided by the surface of the fragment at the beginning of the period. In this way we get the increase in the period in mg. per sq. cm. surface. Next the mean of the increases for the different fragments in a series is calculated; the mean is divided by the number of days in the period, and thus we get the mean increase for a series in mg. per sq. cm. surface and day.

If a fragment breaks, so that there are more than one fragment in a flask, the surface is calculated by adding the surfaces of the single fragments.

Table 1 shows the calculations and results of an experiment with a single fragment for 7 months. Although the increase varies considerably, the accuracy is sufficient to obtain reliable results, when the increase is calculated as a mean of the increases in a series of 5—10 cultures for a prolonged time (cf. Table 2).

Table 1.

1 Weight mg.	2 Surface sq.cm.	3 Increase mg. (7–1)	4 Increase mg. per sq.cm. (3:2)	5 Number of days	6 Increase mg. per sq. cm. per day (4:5)	7 Weight mg.	8 Surface sq. cm.	9 Increase mg.	10 Increase mg. per sq.cm. (9:8)	11 Number of days	12 Increase mg. per sq.cm. per day (10:11)
$^{17}/_{5}$	$^{17}/_{5}$	$^{17}/_{5}$–$^{27}/_{6}$	$^{17}/_{5}$–$^{27}/_{6}$	$^{17}/_{5}$–$^{27}/_{6}$	$^{17}/_{5}$–$^{27}/_{6}$	$^{27}/_{6}$	$^{27}/_{6}$	$^{27}/_{6}$–$^{18}/_{8}$	$^{27}/_{6}$–$^{18}/_{8}$	$^{27}/_{6}$–$^{18}/_{8}$	$^{27}/_{6}$–$^{18}/_{8}$
47	0.63	41.7	66	41	*1.61*	88.7	0.96	48.4	50	52	*0.96*
$^{18}/_{8}$	$^{18}/_{8}$	$^{18}/_{8}$–$^{14}/_{9}$	$^{18}/_{8}$–$^{14}/_{9}$	$^{18}/_{8}$–$^{14}/_{9}$	$^{18}/_{8}$–$^{14}/_{9}$	$^{14}/_{9}$	$^{14}/_{9}$	$^{14}/_{9}$–$^{20}/_{10}$	$^{14}/_{9}$–$^{20}/_{10}$	$^{14}/_{9}$–$^{20}/_{10}$	$^{14}/_{9}$–$^{20}/_{10}$
137.1	1.28	51.1	40	27	*1.48*	188.2	1.59	60.4	38	36	*1.06*
$^{20}/_{10}$	$^{20}/_{10}$	$^{20}/_{10}$–$^{20}/_{11}$	$^{20}/_{10}$–$^{20}/_{11}$	$^{20}/_{10}$–$^{20}/_{11}$	$^{20}/_{10}$–$^{20}/_{11}$	$^{20}/_{11}$	$^{20}/_{11}$	$^{20}/_{11}$–$^{18}/_{12}$	$^{20}/_{11}$–$^{18}/_{12}$	$^{20}/_{11}$–$^{18}/_{12}$	$^{20}/_{11}$–$^{18}/_{12}$
248.6	1.91	38.7	20.3	31	*0.65*	287.3	2.09	70.7	34	28	*1.21*
$^{18}/_{12}$											
358.0	..	..	..	..	..	..	..	..	..	..	..

2. The Influence of Thiamin and Some Amino Acids on the Growth of Tobacco Tissue Cultures.

TATUM and BELL (1946) have shown that different genes are concerned in the synthesis of thiamin in *Neurospora*. The wild type of this fungus does not require thiamin for growth, but four mutant strains have been found, which cannot synthetisize this compound. The strain 18588 can synthetisize pyrimidine, but not thiazole, the strain 9185 can synthetisize both pyrimidine and thiazole, but cannot couple the two compounds. The strains 17084 and 1090 require for growth either thiamin or a mixture of thiazole and pyrimidine. The mutant strains are differentiated from the wild type by single genes.

Thiamin or its precursors are also essential to the growth of roots in organ cultures. For tomato roots growth is obtained by supplying the thiazole portion of the thiamin molecule; hence they must be able to synthetisize pyrimidine and to couple the two compounds (ROBBINS and BARTLEY 1937, 1938, WHITE 1937). The roots of pea require both thiazole and pyrimidine (BONNER 1938). As the green plants are autotrophic we must assume that the thiamin is supplied to the roots from the stem and leaves.

