CULTIVATION OF EXCISED ROOT TIPS AND STEM TIPS UNDER STERILE CONDITIONS[1]

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(WITH FOUR FIGURES)

The growth of higher plants under sterile conditions is a necessary procedure in the investigation of problems involving the direct use by higher plants of organic or inorganic substances which may be altered by bacterial action. Failure to observe sterile conditions throws doubt on the conclusions drawn from the results secured in any experiment where the direct use by plants of an organic compound and some inorganic substances such as ammonium salts or nitrates is investigated.

As indicated by WILSON (15), the methods which have been used to grow plants under sterile conditions have either attempted to grow the entire plant under sterile conditions, or to keep that part of the plant which is of importance in the special investigation in a sterile environment. LUTZ, LAURENT, LEFEVRE, MOLLIARD, GRAFE, RAVIN, KNUDSON (11), BRANNON (4), and others have described methods of cultivating entire plants under sterile conditions. MAZÉ and PERRIER (12), SHULOW, HUTCHINSON and MILLER (9), and WILSON (15) have described in some detail methods by which higher plants may be grown with their tops exposed to the normal aerial environment. MAZÉ and WILSON grew corn plants to maturity with the root systems in sterile water cultures. Isolated and mature plant embryos have been cultivated under non-sterile conditions for longer or shorter periods by BROWN and MORRIS, ANDRONESCU (2), URBAIN (16), and others. KNUDSON cultivated corn embryos, and BUCKNER and KASTLE (3) bean embryos for short periods under sterile conditions. Isolated and immature embryos of species of *Rhapanus* and of *Cochlearia danica* have been grown with some success under sterile and non-sterile conditions by HANNIG (8). HABERLANDT (5) attempted to grow

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the isolated cells of the leaves of higher plants, and succeeded (6, 7) in obtaining some cell division under certain conditions in isolated pieces of the tissue from tubers, stems, and leaves of higher plants. To the writer's knowledge, however, the cultivation in sterile and artificial media of a portion of the meristematic tissue of higher plants has not been accomplished.

In the present paper a method is described by which isolated meristematic tissue (root tips and stem tips) of higher plants may be grown with some success under sterile controlled conditions. There are presented also the results of some preliminary experiments in which the method has been used, and which indicate the possibilities and limitations in growing excised root tips and stem tips under these conditions.

The experiments described were performed for the most part in 1917 at the Alabama Polytechnic Institute and Experiment Station, as the first step in an investigation to define the classes of materials required by a plant shoot or root for continued growth. In order to eliminate the influence of the shoot and its products on the root, or of the root on the shoot, it was considered necessary to grow the root tips isolated from the tops, and the shoot tips isolated from the roots, in artificial media under sterile conditions. It was thought that if the isolated meristematic tissue of a higher plant (such as the shoot tip or the root tip) could be cultivated successfully, such questions as the synthesis of elaborated nitrogen by the non-green parts of higher plants and the relation of chlorophyll and light to it, the necessity of accessory food substances for the growth of green plants, in short, the complete nutrient requirements of the shoot and of the roots of higher plants, and possibly of individual cells of the plant, could be investigated directly.

Method

The method followed in securing sterile root or shoot tips and cultivating the excised tissue was as follows. Seeds were sterilized by WILSON'S (14) calcium hypochlorite method, and transferred without washing to sterile Petri dishes containing a thin layer of 0.8 or 1 per cent plain agar. In transferring, a metal spoon made of aluminum with a wooden handle was used, and this spoon was

dipped in alcohol and flamed in order to sterilize it previous to use. When the seeds had germinated and the roots had reached a length of a centimeter or more, a centimeter or thereabouts of the root tip was cut off in the dish with a sterile scalpel, measured, and transferred with a sterile platinum loop to the described culture medium. The growing tip of the shoot was similarly treated. By this method a part of the plant, including the meristematic region, was excised and placed under sterile controlled conditions.

