THE TOTIPOTENCY OF CULTURED CARROT CELLS: EVIDENCE AND INTERPRETATIONS FROM SUCCESSIVE CYCLES OF GROWTH FROM PHLOEM CELLS

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Daucus carota L. is normally a biennial plant. The familiar storage root of its common cultivars is formed in the first cycle of its growth. After an appropriate period of dormancy and perennation a flowering shoot bears seeds formed in the second cycle of this biennial growth. And so successive life-cycles may be bridged by the formation of seed and viable embryos. As in many biennials temperature (exposure to 5° C.) may promote, to some extent, the premature bolting of the flowering shoot. In some instances the compounds known as gibberellins may substitute for the effect of low temperatures on bolting (Lang, 1957).

Experiments into the culture of the secondary phloem from the storage root, and of free cells derived from it, have shown that this normal sequence of events may now be changed. Growth may be induced in minute explants of the secondary phloem, cut so far from the cambium that they consist only of storage parenchyma that normally would not grow again. When the new growth, stimulated by a medium which contains coconut milk, occurs around a preformed explant, rapid proliferation, without organization, is all that normally occurs. However, by appropriate devices, free cell suspensions can be obtained from the surface of these explants, and the cells so freed will divide when they are suspended in the liquid medium. Such cells give rise to a wide range of growth forms which are not normally encountered either in the cultured explant or the tissue as it occurs normally in the plant body. Two previously unknown responses have been noted with these cells. First, they have been maintained in long continued cultivation as successive liquid cultures, inoculated with freely-suspended cells in the manner of transfer more familiar with micro-organisms. Secondly, the free cells grow into small clusters which readily organize to form roots and, with the formation of organized shoot tips, they form small "embryoids" from which whole plants have been raised. Thus a vegetatively produced "embryoid" grown from cultured phloem cells may bridge two vegetative growth cycles in the manner normally attributable to the setting of seed and the formation of a zygotic embryo.
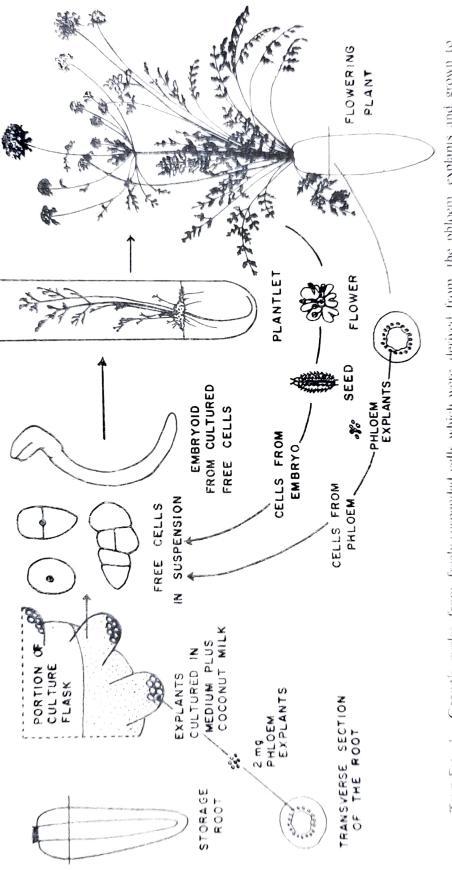
To the first observations of this kind may now be added further examples. Notably, it will be shown that plants bearing carrot roots can be grown from free cells, as heretofore, but that from these storage roots, grown not from seed but from embryoids developed from free cells, the whole process may be repeated. Thus, although the periodic incidence of storage roots may repeatedly give rise to the formation of flowers which set seed, the continuing culture of the strain through successive "life-cycles" may be maintained through vegetatively developed embryoids which originate from cultured cells, instead of from zygotes as in the usual way. The fact that this process may be repeated in yet another cycle of these events is still further evidence of the inherent totipotency of the cultured phloem cells. Moreover, since genetically "pure lines" of carrot are not normally feasible, this may be a technique by which they could be maintained indefinitely and independently of, though concomitant with, the recurrent production of seed.

