Studies in the Physiology of Leaf Growth

I. The Effect of Various Accessory Growth Factors on the Growth of the first Leaf of isolated Stem Tips of Rye

BY

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With three Figures in the Text

INTRODUCTION

IN the present series of investigations, by culturing excised stem tips from rye embryos on artificial media of known composition, information was sought regarding the factors which control the growth and differentiation of leaves. This form of culture makes possible a study of the factors concerned in leaf growth independently of their effect on the growth of roots. The culture of isolated stem tips was first attempted by Robbins (1922), who showed that shoot tips of peas and corn will grow to a limited extent in darkness provided that they are supplied with a source of carbohydrate. White (1933) maintained isolated stem tips of *Stellaria media* in hanging drops of a culture solution containing mineral salts, dextrose, and yeast extract. He was able to demonstrate that such fragments were capable of making a limited amount of further growth. They also had a capacity to differentiate fresh leaf primordia. The culture of isolated leaves was subsequently attempted by Bonner, Haagen-Smit, and Went (1939). These workers maintained immature leaves excised from etiolated pea plants in a synthetic medium containing mineral salts and sucrose. They showed that in such a medium these excised leaves were capable of making a limited amount of growth, which could be further increased by the addition to the medium of a diffusate obtained from peas. Their results led them to conclude that a non-specific leaf-growth hormone may exist which affects the growth of leaves in several species of plants. In this connexion Avery (1935) has stated also that a hormone, probably auxin *a*, is concerned in the leaf growth of Nicotiana.

Some further information regarding leaf growth and differentiation was provided by the investigations of White (1939) and Skoog (1944) of the behaviour of callus cultures of the hybrid *Nicotiana glauca*×*N. langsdorfii*. Both these workers were able to show that a change in physical conditions is by itself sufficient to initiate leaf differentiation in such cultures. They did not postulate the existence of any special leaf-growth-controlling hormone.

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Materials and Methods

Stem growing-points from the embryos of rye grains (var. Petkus) were removed with aseptic precautions and placed on nutrient agar. The position of these excised growing-points in the embryo is shown in Fig. 1. The first leaf primordium, to which attention was mainly directed, is an organ about

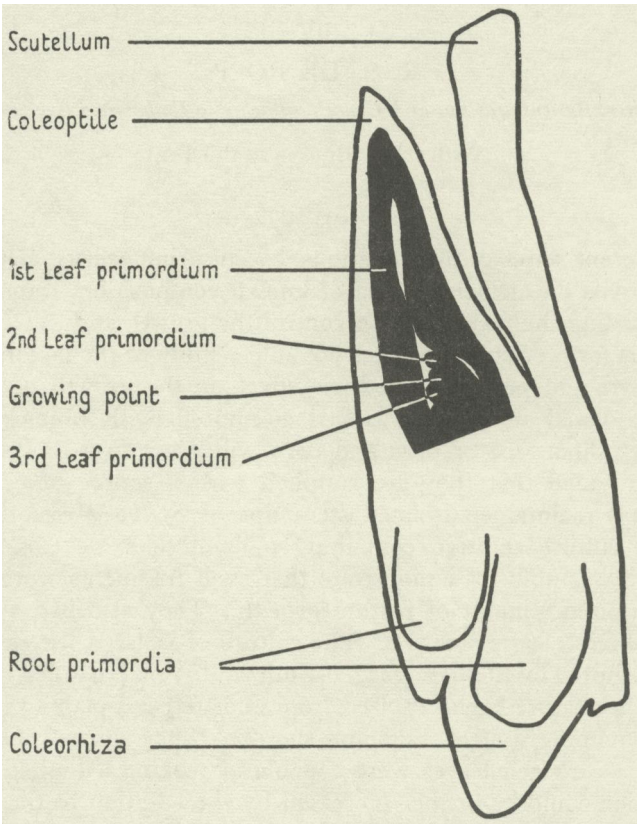

Fig. 1. Longitudinal section of a rye embryo before germination, showing (in black) the form of the stem tip removed in these experiments.