Growth of root tips in sterile nutrient solutions

Using the method described, the root tips of peas, cotton, and corn were placed in (1) a modified Pfeffer's solution,[2] (2) the same solution containing 2 per cent glucose, and (3) the modified Pfeffer's solution containing 2 per cent levulose. Each root tip was transferred to an Erlenmeyer flask of 125 cc. capacity, containing 50 cc. of solution. The flask was set in the dark and allowed to stand at room temperature during the period of the experiment. It was found that in such solution cultures the root tips of peas, cotton, and corn would develop into a considerable root system in the mineral nutrient solution containing carbohydrates, but that little growth occurred in the culture solution to which no carbohydrate was added. For all three plants the greatest growth occurred in the glucose solution. The method of culture and the appearance of root tips of corn in a 2 per cent glucose solution and in a solution lacking carbohydrates at the end of twenty-four days is shown in fig. 1. The detailed results of these early experiments were as follows:

PEAS.—In the experiment with peas, the variety Extra Early was used, and the period of growth was twenty-nine days. Fifteen root tips were used with each sugar solution and thirteen in the mineral solution without sugar. One contamination developed. All of the roots in the levulose solution were darkish brown, particularly at the cut end. Those in Pfeffer's solution were pure white. All were turgid, but the specific gravity of those in the Pfeffer's solution was less than that of those grown in the sugar solution.

[2] The composition of this solution was as follows:
$Ca(NO_3)_2$, 2 gm.; KH_2PO_4, 0.5 gm.; KNO_3, 0.5 gm.; KCl, 0.25 gm.; $MgSO_4$, 0.5 gm.; $FeCl_3$, 0.005 gm.; distilled water, 6000 cc.

The former floated on water, the latter sank. The average growth in length of the roots is given in table I, from which it can be noted

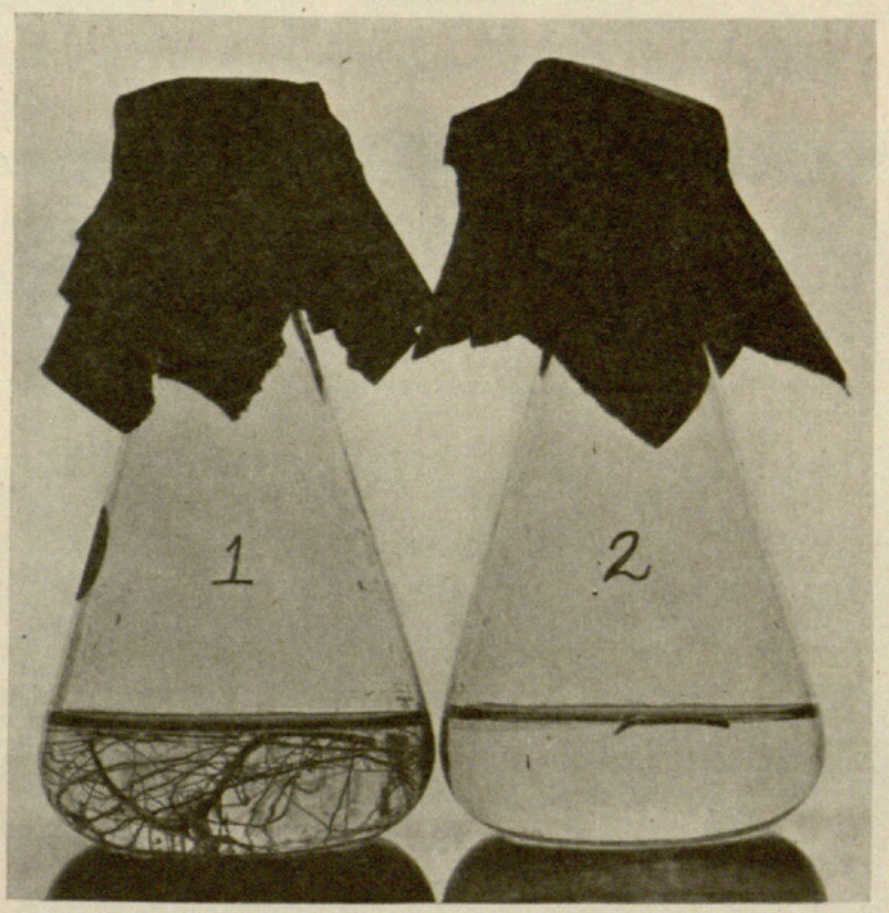

FIG. 1.—Appearance of root tips of corn in Pfeffer's solution at end of twenty-four days; flask no. 1 contains 2 per cent glucose, flask no. 2 contains no carbohydrates; root tip in no. 1 appeared as root tip does in no. 2 at beginning of experiment.