The sequence described above may be represented by the following series of diagrams in Text-Fig. 1, which will serve as the background against which the new observations may be reported.
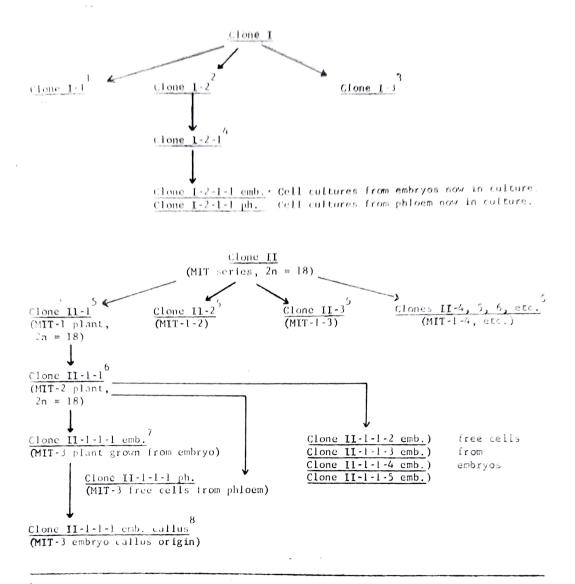
This complete life-cycle of the carrot, which nevertheless bypasses sexual reproduction entirely, has now been traversed several times using different clones of material. These are arbitrarily numbered, as shown in Text-Fig. 2, and summarized as follows:

Clone I–1.—This was derived from a strain of carrot that had been used for purposes of routine assay of the growth factors in coconut milk and by chance it furnished the first cell cultures which were grown freely suspended in the liquid medium. The cytology of the "parent" carrot root was not known. This clone was noteworthy, however, because the freely-cultured cells retained their totipotency and ability to reconstitute whole plants through many (approximately 30) subcultures and a long period of separate cultivation (3 years). Photographs of this first cultured plant, with a small but distinctly orange-colored tap root, were taken and reported by Steward *et al.* (1958 *a*). The storage root was sectioned for photographing and later preserved in alcohol. No derived cultures from this specimen were established.

Clone I–2.—Several other plantlets derived from Clone 1 were also used to repeat the whole cycle. The objective here was to obtain many plants from one cell suspension to see whether they were constant in their morphology. Two of the older plants (designated Clone 1–2 and Clone 1–3) were carried through to mature plants bearing inflorescences. Clone 1–2 is of particular interest as it was the first plant, cultured from cells, that was successfully grown to the flowering stage (Plate 1, Fig. 1 *a–b*) and also because cell cultures were again started from the storage root (Plate 1, Fig. 1 *c*) to begin a new cycle. Although the cytology of the parent stock was unknown, the chromosome number of this reconstituted plant was determined by Dr. J. Mitra as that of a normal diploid (2*n* = 18).

TEXT-FIG. 1. Growth cycles from freely-suspended cells which were derived from the phloem explants and grown to organized cell clusters and mature plants with inflorescences; the cycle then repeated from the free cells derived from the phloem explants and excised embryos from the reconstituted plant.

Clone I

Clone I-1 [1]   Clone I-2 [2]   Clone I-3 [3]

Clone I-2-1 [4]

Clone I-2-1-1 emb.· Cell cultures from embryos now in culture.
Clone I-2-1-1 ph.   Cell cultures from phloem now in culture.

