POTENTIALLY UNLIMITED GROWTH OF EXCISED TOMATO ROOT TIPS IN A LIQUID MEDIUM

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(WITH THREE FIGURES)

Introductory

In the cultivation of isolated tissues, whether plant or animal, it is necessary to distinguish between *growth* at the expense of the culture medium and *survival,* in which the medium plays only an inert or, at most, a secondary rôle. In the first case the nutrient is itself the limiting factor in determining the degree and to some extent the character of the increment obtained. Given a satisfactory nutrient, adequate replacement of exhausted nutrient, and adequate removal of excretory products, growth should continue indefinitely. In the second case increment may also take place for a time at the expense of materials reabsorbed from the older portions of the original explant, the medium acting essentially as an inert substratum only. As these reabsorbed materials are exhausted, the culture will gradually succumb, no matter how often the medium is renewed. An extended period of *growth* ordinarily implies the maintenance of a high level of metabolism. *Survival,* on the other hand, is ordinarily dependent on *minimal* metabolic rates, and is hence prolonged by depressant conditions such, for example, as moderate cold, low oxygen supply, etc.

The literature of the field of plant tissue cultures contains accounts of many experiments which clearly fall in the latter category. Reference need only be made to the work of HABERLANDT, BOBILIOFF-PREISSER, BÖRGER, CZECH, KUNKEL, PRÁT, ÚLEHLA, WHITE, *et al.* This literature has been reviewed more extensively elsewhere (**16**). All of these authors obtained *survival* of isolated tissues under various conditions, often for months, but no true growth. Perhaps the most striking example of this type of result is that of BAILEY and ZIRKLE (**1**) who, using sections of cambium from woody plants immersed in such non-nutrient media as paraffin oil, were able to maintain active protoplasmic cyclosis for many weeks. This is obviously a phenomenon of retarded metabolism only, a mere slowing down of the process of dying.

The experiments of KOTTE (**9**), ROBBINS (**12, 13**), ROBBINS and MANEVAL (**14, 15**), MALYSCHEV (**10**), and WHITE (**17–21**) are less easily classified. In many cultures of these workers active increment did take place, and in the work last cited the nature of the nutrient obviously did contribute quite largely in determining the extent and character of growth obtained. Only comparatively short culture periods were employed, however, and the absolute amounts of increment were small. It therefore is possible that this

increment may also have been dependent in part on materials carried over in the original fragment. The experiments of ROBBINS and MANEVAL (**15**), which represent the most extended periods (five months) and the greatest numbers of passages (ten) reported to date, strongly suggest that this was the case, since the observed mean growth rates decreased rather consistently from about 7.8 mm. per day in the first passage to 0.86 mm. per day in the tenth passage, after which the cultures were lost. Thus the growth rate at any passage bore an inverse relationship to the total amount of tissue formed up to that passage. This would appear to indicate that the rate of increment might have been determined by the concentration of some unknown material furnished to the culture from the tissues of the original fragment and gradually diluted in the multiplication of these tissues. If this be true, these cultures also were cases of *survival,* though for long periods. There are nowhere in the literature of plant tissue cultures accounts of any experiments which can with certainty be said to fall within the first category outlined above.

In view of this uncertainty and of the importance of the concept of *complete nutrients* in the use of a tissue culture method in the study of physiological and pathological problems, it has seemed desirable to determine whether or not a plant tissue can *grow* at the sole expense of a nutrient, without the *quantity* of material contributed by the original fragment entering as a limiting factor. A positive answer to this question will require that such a tissue be maintained in a state of active increase over a long enough period of time and through a sufficient number of passages to insure a dilution of all materials contained in the original fragment beyond the point where they could possibly be effective in determining growth rates. Such has been the purpose of the experiments presented in this paper.

Materials and methods

The present work represents essentially a repetition and extension of the earlier root tip cultures of ROBBINS (**9, 10**) and ROBBINS and MANEVAL (**11, 12**). Tomato was chosen as a test organism, since it was planned to use the tissue culture technique later in the study of certain diseases to which this plant is susceptible. It is assumed that wheat (**18, 19**) would have given similar results.

The methods of culture were approximately those outlined elsewhere (**18, 19**). The seed to be used were removed under aseptic conditions from clean healthy fruit of the Bonny Best variety, grown under greenhouse conditions. They were usually germinated on sterile filter paper. As soon as the roots had attained a length of 5 mm., and some days before the cotyledons appeared, they were severed and placed in 125-ml. Erlenmeyer flasks containing 50 ml. of nutrient each. A single root was placed in each flask.

The nutrient medium used was approximately the same as that developed in previous work (**19**), with some slight modifications. It contained the following salts at the partial concentrations indicated:

SALT	MILLIMOLS
$Ca(NO_3)_2$	0.60
$MgSO_4$	0.30
KNO_3	0.80
KCl	0.87
KH_2PO_4	0.09
$Fe_2(SO_4)_3$	0.006

To this were added 2 per cent. by weight of sucrose, which for tomato was found experimentally to be superior to the dextrose used in previous experiments (**18, 19**), and the filtered extract of 0.01 per cent. by weight of dried brewers' yeast (**18**).