1 mm. long containing ten vascular bundles and enclosing two or three younger leaf primordia as well as the growing-point (Fig. 2).

It was found unnecessary to sterilize the grain before removal of the stem tips provided aseptic precautions were taken during that operation. All instruments used were sterilized by flaming alcohol. Contaminated cultures were rare, not more than 1 per cent., and were rejected. To facilitate the removal of stem tips the grain was soaked for about 30 minutes. Longer soaking rendered the primordium increasingly susceptible to injury. Shorter soaking times made difficult the process of stripping off the coleoptile. The first leaf primordium was very easily damaged and a very small amount of

injury entirely inhibited the growth of the organ. The actual excision was made with a flat-ground, sterile needle, the cut being made immediately beneath the point of attachment of the first leaf.

The medium, to which all accessory factors to be tested were added, was made up with distilled water, prepared in a Pyrex glass still, and contained 2 per cent. sucrose, complete mineral salts in the proportions suggested by White (1943), and 0·3 per cent. agar. The sucrose and mineral salts used were of 'Analar' standard of purity and the agar was extracted before use by the method recommended by Robbins (1939). Usually each isolated stem tip

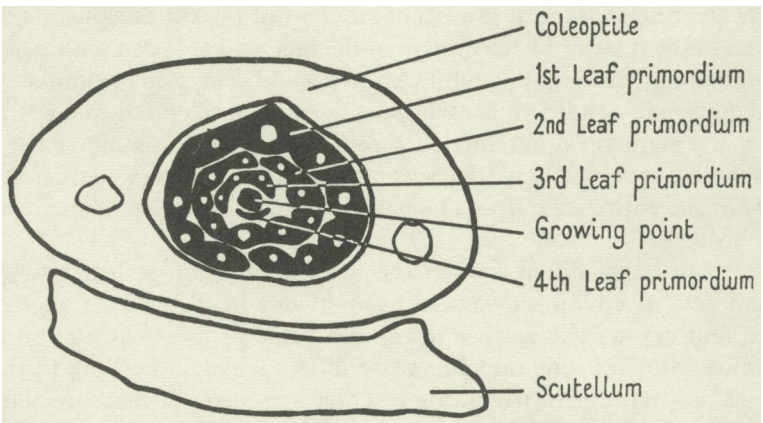

FIG. 2. Transverse section of a rye embryo at the level of attachment of the leaf primordia.

was cultured on 15 ml. of agar contained in a 50-ml. conical flask. The medium was autoclaved for one minute at 10 lb. pressure, which treatment was found to give adequate sterilization. Substances to be tested for their growth-promoting efficacy were added to the agar before or after sterilization according to whether they were heat sensitive or resistant.

The stem-tip cultures were incubated in a constant temperature chamber at 25° C. in complete darkness. In nearly all experiments the period of incubation was 2 weeks, at the end of which time the amount of growth of the first leaf was assessed by measuring its length, fresh weight, and dry weight. For each treatment there were twenty replicates, the minimum number from which a reasonably accurate estimate of the treatment effects could be obtained. White (1943) recommends the same number of replicates for experiments with isolated root tips. The standard errors worked out at about 10 per cent. of the means.

EXPERIMENTAL

1. *Growth of the first leaf on isolated and attached stem tips.*

This experiment was designed to provide a comparison of the growth rate of the first leaf when attached to: (*a*) an excised stem tip cultured on sucrose

mineral agar; (*b*) an excised whole embryo cultured on sucrose mineral agar; (*c*) an embryo in the intact grain cultured on plain mineral agar.

Groups of 120 excised stem tips, excised embryos, and intact grains were placed on the media described above and incubated in darkness at 25° C. From these groups 20 individuals were removed after the following periods of incubation: 3 days, 5 days, 1 week, 2 weeks, 4 weeks, and 6 weeks. The length and fresh weight of each individual was determined. Dry weights were estimated for groups of 5. From these values the means were calculated which, with their standard errors, are shown in Table I.

