

A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures

By

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Introduction

In experiments with tobacco tissue cultured on White's modified medium (basal medium in Tables 1 and 2) supplemented with kinetin and indoleacetic acid, a striking four- to five-fold increase in yield was obtained within a three to four week growth period on addition of an aqueous extract of tobacco leaves (Figures 1 and 2). Subsequently it was found that this promotion of growth was due mainly though not entirely to inorganic rather than organic constituents in the extract.

In the isolation of growth factors from plant tissues and other sources inorganic salts are frequently carried along with the organic fractions. When tissue cultures are used for bioassays, therefore, it is necessary to take into account increases in growth which may result from nutrient elements or other known constituents of the medium which may be present in the test materials. To minimize interference from contaminants of this type, an attempt has been made to develop a medium with such adequate supplies of all required mineral nutrients and common organic constituents that no appreciable change in growth rate or yield will result from the introduction of additional amounts in the range ordinarily expected to be present in materials to be assayed.

As a point of reference for this work some of the culture media in most common current use will be considered briefly. For ease of comparison their mineral compositions are listed in Tables 1 and 2. White's nutrient solution, designed originally for excised root cultures, was based on Uspenski and Uspenskaia's medium for algae and Trelease and Trelease's micronutrient solution. This medium also was employed successfully in the original cultivation of callus from the tobacco hybrid *Nicotiana glauca* × *N. langsdorffii*, and as further modified by White in 1943 and by others it has been used for the

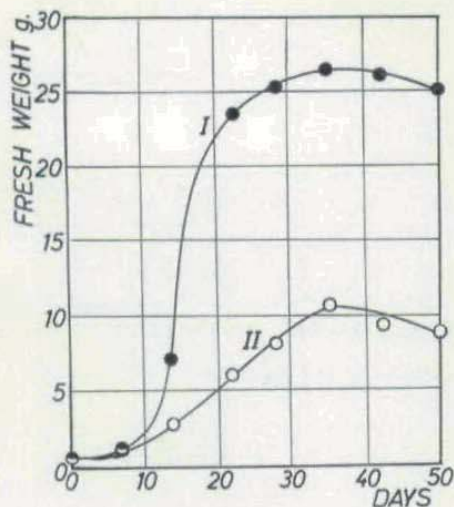


Figure 1.

Figure 1. *Effect of tobacco leaf extract on the growth of tobacco pith tissue (T-2, 10 g/l. of medium: Expt. started Mar. 7, 1958). Curve I with, curve II without leaf extract added to the standard basal medium.*

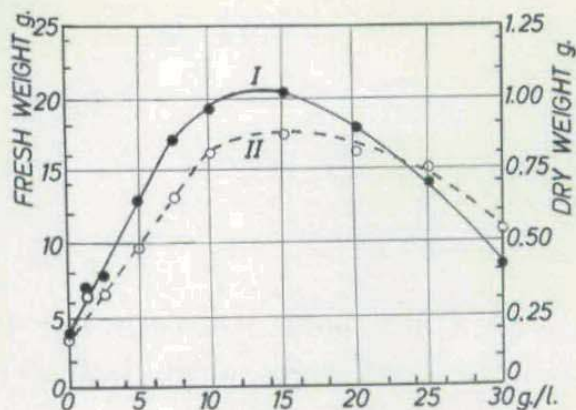


Figure 2.

Figure 2. *Effect of concentration of leaf extract on the yield of tobacco callus tissue (T-2; Growth period, Feb. 13 to Mar. 6, 1958). Curve I, fresh weight; curve II, dry weight.*

cultivation of various tissues from numerous species (See Gautheret's 1959 compilation). Gautheret's medium (1939) was devised by combining a twice diluted Knop's macronutrient solution with a slightly modified Berthelot's micronutrient solution. Hildebrandt, Riker, and Duggar (1946) employed the triangulation technique to improve on White's medium for the cultivation of specific tissues. They reported two new formulae (included in Tables 1 and 2) which they designated as optimal for cultures of the above hybrid tobacco callus and for sunflower crown gall callus respectively. These media, according to Burkholder and Nickell (1949), were still unsuitable for cultivation of virus-induced tumor tissues. By employing the triangulation technique in initial trials and then by testing serial concentrations of each element independently of the others, these investigators devised still another formula based on the medium for sunflower tissue by Hildebrandt *et al.*, and which they reported as more suitable for *Rumex* tissue. Heller (1953) has made detailed studies of the mineral requirements in cultures of carrot and Virginia creeper. He first induced deficiencies of several of the elements by repeated transfers of the cultures to liquid media lacking the element in question and then reintroduced the element in serial concentrations to select satisfactory levels. For the most part the level of each element was varied only in combinations in which the respective levels of all other nutrients were kept unchanged. In comparative tests, Heller found his medium to give two to three times greater yields than White's or Gautheret's media which were employed as controls. The nutrient solution devised more recently by Nitsch and Nitsch (1956) for Jerusalem artichoke is based on the best estimate of available literature for cultures of this species and subsequent modifications

in accordance with results of testing variations in each element separately, but it lacks iron and other trace elements. It is now clear that none of the above media provide nearly the prerequisite amounts of some essential elements for the rapid growth rates and large yields that tobacco tissue is capable of attaining when various organic growth factors are also included in the medium.

Materials and Methods

Source and preparation of tissues. Tobacco tissues, *Nicotiana tabacum*, var. Wisconsin 38, have been used exclusively in this study. Fresh pith was used for the bulk of the work, and continuously subcultured callus was used for the final confirmation tests. Stems were cut from ca. 1 m. tall tobacco plants grown in the greenhouse, the leaves were removed, the stems were swabbed with 95 % ethanol and cut into 5 to 7 cm. long cylinders. Only a 15 cm. region of the stem starting ca. 10 cm. from the tip was used. Cylinders of pith parenchyma were bored from near the center of the excised stems with a sterile No. 2 cork borer. The cylinders were extruded from the borer with a glass-rod, and sliced into discs approximately 2 mm. thick and weighing about 50 mg. each. The ca. half cm. end pieces in each cylinder were discarded as a precaution against contamination. Three discs, each placed with one of its flat surfaces in contact with the medium, were planted in each culture flask. As fresh pith was readily available, it was a convenient material to use in the large scale experiments. However, data obtained from one experiment to the next varied to some extent, possibly due to differences in the nutrient status of the plants from which the pith was excised. For this reason, improvements in yield at successive stages in the development of the nutrient solution and finally the suitability of the revised solution were confirmed in tests with tobacco callus. Firm, white callus from 4—5 weeks old stock cultures was cut into roughly rectangular pieces weighing 40—50 mg. each, and these were planted in groups of three into each culture flask. The stock callus, originally obtained from pith, had been subcultured 10 or more times on medium identical with the basal medium described below except that the kinetin level had been raised to 0.38 mg/l. and inositol had been omitted. The vigorous more compact tissue obtained by four-weekly subculturing on medium modified in this manner gave excellent growth and satisfactory reproducibility of data (within 10 %) from one experiment to another.

Composition of the Basal Medium. The basal medium which has served as control or reference medium throughout this study is a modified White's nutrient solution, which has been routinely employed in this laboratory and with two addenda, *myo*-inositol and Edamin (a pancreatic digest of lactalbumin furnished by Sheffield Chemical Co., Norwich, N.Y.). It had the following composition, in mg/l. of medium: (All salts were reagent grade unless noted to be otherwise.)

- (a) Inorganic salts: NH_4NO_3 , 400; KCl, 65; KNO_3 , 80; KH_2PO_4 , 12.5; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 144; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 72; NaFe-EDTA, 25; H_3BO_3 , 1.6; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 6.5; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.7; and KI, 0.75.
- (b) Organic substances: 3-Indoleacetic acid (IAA), 2.0; kinetin, 0.2 (for fresh pith) or 0.04 (for callus); thiamin · HCl, 0.1; nicotinic acid, 0.5; pyridoxine · HCl, 0.5; glycine (recrystallized), 2.0; *myo*-inositol, 100; Edamin, 1000; sucrose, 20,000; and Difco Bacto-agar, 10,000.

(c) pH adjustment: The pH of all media was adjusted to 5.7–5.8 with a few drops of either 1 N NaOH or 1 N HCl before the agar was added.

Fe was added as agricultural grade sodium-ferric-ethylenediaminetetraacetate obtained from Geigy Agricultural Chemicals, New York, N.Y. or $\text{Na}_2\text{Fe EDTA}$ prepared from $\text{Na}_2\text{-EDTA}$, MW 372.35 (Frederick Smith Chem. Corp. Columbus, Ohio) dissolved and heated in H_2O with an equimolar amount of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

As the NH_4NO_3 content of the basal medium far exceeded that of any other salt, the concentrations of the other elements were varied simply by adding them as either nitrate or ammonium salts and by adjusting the amount of NH_4NO_3 accordingly to attain the specified total inorganic nitrogen level. There was no evident effect on growth from possible changes in pH or in other conditions arising from this procedure.

In Tables 1 and 2 the inorganic constituents of the basal medium are listed in millimoles/l. (mM) or micromoles/l. (μM) of each element. To facilitate comparisons, the concentration of each element is also expressed in mg/l (values in parenthesis). These concentrations will be referred to as the 1 \times levels, and in many experiments modifications will be made in multiples of these, such as 2 \times , 3 \times , etc.