In making up the nutrient, the sequence of mixing the various ingredients appears to be important. The method found most satisfactory was to proceed as follows: The first four salts listed above, $Ca(NO_3)_2$, $MgSO_4$, KNO_3, and KCl were dissolved together in one flask. The KH_2PO_4 and $Fe_2(SO_4)_3$ were each dissolved separately. These were then mixed in a quantity of distilled water such as to make one half of the final volume of nutrient desired, and set aside until the yeast was ready, usually about two hours. The sucrose was dissolved in a quantity of water somewhat less than half the volume of the final nutrient and also set aside. The yeast was boiled for half an hour in 100 to 200 times its volume of distilled water, and filtered twice under suction, using no. 597 Schleicher and Schüll (or no. 1 Whatman) paper. The salts, sugar, and yeast extract were then mixed together, made up to volume, and distributed to the culture flasks, 50 ml. per flask. These were autoclaved immediately at 1 atm. pressure for 20 minutes. They were ready for use as soon as cool.

All cultures made previous to October 5, 1933, were placed on a bench in diffuse sunlight in a potting shed subject during the summer months to the temperature fluctuations of the outdoor atmosphere. On October 5th the cultures were transferred to a northeast laboratory where better temperature control was available. Here also they received diffuse sunlight during the daylight hours. The method of making the original cultures has been outlined above. Subcultures were made by cutting the roots into fragments each bearing one or more growing points. Each cutting was placed in a flask of fresh nutrient.

Experimental results

ROBBINS'S experiments suggested the existence of some limiting factor present in the original fragment of tissue, diluted as this tissue multiplied,

not renewed under the cultural conditions employed, and hence fixing a limit to the amount of growth obtainable in such cultures. In examining further into the probability of the existence of such a factor and hence into the probable limits to which such excised root tips may be grown *in vitro,* data from two clones of cultures, designated as B and C, will be considered. Each clone was derived from a single root tip and was therefore genetically homogeneous.

In the preparation of clone B aseptic seeds of Bonny Best tomato were placed in a series of flasks. This series was started on November 2, 1932. Five days later the seed had begun to sprout and on November 11 the seedling roots were severed. The ages given in the following discussion are measured from this date. The apices were retained in the flasks, the seeds and seedlings being discarded. The fragments retained were about 10 mm. long. After some days the most vigorous examples were selected for further culturing. By November 21, that is 10 days after excision, the particular root under consideration had attained a length of 32 mm., with one branch. The primary root soon stopped growing, and the growing point turned brown, as often happened, especially in the earlier cultures. The lateral, however, continued to grow and to branch further. On November 30 the culture was transferred to a fresh nutrient but without cutting back.

A completely satisfactory experimental complex had not been developed at this time. Rather than risk the loss of the culture by transferring again to a nutrient of uncertain value, the flask was set aside while further experiments on methods were being made. The culture was left undisturbed for 145 days until April 24, 1933. At the end of this time the root had attained a total length, including branches, of 4564 mm., with 181 growing points. It was then removed and divided into 50 pieces, each peice being placed in a fresh flask. Twenty of the 50 subcultures were so made as to contain about 8 mm. of apical meristem each, without any mature tissue. These 20 grew much more satisfactorily than did the apices on larger masses of tissue,

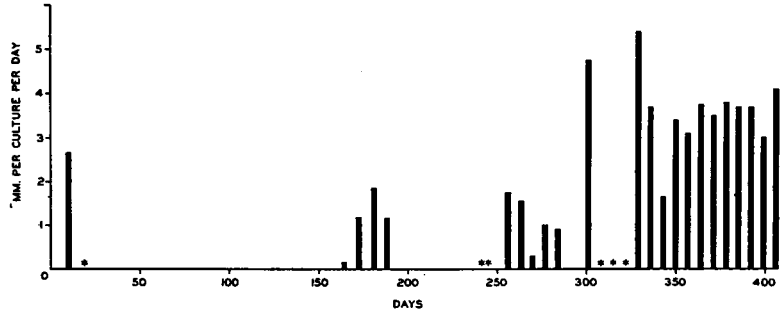

FIG. 1. Increment rates of clone B during 406 days, 29 passages. The asterisks represent passages in which no measurements were taken.

the old tissue apparently exerting a certain inhibitory action. All subsequent cultures were therefore made from these smaller pieces. The cultures grew quite consistently, though not very rapidly (fig. 1), remaining at the bottom of the flasks. On May 2, 11, and 18, subcultures were made, all but about 5 to 10 mm. of meristematic tissue being discarded at each transfer. They were then set aside again until July 10, an interval of 53 days. By this time they were brown, without active growing points, and apparently dead. Nevertheless, upon transfer to fresh nutrient, they resumed growth and put out fresh branches so that by July 14 it was possible to again sever the growing points, discarding all but about 5 mm. of each. Subcultures were made on July 25, August 1, 8, and 15. Up to this time the cultures had remained at the bottom of the flasks, growing only slowly, but branching with great profusion.