One of the most outstanding features of the behaviour of these stem-tip cultures was the absence of growth of any part of the tip except the first leaf. Whereas in the intact embryo growth of the second leaf began within 48 hours of germination, in stem-tip cultures the second leaf primordium remained in an embryonic condition, showing no signs of developing unless, by some chance, the stem tip differentiated a root. Whenever this happened it was accompanied by growth of the second leaf and by renewed meristematic activity of the entire stem tip. The nature of this interaction of stem and root is still obscure.

The quantitative differences in the growth of the first leaf under these different sets of circumstances are brought out in Table I. Length, fresh weight, and dry weight reach a maximum value in most cases after 7 days' incubation. But the amount of increase differs widely according to the condition of the stem tip to which the leaf was attached. If measurements at 0 and 7 days are compared for leaves under these different sets of conditions the ratios obtained are as follows:

	Isolated stem tip.	Isolated embryo.	Whole grain.
Length	18	122	187
Fresh weight . . .	45	180	497
Dry weight . . .	13	18	55

The effect on leaf growth of excising the stem tip may best be assessed by comparing ratios for leaves attached to isolated stem tips with those for leaves attached to isolated embryos. Such a comparison shows that excision of the stem tip resulted in a very considerable reduction of growth in leaf length and fresh weight. Dry-weight growth was reduced to a much smaller extent. The uptake of nutrients thus appears to have been much the same in both cases, the difference being in the uptake of water. A consideration of the values for leaves attached to the whole grain reveals that the presence of the endosperm resulted in a great increase in the uptake both of nutrients and of water by the leaf. Evidently the endosperm provided some factors necessary for leaf growth which were either absent from, or inadequately supplied by, the artificial medium.

Further differences in the behaviour of the three groups occurred when growth was studied for longer than seven days (Table I). Leaves attached to excised stem tips showed no significant change either in fresh or dry weight

TABLE I

Growth of (i) *first Leaf attached to isolated Stem Tip,* (ii) *isolated Embryo, and* (iii) *intact Grain. Incubated in Darkness at 25° C. (Means of 20 estimations)*

Days		0	3	5	7	14	28	42
Isolated stem tip	Length (mm.)	1·00	5·68±0·44	17·50±0·88	18·11±1·54	19·27±1·29	15·70±1·57	14·21±1·58
	Fresh wt. (mg.)	0·25	2·10±0·26	6·60±0·49	11·15±1·22	10·88±0·88	11·10±1·22	11·81±1·88
	Dry wt. (mg.)	0·18	0·42±0·05	1·10±0·19	2·40±0·23	2·00±0·15	2·58±0·11	2·37±0·17
Isolated embryo	Length (mm.)	1·00	35·46±1·72	88·92±2·00	122·20±4·00	136·42±7·16	131·10±5·30	131·80±5·49
	Fresh wt. (mg.)	0·25	13·13±1·14	34·46±1·46	45·00±2·50	46·42±3·39	26·81±3·31	17·21±2·14
	Dry wt. (mg.)	0·18	1·20±0·15	2·70±0·20	3·17±0·25	2·86±0·18	2·56±0·15	2·86±0·17
Whole grain	Length (mm.)	1·00	37·81±3·25	130·00±1·73	187·21±3·91	175·40±6·70	195·61±6·31	173·40±5·39
	Fresh wt. (mg.)	0·25	18·45±2·38	86·40±1·78	124·20±3·57	118·80±8·80	62·01±6·30	17·81±1·81
	Dry wt. (mg.)	0·18	2·18±0·32	7·00±0·21	10·00±0·30	7·00±0·28	6·70±0·27	6·00±0·20

after the seventh day. The apparent decrease in length after the second week did not recur in other experiments. Such leaves remained alive, turgid, and capable of regenerating chlorophyll on exposure to light even after 8 weeks' incubation in darkness. Only after this time did they become brown and die. Leaves attached to isolated embryos were more short-lived. They began to lose water after the second week and were flaccid and dead by the sixth week. Leaves attached to intact grains had an even shorter life. Their dry weight fell rapidly after the first week, and excessive water loss was accompanied by shrinking and browning of the tissues. Within 4 weeks all leaves in this series were brown and dead.

