Reports

Growth in Sterile Culture of Excised Leaves of Flowering Plants

The successful growth in sterile culture of excised leaf primordia of ferns has previously been reported (*1*). It has been shown that fern leaf primordia of various sizes and ages can be grown to maturity on relatively simple agar media. It has remained a problem to extend this technique, with its advantages of growth on defined medium in isolation from the influences of the whole plant, to morphogenetic studies of leaves of the flowering plants. The present report (*2*) records the successful accomplishment of this objective.

In these experiments, the sunflower (*Helianthus annuus* L.) has been employed the most extensively, but some supplementary trials with tobacco (*Nicotiana tabacum* L.) have been carried out. Both species were grown from seed in the greenhouse. In establishing cultures, the outer leaves of the apical bud were carefully removed, and the inner leaves were excised with knives (prepared from sharpened fragments of razor blades) and planted directly on sterile medium in test tubes. No special sterilization methods were employed for the plant material, since these inner leaves are apparently free of microorganisms. In sunflower the first eight leaves on the shoot (P_1 to P_8) were excised, and in tobacco the first five (P_1 to P_5).

Two media were used for the most part in these investigations, both solidified with 0.8 percent agar. In the simpler of these, Heller's solution of mineral salts (*3*) was used with 2 percent sucrose and a mixture of ten B vitamins (*4*). The more complex medium contained, in addition to these constituents, 15 percent autoclaved coconut milk and acid casein hydrolyzate at a concentration of 1 ml/lit.

The major results of these culture studies are shown in Table 1 and indicate that excised primordia of sunflower are capable of making excellent growth in sterile culture on both the simple and the complex media, showing distinct increases in linear dimensions as well as in fresh weight. There is every indication, particularly from a study of sections of leaves grown *in vitro*, that an organized meristematic development is involved in their growth. The leaves grown in sterile culture clearly attain a condition of maturity, but they are smaller than corresponding leaves developed on the intact plant, as reference to Fig. 1 will show, although there is variation in size in natural leaves depending on conditions of growth. Possible explanations for the small size of cultured leaves have been previously considered (*1*) in the study of fern leaf culture, in which a similar phenomenon has been observed. An additional difference was noted in the shortness of the petiole, or in some cases its almost complete absence, in cultured leaves in contrast with the elongate petiole of natural leaves.

It is evident that sunflower leaves will grow to maturity *in vitro* on a culture medium containing only mineral salts and sucrose as nutrients, with a supplement of B vitamins. It is not at all certain, moreover, that the vitamin mixture is necessary, and this problem will be investigated further. On the other hand, the complex additives, coconut milk and casein hydrolyzate, definitely enhance the growth of leaves in culture, especially the younger primordia. They cannot, however, be considered essential. Table 1 shows the extent of development of the fifth to the eighth primordia of sunflower. The second, third, and fourth primordia have also been successfully cultured, although somewhat greater difficulty is encountered because of the small size of the explants. It may also be noted that results which are essentially similar have been obtained with leaves of tobacco (Fig. 1*D*).

Leaves grown on the media described here present one unusual feature which it would be desirable to overcome. When mature, the leaves are for the most part chlorotic—yellow or whitish in color—and they soon die, unlike the cultured fern leaves, which remain alive and healthy for many weeks after reaching maturity. A partial solution to this difficulty has been found in the incorporation of an ammonium salt into the culture medium. The most successful medium thus far devised is a modification of the simple medium in which sodium nitrate is replaced by ammonium nitrate in such a concentration that the total amount of nitrogen supplied is unchanged from the original medium. On this medium the leaves were, for the most part, bright green in color and remained alive and healthy for several weeks after reaching maturity. Some leaves, however, especially the younger primordia, showed a tendency to become disorganized and to produce masses of callus. It is interesting to note that a comparable addition of ammonium nitrogen to the complex medium did not produce an apparent beneficial effect. The improvement of growth in the presence of the ammonium salt in the simpler