Preparation of tobacco leaf extract. The leaf extracts, T-2 or T-3, used in this study are both from *Nicotiana tabacum* variety Wisconsin 38. T-2 is a water extract from 62.4 kg. of greenhouse grown plants and was used here only in the experiments represented by Figures 1 and 2. T-3 is an extract from field grown plants prepared as follow: Healthy green leaves picked from vigorous, nearly mature, ca 1 m. tall plants were packed into plastic bags, sealed, trucked to a cold storage plant, quickly frozen and kept indefinitely. The frozen leaves were then ground in a commercial type meat grinder and thawed. The juice was expressed with a hand press, heated to near 100°C , chilled to $5\text{--}10^\circ\text{C}$, centrifuged and concentrated under reduced pressure. The concentrate was stored in a frozen state in 1 l polyethylene bottles and

Table 1. Macronutrient composition of some plant tissue culture media.

Medium	Element (Concentrations in millimoles per liter of medium)								
	N	K	Ca	Mg	S	P	Cl	Na	Fe
White (1943) ¹	3.2	1.7	1.2	3.0	4.4	0.14	0.9	3.0	0.013
Gautheret ¹	5.5	2.2	2.1	0.5	0.5	0.9	—	—	0.125
Hildebrandt <i>et al.</i> ¹ (Tobacco)	4.2	1.7	1.7	0.7	6.4	0.24	0.9	11.7	0.143
Hildebrandt <i>et al.</i> ¹ (Sunflower)	8.4	3.3	3.4	2.9	3.6	1.0	1.8	1.7	0.018
Burkholder & Nickell ¹ ..	8.0	12.0	6.0	2.0	1.0	8.0	10.0	—	0.009
Heller ¹	7.1	10.0	0.51	1.0	1.0	0.9	11.0	8.0	0.004
Nitsch & Nitsch ²	19.8	39.9	0.23	1.8	1.0	1.8	0.5	1.8	—
Basal Medium	12.05	1.76	0.61	0.29	0.32	0.092	0.87	0.1	0.053
1 \times Level	(169)	(68.8)	(24.4)	(7.10)	(10.6)	(2.85)	(30.9)	(2.3)	(2.94)
Revised	60.0	20.0	3.0	1.5	1.6	1.25	6.0	0.2	0.100
Medium	(840)	(782)	(121)	(36.8)	(52.3)	(39.0)	(212)	(4.6)	(5.57)

¹ Data from Heller's compilation (1953). ² Data from Nitsch and Nitsch (1956). Data in parenthesis are mg/l of medium.

Table 2. Micronutrient compositions of plant tissue culture media.

Medium	Element (Concentrations in micromoles per liter of medium)										
	B	Mn	Zn	I	Cu	Mo	Co	Ni	Te	Be	Al
White (1943) ¹	25	30	10	4.5	—	—	—	—	—	—	—
Gautheret ¹	0.4	4.5	0.2	1.5	0.1	—	0.1	1.0	1.0	0.3	—
Hildebrandt <i>et al.</i> (Tobacco)	6	20	2.2	8	—	—	—	—	—	—	—
Hildebrandt <i>et al.</i> (Sunflower)	50	20	1.0	2.2	—	—	—	—	—	—	—
Burkholder & Nickell ¹	10	2	4.6	—	1.6	1.0	—	—	—	—	—
Heller ¹	16	4.5	3.5	0.06	0.12	—	—	0.13	—	—	0.23
Nitsch & Nitsch ²	—	—	—	—	—	—	—	—	—	—	—
Basal Medium	26	29	9.4	4.5	—	—	—	—	—	—	—
1 × Level	(0.28)	(1.60)	(0.62)	(0.58)	—	—	—	—	—	—	—
Revised	100	100	30	5.0	0.10	1.0	0.10	—	—	—	—
Medium	(1.08)	(5.50)	(1.92)	(0.64)	(0.0064)	(0.096)	(0.006)	—	—	—	—

¹ Data from Heller's compilation (1953). ² Data from Nitsch and Nitsch (1956).

used as needed. T-3 represents 13.6 kg. of solids in 21.6 l of concentrate obtained from 465 kg. of fresh leaves.

Culture conditions and growth measurements. The tissues were grown routinely in 125 ml. erlenmeyer flasks with 50 ml. of medium. Fifteen pieces, three in each of five replicate flasks, were planted for each treatment. The cultures were kept on shelves under low intensity overhead florescent lights in a room at 26–28°C and about 85 % relative humidity.

A growth period of 4 weeks was selected because it extends past the end of the logarithmic phase of growth (see Figure 1), after which time the fresh and dry weights per flask were determined. Since in most cases the dry weights of the tissue were close to 5 % of the fresh weights, only the latter will be reported. However, certain trends in percentage dry weight of the tissue with change in composition of the medium will be considered. Replicate cultures had rather uniform yields, and the

standard error, $\pm \sqrt{\frac{\sum \Delta^2}{n(n-1)}}$, of the average final fresh weights in the 5–20 g range was around ± 0.8 g/flask.

Testing procedure. In most previous work on quantitative nutrient requirements either a single element was varied and the others kept constant, or three elements were varied at a time (the triangulation method). In the present study the requirement of a given element was established by varying its concentration in the presence of several levels of the remaining elements. Tests have been limited to 1 × and higher levels of the elements present in the basal medium. In addition Cu, Co and Mo have been tested.

Results

Preliminary testing of the basal medium showed that doubling and quadrupling the levels of inorganic or organic constituents lead to increases in

Table 3. *Effect of increasing the concentration of the nutrients in the basal medium on the growth of tobacco callus tissue. (Growth period, 11/15-12/18/58.)*

Change in composition of medium	Average final fresh weight g./flask.
None (Basal medium 1 ×)	5.6
All constituents 2 ×	9.9
" " 4 ×	14.9
All organic substances 2 ×	6.3
" " " 4 ×	6.4
All inorganic salts 2 ×	9.7
" " " 4 ×	17.0

Table 4. *Effect of quadrupling the concentration of the inorganic elements of the basal medium all together and each separately on the growth of tobacco callus tissue. (Growth period 12/17/58-1/19/59.)*

Change in composition of medium	Average final fresh weight g./flask.
None (Basal medium 1 ×)	7.1
All elements 4 ×	17.3
N 4 ×	11.6
K 4 ×	11.9
P 4 ×	7.9
Ca 4 ×	7.6
Mg 4 ×	7.6
S 4 ×	7.1
Cl 4 ×	9.6
Fe 4 ×	6.6
B 4 ×	6.8
Mn 4 ×	7.0
Zn 4 ×	7.5
I 4 ×	6.7

yield of tissue (Table 3) and that the principal, although not always the entire improvement was from the inorganic salts. When each element was increased separately to 4 times its level in the basal medium (4 ×), each major element except S gave appreciable improvement in yield (Table 4). N and K were especially effective, but increasing all the salts to the 4 × level definitely had a still greater effect than increasing any one element alone. Of the minor elements, excepting perhaps Cl, none was significantly better at the 4 × than at the 1 × level.

The requirements for N, K, and P

In view of the above results further tests were carried out with possible combinations of N, K, and P raised to 2 ×, 4 × and 8 × levels. In addition these combinations of N, K and P were tested with the remaining elements increased to 2 ×, 4 × and 8 × levels. The greatest increase in tissue growth was obtained when N was increased to 4 ×, K to 8 ×, P to 8 ×, and all other elements

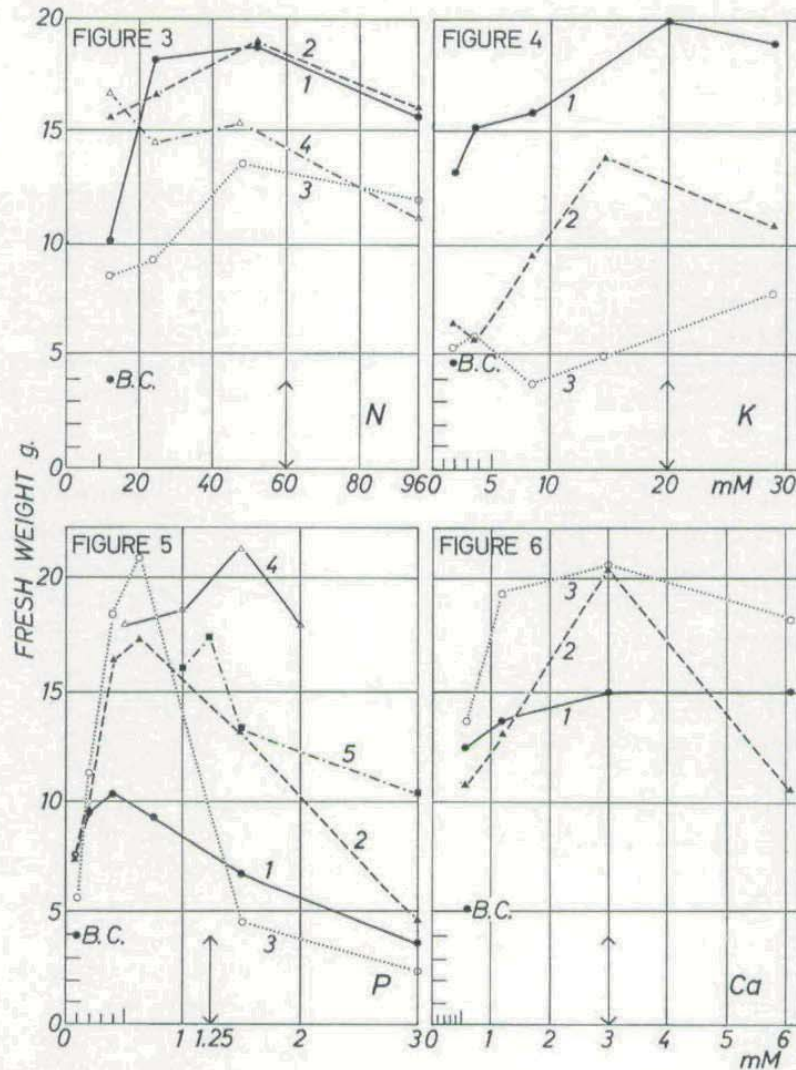
were increased to $2\times$ or $4\times$, *i.e.* under the more favorable conditions for growth the optimal levels of N, K and P would appear to be about 50, 15 and 1.0 mM respectively.