In leaves attached to the intact embryos and grown in the dark, a red or yellow colouring matter developed which attained its maximum intensity about 7 days after germination and then gradually disappeared. In leaves attached to isolated stem tips this colour hardly appeared at all; a trace of yellow could sometimes be seen near the tips of such leaves but they were, for the most part, quite colourless. They also differed from the attached leaves in that only the lamina developed, the sheath being entirely absent.

2. *Effect of sucrose and mineral salts on the growth of the first leaf attached to an isolated stem tip.*

The two main components of the nutrient medium, sucrose and mineral salts, were tested separately and together to determine their effect on the growth of the first leaf in stem-tip cultures (Table II). In the absence of

TABLE II

Effect of Sucrose and Mineral Salts on Growth of first Leaf of isolated Stem Tip. Incubated 2 Weeks in Darkness at 25° C. (Means of 20 estimations)

	No sucrose or mineral salts.	Mineral salts alone.	Sucrose alone.	Sucrose plus mineral salts.
Length (mm.)	2·15±0·04	2·26±0·04	6·17±0·53	18·73±2·38
Fresh wt. (mg.)	0·45±0·01	0·61±0·02	4·02±0·25	11·73±1·00
Dry wt. (mg.)	0·10*	0·15*	0·71±0·02	2·1±0·15

* Replicates bulked before drying.

sucrose no growth could be observed whether mineral salts were supplied or not. When both were supplied together growth was significantly greater than it was in the presence of sucrose alone, a fact which indicates that a supply of carbohydrate was not by itself sufficient to ensure maximum growth. The different appearances of these stem-tip cultures grown on different media is shown in Fig. 3.

3. *Effects of crude plant extracts on growth of the first leaf attached to an isolated stem tip.*

The effect of plant extracts on the growth of isolated plant organs has been studied by several workers. Robbins and White (1937) recorded that an