Clone II
(MIT series, 2n = 18)

Clone II-1 [5]   Clone II-2 [5]   Clone II-3 [5]   Clones II-4, 5, 6, etc. [5]
(MIT-1 plant,   (MIT-1-2)   (MIT-1-3)   (MIT-1-4, etc.)
2n = 18)

Clone II-1-1 [6]
(MIT-2 plant,
2n = 18)

Clone II-1-1-1 emb. [7]   Clone II-1-1-2 emb.)   free cells
(MIT-3 plant grown from embryo)   Clone II-1-1-3 emb.)   from
                                           Clone II-1-1-4 emb.)   embryos
          Clone II-1-1-1 ph.   Clone II-1-1-5 emb.)
          (MIT-3 free cells from phloem)

Clone II-1-1-1 emb. callus [8]
(MIT-3 embryo callus origin)

[1] First cell culture to develop an organized tap root (Amer. Jour. Bot. 45: p. 706).
[2] First cell culture to develop into plant with inflorescence.   (Referred to in
    Fig. 3a-d.)
[3] Second plant from cells to produce an inflorescence.
[4] Another growth cycle from phloem cells leading to plant with inflorescence (Fig. 3h).
[5] Plants grown from cells from phloem explants -- known cytology (Fig. 4e).
[6] Plants raised from cells to maturity. Plant used to start a new growth cycle
    : from phloem and embryo cells (Fig. 4f).
[7] Zygotic embryo from [6] raised to mature plant (Fig. 4g).
[8] Embryoids from callus grown from the embryo culture of [7] (Fig. 4h and i).

TEXT-FIG. 2. Nomenclature of clones under investigation.

In order to establish a cell culture from this derived plant of Clone I-2 (Plate I, Fig. 1 a-d), small explants (3·5 mg) from the phloem of the storage root were again grown in White's basal medium supplemented with coconut milk (10% by volume). These were first grown in culture tubes on June 30, 1959 and were later transferred to large flasks with 250 ml of the medium to obtain a larger population of freely-suspended cells (Plate I, Fig. 1 e). Transfers of the rooted clusters were made to agar tubes containing coconut milk medium and the

tubes were kept stationary in the culture room. Shoots with typical carrot leaves appeared after a few months (Plate I, Fig. 1 *g*). The plantlets were maintained in stock culture and transferred to a fresh agar medium when needed. The plants were later (fall of 1961) grown in vermiculite and, when older, transferred to soil in the greenhouse. During this period the plants were nourished occasionally with Hoagland's solution. In January 1963, when the storage root was well developed, the plant produced a flowering stalk (Plate I, Fig. 1 *h*). This shift to the flowering stage without any prior cold treatment or vernalization has been noted, and, although the first plants flowered coincident with the flowering of the wild carrot (*Daucus carota*), the plants did so only after the large storage root was formed.

Clone II–1.—This strain was established from a carrot root of precisely known origin and of determined cytology. A garden variety (Nantes) was selected for this study, and the plant used and the cell cultures derived from it were arbitrarily designated MIT–1. Cultures were started on October 23, 1958 from the phloem tissue which was explanted at a distance of 1–2 mm from the cambium. Free cells (Plate II, Fig. 2 *a*) and cell clusters were obtained in the usual manner and their cytology studied and reported (Mitra *et al.*, 1960).

The first visible root from a cell cluster was observed on February 3, 1959. When the cultures grown from free cells began to show some large cell clusters, one of the flasks was placed in total darkness in the cold room (4–5° C.) on March 5, 1959. After a period of 5 months the flask was brought out for examination and the cell clusters showed abundant roots (Plate II, Fig. 2 *c*). From this flask and from several others rotated on the wheels of the culture apparatus (*cf.* Steward and Shantz, 1956), about 100 of the rooted clusters were transferred to a semi-solid medium containing 2%, 5% or 10% coconut milk on August 26, 1959. The first shoots were noticed in cultures grown in 2% or 5% coconut milk. Some of the cultures transferred to the medium which contained 10% coconut milk continued to proliferate. The plants grew slowly, and as they outgrew the tubes and flasks they were transferred, as required, to vermiculite in the greenhouse.