that in the mineral nutrient solution alone the average gain in length was 0.84 cm., and no secondary roots were produced. In levulose the average gain was 2.04 cm. with 2.3 secondary roots;

TABLE I

ROOT TIPS OF PEA GROWN TWENTY-NINE DAYS IN DARK

Solution	Number root tips used	Average original length (cm.)	Gain in length 29 days (cm.)	Side roots, average number per root
Pfeffer's solution	13	1.58	0.84	0
Pfeffer's plus glucose	14	1.49	4.27	3.5
Pfeffer's plus levulose	15	1.54	2.04	2.3

in glucose the average gain was 4.27 cm. with 3.5 secondary roots. Considerable variation in growth was evident. Some of the roots

in the sugar solution made little or no growth, others grew well (fig. 2). These roots which showed little growth lowered the average for the lengths in the sugar solutions. The maximum growth in glucose of a root tip originally 1.5 cm. long was 13.5 cm. with

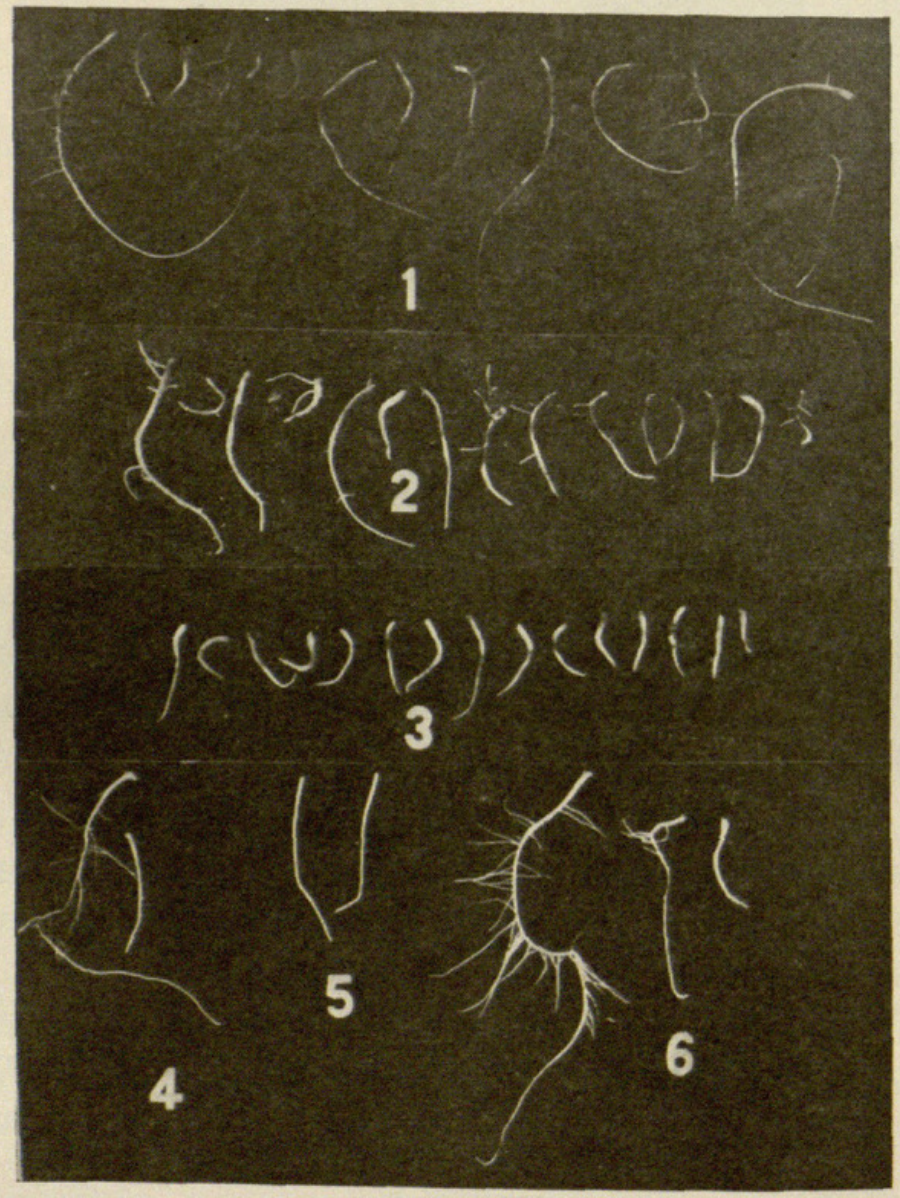

FIG. 2.—(1) Root tips of pea grown in dark in Pfeffer's solution plus 2 per cent glucose; (2) plus 2 per cent levulose; (3) plus no carbohydrate; (4) corn root tips grown in Pfeffer's solution plus 2 per cent levulose; (5) plus no carbohydrate; (6) plus 2 per cent glucose.

thirteen side roots, in levulose of a root tip originally 2.6 cm. the maximum growth was 7.5 cm. with nine secondary roots.

The general appearance of the pea roots was not entirely normal. The brownish color, especially noticeable in the levulose solutions, and the failure of some of the roots in the sugar solution to make much growth while others grew fairly well, suggest that the sterili-

zation of the seeds, the method of handling the roots, or the culture solution was injurious or at least unfavorable. It is of interest to note that when the root curved in its growth the lateral roots were produced on the convex side of the root. This was found to be true also in the roots of all three kinds of plants, and it can be noted in the case of corn and pea roots in fig. 2. JOST (10) states that by bending the main root, development of lateral roots may be prevented from the concave side, and cites the lupine as an example after NOLL.

CORN.—Using the same methods, corn roots were investigated. The period of growth was eleven days in the dark, and the number of roots used in this preliminary experiment was three in the glucose solution, two in the levulose solution, and two in the mineral nutrient solution without sugar. The corn root tips made a much greater growth than the peas, and did not show the browning so evident in the pea roots in the sugar solution, but were white in both the sugar solution and the mineral nutrient solution at the end of the experiment. The data in table II show that the average