Thus a similar difference exists as to the ability to synthetisize thiamin on the one hand between the wild type of *Neurospora* and the mutant strain 18588, on the other hand between the leaves and the root of a tomato plant. In the first case the difference can be explained by the mutation of a gene. Even if it is probable that genes also are concerned in the synthesis of thiamin in the tomato plant, the inability of the root to build this substance cannot be explained by a difference in the genes in the leaves and the root, because the nuclei in these organs are equivalent. We must therefore assume that besides the genes also a cytoplasmatic factor or a surrounding factor, the effect of which is different in leaves and root, is concerned in the synthesis of thiamin.

It may be possible to elucidate the nature of this factor through studies on the requirement of thiamin in tobacco tissue cultures. We can prepare tissue cultures both from the stem and the root and can therefore investigate if such cultures differ as

to their need of thiamin and if the synthesis of thiamin is influenced by external factors (e.g. light).

Previously HILDEBRANDT, RIKER, and DUGGAR (1946) have studied the influence of thiamin on the growth of tobacco tissue cultures. They found: "thiamine and glycine thus seemed beneficial for tobacco tissue, but, since the L. S. D. values for these media were not significant, the necessity of these vitamins and glycine for these tissues is questionable."

With the method described above I have investigated the necessity of thiamin, nicotinic acid, and some amino acids for the growth of tobacco tissue cultures in light. The results of the experiments are rendered in Table 2.

The basal medium contains the ordinary salts + sucrose + thiamin + nicotinic acid + glycine + asparagine. The difference in the increase for the different strains is not great. The highest increase is found for strain 18, prepared from sterile seedlings.

An omission of one of the vitamins or amino acids does not diminish the increase materially.

The most significant fact is that the increase on a nutrient only containing inorganic salts and sucrose is about the same as in the basal medium (1.26 mg. per sq. cm. per day against 1.31 and 1.35).

The experiment lasted 7 months, during which time the weight of one of the cultures was augmented from 47 to 358 mg. As a diminution of the increase could not be observed (cf. Table 1), a reserve of thiamin in the fragment at the beginning of the experiment cannot be held responsible for the growth of the tissue.

Hence we may conclude that tobacco tissue from the stem can grow in light without any supply of thiamin; therefore it must be able to synthetisize this compound. Still it must be remembered that the increase of a tissue culture is rather small, and the possibility exists that the thiamin produced by the cells would not suffice if the increase was of the same magnitude as in normal plants.

I have made few experiments with roots. SKOOG (1944) remarks that experiments with excised roots of the tobacco hybrid could not be continued "as in all cases roots would eventually produce callus and would then cease to grow". I have got an impression that it will be possible to obtain an unlimited growth

Table 2. Increase, mg. fresh weight per sq. cm. surf. per day.

Strain	Composition of the nutrients	Number of cultures	c. $^{17}/_{5}$ – $^{27}/_{6}$ 1944	c. $^{27}/_{6}$ – $^{19}/_{8}$	c. $^{19}/_{8}$ – $^{15}/_{9}$	c. $^{15}/_{9}$ – $^{20}/_{10}$	c. $^{20}/_{10}$ – $^{18}/_{11}$	c. $^{18}/_{11}$ – $^{17}/_{12}$	c. $^{17}/_{12}$ – $^{26}/_{1}$ 1945	Mean	Composition of the nutrients
18	Ib + II + $IIIa_1$ + $IIIa_2$	5	1.59	2.30	2.51	1.40	1.97	1.45		1.87	} basal medium
18	Ib + II + $IIIa_1$ + $IIIa_2$	3	0.92	2.62	1.70	1.66	2.10	1.81		1.80	
23	Ib + II + $IIIa_1$ + $IIIa_2$	4	1.12	1.26	1.48	1.37	1.76	0.87		1.31	
24	Ib + II + $IIIa_1$ + $IIIa_2$	3	0.76	1.17	2.41	0.97	2.21	0.58		1.35	
25	Ib + II + $IIIa_1$ + $IIIa_2$	5	0.91	1.38	1.73	1.89	1.63	2.30	0.97	1.54	
18	Ib + II + $IIIa_6$	6	1.19	1.55	2.17	2.72	2.36	3.36	2.41	2.25	bas. med. – Glycine
18	Ib + II + $IIIa_5$	6	1.61	1.47	2.00	2.14				1.81	– asparagine
18	Ib + II + $IIIa_7$	5	1.32	0.84	1.30	1.89	1.68	1.61	1.44	1.44	– (glycine + asparagine)
24	Ib + II + $IIIa_3$	5	0.83	1.00	1.22	1.59	1.07	1.30	2.02	1.29	– thiamin
18	Ib + II + $IIIa_4$	6	1.41	2.36	1.63	2.17	2.11	2.68	2.41	2.11	– nicotinic acid
23.24	Ib + II	5	1.32	1.06	1.67	0.92	1.13	1.43		1.26	– (glycine + asparagine + thiamin + nicot. acid)