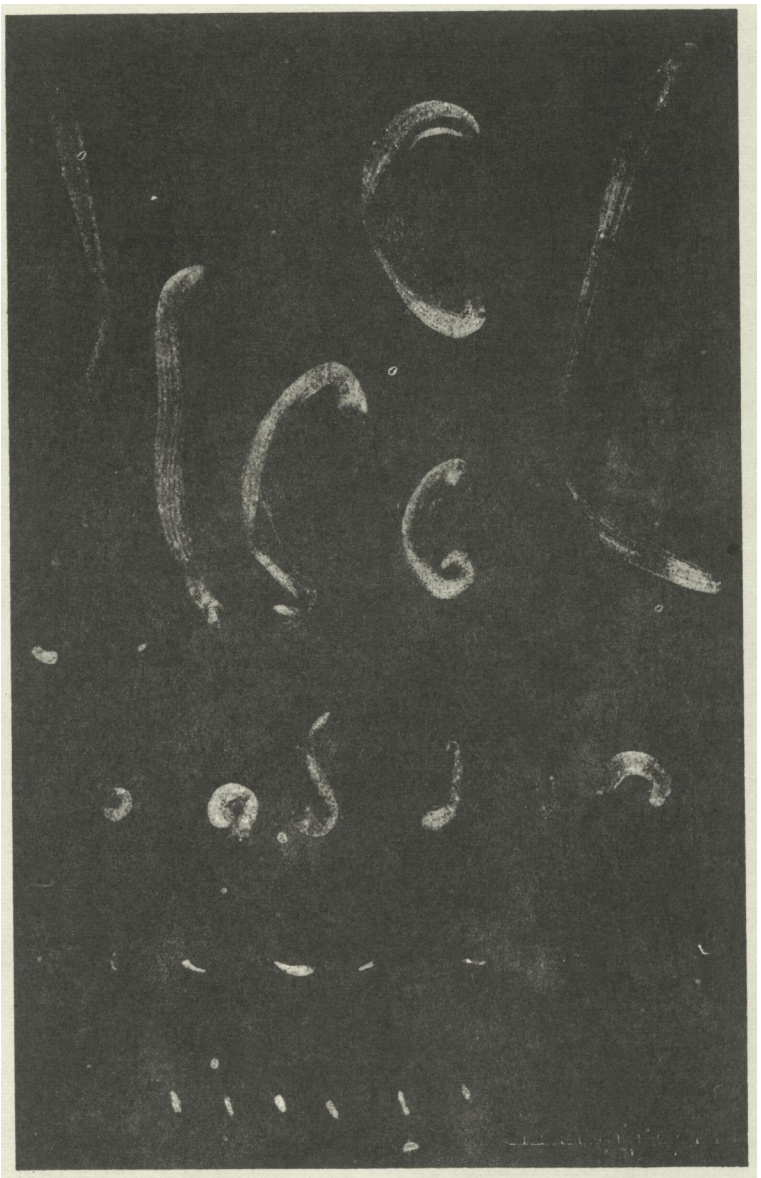

FIG. 3. Effect of sucrose and mineral salts on the growth of isolated rye stem tips on artificial media. Top row, sucrose and mineral salts present; second row, mineral salts absent; third row, sucrose absent; bottom row, tips on removal from the grain. ($\times 2\frac{1}{2}$) (Photograph by V. Stansfield.)

extract of maize grain slightly increased the growth of isolated corn roots. Van Overbeck *et al.* (1944) have stated that a factor affecting the growth of isolated Datura embryos is present in coconut milk. The claims of Bonner, Haagen-Smit, and Went regarding the growth-promoting efficacy of pea

diffusate have been previously mentioned. The effect of an extract of yeast on root growth is well known from the work of White and others.

In the present work the following crude extracts were tested to determine their effect on the growth of the first leaf in isolated stem-tip cultures: (*a*) Pea diffusate prepared by the method described by Bonner, Haagen-Smit, and Went, added to agar, after autoclaving, to give a final concentration of 1 per cent. (*b*) Hot-water extracts of sprouted and unsprouted rye grain added to agar, after autoclaving, to give a concentration of 1 per cent. (*c*) Cold-water extracts similar to (*b*). The grain was ground and extracted with water at 1° C. for 24 hours, the sprouted grain having been first dried in the frozen state. This extract was sterilized by filtration and added to the agar just before it cooled to give a final concentration of 1 per cent. (*d*) Hot-water extract of dried yeast added to agar to give a final concentration of 1 per cent. (*e*) Endosperm digest, obtained by placing an excised fragment of endosperm, with the scutellum attached, on the surface of the nutrient agar. Such fragments were rendered sterile by carefully stripping them of the outer integuments. On the surface of the agar they underwent digestion by enzymes produced by the attached scutellum. The soluble products of this digestion could diffuse out into the agar on which an excised stem tip was then placed in the usual way.

This experiment was set up in three sections each having its own control, and the results are shown in Table III. It is clear that neither extracts nor endosperm digest had any effect on the growth of the first leaf attached to these stem tips. The differences between separate treatments are no greater than those between untreated controls. The pea diffusate, to which Bonner, Haagen-Smit, and Went were inclined to attribute a rather general leaf-growth-promoting efficacy, had no effect on this material.