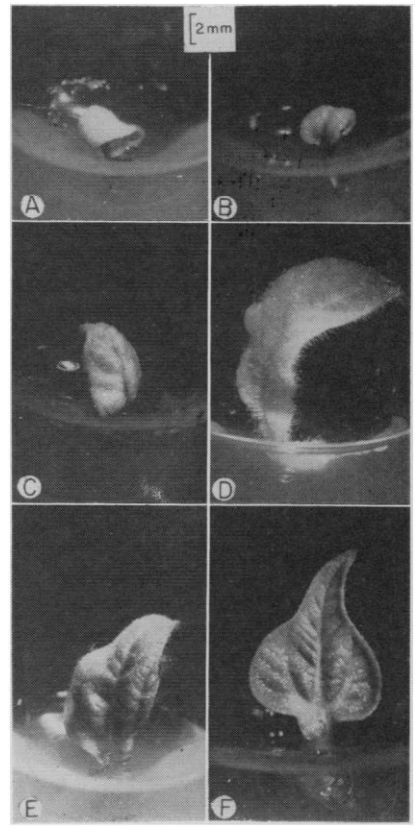

Fig. 1. Excised leaves grown in sterile culture. (*A*) *Helianthus* P_6 on simple medium; (*B*) *Helianthus* P_6 on complex medium; (*C*) *Helianthus* P_6 on simple medium containing an ammonium salt; (*D*) *Nicotiana* P_5 on complex medium; (*E*) *Helianthus* P_8 on simple medium; (*F*) *Helianthus* P_8 on complex medium.

All technical papers and comments on them are published in this section. Manuscripts should be typed double-spaced and be submitted in duplicate. In length, they should be limited to the equivalent of 1200 words; this includes the space occupied by illustrative or tabular material, references and notes, and the author(s)' name(s) and affiliation(s). Illustrative material should be limited to one table *or* one figure. All explanatory notes, including acknowledgments and authorization for publication, and literature references are to be numbered consecutively, keyed into the text proper, and placed at the end of the article under the heading "References and Notes." For fuller details see "Suggestions to Contributors" in Science **125**, 16 (4 Jan. 1957).

Table 1. Growth of excised leaf primordia of *Helianthus annuus*.

Primordium	Medium	Initial		Final		
		Length (mm)	Fresh wt. (mg)	Length (mm)	Width (mm)*	Fresh wt. (mg)
P_5	Simple	1.1	< 0.1	3.0	1.2	1.2
P_5	Complex	1.1	< 0.1	4.7	2.1	5.8
P_6	Simple	1.8	< 0.1	5.3	2.1	4.2
P_6	Complex	1.8	< 0.1	6.6	3.0	5.9
P_6	Ammonium simple	1.8	< 0.1	9.6	4.2	16.1
P_7	Simple	2.5	0.3	6.2	3.2	8.2
P_7	Complex	2.5	0.3	11.9	5.0	16.8
P_8	Simple	3.4	0.7	14.5	5.4	17.9
P_8	Complex	3.4	0.7	12.3	4.4	18.2

* Width measured at widest point of lamina.

medium is somewhat surprising, since, in general, nitrate is more suitable for the growth of plant tissue cultures (*3, 5*). It is possible that nitrogen in a reduced form is more easily used by small explants having no roots. On the other hand, it may be that the effect is indirect, through an influence on *p*H which makes certain other ions more readily available. Preliminary experiments suggest that the latter is not the case, but further work on this question is in progress.

The studies described in this report indicate that the technique of sterile leaf culture may now be extended to the flowering plants. As a result, the advantages of growth in sterile culture, on defined medium and isolated from the influence of the whole plant, may now be applied to developmental and physiological studies on angiosperm leaves. In addition, it has been demonstrated in these preliminary experiments that in the flowering plants, as in the ferns, the complex pattern of leaf development is self-controlled within the leaf, once the leaf has been determined at the shoot apex, and is not dependent on continued association with the plant. The youngest primordia cultured, down to the second on the shoot apex, have always, if they have grown at all, developed as leaves, except for a few cases in which a callus was produced. A medium permitting satisfactory development to maturity has been devised, but it is probable that further improvements can be introduced. This is the subject of investigations now in progress.