N. The N requirement was examined in more detail by testing the growth of pith tissue on media containing varying amounts of NH_4NO_3 to give N levels of 12, 24, 48 or 96 mM in combinations with 14.4 or 28.8 mM K and 0.74 or 1.5 mM P. All other inorganic constituents were kept at their $3\times$ levels. The average final fresh weights of tissue obtained in one experiment are plotted against the N level in Figure 3. It may be noted that under the conditions of this experiment the increase in K for unknown reasons resulted in a marked increase in yield at the low N levels. In this experiment the high phosphate level (1.5 mM) depressed growth except perhaps in the case of high K and low N media. In experiments with other lots of plants, P levels up to 2.0 mM were sometimes favorable, but generally P levels of 1.5 mM or higher have tended to depress the yield of either pith or callus tissue.

The data show that 50 mM is close to optimal for N irrespective of the K and P concentrations, and that the N content may be increased somewhat above this level with no appreciable effect on the yield. However, N levels of 80 mM or higher consistently were found to depress the yield. Hence, a level of 60 mM N has been selected as satisfactory.

K. The K requirement was determined with N kept at the $4\times$ level (48 mM); P kept at three levels, $8\times$, $16\times$ and $32\times$ (0.74, 1.5 and 3.0 mM respectively), and all other minerals kept at their $3\times$ levels. As shown in Figure 4, the yields presented in curves 1 and 2 increased with the concentration of K up to 15 mM and fell only slightly at 30 mM, the highest tested K level. In curve 3 the yields are low throughout, probably due to toxicity of the high P level (see above). A 20 mM K level has been selected.

P. The P requirement was determined with N at the $4\times$ level (48 mM) with K at three levels (7.2, 14.4 and 28.8 mM) and with all other inorganic constituents at their $3\times$ levels. The results are shown in Figure 5. It may be seen that the optimal requirement for P was strikingly dependent on the level of K, but with an ample supply of K it lies in the region between 0.7 and 1.5 mM. Because, as stated, high P levels were toxic to the pith explants in this particular lot of plants, the experiment was repeated in part with several other lots of plants. In these cases the K level was set at 20 mM and as shown by typical results, curve 4 in Figure 5, the optimum P level was 1.5 mM. However, sometimes this level and frequently higher levels were growth inhibitory even though very healthy looking tissue might be produced. Because of these difficulties six additional experiments were done, three with pith and three with callus cultures. P levels were adjusted to 1.0, 1.25, 1.5, 1.0 and 3.0 mM. Other elements were as specified for the revised medium (Tables 1 and 2) except that Fe was used at both the 0.10 and 0.25 mM levels. Of the organic constituents (Table 6 B) Edamin was omitted, and for some treatments sucrose was used at either the 2% or 4% level as well as at the specified 3% level. Data from one experiment with pith, typical of the results obtained, are presented in curve 5 in Figure 5. It appeared that the P requirement may vary with the levels of Fe and sucrose as well as K. Nevertheless, the results showed conclusively that 1.25 mM is close to the optimal P level;



Figures 3 to 10 inclusive. *Fresh weight yields of excised tobacco pith cultures in response to increased concentrations of nutrient elements.*

Figure 3. *Effect of N.* (Growth period, May 5 to June 20, 1959). All elements at 3 × levels except K and P.

Curve 1: K 14.4, P 0.74 mM. Curve 2: K 28.8, P 0.74 mM. Curve 3: K 14.4, P 1.5 mM. Curve 4: K 18.8, P 1.5 mM. BC, basal control.

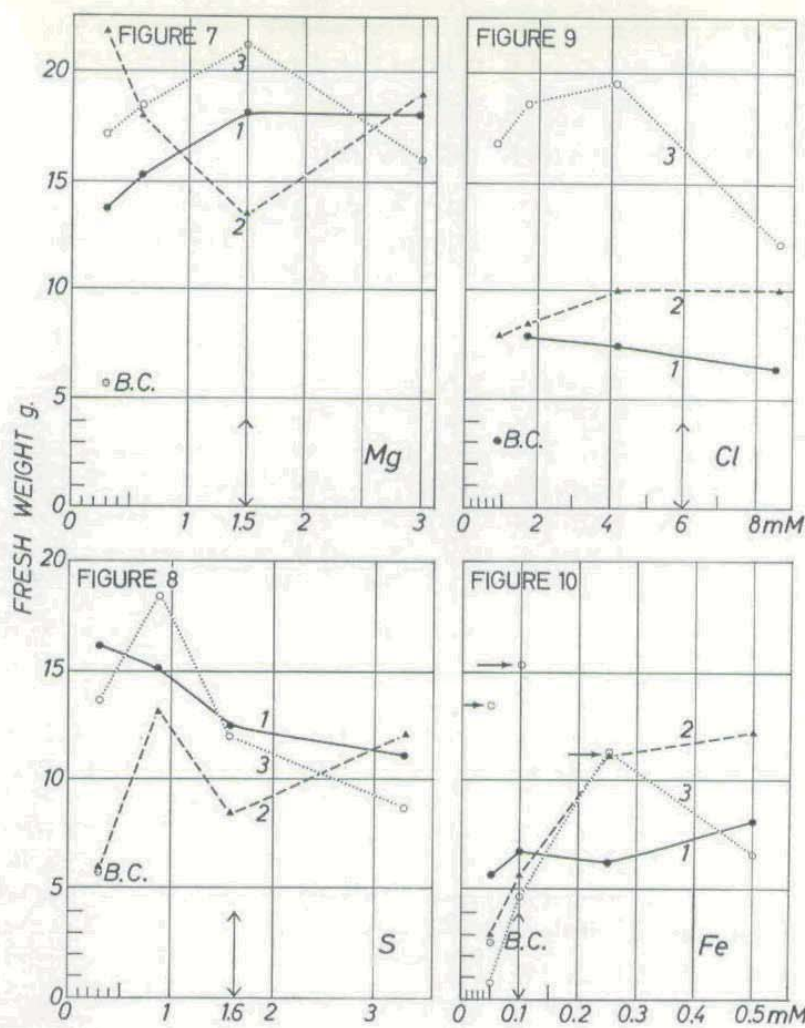
Figure 4. *Effect of K.* (Growth period, May 19 to June 16, 1959). N kept at 4 × (48.8 mM) and all other elements at 3 × levels except P.

Curves 1, 2 and 3, the level of P was 0.74, 1.5 and 3.0 mM respectively. BC, basal control.

Figure 5. *Effect of P.* (Growth period, Curves 1–3, May 20 to June 17, 1959; Curve 4, 1960; Curve 5, July 19 to Sept. 1, 1962).

N kept at 4 × and all other elements at 3 × levels except K. Curves 1, 2, 3 and 4, the level of K was 7.2, 14.4, 28.8, and 20.0 mM respectively. Curve 5, see text. BC, basal control.

Figure 6. *Effect of Ca.* (Growth period, July 9 to Aug. 10, 1959) N kept at 55 mM, K at 20 mM, P at 1.0 mM, and micronutrients B, Fe, Mn and Zn at 3 × levels. Mg, S, and Cl varied. Each kept at its 1 ×, 2 ×, and 5 × levels in Curves 1, 2 and 3 respectively. BC, basal control.