TABLE III

Effect of Crude Plant Extracts on Growth of first Leaf attached to Isolated Stem Tip. Incubated in Darkness at 25° C. for 2 Weeks. (Means of 20 estimations)

Extract.	Length (mm.).	Fresh wt. (mg.).	Dry wt. (mg.).
Sprouted grain (Hot water extract)	16·31±1·20	12·71±1·41	2·44±0·21
Unsprouted grain (Hot water extract)	15·98±1·67	11·76±1·13	2·45±0·12
Yeast (Hot water extract)	13·01±1·22	9·52±0·81	2·00±0·11
Control (no extract)	15·56±1·11	9·81±1·06	1·84±0·14
Sprouted grain (Cold water extract)	15·43±1·06	11·62±1·32	2·40±0·19
Unsprouted grain (Cold water extract)	19·21±1·13	10·89±1·20	2·05±0·16
Endosperm digest	14·61±1·31	10·22±0·96	2·65±0·14
Control (no extract)	17·26±0·91	11·05±0·81	2·21±0·20
Pea diffusate	15·96±1·72	10·10±1·31	2·07±0·13
Control (no diffusate)	14·98±1·23	11·80±1·14	2·71±0·17

4. *Effect of vitamins of the 'B' groups on the growth of the first leaf attached to an isolated stem tip.*

Thiamin, nicotinic acid, and pyridoxine have all been shown to be necessary for the growth of isolated plant roots (White, 1937; Robbins and Schmidt, 1939; Bonner, 1943). Few investigators have concerned themselves with the possible role of these substances in leaf growth, though Bonner, Haagen-Smit, and Went (1939) tested the effects of thiamin, riboflavin, and biotin on the expansion of fragments of Raphanus leaves and found all of them without effect. In the present work thiamin, nicotinic acid, riboflavin, calcium pantothenate, pyridoxine, and biotin were each tested separately for their effect on the growth of isolated stem tips, and thiamin, pyridoxine, and nicotinic acid were tested in combination. The individual vitamins were added to sucrose mineral agar before autoclaving to give the following final concentrations (γ/c.c.):

Thiamin.	Nicotinic acid.	Riboflavin.	Ca pantothenate.	Pyridoxine.	Biotin.
0·5	0·1	0·1	0·1	0·1	0·1

The growth of the first leaf in stem-tip cultures containing these individual substances is shown in Table IV. Thiamin appears to have had a significant effect but this was not observed again in later experiments. None of the other vitamins had any effect.

TABLE IV

Effect of 'B' Vitamins on Growth of first Leaf attached to Isolated Stem Tip. Incubated in Darkness for 2 Weeks at 25° C. (Means of 20 estimations)

Vitamin.	Length (mm.).	Fresh wt. (mg.).	Dry wt. (mg.).
Thiamin . .	20·91±1·38	12·32±1·06	2·51±0·22
Nicotinic acid . .	19·65±1·67	11·37±1·32	2·00±0·11
Riboflavin . .	19·30±1·11	10·15±1·01	2·00±0·14
Ca pantothenate .	19·37±1·06	9·06±0·86	1·75±0·16
Pyridoxine . .	15·35±0·91	10·00±1·73	2·06±0·20
Biotin . . .	17·91±1·72	9·27±1·92	1·90±0·15
Thiamin, pyridoxine and nicotinic acid combined			
10 γ per c.c. . .	13·01±1·18	9·31±0·78	2·21±0·21
1 γ per c.c. . .	14·92±0·76	11·15±0·66	2·39±0·18
Control (no vitamin)	18·18±1·28	10·55±0·97	2·05±0·20

To test the effect of thiamin, nicotinic acid, and pyridoxine in combination the vitamins were added to nutrient agar to give final concentrations of 1, 10, and 100 γ per c.c. of each component. Growth was entirely inhibited by the highest concentrations of these substances. The lower concentrations produced no significant effect (Table IV).

