the available photographs it is composed of approximately 200 members, scattered somewhat irregularly over an area of 1.5 square degrees. On the assumption of three-tenths of a magnitude as possible space absorption, its distance is about forty megaparsecs.

* 115 million light years.

[1] *H. A.*, **105**, Paper No. 8, 1937.

[2] Shapley and Boyd, *H. C.*, **411**, 1936; *H. A.*, **105**, Paper No. 13, 1937.

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## *THE GROWTH OF PLANT EMBRYOS IN VITRO. PRELIMINARY EXPERIMENTS ON THE RÔLE OF ACCESSORY SUBSTANCES*

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The aseptic culture of plant embryos isolated from the seed dates back to the work of Brown and Morris,[1] Hannig[2] and Dietrich.[3] More recent contributions to our knowledge concerning the culture *in vitro* of excised embryos have been made by Tukey,[4] Brunner[5] and LaRue,[6] among others. It has been recognized by, for example, Ray[7] that the embryo culture technique offers a useful tool for biochemical investigations, and it has also been recognized[4,8] that it may be used as a practical measure to circumvent the abortion of embryos. It has, however, been found that in general the growth of the excised embryo, even upon a medium containing essential inorganic materials and sugar, is far less than that of normal intact seedlings. This has led to the suggestion[5] that "accessory growth factors" which are needed in minute amounts, are required by the developing plant as they are by the developing animal organism. The present work, as well as that of Kögl and Haagen-Smit,[9] furnish final proof that this is the case; that these accessory substances, although normally furnished by the seed, may be replaced to some extent by pure compounds added in small amounts to the embryo culture medium. These investigations, taken up early in 1936, are concerned particularly with orienting experiments undertaken with an ultimate view toward the elucidation of the nature and mode of action of these accessory growth factors. The embryo culture technique is here to be used as a tool in the "hormonal" analysis of plant development.

*Materials and Methods.*—Pea seeds of the "Perfection" variety were used for all of the principal experiments. A few of the preliminary experiments were done with the "Alaska" variety. Seeds were sterilized

by the usual technique of this laboratory (washed in 95% alcohol, soaked for 20 minutes in 0.1% $HgCl_2$) and then soaked in water for 6 (in some cases 9) hours in sterile Petri dishes. The embryos were then removed and cultured under sterile conditions. Infections were very rare. The basic culture medium was that which has been found to be highly satisfactory for the growth of excised pea roots[10] and contained the necessary inorganic salts with nitrate as the nitrogen source, as well as four per cent sucrose. This concentration of sucrose was found to be superior to lower concentrations for the growth of embryos as well as for excised roots. Agar medium was markedly superior to liquid medium. In the latter the growth of the shoot in particular was greatly diminished. Pyrex culture vessels were also superior to those of soft glass. It is worthy of note that while the embryos were able to make some growth either on liquid medium in Pyrex or on agar medium in soft glass, they were completely inhibited on liquid medium in soft glass. A comparison of the various types of possible culture vessels showed that flasks were somewhat superior to test tubes and hence Pyrex flasks were used for all of the cultures reported here. Twenty embryos were grown in each of the media tested in a single series. The individual values given in the tables below are thus each the mean from twenty plants. All cultures were grown in the dark and measured weekly with a flexible millimeter scale. At the end of four weeks each series was stopped and a final, more accurate measurement of root and shoot growth made.

*Principal Experimental Results.*—An attempt was first made to cultivate the excised apical meristem of the pea embryo. Such meristems grew, however, very poorly Additions of cotyledons, yeast extract, malt, peptone, etc., did not improve this growth but it was noted that those fragments which regenerated roots at their basal ends grew in the mean 2.5 mm. in two weeks, while those which did not form roots grew only 1.3 mm. in the same time. This was taken to indicate that roots *per se* are beneficial to stem growth. That this is actually true has been shown in other ways by Went.[11] That it is true in the present case is also shown by many other experiments in which the excised embryonic shoot grew less than one-half as rapidly as the entire embryo under the same conditions. This factor coming from the root and necessary for shoot elongation is apparently transported only through living tissue[11] and it was impossible to cause it to diffuse from excised shoot to excised root. It was also impossible to replace it by any of the extracts or substances which have been added to the medium. The further experiments were therefore made with the entire embryo.

Pea embryos when cultured on the basic medium attained a shoot length of 19 to 36 mm. in four weeks' time under the conditions used. In three series only did the growth on the basic medium exceed this amount and in

all three of these cases the relative response to added accessory factors was proportionately diminished. It seems reasonable to suppose that in these three series the embryos had for some unknown reason "mobilized" a larger portion of accessory substances from the cotyledon before excision.

Several series in which crystalline vitamin $B_1$[12] was added in varying amounts to the basic medium gave substantially identical results and as an example the measurements from a portion of a typical series are given in table 1. The effect of $B_1$ upon the root growth of the cultures was very marked, and it is possible that the effect upon shoot growth is therefore indirect. The optimum vitamin $B_1$ concentration was found to be roughly 0.13 $\gamma$ per cc. and this concentration was used for the later combination experiments.

Vitamin C, also, caused a distinct increase in the growth rate of both root and shoot, as is shown in table 1. The concentration of 0.05 mg. per cc. was superior to 0.1 mg. or to 0.025 mg. per cc. and was also used in the later experiments.