- Figure 7. *Effect of Mg* (Growth period, July 9 to Aug. 10, 1959). N kept at 55 mM, K at 20 mM, P at 1.0 mM, and micronutrients B, Fe, Mn, and Zn at 3 × levels. Ca, S and Cl varied. Each kept at 1 ×, 2 × and 5 × levels in Curves 1, 2 and 3 respectively. BC, basal control.
- Figure 8. *Effect of S* (Growth period, July 10 to Aug. 10, 1959). N kept at 55 mM, K at 20 mM, P at 1.0 mM, and micronutrients B, Fe, Mn, and Zn at their 3 × levels. Ca, Mg, and Cl varied. Each kept at 1 ×, 2 × and 5 × levels in curves 1, 2 and 3 respectively. BC, basal control.
- Figure 9. *Effect of Cl* (Growth period, July 10 to Aug. 11, 1959). N kept at 55 mM, K at 20 mM, P at 1.0 mM, and micronutrients B, Fe, Mn, and Zn at their 3 × levels. Ca, Mg, and S varied. Each kept at its 1 ×, 2 × and 3 × levels in curves 1, 2 and 3 respectively. BC, basal control.
- Figure 10. *Effect of Fe* (Growth period, July 15 to Aug. 12, 1959). Nutrients kept at the following levels: N 55, K 20, P 1.0, Ca 3.0, Mg 1.5, S 1.6 and Cl 6.0 mM. Microelements B, Mn, and Zn varied. Each kept at its 1 ×, 2 × and 5 × levels in curves 1, 2, and 3 respectively. Horizontal arrows represent yields attained in later experiments, see text. BC, basal control.

1.0 is on the low side, 1.5 on the high side, and 2 or more mM definitely too high. For this reason, a 1.25 mM P level has been selected.

Requirements for Ca, Mg, S, and Cl

When the requirements for Ca, Mg, S, and Cl first were evaluated, the N, K, and P levels of the media were kept at 55, 20 and 1.0 mM respectively; *i.e.*, close to the values selected above as satisfactory for each of these elements. Each of the four elements was tested at four levels (1×, 2×, 5×, and 10×) and in conjunction with three levels (1×, 2×, and 5×) of the other three elements. The micronutrients B, Fe, Mn and Zn were provided at their 3× levels.

Ca. By comparison of the curves in Figure 6 the requirement for Ca would appear to be relatively higher with the 2× than with the other levels of Mg, S and Cl. With these elements at the 5× level the Ca concentration curve forms a broad plateau between 1.5 and 6 mM. A 3.0 mM Ca level has been selected.

Mg. Results from one of the experiments with Mg are shown in Figure 7. Both with the 1× and 5× levels of Ca, S and Cl (curves 1 and 3) tissue yields increased as the Mg concentration was increased from 0.3 to 1.5 mM. The 3.0 mM level of Mg was without further stimulating effect when the levels of Ca, S and Cl were 1× and it was perhaps inhibitory when the levels of these elements were 5×. With 2× levels of Ca, S, and Cl, the final fresh weights of the pith explants fell as the Mg concentration was raised from 0.3 to 1.5 mM but was up again when the Mg concentration was 3.0 mM. Although in this case the lowest concentration of Mg resulted in the highest yield it should be noted that an imbalance existed as the cultures in this particular treatment became very necrotic. The 1.5 mM level of Mg was selected.

S. As is shown by curve 1 in Figure 8, with 1× levels of Ca, Mg and Cl, the lowest concentration of S (0.3 mM) was optimal and higher S concentrations caused a steady reduction in tissue yields. However, with the 2× or 5× levels of Ca, Mg and Cl, the 0.3 mM S level was less than adequate, and a substantial increase in yield was obtained at the 0.66 mM level. S concentrations higher than 0.66 mM were inhibitory to the pith explants. In subsequent tests with callus, on the other hand, no toxicity was obtained even with S concentrations as high as 13 mM. On the basis of all results and even though it may be slightly higher than optimal for excised pith cultures, the 1.6 mM level of S has been selected.

Cl. With Ca, Mg and S at their respective 1× levels, increases in Cl concentration had no appreciable effect on growth of pith explants (Figure 9), whereas with Ca, Mg and S at their 2× or 5× levels, increases in the Cl level up to 4.4 mM were stimulatory. A higher level of Cl (8.7 mM) gave neither a further increase nor inhibition of growth in the presence of 2× levels of Ca, Mg and S, but it was far less stimulatory than the lower Cl levels in the presence of 5× levels of Ca, Mg and S. With callus instead of pith, Cl concentrations as high as 12 mM were without inhibitory effect. A Cl level of 6 mM has been selected.

Micronutrient requirements

Preliminary studies indicated that $3\times$ to $5\times$ levels of B, Fe, Mn and Zn were not inhibitory, and that in combinations with increased NaFe-EDTA levels they were sometimes stimulatory. In these experiments the other elements were supplied in the concentrations selected as optimal or very close to these levels.

Fe. Availability of Fe has long been recognized as a critical requirement for vigorous and prolonged growth of plant tissues. The use of chelating agents, citric or tartaric acid and more recently especially the use of EDTA has greatly improved the available supply of Fe and possibly some other micronutrients in culture media. In the present experiments Fe was supplied at first as a commercial NaFe-EDTA chelate which was tested in combination with $1\times$, $2\times$, and $5\times$ levels of B, Mn and Zn. As shown in the curves in Figure 10, increases in the Fe chelate from 0.05 to 0.25 mM resulted in increased yields of tissue especially in the presence of $2\times$ and $5\times$ levels of the other three trace elements. Increasing the NaFe-EDTA level to 0.5 mM resulted in a definite decrease in yield when the other trace elements were raised to their $5\times$ levels. A higher purity preparation of $\text{Na}_2\text{-Fe-EDTA}$ was then used, which in a large number of tests with both callus and pith tissues consistently gave optimum yields at the 0.10 mM level. Even 0.05 mM Fe permitted excellent growth, and sometimes close to optimum yields, of both types of tissue, whereas 0.25 mM Fe generally gave considerably lower yields. Representative yields at the 0.05, 0.10 and 0.25 mM levels obtained in a series of three experiments with pith tissue are shown by horizontal arrows in figure 10. In view of these results the Fe content of the commercial Fe-chelate was verified by chemical analysis. Considering the complex interaction between the Fe-chelate and other elements in the nutrient medium various plausible reasons might account for the difference in the results obtained. The curves in figure 10 suggesting an optimal level of 0.25 mM are included for the sake of consistency with the data for the other elements, even though on the basis of all results this level is too high and a 0.10 mM of $\text{Na}_2\text{Fe-EDTA}$ has been selected as optimal.

B, Mn, and Zn. Considering the likely presence of impurities, the B, Mn and Zn requirements were finally tested with callus grown on media made up with Difco purified agar and without addition of Edamin. Each element and all three in combination were tested in serial concentrations up to their $5\times$ levels, and $\text{Na}_2\text{Fe-EDTA}$ was kept at the 0.25 mM Fe level. There was no inhibition from their presence, and perhaps a stimulation, at least when all three test elements were increased to their $5\times$ levels (See Table 5). From these and other data the levels 0.10 mM B, 0.10 mM Mn, and 0.030 mM Zn have been selected.

Other Elements (Cu, Mo, Co, I, and Na). Cu added in the range from 0.00003 to 0.03 mM was without effect on the growth of the tobacco tissue, and so was Mo, 0.001 mM, the concentration employed by Torrey (1954) with pea cultures. Iodine, even when raised to the $4\times$ level (0.02 mM), and Na tested at the 5 and 10 mM levels, *i.e.*, in the range used by Heller (1953) and by Hildebrandt *et al.* (1946), similarly were without effect on yields.

In spite of the above negative results these elements have been included in

Table 5. *Effect of increased levels of B, Mn and Zn on the growth of tobacco callus tissue.*
(Growth period 9/15-10/12/59.)

Change in composition of medium	Average final fresh weight g./flask.
None (Basal medium 1 ×)	12.5
B 5 ×	11.6
B and Mn 5 ×	12.6
B and Zn 5 ×	12.6
B, Mn and Zn 5 ×	14.3
Mn 5 ×	13.7
Mn and Zn 5 ×	12.2
Zn 5 ×	14.0

the revised medium. Cu and Mo are added because they are known to be essential elements for plant growth. Similarly Co is included for reasons of its requirement in lower plants (Holm-Hansen *et al.* 1954) and the possible role of cobaltous ion in morphogenesis of higher plants (Miller 1954, Salisbury 1959). A test of CoCl_2 in eight serial concentrations between 0.0001 and 0.16 mM and all other elements at the 1 × levels was included in an early experiment. No stimulatory effect of Co was obtained at any level but rather a toxic action at the two highest levels, 0.08 and 0.16. The selected levels are: Cu 0.0001, Mo 0.001 and Co 0.0001 mM. Iodine arbitrarily is retained at 0.005 mM. Na, ostensibly nonessential but perhaps not entirely devoid of its often claimed stimulating effect on plant growth, being a part of the FeSO_4 - Na_2 EDTA solution, therefore, is supplied at the 0.20 mM level.

pH. The pH of the medium was adjusted to 5.7-5.8 with a few drops of 1 N HCl or with NaOH or KOH either before or after the agar was added and had been heated a few minutes in the autoclave. The value was selected on the experience that the reaction of either more acid or more basic media tends to drift toward this region during the heat treatment for sterilization and subsequently with time even in uninoculated flasks. Preheating with the agar present avoids any appreciable change in pH during the subsequent autoclavation. The value 5.7 to 5.8 is suitable for maintaining all the salt in soluble form even with relatively high phosphate levels and low enough to permit rapid growth and differentiation of the tissue. The amounts of Cl, Na or K introduced in the process are not included in the stated levels, 6.0, 0.2 or 20 mM respectively of these ions.