At least 100 plants were grown in the laboratory and greenhouse in order to record any variations in gross anatomy and chromosome complement. Thus far the chromosome numbers of the organized roots grown from cultured cells have all been of normal diploid number ($2n = 18$), similar to that in the original cells from which the cultures were grown in the first place. Plate II, Fig. 2 *e* shows an assortment of cultured plants, from older ones showing inflorescences and the newly transplanted ones from agar. Probably due to lack of active pollination under greenhouse conditions, only a few flowers per umbel developed seeds.

Clone II–1–1.—This strain of cells was established from the carrot storage roots grown at the end of the first growth cycle of one of the reconstituted plants of MIT–1 (Clone II–1) as these were raised from

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cells. This strain was designated MIT–2 in the laboratory record. It will be shown that whole plants, carrot storage roots, and, in the second cycle of growth, an inflorescence bearing viable zygotic embryos have all been grown from the free cells of this strain. Therefore, starting with a carrot root of known origin and of defined cytology the complete "life-cycle", illustrated in the diagram (Text-Fig. 1) has twice been carried completely through. Thus vegetatively produced embryoids grown from free-cell suspensions have twice linked successive "life-cycles" of the carrot plant in the manner normally achieved by fertilization and seed formation. When the plants so grown form flowers they also bear viable embryos. There can, therefore, be no doubt that the cultured carrot phloem cells which linked one "generation" of carrot to another retained the full totipotency equivalent to that in a zygote.

The evidence upon which these statements are based is as follows. In March 1959 one of the first rooted clusters from the cell culture of MIT–1 (Clone II–1) was transferred to a semi-solid medium containing coconut milk. Applying the now usual procedure for growing freely-suspended cells and cell clusters, the rooted cluster and eventually the plantlet derived from it was transferred to vermiculite and then to soil under greenhouse conditions. In September 1960 cell cultures (Plate II, Fig. 2b) were started from the storage root and maintained in stock as MIT–2 (i.e., Clone II–1–1). Again when the cell clusters showed roots and later typical carrot shoots (Plate II, Fig. 2d), one of the plantlets was transferred to vermiculite and nourished with Hoagland's solution until it could be grown in soil in the greenhouse. In June 1962 the first flowers opened and the flowering period continued through the summer (Plate II, Fig. 2f).

Clone II–1–1–1.—On September 21, 1962 several seeds from a plant in flower (Clone II–1–1), mentioned above and shown in Plate II, Fig. 2f, were brought into the laboratory for examination. The fertile seeds in the umbel that were swollen and larger than the rest were excised aseptically for transplanting the embryos to agar plates containing coconut milk. The embryos developed roots and shoots in the normal manner and produced normal-appearing plants which were designated MIT–3 (i.e., Clone II–1–1–1 emb.). One of them is shown in Plate II, Fig. 2g. Thus another cycle from cultured root, to freely-suspended cells, to organized clusters and eventually to a plant in flower and bearing normal seeds has been completed; in this clone, therefore, two full "life-cycles" have been completed.

A recent and particularly interesting development is as follows. Embryos, such as those borne on the plant (Clone II–1–1, Plate II, Fig. 2f) which was itself grown from free cells, can give rise to a proliferating culture when they are grown aseptically in a medium which contains coconut milk. These embryo cultures readily give rise to cells and these seem to form embryoids with remarkable ease. In fact, as they do this, heart-shaped and torpedo-stage embryoids (Plate II, Fig. 2h and i), indistinguishable from those that develop from zygotes, have been seen.