TABLE 1

THE GROWTH *in vitro* OF EXCISED PEA EMBRYOS UPON MEDIA CONTAINING VITAMINS $B_1$ AND C AS ACCESSORY GROWTH FACTORS

| MEDIUM | 1 WEEK | 2 WEEKS | 3 WEEKS | 4 WEEKS STEM | 4 WEEKS ROOT |
|---|---|---|---|---|---|
| Control | 10 | 18 | 25 | 34 | 85 |
| $B_1$, 0.13/cc. | 13 | 25 | 39 | 52 | 106 |
| $B_1$, 0.013/cc. | 8 | 18 | 30 | 44 | 104 |
| Control | 9 | 17 | 28 | 34 | 83 |
| C, 0.05 mg./cc. | 13 | 25 | 41 | 48 | 108 |

It has been found that a complex mixture of amino acids is necessary for the continued growth of excised roots[10,13] and such a mixture was therefore added to the basic medium as a co-growth factor for vitamin $B_1$. In none of the concentrations tested was the growth of the pea embryos increased over that brought about by vitamin $B_1$ alone. Inositol in conjunction with vitamin $B_1$ was also without effect.

Pantothenic acid,[14] however, was found to be a potent growth factor for these embryos. The optimum concentration of 0.15 gamma per cc. caused an increase in stem growth rate at least as great as that due to vitamin $B_1$ (table 2). The influence upon the root was less marked, however. Roots of embryos grown in pantothenic acid medium were longer than those in basic medium alone, but were neither as long nor as luxuriant as those grown on vitamin $B_1$ medium.

Folliculin was the fourth of the active substances investigated, and it also caused considerable increase in both root and shoot. Concentrations

TABLE 2

THE GROWTH *in vitro* OF EXCISED PEA EMBRYOS UPON MEDIA CONTAINING ACCESSORY GROWTH FACTORS ALONE AND IN COMBINATION

(Concentrations given in text)

| MEDIUM | 1 WEEK | 2 WEEKS | 3 WEEKS | 4 WEEKS STEM | 4 WEEKS ROOT |
|---|---|---|---|---|---|
| Control | 10 | 21 | 24 | 34 | 80 |
| Pantothenic acid | 13 | 26 | 34 | 54 | 95 |
| Vitamin $B_1$ | 9 | 20 | 33 | 51 | 109 |
| Folliculin | 13 | 31 | 40 | 51 | 102 |
| $B_1$ + pant. acid | 15 | 29 | 43 | 63 | 100 |
| $B_1$ + pant. acid + C | 15 | 39 | 51 | 68 | 141 |
| $B_1$ + pant. acid + C + folliculin | 16 | 40 | 54 | 65 | 149 |

of 0.13 to 0.67 gamma per cc. showed no considerable difference in growth-promoting activity, but were more beneficial than either higher or lower concentrations.

If two or more of the active accessory factors were added simultaneously to the basic medium the effect was greater than that of either alone but was not equal to the sum of the individual effects. This is shown clearly in table 2, where the results of series with vitamin $B_1$, pantothenic acid and folliculin are given, tested alone and in combination, as well as in combination with vitamin C. There are some irregularities but it is clear that there is a maximum average shoot length of about 65 mm. (although individual shoot lengths of as great as 95 mm. in four weeks have occasionally been recorded). The great effect upon root length of vitamin C in combination with $B_1$ and pantothenic acid is also worthy of note.

Leaf development was markedly less than that of the intact etiolated seedling and was not greatly influenced by any of the added accessory substances.

*Discussion.*—All of the four substances which have been shown to increase the growth of excised pea embryos are known to be natural plant products. Thus vitamin $B_1$, pantothenic acid and folliculin are known to occur in seeds, while vitamin C is formed during the germination process from sugars stored in the cotyledons.[7] It need not, then, occasion surprise that these substances apparently play a part in the development of the young plant, a conclusion which has been arrived at also by others. Thus Kögl and Haagen-Smit[9] in their work found an effect of vitamin $B_1$ upon the growth of the excised embryo similar to that recorded here. They have shown in addition that biotin, another normal constituent of the pea seed, produces much the same effect as does the related pantothenic acid used here. In their work just as in the present investigation, shoot growth in particular was increased by the bios II factor. Kögl and

Haagen-Smit have, however, reported that vitamin C was without influence upon the growth of the pea variety which they used. It seems not unlikely that varieties may differ in their ability to synthesize this substance for themselves; an ability which Ray[7] has shown that excised pea embryos do possess. In the present case vitamin C clearly acts as an accessory growth factor, in agreement with the results of von Hausen,[15] Havas[16] and Davis, Atkins and Hudson.[17] That folliculin exerts beneficial effects upon the growth of intact plants can no longer be doubted.[18] The present work showing that it is beneficial to the growth of excised embryos is also in accord with the findings of Kögl and Haagen-Smit.[9]

It is not surprising that the combination of several of the accessory growth factors should fail to yield additive growth responses. It is quite possible that as the growth rate is increased by the addition of successive factors, still other factors or conditions as yet unknown become limiting. It is also possible that the optimum concentration of one factor alone is not the same as the optimum concentration of the same factor in the presence of others. Both of these possibilities may well play a part in the determination of the upper growth rate limit shown in table 2. It is in any case clear that a "normal" growth of the excised embryo can be arrived at only by a long series of successive approximations.

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