Composition and growth effects of the revised medium. The kinds and amounts of mineral salts finally adopted for the revised medium are listed in Table 6 A. Quantities have been adjusted upward, beyond actual needs in some cases to provide total concentrations of all elements in round numbers as shown in Tables 1 and 2.

The yield of tobacco tissue on the revised medium has been compared with those on the original basal medium (1 ×) and other media as shown in Table 7 and in Figure 11. Not only were the highest yields obtained consistently on the revised media but the pronounced tissue necrosis characteristic of old cultures on all other media was avoided as is also evident in Figure 11.

Table 6. *Composition of the revised medium. pH adjusted to 5.7-5.8 with HCl, KOH, or NaOH (see text).*

A. Mineral salts

Major elements			Minor elements		
Salts	mg/l.	mM	Salts	mg/l.	μ M
NH ₄ NO ₃	1650	N 41.2	H ₃ BO ₃	6.2	100
KNO ₃	1900	18.8	MnSO ₄ · 4H ₂ O	22.3	100
CaCl ₂ · 2H ₂ O	440	3.0	ZnSO ₄ · 4H ₂ O	8.6	30
MgSO ₄ · 7H ₂ O	370	1.5	KI	0.83	5.0
KH ₂ PO ₄	170	1.25	Na ₂ MoO ₄ · 2H ₂ O	0.25	1.0
Na ₂ -EDTA	37.3 ¹	Na 0.20	CuSO ₄ · 5H ₂ O	0.025	0.1
FeSO ₄ · 7H ₂ O	27.8 ¹	Fe 0.10	CoCl ₂ · 6H ₂ O	0.025	0.1

¹ 5 ml/l of a stock solution containing 5.57 g FeSO₄ · 7H₂O and 7.45 g Na₂-EDTA per liter of H₂O.

B. Organic constituents

Sucrose	30 g/l.	Agar	10 g/l.
Edamin (optional)	1 g/l.	<i>myo</i> -Inositol	100 mg/l.
Glycine	2.0 mg/l.	Nicotinic acid	0.5 mg/l.
Indoleacetic acid ²	1-30 mg/l.	Pyridoxin · HCl	0.5 mg/l.
Kinetin ²	0.04-10 mg/l.	Thiamin · HCl	0.1 mg/l.

² See text and figures 12 and 13.

Table 7. *Comparison of yields of tissue on different media*

A. Pith Tissue (Growth period 10/2-11/3/59).

Medium	Fresh weight in g/flask		Dry weight in g/flask	
	Without Edamin	With Edamin	Without Edamin	With Edamin
Revised.....	17.3	24.5	0.47	0.49
Basal.....	4.1	7.1	0.13	0.26
Heller	5.0	17.9	0.19	0.43
Nitsch	1.9	9.7	0.10	0.34
Hildebrandt <i>et al.</i>	5.3	9.2	0.20	0.37

B. Callus Tissue (Growth period 8/31-10/10/59).

Medium	Fresh weight in g/flask		Dry weight in g/flask	
	Without Edamin	With Edamin	Without Edamin	With Edamin
Revised.....	21.5	22.7	0.48	0.46
Basal.....	5.1	10.8	0.22	0.35
Heller	3.6	16.2	0.24	0.38
Nitsch and Nitsch	3.0	17.6	0.28	0.48
Hildebrandt <i>et al.</i>	2.8	9.8	0.18	0.37

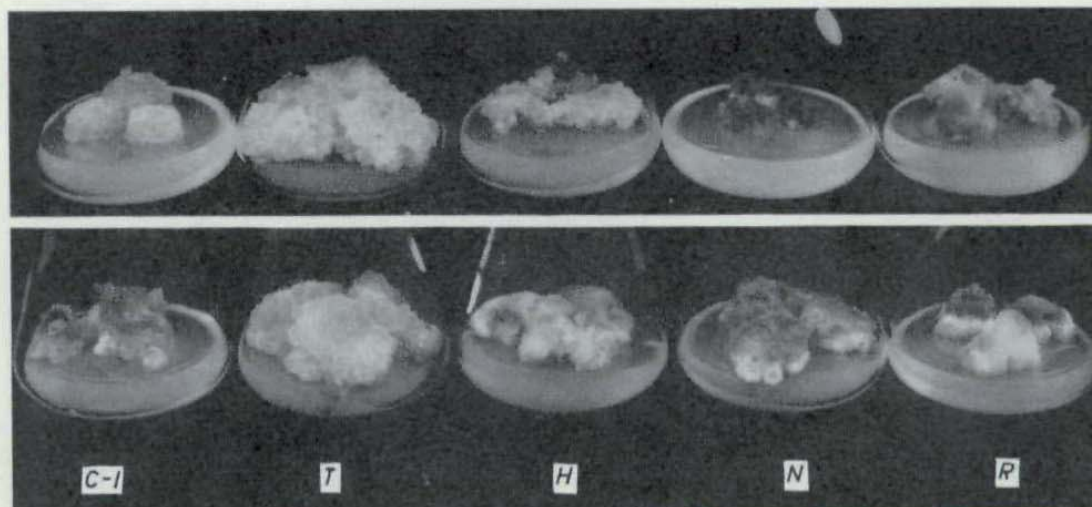


Figure 11. Comparison of yields of tobacco pith tissue cultured on various media (Growth period, Oct. 2 to Nov. 3, 1959). Upper row without, lower row with Edamin 1.0 g/l. Media from left to right: C-1, basal medium 1 ×, T, revised; H, Heller (1953), N, Nitsch and Nitsch (1956), and R, Hildebrandt *et al.* (1946).

Organic Constituents

The required levels of organic constituents in the basal medium have been tested only to a limited extent.

From the results in Table 3, no large increase in yield would be expected from increased concentrations of the specified organic ingredients, although with improved mineral supplies some beneficial effect might be obtained by modifications in these. The list of other potentially stimulatory organic substances and favorable combinations of these is of course inexhaustible.

Vitamins. The levels of the following vitamins have been retained unchanged from the basal medium: thiamin-HCl 0.1 mg/l, nicotinic acid 0.5 mg/l, pyridoxine · HCl 0.5 mg/l. On the basis of experience by others (Braun 1958, Steinhart *et al.* 1961, 1962) *myo*-inositol was tested and found to promote growth in cultures when other conditions would permit high yields. A level of 100 mg/l of *myo*-inositol has been selected.

Nitrogenous compounds. The combination of amino acids in yeast extract and also various simpler combinations are known to promote growth of tobacco callus on the basal medium (Sandstedt and Skoog 1960, La Motte 1960). Glycine has been retained without further testing at 2:0 mg/l. The casein hydrolysate, Edamin, was tested extensively. In the comparative tests with different media it substantially increased the yield on all except the revised medium (Table 7). The strikingly lower response on the latter medium probably is due mainly to the relatively high N content. The small increase which did result from addition of Edamin perhaps is due in part to unknown organic factors. An additional amount of NH_4NO_3 equivalent to the N content of the Edamin failed to replace it and instead lowered the yield. However, $\text{NH}_2\text{-N}$ is not excluded by these results as being the principal active

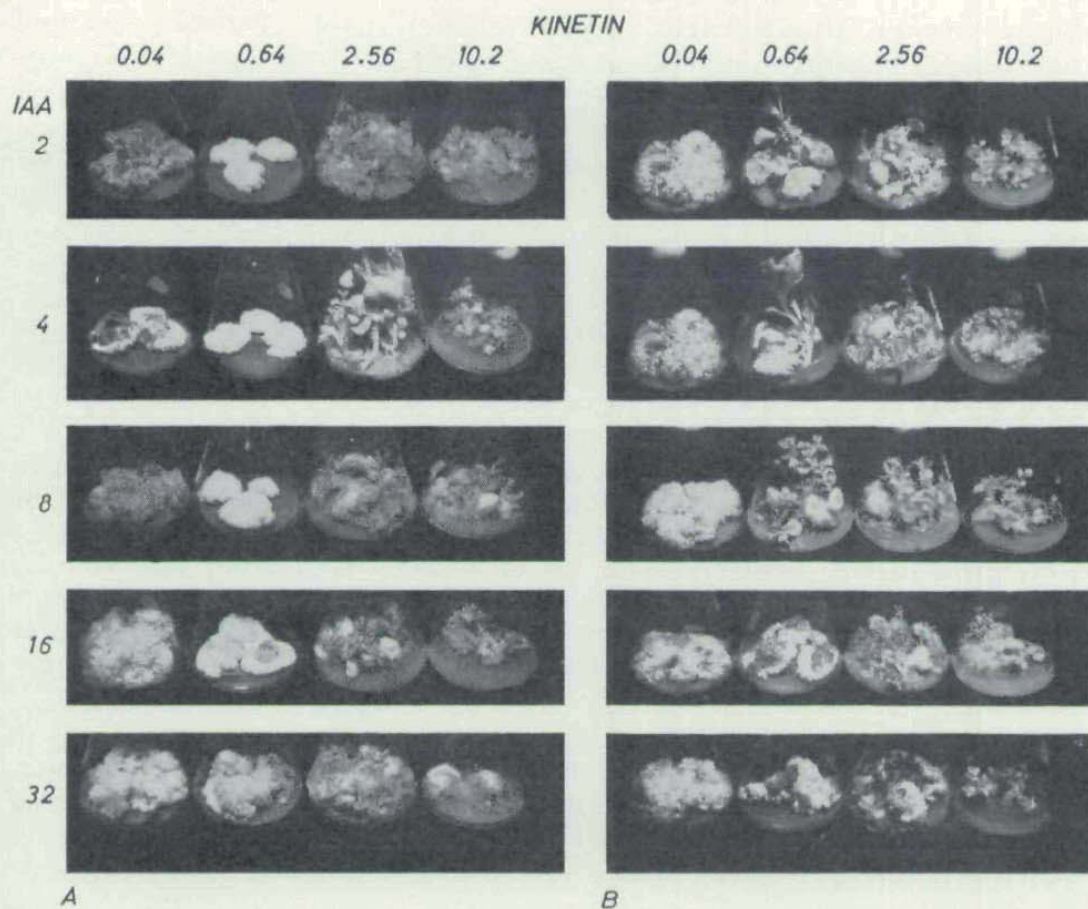


Figure 12. Tobacco callus cultures grown on revised medium with increasing levels of IAA (ordinate, mg/l.) and kinetin (abscissa, mg/l.). A without Edamin; growth period, Oct. 6 to Dec. 22, 1961. B with 1 mg/l. Edamin; growth period Oct. 11 to Dec. 22, 1961.