The low increment rate and high branching rate of those cultures which remain at the bottom of the flasks appear to be correlated with low respiration rate, but whether the fact of sinking to the bottom is itself the cause or an effect of some unknown condition remains to be determined. The two conditions are concomitant. Of the 50 cultures made on August 15, three floated to the top of the nutrient. These were all derived from no. 17 of the previous (12th) passage, and were designated as nos. 4, 5, and 6. Coincident with the floating of these individuals to the top of the nutrient, their increment rates quadrupled as compared with their previous values, the branching rate showing a corresponding fall. No. 4, for unknown reasons, ceased to grow after a week's time. No. 6 became contaminated with bacteria and had to be discarded. No. 5 remained in good condition and ultimately became the foundation for the entire clone. Although transfers on September 1 (14th passage) included material from 6 examples of the previous passage, all except the descendants of this one actively growing culture were eliminated by selection in the next two weekly passages. Detailed daily records were begun on September 28 in the 18th passage, when the culture was 322 days old, and were continued until it had reached the age of one year. In passages after that date the lengths of the roots at the beginning and end of each passage were recorded, giving a measure of the increment rates for each week, but without the daily detail. Records of the clone were discontinued at the end of the 28th passage, at an age of 405 days, but the clone is, at the time of preparation of this paper, alive and growing normally at an age of over 500 days.

Figure 1 shows the results of observations throughout 406 days of the life of this clone. Following the elimination of the slower growing members, the last 16 passages show a markedly *increased* mean growth rate over that of the previous 9 months. Figure 2 is of a single subculture photographed at the age of 367 days (passage, 12 days) and again 4 days later.

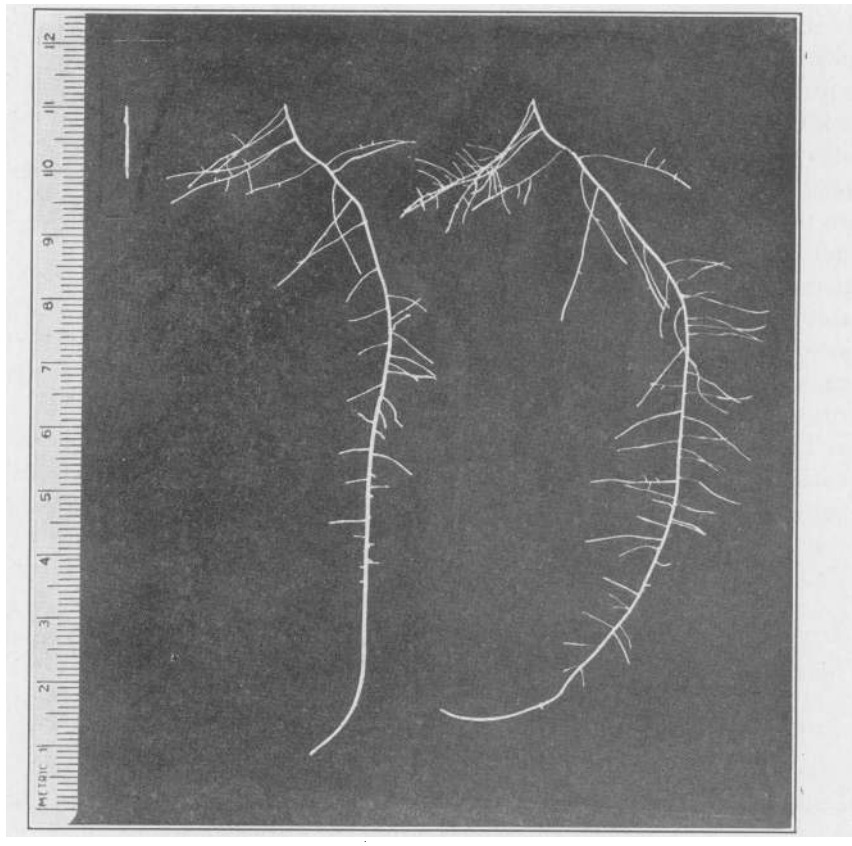

FIG. 2. A typical subculture of clone B, passage 23. The figure at the upper left shows the size of the fragment at the beginning of the passage, aged 355 days. At the center 12 days later, and at the right the same culture after 4 days more.

The increase both in length and in number of branches is evident, and represents what may be considered as approximately normal growth for tomato root tips. The fact that an isolated root tip can continue to maintain approximately normal activity at the end of a year's time in an artificial environment, is good presumptive evidence of its capacity to grow indefinitely under such conditions.