5. *Effect of β indole acetic acid and α naphthalene acetic acid on growth of the first leaf attached to an isolated stem tip.*

The capacities of these substances to act as growth-promoting factors for plants have been variously reported by different workers. Smith (1940) has

stated that they inhibit leaf growth on excised stem tips of *Helianthus annus*, and Ball (1944) has found that they produce abnormalities in leaf growth when applied to the attached shoot apex of *Tropaeolum majus*. Spoehr (1942) has shown that they have no growth-promoting effect on the leaves of albino maize plants. Avery (1935) has claimed that auxin is involved in the growth of leaves of Nicotiana, but the substance in question was more probably auxin *a* than either indole or naphthalene acetic acid. Berger and Avery (1944), however, have isolated indole acetic acid from a precursor in dormant maize grains which suggests that this substance may play a part in the growth of the embryo of the Gramineae.

In these experiments two concentrations of each acid were used. Weighed amounts of the dry substance were dissolved in small quantities of absolute alcohol, diluted with sterile water and added to the agar after autoclaving. The final concentrations of growth substance in the agar were 10^{-4} and 10^{-7}. The growth made by isolated stem tips cultured on this medium is shown in Table V. The higher concentration of growth substance inhibited growth entirely and no results have therefore been recorded. At the lower concentration they had no significant effect on the growth of the first leaf.

TABLE V

Effect of Various Accessory Factors on the Growth of the first Leaf attached to Isolated Stem Tip. Incubated for 2 Weeks in Darkness at 25° C. (Means of 20 observations)

Vitamin concentration.	Length (mm.).	Fresh wt. (mg.).	Dry wt. (mg.).
β indole acetic acid 1/10^7	18·29±1·16	9·88±1·65	1·50±0·18
α naphthalene acetic acid 1/10^7	17·63±1·01	11·47±1·33	1·65±0·20
Ascorbic acid 100 γ per c.c.	12·52±1·50	8·82±1·00	1·53±0·25
Ascorbic acid 10 „ „	17·94±1·24	10·22±1·00	1·72±0·25
Ascorbic acid 1 „ „	21·00±2·00	11·68±0·96	2·37±0·27
Vitamin E	17·31±1·71	10·31±0·58	2·12±0·20
Vitamin K	11·60±1·56	8·93±0·75	1·93±0·14
Adenine	11·28±1·54	10·42±1·01	2·28±0·13
Guanine	17·00±2·56	12·42±2·71	2·24±0·21
Uric acid	16·87±1·62	12·00±2·00	2·50±0·16
Caffein	14·36±1·73	9·37±1·02	1·87±0·18
Control (no growth factor)	17·17±1·12	11·18±2·01	2·14±0·18

6. *Effect of ascorbic acid on growth of the first leaf attached to an isolated stem tip.*

Ascorbic acid has been shown to be present in relatively large quantities in practically all green leaves (Bessey and King, 1933) and its function has been associated with biological oxidation (Carrol, 1943). Havas (1935) has stated that the vitamin may function as a growth-promoting substance in wheat, but this statement lacks confirmation. Clark (1937) has found it to be without any growth-promoting effect on the Avena coleoptile.

In the present work recrystallized ascorbic acid was dissolved in distilled water, sterilized by filtration and added to nutrient agar after autoclaving to

give final concentrations of 1, 10, and 100 γ per c.c. No significant increases in growth were produced by the presence of this vitamin. At the highest concentration used it had a depressing effect on growth (Table V).

7. *Effect of vitamins E and K on the growth of the first leaf attached to an isolated stem tip.*

The presence of vitamin E (α tocopherol) in lettuce leaves and wheat-germ oil (Olcott and Mattill, 1934), and of vitamin K in green leaves of various plants (Dam and Nielsen, 1940), suggests that both these substances may play some role in leaf metabolism. An experiment was set up to determine whether either substance was capable of affecting the growth of the first leaf attached to an isolated stem tip. The vitamins were added to nutrient agar to give a final concentration of 10 γ per c.c. Vitamin E was dissolved in a small amount of alcohol before adding to the agar with which it formed a stable suspension. Vitamin K was added in the form of the water-soluble analogue 'Synkavit' (tetra sodium salt of 2-methyl-1 : 4-naphthohydroquinone diphosphate). Neither of these vitamins produced any effect on the growth of the first leaf under the conditions of the experiment (Table V).