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References and Notes

1. I. M. Sussex and T. A. Steeves, *Nature* **172**, 624 (1953); T. A. Steeves and I. M. Sussex, *Am. J. Botany*, in press.
2. This work was supported in part by a grant to R. H. Wetmore and T. A. Steeves from the American Cancer Society, Inc., upon the recommendation of the Committee on Growth. H. P. Gabriel was aided by a summer grant from the National Science Foundation.
3. R. Heller, thesis, University of Paris (1953).
4. Modified from G. Morel and R. H. Wetmore, *Am. J. Botany* **38**, 138 (1951). Contents and concentrations per liter are as follows: thiamine hydrochloride, 10.0 mg; pyridoxine hydrochloride, 1.0 mg; nicotinic acid, 5.0 mg; calcium pantothenate, 5.0 mg; riboflavin, 0.5 mg; folic acid, 0.1 mg; biotin, 0.01 mg; choline chloride, 1.0 mg; para-aminobenzoic acid, 0.5 mg; inositol, 10.0 mg.
5. R. J. Gautheret, *Ann. Rev. Plant Physiol.* **6**, 433 (1955).

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Paleobotanical Studies of Canadian Pleistocene Nonglacial Deposits

During recent years I have made a palynologic study of certain nonglacial deposits in the St. Lawrence lowland, Quebec, and in the James Bay lowland, Ontario. The results warrant an assessment of the climate of the nonglacial interval and of the stratigraphic positions of the deposits (*1*).

In the St. Lawrence lowland, the nonglacial sequence is exposed in several sections along the St. Lawrence River and its tributaries, over a distance of 70 mi, from Pierreville to Donnacona, about 25 mi west of Quebec City. In the James Bay lowland, the exposures of nonglacial deposits have been observed on the Missinaibi, Opasatika, Soweska, and Albany rivers (*2*). A sound Pleistocene geologic background for the palynologic studies has been provided by N. R. Gadd (*3*) and O. L. Hughes of the Geological Survey of Canada, who have mapped the Pleistocene deposits in the St. Lawrence lowland and Cochrane areas, respectively.

By oversimplifying matters considerably, it may be stated that the stratigraphic sequence in both areas is composed of a basal set of glacial deposits of unknown age, overlain by a nonglacial sequence which is in turn overlain by younger glacial deposits. Marine and alluvial deposits overlie the uppermost glacial deposits. Radiocarbon determinations made on samples of peat and wood gave an age of more than 38,000 yr (sample W-242) for the nonglacial deposits on the Missinaibi River and more than 40,000 yr (sample W-189) for the nonglacial deposits in the St. Lawrence lowland (*4*). The palynologic evidence indicates that boreal conditions prevailed during most of the nonglacial interval in both areas and that the temperature did not reach a maximum as warm as that of the present. An arctic and subarctic environment is evident at the beginning and close of the interval (relatively high percentages of birch, alder and nontree pollen). Black spruce (*Picea mariana*) and white spruce (*Picea glauca*) were the predominant trees in the early and late parts of the interval. Higher values (40 to 60 percent) of pine pollen (*Pinus banksiana*) in the middle of the interval probably indicate slightly warmer conditions. Alder and birch pollen percentages were low during most of the interval.

In the St. Lawrence lowland, small amounts of white pine pollen (*Pinus strobus*) have been found in samples from the middle and lower parts of the nonglacial peat sequence. In the lower middle part of the sequence, there is a scattered but significant presence (2 to 5 percent) of pollen of temperate deciduous trees (*Quercus, Ulmus, Acer, Fraxinus, Carya, Carpinus, Tilia*). Hemlock pollen (*Tsuga canadensis*) is apparently absent, whereas it is present in all pollen diagrams of post-glacial sediments from the same area. Judging by the geologic and palynologic evidence, a drainage system was established prior to the deposition of the peat sequence. The peat, silt, and silty peat sequence was most likely deposited along wide river valleys.

An attempt to estimate the length of the nonglacial interval in the St. Lawrence lowland is admittedly precarious, but, judging by the findings of Zumberge and Potzger (*5*), it is probable that the peat alone represents a period of 3000 to 4000 yr. Some 500 to 700 yr are represented by the varved clays, both above and below the nonglacial deposits. The period of erosion, previous to deposition of the nonglacial deposits, is of undetermined length, but it is probable that only 2000 to 3000 yr are represented by this hiatus; otherwise, evidence should have been found of a type of vegetation more closely resembling that of the present. It is likely that there was not enough time available for migration of hemlock into the St. Lawrence lowland during the nonglacial interval, which was named the St. Pierre interval by Gadd (*3*).

The chronologic position of the St. Pierre interval is still subject to some doubt. It is older than any of the recognized intervals of the Wisconsin stage.