The records kept of clone C are somewhat more complete and continuous. Seeds of Bonny Best tomato were germinated aseptically on filter paper and on March 1, 1933, 25 severed root tips were placed in flasks of a nutrient (not the standard described above) which later proved only partially satisfactory. A single one grew, and this only poorly. On March 13 this one root was transferred to a more satisfactory nutrient in which it grew actively though somewhat unevenly. On March 29 it was divided into

16 subcultures and on April 21 two of these were selected and again divided into 42 and 24 pieces respectively. Neither the nutrient solution nor the culture method was at this time entirely satisfactory and experiments were conducted aiming at their improvement, using the cultures of this clone as experimental material. In the next 7 passages, therefore, the transfer periods and nutrients used were not uniform. With the 11th passage, at an age of 127 days, the nutrient solution and method of preparation outlined at the beginning of this paper were established as standard, and a period of one week chosen for the length of passage. The actual data must therefore be divided into two groups, the first embracing the first 10 passages, the second all passages subsequent to the 10th. The latter group should be much more nearly homogeneous and more reliable as a basis for drawing conclusions. Since at the date of preparation of this paper the clone is still being maintained, so that the data cannot be considered as a completed whole, a period covering 52 passages has been arbitrarily chosen for consideration in detail. The second homogeneous group of data, from the 11th to the 52d passage, thus embraces 294 days under standard conditions. These data are summarized in table I.

TABLE I

LINEAR INCREMENTS AND NUMBERS OF BRANCHES PRODUCED FROM A SINGLE ROOT DURING 52 PASSAGES

PASSAGES	TOTAL OBSERVED INCREMENT	TOTAL OBSERVED NUMBER OF BRANCHES	NUMBER OF PASSAGES	NUMBER OF DAYS RECORDED	MEAN PASSAGE LENGTH	MEAN BRANCHING RATE PER DAY	MEAN INCREMENT RATE PER DAY
	mm.						*mm.*
1–10	14,319	3,044	10	127(–52)*	13	0.385	1.91*
11–17	18,379	3,505	7	49	7	0.716	3.75
18–24	18,759	3,756	7	49	7	0.766	3.83
25–31	26,769	6,582	7	49	7	1.303	5.48
32–38	26,158	6,657	7	49	7	1.383	5.33
39–45	23,084	5,143	7	49	7	1.064	4.71
46–52	27,379	6,912	7	49	7	1.091	5.59
Total ...	154,847	35,399	52	421	.....	0.958	4.37

* Detailed linear increment measurements were not made in the first 2 passages totaling 52 days. For that reason, although the first 10 passages totaled 127 days, the calculation of mean increment rate is based on 127–52, or 75 days only.

In 52 passages (421 days) the clone made a total measured increment, exclusive of all branches, of 154,847 mm. To this are to be added the increments of 35,399 branches 1 mm. or more in length. Since these average at least 5 mm. each, their total length would be about 175,000 mm. The total observed increment for 52 passages was, therefore, about 430 meters, or about 1300 feet. This was all derived from a single growing point approximately 10 mm. long. That a single root-tip can thus be maintained and made to multiply at approximately normal rates through 52 passages, is again strong presumptive evidence that it can be maintained indefinitely.

The details of the data further support this general presumption. In figure 3, A, the mean linear increment rates (exclusive of branches) are plotted as ordinates against the passage numbers as abscissas, while in figure 3, B, the mean numbers of branches formed per culture per passage are similarly shown. Each point on the curve of linear increments represents the mean value obtained from daily observations on 100 cultures over individual passages of 3 to 28 days. Since all passages after the 10th were uniformly 7 days in length, each point for the last 42 passages thus represents the mean of 700 observations. Each point on the curve of branching rates represents the mean number of branches on 100 cultures.

That portion of each curve representing the first ten experimental passages need not be considered in detail. The remainder, covering 42 passages under standard conditions, may be divided into five parts. Passages 10–14 show a steady rise both in increment rate and branching rate believed to have been brought about by gradual selection within the clone. Passages 14–17 are marked by an equally regular and very sharp fall in both indices. The cause of this was found to be lead poisoning from a faultily repaired water still. After this was corrected, passages 17–26 showed a quite regular increase, broken somewhat at the 19th and 20th passages, when transfers were made by one of the writer's colleagues,[1] and at the 24th and 25th passages when drastic culling, carried out in an effort to eliminate a troublesome brown discolored condition present in some cultures[2] resulted in discarding many cultures, thus lowering the average increments as estimated on the basis of the initial 100. Passages 26–39 then showed comparatively uniform growth indices, the mean linear increments oscillating quite regularly between extremes of 4.5 and 6.8 mm. per culture per day. The branching indices fluctuated somewhat more widely and erratically. A second

[1] It is a pleasure to acknowledge the help of Dr. L. O. KUNKEL who undertook the task of making the transfers, and of Dr. E. L. SPENCER who made the necessary measurements during this period.

[2] This condition was of such a nature as to suggest the presence of a bacterial infection. Attempts to find any organism, either by microscopic examination or by plating on various standard bacteriological culture media all failed, however. The incidence of this condition was greatly reduced by repeated selection, but its cause is still unknown.