DISCUSSION

The results here described represent the fulfilment of Haberlandt's vision of raising higher plants from cells. In Braun's experiments with tobacco tumors another example is to be seen (Braun, 1958). Single cells of tumorous origin were grown into small clusters which were then grafted on the decapitated shoots of normal plants. By repeated grafts the tissues which originated from tumorous cells retraced their development from the tumorous state until they could give rise again to a normal shoot. This only occurred under some environmental conditions contributed by the normal plant. In the case of carrot the conversion of free cells of adult origin to embryoids requires first a measure of freedom from any of the restrictions superimposed by organic contact with preformed and differentiated tissue, and secondly it requires the nearest equivalent of the biochemical environment of a zygote. Under these circumstances any diploid carrot cell is potentially totipotent and can behave like a zygote. The parallelism with embryogeny is close because, *enroute* from carrot cell to carrot plants, embryoids are formed (*cf.* Fig. 6 of Steward and Mohan Ram, 1961). The data here reported show that plants grown from embryoids originating from cells are normal in every respect and the growth cycle of the carrot plant may be repeatedly passed through without the intervention of seeds. Moreover the early stages of development in the vegetatively produced embryoids resemble key stages in normal embryogeny.

While the totipotency is present in any cell, its full expression it evoked more easily if the cell has access to a medium which is, as is were, "conditioned" by the substances released from other growing cells. Unpublished work of this laboratory shows that free cells, spread thinly on an agar medium like bacteria, spring into growth more readily if, over and above the coconut milk in the medium, they are in the near proximity of some previously cultured explants. This shows that a vigorously growing colony of carrot cells will release to the medium something which is even more potent than is coconut milk itself (work of Blakely with Steward). However, it is now very clear that the conditions under which cells divide most rapidly (*i.e.*, when they are attached to an existing piece of explanted tissue) are *not* those best for their morphogenetic development since this occurs when they can be grown free, albeit at a slower rate. Thus the environment of the cultured explant superimposes some controls or limitations upon the otherwise totipotent cell.

One might suppose that the cells which grow so freely into plants have been changed fundamentally (*e.g.*, cytologically) from those that existed in the carrot plant from which they were derived. While this may yet be so, in detail, all the evidence is against this. Cytological examination of carrot plants derived from free cells always shows (at least as yet) that they are normal diploids ($2n = 18$) and no visible morphological variations exist between them. This is the more surprising because different clones or strains of cells, cultured from free

cells, do show spontaneous variations (Steward *et al.*, 1958 *b*). Presumably the variants are not viable enough as organized structures to be raised to whole plants by existing methods.

On this point of variation in the cultured cells it is significant that by long cultivation a strain of free cells (which originated from MIT–1, *i.e.*, Clone II) has tended to lose its ability to organize although *not* its ability to grow freely. This change has occurred after 14 months and 12 transfers, for this strain only occasionally produces roots and forms shoots with even greater difficulty. It may well be that the conditions of long cultivation tend to select some aberrant or less than totipotent cells. However, if one passes the organism through the vegetative cycle shown in Text-Fig. 1, it seems that the free cells derived from the storage organ in each successive vegetative cycle do retain their full totipotency.

Two other possibilities suggest themselves. The free cells that are derived from different tissues and organs that have already differentiated, such as pith, cambium, cortex, xylem parenchyma, phloem parenchyma of root or shoot, etc., may reveal their genetic totipotency with greater or less difficulty according to the tightness of the control which their differentiation imposed upon them. Although this point is under investigation, data cannot yet be given. A corollary is, however, that cell cultures derived from embryos, particularly immature embryos, might well be an exceptionally free source of totipotent cells that will grow, *via* embryoids, into plants. The first culture of this sort which was examined was one derived from tobacco (*Nicotiana rustica*) embryo and supplied by Dr. Mohan Ram of the University of Delhi, India. Although this culture has grown as a free-cell culture, under our conditions, with great vigor it has failed to organize, possibly because of its long history in culture. It is, therefore, of great significance that a free-cell culture from carrot embryos (or plants also grown from cells) does readily organize and grow (*see* Text-Fig. 1 and Plate II, Fig. 2 *h* and *i*). Furthermore, the comparative ability of the wild *Daucus carota*, unmodified by breeding, to exhibit these morphogenetic responses in its various cells is also being investigated.

Now that one can produce embryoids from free cells of vegetative origin, another intriguing possibility exists. If by environmental or growth substance treatment the minute embryoids could be vernalized, the normal biennial growth cycle of the carrot could be by-passed. This has, as yet, only been subjected to preliminary tests.