8. *Effect of purine derivatives on the growth of the first leaf attached to an isolated stem tip.*

That certain purine derivatives, particularly adenine, are capable of increasing leaf growth has been claimed by Bonner and Haagen-Smit (1939), but the effects recorded by these workers were very small and appear of doubtful significance. In the present work the purine derivatives, adenine, quanine, caffein, and uric acid were tested to determine their effect on the growth of rye leaves attached to isolated stem tips. They were added to the nutrient agar to give a concentration of 10 γ per c.c. None of these substances was capable of producing a significant increase in the growth of the first leaf under these conditions (Table V).

Discussion

The experiments described in this paper show that, when the entire growing-point was excised from a rye embryo and cultured in darkness on 2 per cent. sucrose and mineral salts, a limited amount of growth resulted which was almost entirely confined to the first leaf. The growth of the first leaf primordium attached to the isolated stem tip differed in several respects from the growth of the primordium when attached to the embryo, whether the embryo was cultured on an artificial medium or attached to its own endosperm. These differences may be listed as follows: (1) smaller increase in fresh and dry weight and particularly in length; (2) absence of red or yellow pigment; (3) failure to form a leaf sheath.

It is clear from (1) that the isolated stem tip is able to utilize the nutrients supplied only to a limited extent. This might be due to the absence from the medium of some factor essential for growth, or to a physical inability on the

part of the stem tip to continue absorbing nutrients at a sufficient rate to maintain growth.

The nutrient medium used in this work supplied both major and minor mineral elements, carbon as sucrose and nitrogen as nitrate. It is possible that sucrose and nitrate were not the best forms in which to supply these essential nutrient elements, and that better growth would have been obtained with some other source of carbon and nitrogen. Possibly again some factor of a vitamin or hormone nature essential to the nutrition of the shoot was lacking from the nutrient solution. The present investigation was concerned with this aspect of the problem. Seven different crude plant extracts and thirteen pure substances were tested, but none enabled the isolated stem tip to make any greater growth than with sucrose and mineral elements alone.

Nevertheless, it would not be safe to conclude that none of the substances tested in these experiments plays any part in leaf growth. In all cases in which an isolated plant organ is grown on an artificial medium, interpretation of results is complicated by questions regarding the ability of such an organ to absorb all the nutrients it requires from the medium. The stem tip is not physiologically adapted to absorb already-elaborated nutrients direct from an external medium. Growth, therefore, may be limited by this simple disability.

Attention has already been drawn to the immediate effect on the growth of these isolated stem tips of the development of a root system. When such development occurs the meristematic activity of the entire growing point is awakened and new leaves are developed in which the leaf sheath is differentiated and yellow colouring matter formed. The nature of this stimulating action is at present unknown.

Summary

The growth of isolated stem tips excised from rye embryos on a culture medium containing 2 per cent. sucrose and mineral salts was studied.

Growth on this medium was found to be almost entirely confined to the first leaf and presented several abnormal features. In the absence of mineral salts growth was much reduced. In the absence of sucrose no growth occurred.

The following substances added to the medium failed to produce any significant increase in leaf growth: (*a*) Crude extracts of peas, rye grains both sprouted and unsprouted, yeast, and a digest of rye endosperm; (*b*) the 'B' vitamins thiamin, nicotinic acid, calcium pantothenate, pyridoxine, riboflavin, biotin; also ascorbic acid, vitamins E and K, indole acetic and naphthalene acetic acids, and the purine derivatives adenine, guanine, uric acid, and caffein.

It was observed that if any isolated stem tip developed a root the entire growing point was stimulated to meristematic activity, and leaves normal in form and size developed.

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