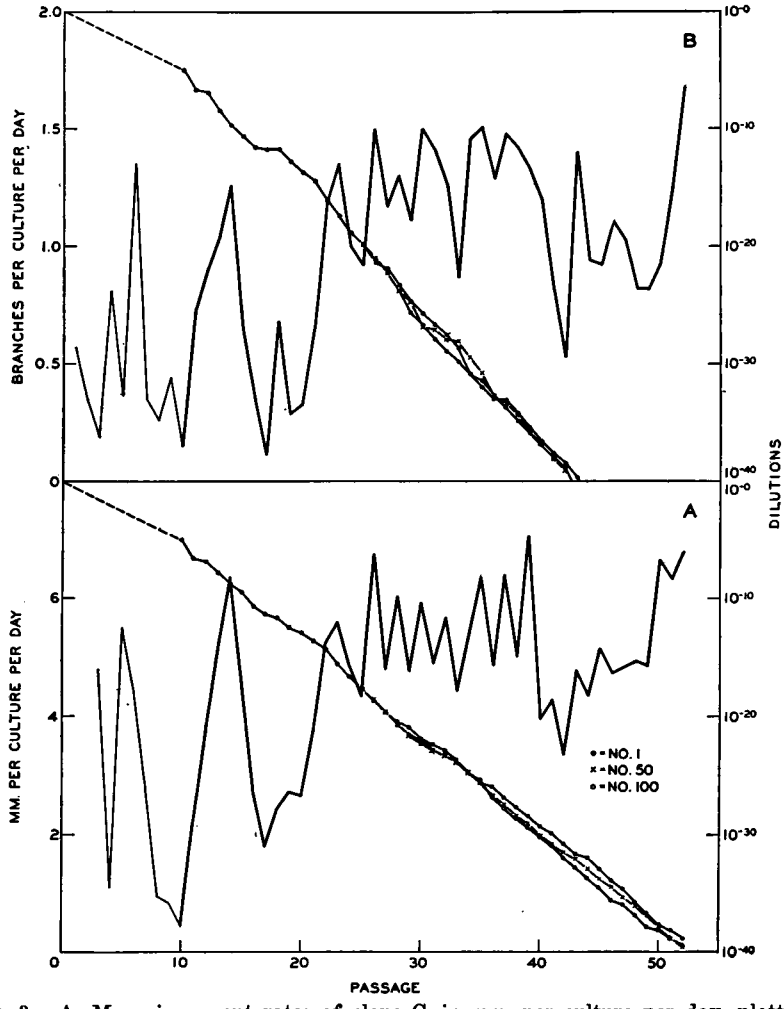

FIG. 3. A. Mean increment rates of clone C, in mm. per culture per day, plotted by passages. The corresponding dilutions of any material from the initial fragment distributed uniformly to all tissues formed are plotted for three individual cultures for comparison. B. Branching rates of clone C in numbers of branches formed per culture per day. The corresponding dilutions of any material from the initial fragment distributed uniformly to all growing points are plotted for the three examples as in A.

sharp drop in indices occurred in the 40th passage. The reason for this has not yet been satisfactorily traced, but the fact that both indices have subsequently recovered makes it clear that the drop was due to some uncontrolled external factor. The character of growth obtained at the end of the experiment was quite normal, the roots being clean, white, straight, with normal branching habit, and growing at rates which were not markedly sub-

normal. Individual cases of roots growing 30 to 40 mm. per day were fairly frequent. The factors which affect growth rates will be discussed more extensively elsewhere. The important fact brought out by these curves is that, unlike ROBBINS's cultures, the changes in growth rates can in no way be correlated with number of passages and can therefore *not* be interpreted as resulting from a time or dilution factor. This is perhaps even more clearly brought out by a comparison of clones B and C from the point of view of the relative effects of age and previous treatment. Clone B was approximately 200 days old in the 6th passage, while clone C had a similar age in the 21st passage. The linear indices at this age were for clone B 0.7, for clone C 3.7 mm. per culture per day. On the other hand, 134 days later the *simultaneous* indices were: clone B (18th passage) 5.3, clone C (23d passage) 5.6 mm. per culture per day. It is obvious from these figures that neither age nor member of passages, but simultaneity (and hence identity in environmental conditions) was the crucial factor in determining growth rates. It is the precise details of the environmental complex rather than any characteristics, other than genetic ones, inherent in the material of the explant, which determine the rates of growth at any moment.

Two strains of cultures have then been maintained for more than a year, one through 52 passages and the other through 30 passages. The measured tissue increment has been many thousands of times the original value. The mean growth rates have been clearly independent of origin, age, number of passages, previous treatment, etc., but closely dependent on the detailed variants in the artificial environmental complex. Hence it may be concluded with reasonable safety that the cultures have ceased to be dependent on any material factors carried over from the parent plant. The environmental complex used is therefore adequate for all requirements for growth of such tissues. The cultures have been shown to be capable of unlimited growth in such an environment.