TABLE II

ROOT TIPS OF CORN GROWN ELEVEN DAYS IN DARK

Solution	Number roots used	Average original length (cm.)	Gain in length 11 days (cm.)	Average number secondary roots
Pfeffer's plus 2% glucose...	3	2.3	8.33	22
Pfeffer's plus 2 % levulose..	2	3.75	5.95	20
Pfeffer's solution.....	2	4.15	1.60	0

increase in length in the Pfeffer's solution was 1.6 cm. with no secondary roots, the average increase in length in the levulose was 5.95 cm. with twenty secondary roots, and in glucose 8.33 cm. with twenty-two secondary roots. The appearance of the corn roots at the end of the experiment is shown in fig. 2.

This preliminary experiment does not indicate, however, the maximum amount of growth which corn roots may make under the conditions described. Since the experiment was performed, several hundred corn roots have been grown in sterile nutrient solutions. Corn roots with a length of 14–17 cm. and with 80–125 secondary

roots have frequently been grown in two weeks in the dark in Pfeffer's solution containing 2 per cent glucose. The maximum length has been that of a tip originally 2.0 cm. long which attained a length of 32.5 cm. and 131 secondary roots, in forty-three days.

COTTON.—Root tips of cotton were grown seventeen days in the three solutions, and with them even more striking results were secured than with the corn. Tips of ten roots were used in glucose, ten in levulose, and eleven in Pfeffer's solution without sugar. The root tips in glucose lengthened very rapidly, and at the end of seventeen days, as indicated in table III, had attained an average

TABLE III

GROWTH OF ROOT TIPS OF COTTON IN STERILE NUTRIENT SOLUTIONS

Solution	Number of roots	Average original length (cm.)	Gain in length 17 days (cm.)	Average number secondary roots per root	Dry weight per root (gm.)
Pfeffer's plus 2% glucose.....	10	3.06	31.76	61	1224
Pfeffer's plus 2% levulose.....	10	3.11	2.34	29	0.0728
Pfeffer's solution.....	11	2.99	1.92	0.1	0.1240

length of 34.8 cm., while originally they were but 3.06 cm. long. The cut end of the roots in glucose was very dark brown, almost black for 1–1.5 cm., and brownish for 3–4 cm. The root cap was black. In levulose the increase was much less, only 2.34 cm., and the whole root was dark brown. The increase in length in the mineral solution alone was still less, being only 1.9 cm. The roots were pure white. The maximum growth in the glucose solution was that of a root tip 3.2 cm. long which attained a length of 42.2 cm. with seventy secondary roots. In levulose the maximum growth was that of a root tip 2.1 cm. long which grew to 13.4 cm. with thirteen secondary roots.