agent contributed by the hydrolysate, for it has been shown (Steinhart *et al.* 1961) that spruce tissue which is unable to survive on a medium with NH_4NO_3 as the sole N-source grows well on the same medium supplemented with either urea, arginine or other basic amino acid. The influence of different kinds of casein hydrolysate (kindly supplied by the Sheffield Chemical Co.) is quite variable, Edamin being among the more active preparations. The influence of Edamin itself seems to vary to some extent, increasing with age of the cultures and being even more striking with respect to the development of vigorous shoots and root systems than the increase in yield. As may be seen from comparisons of corresponding cultures under A and B in Figure 12, the presence of Edamin has resulted in a broader range of IAA and kinetin levels which permit vigorous organ development. It has shifted the levels and to a slight extent perhaps also the ratio of the two substances required for a particular developmental pattern to emerge. However, essentially no effects were obtained in the presence of Edamin that could not be obtained also in its absence by suitable modifications of the kinetin and IAA levels.

Carbohydrates. Although tobacco cultures are capable of utilizing various carbohydrates and organic acids to some extent as energy sources, sucrose is as favorable as any known material of this type. Concentrations of 2, 3, and 4 % are almost equally suitable for cultures with low yields. However, in several large series of comparative tests 3 % was definitely better than 2 %, and 4 % was often somewhat less effective than 3 % in cultures with moderate to high yields. For example in one experiment fresh weight yields of cultures with 2, 3 and 4 % sucrose were 14.3, 15.9 and 12.9 g/flask respectively and the corresponding dry weights were 0.44, 0.67, and 0.62 g/flask. Dry weights often were somewhat higher per fresh weight in cultures with 4 % sucrose than in the others, but on the average also the total dry weight per flask was less in these than in cultures with 3 % sucrose. The level of sucrose in the revised medium, therefore, has been set at 3 %.

Agar ordinarily is kept at 1 % to give a suitably moist but rigid medium. The concentration may need to be varied to some extent depending on the preparation and the salt content, pH, etc. of the medium. In the case of weakly growing tissue cultures agar content may be critical for survival, but for vigorously growing tobacco cultures it was unimportant. In liquid cultures, on the other hand, changes in composition of the medium definitely are required for vigorous bud development to occur (Loewenberg *et al.* unpublished.)

Indoleacetic acid and kinetin. The optimal level of IAA or kinetin will depend on the type of growth that is desired. It will vary with the endogenous contents of auxins and kinins and other factors influencing the sensitivity of a particular strain or clone of tissue to these substances.

The curves in figure 13 representing fresh weight yields in two experiments with different IAA-kinetin combinations clearly show that the optimal requirement for either one increased with the concentration of the other. The effect of a given combination, especially in the intermediate concentration range, varied from one experiment to the next as illustrated by the two examples in figure 13. Note that the response to 3 mg/l. IAA is much greater in one experiment than the response to 4 mg/l. in the other. Remarkable developmental patterns, differences in form and texture of the callus, extent of organ formation, number and size of organs, vigor and longevity of the cultures are associated with specific levels and ratios of IAA and kinetin. An impression of the range in size and form of the cultures may be gained from figure 12. Details of the strict quantitative aspects and other characteristics of the growth responses which have emerged as functions of the IAA-kinetin concentrations will be presented elsewhere. In general the following applied. For continuous growth of firm, healthy tobacco callus in sub cultures, 0.2 mg/l. of kinetin and 2.0 mg/l. of IAA have been used with excellent results for several years in our laboratory. However, 0.04 mg/l. of kinetin and 1.0 mg/l. of IAA has given much faster growing, loosely packed masses of cells which could be transferred and maintained as callus even though vigorous root formation ordinarily occurred in these cultures after 2 to 3 weeks. For long lasting cultures (2 to 6 months) higher kinetin levels were required, and for vigorously growing such cultures high levels of both kinetin (3 to 6 mg/l.) and IAA (up to 30 mg/l.) were needed. For induction of buds the IAA level

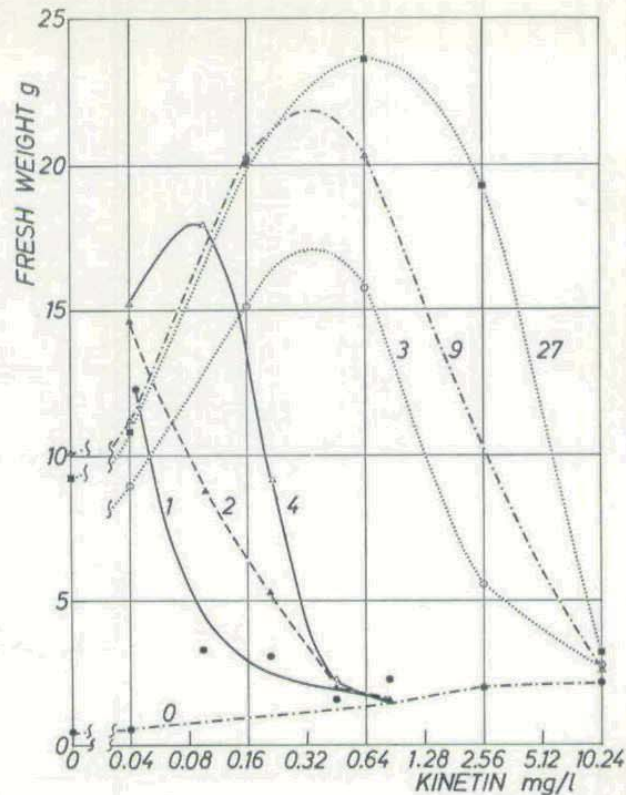


Figure 13. Effects of increasing concentrations of IAA and kinetin on fresh weight yield of tobacco callus cultured on revised medium (Growth period, 4 weeks, Aug. to Oct. 1960). Ordinate: average fresh weight per flask, abscissa: kinetin concentration. The numbers on the curves 0, 3, 9, 27 and 1, 2, 4 correspond to the concentration of IAA added in each case. Results are from two experiments represented by solid and broken lines respectively.

must be kept relatively low, between 1 and 5 mg/l., depending on the kinetin level.

Naphthaleneacetic and 2,4-dichlorophenoxyacetic acids have been tested as substitutes for IAA. In tissues with high IAA inactivation rates they were preferable if not essential for growth. Levels of 0.05 to 0.2 mg/l. have been satisfactory for the growth of callus. These substances are so much stronger or longer lasting auxins than IAA, that all but extremely low concentrations prevented bud formation.

Re-test of the tobacco leaf extract. In accordance with the objective to insure an adequate supply of minerals in tissue culture bioassays of organic growth promoting substances in complex extracts, the stimulating effect of tobacco leaf extract was now re-tested with the revised medium. The extract again promoted growth (Figure 15), but in contrast with the earlier tests on the basal medium, on the revised medium the ash of the extract was without significant effect. For example, in one experiment the fresh and dry weight yields on the revised medium alone were 13.6 and 0.43 g/flask respectively; with 3.75 g/l. T-3 extract they rose to 21.7 and 0.58 g/flask respectively; whereas the ash of this extract gave only 14.6 and 0.46 g/flask respectively.

The promotion of growth by the T-3 extract in this case, therefore, can be attributed to its content of organic substances rather than inorganic salts.