The conclusion just drawn, that isolated root tips can be grown indefinitely under the experimental complex employed, may at first seem acceptable without further proof. Nevertheless, the data in the form presented above still leave a logical doubt as to its validity. The dilution of materials derived from the original explant, calculated as the ratio between initial tissue volume and total observed increment, has, even in clone C, been only of the order of $10/150{,}000 = 6 \times 10^{-5}$. It is well known that many materials: hormones, enzymes, viruses, etc., are effective at much greater dilutions than this. Even some of the essential nutrient elements, such as boron, become toxic above concentrations of the order of molar $\times 10^{-5}$. Copper, zinc, iodine, etc., are effective in even smaller quantities. It is hence quite conceivable that even at a dilution of 10^{-5} there may still remain sufficient quantities of limiting materials carried over from the original frag-

ment to be effective, although complete elimination of these materials would prevent further growth. In order to remove this doubt, it is therefore necessary, as stated in the introduction to this paper, to answer the question: "Can it be shown that the greatest possible crucial dilution for all conceivable materials of the original fragment must have been surpassed in the experiments under consideration?" This question has certainly not been answered in the foregoing section.

The experimental data do, however, furnish an affirmative answer if treated in a manner slightly different from that used above. If the pedigrees of three representative cultures of the last (52d) passage, nos. 1, 50, and 100, are traced back, it is found that all three were derived from culture no. 99 of passage 28, and further back from culture no. 57 of passage 11 when the standard environmental complex was introduced. Their histories from passage 11 to passage 28, through 17 passages, were thus identical. The detailed records show that this initial culture of the 11th passage, no. 57, was, when started, 7 mm. long, growing during the passage to a length of 36 mm. In passage 12 it was initially 11 mm. long, growing to 65 mm., the corresponding figures being 14 and 67 mm. for passage 13. The dilution of any material derived from this initial culture and uniformly distributed to all tissue formed therefrom during these three passages would thus be $7/36 \times 11/65 \times 14/67$, or 7×10^{-3}. The dilution obtained in this way for the 17 passages in which the history of the three examples was identical was about 3×10^{-12}, and the final dilutions for the three at the end of the 42 passages under standard conditions (52d passage) were 1.5×10^{-34}, 4.5×10^{-36}, and 5.3×10^{-36}. If, instead of considering the increment in volume of tissue, the numbers of growing points formed are taken as a measure of the dilution, the values obtained are still higher. During passages 11 to 15 the numbers of branches formed were 4, 8, 3, 22, and 19, giving a dilution of 2.5×10^{-5}. The final dilutions based on numbers of branches formed would then be 1.9×10^{-45}, 2.3×10^{-46}, and 1.6×10^{-45}. None of these figures include any of the dilution occurring during the first 10 experimental passages, which is roughly estimated to have been of the order of 10^{-5}. It is thus evident that whether this hypothetical limiting substance be considered as distributed to all tissues or only to growing points, its "dilution" at the end of the 52 experimental passages would be not less than approximately of the order of 10^{-40}.

Now, the pieces of tissue employed in starting each subculture weighed on the average about 2 mg. or 2×10^{-3} gm. fresh weight. A molecule of water has a mass of approximately 3×10^{-23} gm. If the root tips used as subcultures be considered to be made up of water only, such a piece would thus contain approximately $2 \times 10^{-3}/3 \times 10^{-23} = 0.67 \times 10^{20}$ molecules. If this be diluted as has been shown to have been the case in the cultures under

consideration to a value of the order of 10^{-40} times its original value, it is evident that there could still be only $10^{20} \times 10^{-40} = 10^{-20}$ part of one molecule of the material of the original root tip present in each culture of the 52d passage. This result obviously represents a *reductio ad absurdum,* and since the increment rate (fig. 3, A) at the end of the 52d passage has been equalled in only two previous passages, nos. 26 and 39, and the branching rate (fig. 3, B) is higher than at any previous time in the entire 400 odd days, the idea of any limiting factor in the form of a "hormone" or other material furnished to the culture from the parent plant is untenable. The results of the experiments of ROBBINS and MANEVAL, which seemed to indicate the existence of such a factor, must be explained in some other way.

This conclusion, of course, in no way affects the possibility of hormones being manufactured by the root itself. But if such exist they are not factors limiting the potential amount of growth obtainable in culture, and hence of no particular interest in the problem at hand. Moreover, so long as all the observed facts in a given series of experiments are, as in the present case, explainable without resorting to such hypotheses, and since the existence of such hormones can be demonstrated only by the behavior of cultures in their absence—a condition not demonstrably obtained in these cultures—the introduction of such hypotheses appears to the author to be an unnecessary encumbrance.

It appears, then, to have been demonstrated that the nutrient and the environmental complex employed in these experiments are adequate to sustain normal growth of such root tips indefinitely.

Discussion

It is important to note that in thus isolating a plant root from the parent plant and placing it in an artificial nutrient which is demonstrably adequate for its continued growth, a method is presented for an analysis of those factors by which the parent plant under natural conditions nourishes and controls this organ. It has been generally supposed, though without experimental demonstration, that the reciprocal relationship by which the aerial portions of the plant are supplied with salts and water by the roots and these in turn are supplied with the products of photosynthesis by the tops, was one necessary for the continued well being of both. The exact form in which these photosynthetic products were furnished to the roots has remained in doubt. It has also been doubtful just where the seat of synthesis of proteinaceous materials lay, whether in roots or aerial organs, and whether this process might not likewise be dependent on photosynthesis.