Growth of root tips in agar

The excised root tips of corn have also been grown in 1 per cent agar. Fig. 3 shows the growth of an excised root tip of corn at the end of two weeks in Pfeffer's solution containing 1 per cent

agar, and in Pfeffer's solution plus 2 per cent glucose containing 1 per cent agar. It can be noted from the figure that the root tip not supplied with glucose has made little growth, while the root tip supplied with glucose has made considerable growth, has responded normally to gravity, and has produced a considerable number of secondary roots.

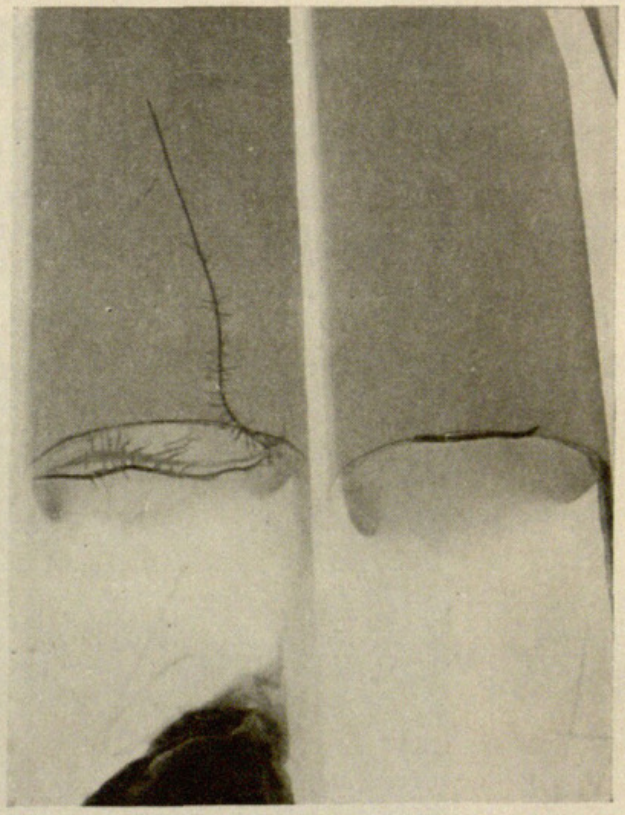

FIG. 3.—Growth of root tips in Pfeffer's solution plus 1.0 per cent agar; tube on left no glucose, tube on right 2 per cent glucose.

Growth of shoot tips in sterile nutrient solutions

The shoot tips of pea, corn, and cotton were placed in the three solutions and grown in the dark. While growth was secured in the carbohydrate solution with cotton, the development was abnormal and measurements were not made. The shoots of peas and corn developed more normally in the carbohydrate solution, and in many cases produced roots. The plants, however, were chlorotic and showed the elongation and small leaf development typical of plants grown in the dark (fig. 4). In both cases, as indicated in table IV, the greatest increase in length occurred in the glucose solution and least in the Pfeffer's solution without sugar. The relative growth of the shoot tips in the three solutions was therefore the same as that of the root tips. Starch was found in the tissue grown in the glucose and levulose solutions, and in the guard cells of the shoots in the Pfeffer's solution.

From these experiments it is evident that in the dark, in solution cultures containing the mineral salts commonly accepted as essential for the growth of green plants and a soluble carbohydrate, the root tips of corn, cotton, and peas, and the stem tips of corn, cotton, and peas make considerable growth. In the same solutions lacking

carbohydrates, only a slight increase in length occurs, probably at the expense of carbohydrate originally present in the root tip.