of the roots by culture on an oblique surface of an agar nutrient in test tubes. Unfortunately I shall not be able to continue these experiments in the future.

3. Differentiation in Tobacco Tissue Cultures.

WHITE (1939) has shown that tobacco tissue cultures when immersed in a liquid nutrient can form leafy branches. He supposes that diminution of oxygen calls forth differentiation. SKOOG (1944) found that also temperature and light influence the organ formation in tissue cultures. In experiments carried out at 33°, 25°, 18°, 12°, and 5° the amount of differentiation reaches an optimum at 18°. Tissue developed in strong light is relatively undifferentiated, in darkness and in weak light development of buds occurred generally.

Also the differentiation in higher plants is influenced by the surrounding factors. Germinating fern spores in weak light develop an undifferentiated tube, in stronger light cell divisions occur, and a prothallium arises. In seedlings of dicotyledonous plants light inhibits elongation of the internodes of the stem, but promotes differentiation. In shoots developed from a stub of a tree and therefore supplied with nutrients and water in abundance the leaves are abnormally large, but the differentiation is inhibited. Humid air and 3-indole acetic acid stimulate the growth of cells and can promote formation of undifferentiated tissue.

Even if there are exceptions a certain correlation seems to exist between growth and differentiation. Factors accelerating growth (weak light, nutrients and water in abundance, stimulants) inhibit differentiation and vice versa. But also other factors are concerned with differentiation. Thus Ca-ions seem to promote formation of root hairs.

SKOOG (1949) has found that tissue cultures never produce roots. In some instances formation of root systems on shoots produced from a tissue culture occurred. I can confirm these results.

I have tried to produce a normal plant from a tobacco tissue culture. The first step was to make the culture grow auto-

trophically. At intervals of 14 days the cultures were transferred from a 2 per cent. sucrose solution to solutions with 1 per cent., 0.5 per cent., and 0.25 per cent. sucrose and at last to a pure inorganic nutrient. The cultures were maintained in rather weak light.

In order to supply the tissue fragments with CO_2 they were placed in Erlenmeyer flasks on the surface of a semisolid substratum which besides the usual salts contained 0.75—1 per cent. agar. The flasks were placed in Fresenius desiccators, which were evacuated and filled with pure CO_2 (prepared from pure Na_2CO_3 and H_2SO_4, washed with a $NaHCO_3$ solution and stored in a flask above $NaHCO_3$) until a pressure of 200 mm. The CO_2 content was renewed twice weekly. The desiccators were maintained in open air in shadow. Gradually the cultures assumed a deep green colour, but they were relatively undifferentiated. An addition of $CaSO_4$ to the nutrient made the cultures form a great number of leafy branches with a length of 1—2 mm., but no roots were produced. Two of the biggest shoots were severed off, and the basal part was covered with a paste containing 3-indole acetic acid to promote the formation of roots. The shoots were planted in soil and covered with a beaker. In some weeks they produced 3—4 small leaves; in the meantime the autumn had come and the experiments had to be finished. One of the shoots produced two roots.

Thus it was possible from an undifferentiated culture to produce an autotrophic plant with leaves, stem, and root. But the growth was very slow, the plant seemed to lack some, probably cytoplasmatic, factor.

4. Summary.

1. A method is described which permits consecutive measurements of the increase of the same fragment of a tissue culture.

2. A tobacco tissue culture from the stem can grow indefinitely in light in a nutrient containing only inorganic salts and sucrose. No addition of amino acids or vitamins is needed.

3. It was possible to produce a normal autotrophic plant from an undifferentiated tobacco tissue culture; but the plant was only a few mm. high, and the growth was very slow.

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