The present investigation has brought answers to some of these questions and furnishes a means not previously available of obtaining data pertinent to others. The interdependence of top and root is *not* a necessary one, at

least as far as the root is concerned, since these roots have shown unmistakably that they can be grown indefinitely without ever, even as seedlings, having been under the influence of tops. The artificial medium has provided adequate substitutes for whatever products the tops ordinarily send down to the roots. The photosynthetic product has been replaced by sucrose. The salts supplied have been those used for entire plants and, as was to be expected, their assimilation is quite independent of green tissues. The proteins required have evidently been manufactured in the roots, from the elements supplied in the nutrient. There remains, aside from possible traces of impurities, only one factor in the environmental complex supplied which is recognized as an unknown, namely, the extract of dried yeast. Whether this is itself replaceable by more easily analyzable materials remains to be seen.

It is interesting to compare the nutrient used in these experiments which has proved adequate to supply all the needs of a plant tissue, with the corresponding nutrients used for similar cultures of animal tissues. A great part of the work on the latter material has followed the methods introduced by HARRISON (8) and developed by CARREL. These methods will, therefore, be considered for comparison. In the work outlined above, the nutrient has consisted of three categories of materials: (1) the salt solution, (2) the carbohydrate, and (3) the organic material added as yeast extract. Of these, the exact constitution of the first two, representing 99.99 per cent. of the mass of the nutrient, is, aside from possible impurities, precisely known and can be varied at will. Only one component, the yeast extract, representing 0.01 per cent. of the entire mass, is of unknown constitution.

The nutrient generally used by CARREL (see CARREL, EBELING, FISCHER, ERDMAN, *et al.*), on the other hand, contains *no salt solution* and *no carbohydrate*. In its usual form it consists of two components, chicken serum making up from 50 to 70 per cent., and embryonic juice from 30 to 50 per cent., of the mass of the nutrient. A satisfactory modification used by EBELING (5) contained fibrinogen (prepared according to MELLANBY, 11) 12.5 per cent., chicken serum 37.5 per cent., and embryonic juice 50 per cent. Dry weights are not usually given for these constituents, so that they cannot be directly compared with the constituents of the plant culture nutrient. But it is clear that the animal nutrient differs radically from the plant nutrient in being made up entirely of complex organic materials. DREW (4) has replaced the serum of CARREL's medium with a salt solution consisting of NaCl, $CaCl_2$, KCl, $NaHCO_3$, $MgHPO_4$, and $CaH_2(PO_4)_2$, and has replaced the embryonic juice with autolyzed tissue extract or tumor extract, with satisfactory results. In this case, the known elements are somewhat greater, but still represent less than half of the mass of the entire nutrient. It has also been shown that the embryonic juice can be replaced

by a fibrin digest prepared either by the action of pepsin (BAKER and CARREL, **2**) or by controlled bacterial digestion (FISCHER and DEMUTH, **7**). These represent, to a certain extent, attempts to reduce the medium to simple components which can be adequately analyzed. But even in the case of DREW'S salt solution it should be noted that, although the cations Na, Ca, K, and Mg are supplied, there has been no attempt to supply a balanced *anion* portion, the SO_4 and NO_3 ions being entirely omitted, and the PO_4 and CO_3 ions introduced only because of their buffer action. The other salt solutions occasionally used in such work: RINGER'S, TYRODE'S, etc., are, like DREW'S solution, made up of chlorides only, with PO_4 or CO_3 or both added only as buffers. Such unbalanced salt solutions, as has been experimentally demonstrated elsewhere (**19**), would be entirely inadequate for plant tissues.

It is, of course, difficult if not impossible to determine the exact constitution of such complex, essentially organic nutrients, while the determination of the constitution of the simple, essentially inorganic nutrient used in these plant cultures is comparatively simple. It is equally impossible to vary the concentrations of the active constituents, except in a very gross way. So long as such complexes as blood serum and embryo juice make up the greater part, or all, of the nutrient, the only nutrient variants available to the experimenter are varying proportions of these complexes. CARREL, BAKER, and others have made analyses which indicate that the proteoses play an important rôle in the activity of embryo juice, but these are again complex substances of undetermined constitution (FISCHER, 6). The complex nutrients have been retained because they fulfilled the two basic requirements of (1) complete adequacy for maintenance of unlimited growth, and (2) simplicity and duplicability of preparation. Perhaps if such a natural nutrient had been found in the early development of plant tissue cultures, it too would have been retained and used without further search. The latter field has, however, had a history quite the reverse of that of the animal field. Every attempt to find a natural culture medium comparable to that of CARREL, prepared from embryo juices, pressed saps, sieve tube exudates, endosperm fluids, digests, etc., has consistently failed with, in most cases, marked evidence of toxicity (see HABERLANDT, THIELMANN, ÚLEHLA, PRÁT, ROBBINS, MAYER, and others). Because of this failure, the workers in the plant field have been forced to adopt the reverse method, of starting with a simple medium of known constitution and, by a study of the individual constituents of this medium in their effects on growth and by the addition of new constituents, to build up an increasingly adequate, and at the same time precisely known, substratum and general complex for the maintenance of the cultures. The result has been a cultural complex which, as shown in the present paper, possesses both of the essential char-

acteristics of the animal complex, complete adequacy for the maintenance of unlimited growth, and simplicity and duplicability of preparation, and which in addition, as shown elsewhere (**18, 19**), possesses a flexibility far greater than that of the complex used in the older field.