Glucose is apparently a better source of carbon than levulose for all three plants and for both tops and roots. This is particularly

FIG. 4.—(1) Corn shoot tips grown in dark in Pfeffer's solution plus 2 per cent levulose; (2) plus no carbohydrate; (3) plus 2 per cent glucose; (4) pea shoot tips grown in dark in Pfeffer's solution plus 2 per cent levulose; (5) plus 2 per cent glucose; (6) plus no carbohydrate.

noteworthy in the case of cotton, where the increase in length of the root tips in glucose, as indicated in table III, was thirteen times the gain in levulose, twice as many secondary roots were produced,

and the gain in dry weight compared with the check was twice as great. Although the number of corn root and stem tips used in this experiment was small, the roots grown in glucose were better than those in levulose. No comparison between the two sugars could be made in the case of cotton stem tips. The contrast between the relative effect of glucose and levulose on the amount of growth made by the tissue of these seed plants and their effect upon the

TABLE IV

GROWTH OF SHOOT TIPS OF PEA AND CORN IN DARK, TWENTY-NINE AND ELEVEN DAYS RESPECTIVELY, IN STERILE NUTRIENT SOLUTIONS

Additions to modified Pfeffer's solution	Number shoot tips used	Average original length (cm.)	Gain in length (cm.)	Total number roots
	Peas			
Glucose (2%)	15	1.75	12.77	7
Levulose (2%)	15	1.72	3.58	3
None	14	1.75	0.88	14
	Corn			
Glucose (2%)	3	2.16	17.5	13
Levulose (2%)	3	2.90	12.43	8
None	2	3.75	4.50	1

growth made by *Ceratodon purpureus* should be noted. In the case of the latter plant, as reported earlier (13), the amount of dry matter produced with levulose as the carbon source was 2–7 times as great as the amount produced with glucose. The fact that the stem tips in the dark, even in the presence of 2 per cent glucose, are morphologically like etiolated shoots, would also indicate that it is not an absence of available carbohydrate which causes the stem elongation and small leaf development of plants grown in the dark.

Continued growth of root tips in culture solutions

The fact that the root tips in these experiments grew in solutions containing carbohydrates, and made very little growth in the same solutions lacking sugar, would suggest that the complete requirements for the growth of roots are water, mineral salts, carbohydrates, and free oxygen. If such were the case we should expect

that, furnished with sufficient quantities of these materials in the proper proportions, a root would continue to grow indefinitely. The excised roots of corn, however, will not grow indefinitely under the conditions described. Their development is rather definitely limited in the dark in the culture solutions used, as can be noted from the following experiment.

Root tips of corn were grown in the dark in the Pfeffer's solution containing 2 per cent glucose. At the end of eight days the tips of the roots were cut off and transferred to a fresh solution of the same composition, where they were allowed to grow for ten days. At the end of that time the tip was again cut off and transferred to a new solution. Growth took place there for ten days. If the nutrient solution were complete and no inhibiting or injurious factors active, by continued transfers we should be able to keep a root tip growing indefinitely in the same way as cultures of bacteria, molds, or yeasts are kept growing indefinitely by continued transfers to fresh media.

In this experiment the growth during the first period was excellent, and the number of secondary roots large (table V). During

TABLE V

GROWTH OF ROOT TIPS OF CORN IN NUTRIENT SOLUTIONS: ROOT TIPS IN GLUCOSE SOLUTION CUT OFF AND TRANSFERRED AS INDICATED

Period of growth	Number roots used	Average original length (cm.)	Gain in length (cm.)	Number secondary roots per root
	Pfeffer's solution plus 2 per cent glucose			
June 30–July 7	16	3.43	8.98	64.8
July 7–July 17	16	3.48	2.04	20.8
July 17–July 28	13	1.86	0.14	00.0
	Pfeffer's solution			
June 30–July 28	10	3.65	0.90	00.0

the second period the increase in length was one-fourth that of the first period, and the number of secondary roots one-third. In the third period hardly any increase in length was found, and no secondary roots were produced.