Effect of volume of medium. Further experiments revealed tissue yield to

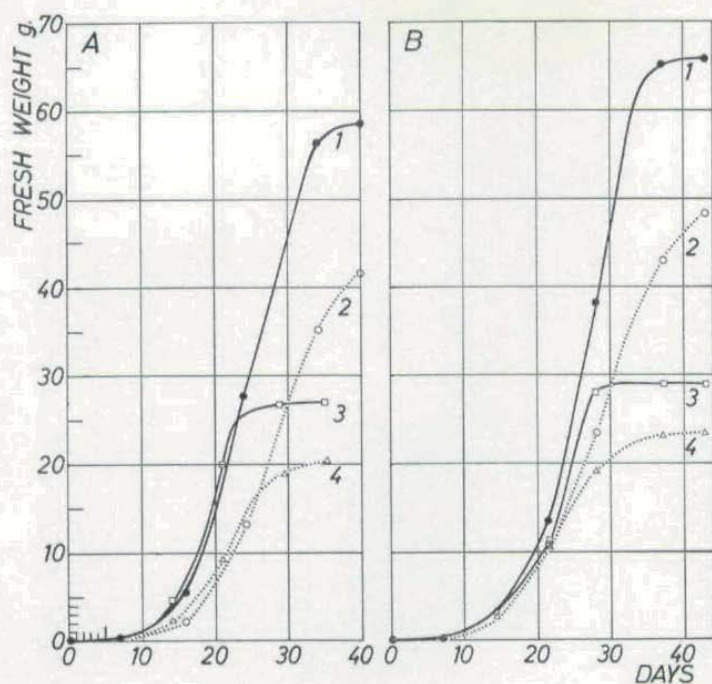


Figure 14. Effects of the volume of revised medium and of the addition of tobacco leaf extract on fresh weight yield.

A. Tobacco pith sections (planted Oct. 1, 1959).

B. Tobacco callus (subcultured Sept. 29, 1959).

Curves 1 and 2, 100 ml of medium in 250 ml Erlenmeyer flasks.

Curves 3 and 4, 50 ml of medium in 125 ml flasks.

Curves 1 and 3 with T-3 extract, 3.75 g solids/l of medium.

Curves 2 and 4 without T-3 extract.

be related not only to extract concentration, but also to the volume of medium used. It can be seen in Table 8, that at least at the higher extract levels, the yield was almost directly proportional to the volume of the medium. More important, yield differences between extract levels were pronounced in the 100 ml. volumes, much less in the 75 ml. volumes and only slight in the 50 ml. volumes of medium. Yields of tobacco tissue was increased from 30 to 60 g/flask by adding the optimum extract level and using 100 instead of 50 ml. of medium per flask. Figure 14 reveals, furthermore, that the tobacco extract enhanced the rate of growth of tissue, whereas increase in medium volume essentially prolonged the logarithmic growth period. It may be concluded that stoppage of growth is due to exhaustion of the entire medium, perhaps mainly the water (see figure 16). The greater final fresh weights of cultures with extract may in fact be due in part to the earlier absorption of most of the available water by the tissue and consequent lower loss by evaporation from the medium.

Although the fresh and dry weights increased in about the same manner, it is clear from the data in Table 8 as from other experiments that the dry weight continues to increase with the concentration of extract considerably beyond the level which is optimal for increase in volume of the tissue. The extent to which this additional increase in dry weight reflects biosynthesis of new cell materials or merely continued absorption of solids from the medium has not been determined.

Attempts to replace the leaf extract by known substances. Optimal fresh weight yields on 50 ml. volume of revised medium often have approached but rarely exceeded 25 g/flask, whereas with leaf extract added under otherwise the same conditions yields went over 30 g and once reached 35 g/flask.

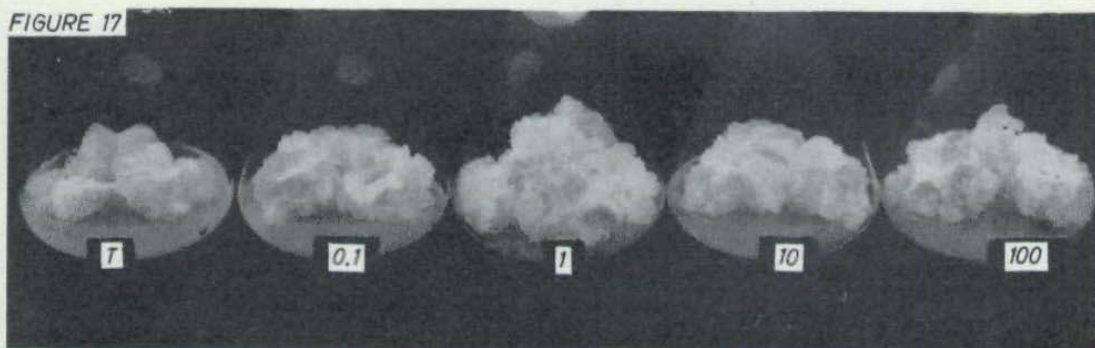
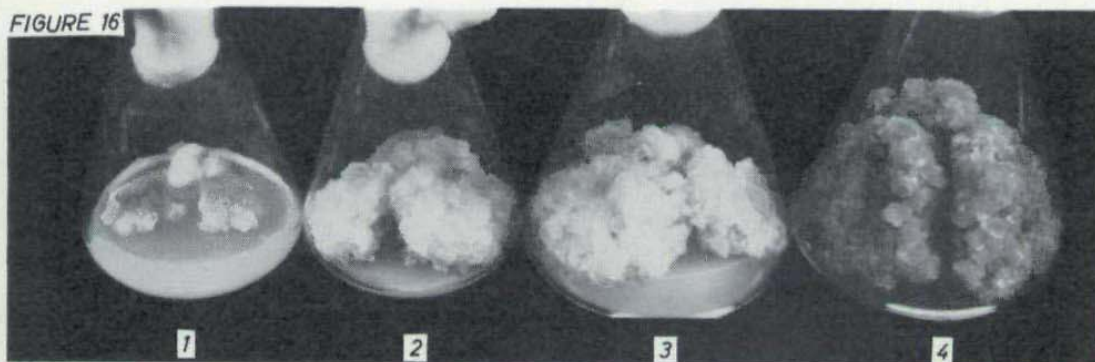
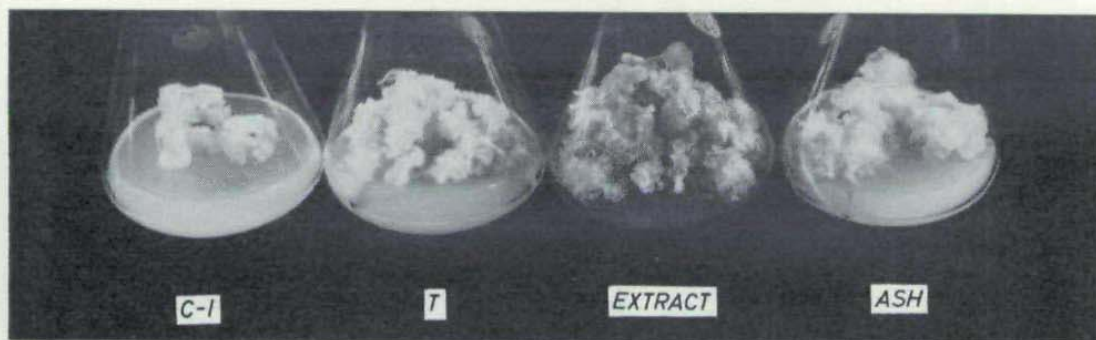


Figure 15. *Effects of tobacco leaf extract and its ash content on the growth of tobacco callus tissue* (Growth period, Sept. 3 to Oct. 2, 1959). From left to right: Basal control, revised medium alone, with T-3, 3.75 g/l, and with ash from the corresponding amount of T-3.

Figure 16. *Cultures illustrating growth and utilization of media of different composition and volume.* (Growth period, July 2 to 30, 1959.) Flask 1, basal medium 50 ml; flask 2, revised medium 50 ml; flask 3, revised medium 100 ml; and flask 4, revised medium with leaf extract 100 ml.

Figure 17. *Effect of concentration of gibberellic acid* (Growth period, Oct. 29 to Nov. 19, 1959). Numbers denote mg/l.

Table 8. *Effects of concentration of extract and volume of medium on tissue yield on revised medium (Growth period, 7/2-7/30/59).*

Volume ml/flask	T-3 Extract (solids, g/l)				
	0	0.9	1.9	3.8	7.5
	Fresh weight g/flask				
50	25.7	24.7	24.3	30.7	28.5
75	31.0	30.3	32.3	47.9	47.9
100	32.0	39.4	51.1	58.5	54.2
	Dry weight g/flask				
50	0.65	0.67	0.70	0.77	0.86
75	0.90	0.82	0.87	1.17	1.36
100	0.93	1.08	1.31	1.41	1.55

With larger volumes of medium the differences in yield in the presence and absence of leaf extract were still more marked (see Table 8). About the same can be said for dry weight yields.

As a preliminary step in diagnosing the stimulatory effect of the extract various substances have been tested which are known to enhance the growth rate of tissue cultures.

"*Braun's supplements*" (cytidylic acid 200 mg/l., guanylic acid 200 mg/l., L-asparagine 500 mg/l. and L-glutamine 500 mg/l.: private communication) in some cases where the controls were relatively low, gave up to nearly 50 % increase in fresh weight but never more than between 25 and 30 g/flask. Furthermore, there were no corresponding increases in dry weight, so that apparently these addenda influenced mainly the rate of water uptake and cell expansion.