Summary and conclusions

Tomato root tips, excised, and grown in a simple liquid medium of known constitution, have been maintained for over a year, and through 52 passages, in continuous active growth as measured by increase in number of growing points and total volume of tissue. One isolation has produced *in vitro* approximately 35,000 growing points and more than 400,000 mm. of tissue, from an initial fragment 10 mm. long. Calculations based on averages from approximately 40,000 separate observations on this clone give the theoretical dilution of any material of the original fragment as well beyond 10^{-40}, hence beyond the limits of molecular dilution. The entire material of most, if not all, of the ultimate cultures is therefore necessarily derived from the nutrient. This nutrient is thus demonstrated to be adequate for *all* growth requirements of such tissue. The growth rates have shown no consistent tendency to fall off, there being, on the contrary, a consistent increase in the early passages from 1.5 mm. per culture per day in the 1st to 6.8 mm. in the 26th passage, the corresponding value at the 52d passage being also 6.8 mm. per culture per day. It is thus proved that tomato root tips are capable of maintaining an independent, active, apparently normal existence *in vitro* for potentially unlimited periods of time, under such an artificial environmental complex as has been described here.

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LITERATURE CITED

1. BAILEY, I. W., and ZIRKLE, C. The cambium and its derivative tissues. VI. The effects of hydrogen ion concentration in vital staining. Jour. Gen. Physiol. **14**: 363–383. 1931.
2. BAKER, LILLIAN E., and CARREL, A. The effect of digests of pure proteins on cell proliferation. Jour. Exp. Med. **47**: 353–370. 1928.
3. CARREL, A., and BAKER, LILLIAN E. The chemical nature of substances required for cell multiplication. Jour. Exp. Med. **44**: 503–521. 1926.
4. DREW, A. H. A comparative study of normal and malignant tissues grown in artificial culture. Brit. Jour. Exp. Path. **3**: 20–27. 1922.

5. EBELING, A. H. Fibrin and serum as a culture medium. Jour. Exp. Med. 33: 641–646. 1921.
6. FISCHER, A. Gewebezüchtung. Handbuch der Gewebezellen *in vitro*. 3 Ausgabe. Müller and Steinicke. München. 1930.
7. FISCHER, A., and DEMUTH, F. Eiweissabbauprodukte als wachstumsfördernde Substanzen. Arch. exp. Zellf. **5**: 131–142. 1928.
8. HARRISON, R. G. Observations on the living developing nerve fiber. Proc. Soc. Exp. Biol. and Med. **4**: 140–143. 1907.
9. KOTTE, W. Kulturversuche mit isolierten Wurzelspitzen. Beitr. allg. Bot. **2**: 413–434. 1922.
10. MALYSCHEV, N. Das Wachstum des isolierten Wurzelmeristems auf sterilen Nährböden. Biol. Zentralbl. **52**: 257–265. 1932.
11. MELLANBY, J. The rate of formation of fibrin ferment from prothrombin by the action of thrombokinase and calcium chloride. Jour. Physiol. **51**: 396–403. 1917.
12. ROBBINS, W. J. Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz. **73**: 376–390. 1922.
13. ———. Effect of autolized yeast and peptone on growth of excised corn root tips in the dark. Bot. Gaz. **74**: 59–79. 1922.
14. ———, and MANEVAL, W. E. Further experiments on the growth of excised root tips under sterile conditions. Bot. Gaz. **76**: 274–287. 1923.
15. ———, and ———. Effect of light on growth of excised root tips under sterile conditions. Bot. Gaz. **78**: 424–432. 1924.
16. WHITE, P. R. Plant tissue cultures. The history and present status of the problem. Arch. exp. Zellf. **10**: 501–518. 1931.
17. ———. Plant tissue cultures. A preliminary report of results obtained in the culturing of certain plant meristems. Arch. exp. Zellf. **12**: 602–620. 1932.
18. ———. Influence of some environmental conditions on the growth of excised root tips of wheat seedlings in liquid media. Plant Physiol. **7**: 613–628. 1932.
19. ———. Concentrations of inorganic ions as related to growth of excised root tips of wheat seedlings. Plant Physiol. **8**: 489–508. 1933.
20. ———. Plant tissue cultures. Results of preliminary experiments on the culturing of isolated stem-tips of *Stellaria media*. Protoplasma **19**: 97–116. 1933.
21. ———. Liquid media as substrata for the culturing of isolated root tips. Biol. Zentralbl. 53: 359–364. 1933.