The maximum gain in the first period was that of a root tip originally 4.3 cm., which increased to 18.0 cm. and produced ninety-seven secondary roots. The maximum gain in the second period was that of a root tip 2.7 cm. long originally, which increased to 7.4 cm. and produced twenty-one secondary roots. In the third period only three out of thirteen roots showed any increase in length, the maximum being 0.9 cm. This experiment has been repeated many times, and many root tips of corn have been carried through the three periods of culture given. Five varieties of corn have been used: a dent variety from Alabama; Longfellow flint from New York (kindly furnished by Dr. J. K. WILSON); Boone County White and Reed's Yellow dent from Missouri; and Funk's Yellow dent from Illinois. The stoppage of growth in the third period in Pfeffer's solution plus 2 per cent glucose has occurred in every case. No single root tip thus far has made more than a slight amount of growth in the dark in the third period.

Not only does a diminution of growth rate, and a reduction in the production of secondary roots ending in a cessation of growth in the third period take place in the course of these transfers, but the diameter of the root tip continually decreases, until in the third period its diameter is one-fourth or less that of the original root tip. When stoppage of growth takes place, however, the root tips may apparently be normal in macroscopic and microscopic appearance, showing vascular bundles and root hair development.

The failure of an excised root to continue growth when repeated transfers of the root tip are made, at once suggests that the seedling root contains some material derived from the seed other than glucose, the mineral salts of Pfeffer's solution, water, and free oxygen which are necessary for continued growth and which the root cannot synthesize in the dark in solution cultures from the material supplied. Such material or materials would be fractionated by the continued transfers of the root tips.

Other explanations may be suggested. The stoppage of growth may be due to an unbalanced condition of the nutrient solution in which the roots are grown. The root tips, however, at the time of growth stoppage may show no macroscopic evidence of injury, and the fact that root hairs may be present on the root

tip in the third period also would indicate that the stoppage of growth is not due to the toxicity of the solution. The dextrose may penetrate too slowly to furnish sufficient carbohydrate to the root cells for continued growth. This would not appear to be a feasible explanation, however, because the early growth of the root tip is rapid, and the decrease in growth appears progressively greater. The early growth must occur at the expense of glucose which penetrates the root cells, because the root tips furnished with no glucose in the nutrient solution grew very little. It would seem that if the rate of penetration of the glucose determines the stoppage of growth one would have to assume a continuously increasing difficulty of penetration, terminating in entire impermeability of the root cells to glucose. The limited oxygen supply in the solution cultures may account for the growth stoppage. Here again, however, no better supply of oxygen is supplied in the first period than in the later periods, and yet the growth in the first period is the most rapid. If the limited oxygen supply is the factor which eventually causes the stoppage of growth, it must be a cumulative effect, due either to the development of deleterious materials or failure to synthesize some necessary material. It should also be noted that, although the aeration of water cultures of entire corn plants favors root and top development (ANDREWS and BEALS 1), the roots of an entire plant in unaerated water cultures do not show the stoppage of growth evident with the excised roots in solution cultures. It would seem reasonable to assume, therefore, as a working hypothesis from the experiments described, that oxygen, the mineral salts of Pfeffer's solution, glucose, and water are insufficient for the continued growth of excised corn roots.

Summary

1. A simple method of growing the isolated meristematic tissue of higher plants, excised root tips and stem tips, under sterile conditions is described.

2. The excised root tips of peas, corn, and cotton make considerable growth in the dark in solution cultures containing mineral salts and glucose or levulose.

3. The excised root tips of peas, corn, and cotton make little growth in the dark in solution cultures containing mineral salts and lacking carbohydrate.

4. The growth of the isolated root tips of peas, corn, and cotton is markedly greater in solution cultures containing glucose than in those containing levulose.

5. The excised roots of corn respond normally to gravity when grown on agar containing mineral salts and glucose.

6. The isolated shoot tips of peas and corn make considerable growth in the dark in sterile solution cultures containing mineral salts and glucose or levulose, but little in the absence of carbohydrates.

7. The excised shoot tips of corn and peas grown in sugar solutions remain chlorotic, and those of peas show the stem elongation and small leaf development characteristic of plants grown in the dark.

8. When the excised root tips of corn are grown for ten days or two weeks in the dark in a solution culture containing glucose and mineral salts, and the tip is then cut off and transferred to a fresh solution of the same type, the amount of growth in the second period is less than that in the first, and ceases in the third period.

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