Gibberellic acid added to the basal medium (X) resulted in little or no growth stimulation. In contrast, with the revised medium cold-sterilized gibberellic acid markedly increased the growth rate. An example of the effect of increasing concentrations is shown in figure 17. In this case 1 mg/l. appears to be optimal, but generally perhaps higher levels may be preferable. As shown by the data in table 9 the gibberellin effect, except perhaps of very high levels, in these experiments was entirely on fresh weight. It was most

Table 9. *Influence of gibberellic acid on yield of tobacco callus tissue (Growth period, 10/29-11/27/59).*

GA mg/l	Yield, g/flask	
	Fresh weight	Dry weight
0	15.5	0.55
0.1	26.8	0.53
1.0	27.7	0.53
10	26.3	0.56
100	25.3	0.64

noticeable in the early stages of growth. In some cases where the tissues did not quickly exhaust the available medium and were kept for 8 weeks or longer the controls seemed to catch up with the treated cultures.

In pith cultures supplied with gibberellic acid, kinetin and IAA singly and in combinations it was clear that neither gibberellic acid nor kinetin alone, or in combination of the two, stimulated growth. In contrast, IAA alone of course gave rise to marked tissue expansion which effect of IAA was enhanced by gibberellic acid. IAA together with kinetin permitted continuous growth of the tissue and the rate of this callus development was speeded greatly by the further addition of gibberellic acid. Thus, on a suitable medium, the three substances in combination exerted growth stimulatory effects which readily could be distinguished each one from the others.

With the addition of all the above mentioned organic materials to the revised medium, rapid early growth rates comparable with those on medium with leaf extract added have been obtained, but so far the final yields, especially the dry weights, have been lower. In spite of this it is possible that the effect of the leaf extract may be accounted for in terms of its contents of gibberellins, purine derivatives and other known constituents. In fact, it now seems unlikely that its growth promoting activity is due to an unknown growth factor of major importance, although this possibility is not entirely excluded by the present results.

Discussion

The use of tissue cultures as a tool in problems of inorganic nutrition of plants has been much advocated but so far for several obvious reasons has proved fruitless in practice. Their use for the detection and study of biologically active organic substances, natural and synthetic has been more purposeful. It is clear, however, that biological activity of any one substance not only varies with the dosage but depends greatly on the milieu in which it is placed. Conclusions as to the presence or function of a particular substance on the bases of growth responses must be drawn with caution. In bioassays for growth factors practically an all-or-none response is needed as evidence of a new active agent. Most claims for a new factor based solely on quantitative increases in yield or growth rate from the administration of unknown mixtures of materials have no validity and limited usefulness. Even in cases of unknown factors supplied in mixtures or in unknown amounts, very large differences between treatments and controls may be required for significant results. The medium specified in table 6 has been designed to minimize the response of the tobacco tissue employed to variations in its constituents and to inorganic salts, sugars, etc. commonly present in tissue extracts. A positive growth response, therefore, is likely to reflect the presence in the medium of growth promoting organic substances. This medium has provided for excellent growth of either tobacco callus or excised pith tissue under ordinary environmental conditions. Replicate cultures within a given treatment of an experiment were astonishingly alike in size and external appearance. The reproductibility of results from one experiment to the next also has been satis-

factory, but some variability was observed in total yields and in morphogenic patterns in response to a given treatment. At least in part this variability can be ascribed to differences in environmental conditions and treatment of the stock cultures.

The reported optimal yields were obtained with the temperature of the culture room kept between 26 and 28°C., but the temperature was not carefully controlled nor was its influence systematically studied.

The cultures were kept on tiers of ca 1 meter wide shelves open only on one side, so that they were exposed to continuous weak and somewhat variable diffuse light from fluorescent tubes and Mazda lamps mounted on the ceiling. It has been shown earlier that growth and organ formation in response to IAA and kinetin treatments are obtained in darkness as well as in the light, but light is not without modifying effects. In general weak diffuse artificial light is employed. With increase in its intensity the tissue becomes more compact, the fresh weight tends to decrease and eventually also the dry weight is lowered. More uniform responses might be obtained by close regulation of the light intensity. It is likely also that more rapid and uniform growth might be obtained through suitable choices of light quality and especially of day length.

Variability in results also derives from disuniform planting material. Stock cultures of different age or grown on media with different levels of IAA or kinetin, for example, give sub-cultures which differ both in growth rate and yield. A standard pretreatment of stock cultures is needed but difficult to specify in advance, as it will depend on the type of growth desired and will vary with conditions and changes in the stock itself. Requirements of inorganic nutrients probably remain relatively fixed, but the need for organic factors may fluctuate widely. This is strikingly illustrated by the sporadic appearance in control cultures of tissue pieces which have the capacity to grow in the absence of IAA or kinetin and occasionally without either of these in the medium. That increased rates of biosynthesis is responsible for this behavior is well known in the case of auxin from the work of Gautheret (1959) and collaborators; and E. J. Fox in our laboratory recently has shown a relatively high kinin content in one tissue which grew without kinetin. Acquired capacity for increased rates of synthesis of both auxin and kinin is of course also known from Braun's studies of crown-gall in tobacco.

A significant case of acquired, graded capacity of tissue development is the report by Chouard and Aghion (1961) that stem segments from the apical region of a flowering tobacco plant produced buds with flowers *in vitro* whereas segments from the basal part of the stem produced only vegetative buds. Acquired differences in accumulative or biosynthetic capacities must be responsible for this difference in behavior. It is especially noteworthy that in this case the change in morphogenetic capacity is associated with normal ontogeny. It is not brought about by an exogenous agent in the same sense as is the case in tumor induction; nor is it an act that at present would find respectable status in the category of so called "biochemical mutations".

In the light of the above considerations it is to be expected that tissues even from the same plant may have different quantitative nutrient requirements for optimal growth *in vitro*, and that these may change with time and conditions. In fact it is surprising that cultures from all parts of the plant kingdom

apparently have rather similar requirements, and that often their growth is limited by one or a few of a small group of common growth factors, as for example by auxin or vitamin B₁.

The positive response of the tobacco tissue to gibberellic acid in the presence of the other growth factors on the revised but not on the basal medium is of special interest in showing the futility of attempts to classify tissues of various origin in terms of their requirements for a particular growth factor when cultured on a medium of unknown general adequacy for their growth.

During the five years this work has been in progress the medium in different stages of its revision has been used for cultivation of various species and strains in our and other laboratories. The results have varied from very good growth to no growth at all. In no case have yields approached those for the usual tobacco tissue. It seems that the medium must be modified to accommodate the needs of any given tissue. Furthermore, several passages may be required for the tissue to adapt to high nutrient levels.

The organic addenda in this connection may serve not merely to provide specific growth factors but also in part to buffer against excessive electrolyte activities. Complex formation by amino acids is mentioned as an example of this which might be important especially in early stages. In the course of its growth the tissue itself undoubtedly synthesizes and releases metabolites which not only react with specific constituents but may be said to modify the general physical and physiological properties of the medium.

The mechanisms which enable the tissue to derive nourishment for persistent growth, representing doubling in weight on the average every 2 days, for a 3 to 4 weeks period, are unknown and difficult to imagine. The completeness of the incorporation of water from the revised medium into tissue, especially in the presence of leaf extract, would appear to be a "new" and remarkable phenomenon. In cases of the highest yields, nearly 35 g. fresh weight on 50 ml. and 70 g. on 100 ml. of medium, the dry weights were ca. 0.9 and 1.5 g. respectively. Therefore, about 70 % of the water furnished originally was incorporated into living material and most of the remaining 30 % was lost by evaporation and transpiration during the four week growth period. As compared with this, high yielding dense suspensions of algae have been recorded to utilize about 10 to 15 % of the water available in the nutrient solution for the production of cells. It may be seen in figure 16 that very little medium remained in the 50 ml. revised nutrient solution and practically none in the 100 ml. revised medium with added leaf extract. The tissues must make intimate contact with the agar surface. In fact, breaks occur in the agar itself, and flakes adhere to the tissue as it "burrows" through the medium. Oxygen availability would appear to be no problem in these vigorously growing cultures. During the later stages of growth the osmotic forces required for transfer of water must be huge, *i.e.* of the same order as develop in root systems.

Summary

A several fold increase in yield of excised pith or callus cultures of *Nicotiana tabacum*, variety Wisconsin 38 was obtained by addition of leaf extract to the standard modified White's nutrient medium.

In part the increase was due to inorganic constituents of the extract, especially N and K, which could be substituted for by either the ash of the extract or raised levels of N and K salts in the medium.

In part the increase was due to organic constituents of the extract which increased both the growth rate and final fresh weight of the cultures.

Similar increases in growth rate but not in final yield were obtained by adding gibberellic acid and Braun's supplements of purines and amino acids to a revised medium.

A revised medium has been developed (Table 6) with each element provided in sufficient quantity to insure that no increase in yield will result from the introduction of additional amounts in the range ordinarily to be expected in plant tissue extracts, etc.

The organic constituents have been retained unchanged except that sucrose has been raised to 3 per cent, *myo*-inositol has been added as a regular constituent and Edamin has been introduced as an optional constituent.

The revised medium is designed for use in bioassays of organic growth factors. It provides for rapid growth rate, increased response to organic growth factors and minimal interference from inorganic and common organic nutrients.

In the presence of plant extracts fresh weight yields of up to 35 g. on 50 ml. of medium have been obtained. Under these conditions water appears to be the limiting factor for growth of the tobacco tissues employed.

Aspects of general application, limitations and behavior of plant tissue cultures are discussed.

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