This work clearly raises some profound problems with respect to the controls which must exist to limit or determine the full expression of the genetic totipotency which is clearly inherent in any diploid carrot cell. Although this point is made here with respect to morphogenetic expression it could be made with equal effect in terms of biochemical and metabolic characteristics. The different organs, derived ultimately from free cells, have different biochemical composition and metabolism.

A current view is that there are mechanisms which in effect "turn on or off" the action of specific genes, or groups of genes. Some believe that histones play a part in this respect (Gifford and Tepper, 1962; Huang and Bonner, 1962). Some also regard it as an adequate explanation to attribute this role to particular parts of the genetic machinery which in effect act as the controls that determine whether certain genes are active or quiescent. Whatever the machinery, however, the facts of plant development require that both environment and exogenous growth substances must have access to the centers where these controls operate. In fact, a feasible but as yet unproven explanation is that the growth-regulating substances which evoke cell division, promote cell enlargement and control morphogenesis may activate particular areas of the genetic apparatus. Unfortunately we cannot yet associate visible areas of activity along plant chromosomes with known stages of morphogenetic development in the manner that has been so dramatically possible in the salivary gland chromosome of certain insect larvae (Breuer and Pavan, 1955). These visible effects on the chromosome, as shown by the now well-known "puffs", are evidence enough of activity evoked by, or concomitant with, some developmental stimuli. In plants, similarly localized activity on the chromosomes may not be accompanied by the same degree of visible expression; even so it may exist.

On these views one could regard a given morphogenetic stimulus as the cause of a temporary release of some particular form of RNA which would make its imbalanced impact upon metabolism and development.

It would have been a happy outcome of the work on the coconut milk stimulus to cell division and morphogenesis if cells synthesized different RNA's in their activated and quiescent states. So far as present data in this laboratory go, this is unlikely to be so. (The base ratios of the RNA from quiescent and activated cells do not seem to be different.) Moreover, even in a complete basal medium, unsupplemented by coconut milk, cells which do *not* divide synthesize RNA to a high content per cell. It seems, however, that the RNA so synthesized cannot be effective as a template for synthesis and thus lead on to cell division and growth. What the coconut milk growth factors do is to allow the RNA so made to become effective in synthesis, growth and the division of cells. Thus there is here an area in which exogenous growth-regulating substances may, without activating genes directly, nevertheless determine indirectly the extent of their effect. This may be so inasmuch as they control the degree to which the RNA's which the genes produce will affect metabolism. Conversely, inhibitors of growth factors, like the factors in differentiation which limit morphogenetic expression, may not exert their effect upon the action of the genes directly, so much as upon the expression of the RNA's through which the genes normally mediate their effect.

Thus, although the totipotency of single diploid carrot cells is now amply demonstrated, the biochemical means by which this is controlled

and constrained during differentiation is now a more pressing problem than ever.

REFERENCES

BRAUN, A. C. 1958. A physiological basis for autonomous growth of the crown gall tumor cells. *Proc. nat. Acad. Sci., Wash.* **44**: 344–49.

BREUER, M. E. AND PAVAN, C. 1955. Behaviour of polytene chromosomes of *Rhynchosciara angelae* at different stages of larval development. *Chromosoma* **7**: 371–86.

GIFFORD, E. M. JR. AND TEPPER, H. B. 1962. Histochemical and autoradiographic studies of floral induction in *Chenopodium album*. *Amer. J. Bot.* **49**: 706–14.

HUANG, R. C. AND BONNER, J. 1962. Histone as suppressor of chromosomal RNA synthesis. *Proc. nat. Acad. Sci., Wash.* **48**: 1216–22.

LANG, A. 1957. The effect of gibberellin upon flower formation. *Ibid.* **43**: 709–17.

MITRA, J., MAPES, M. O. AND STEWARD, F. C. 1960. Growth and organized development of cultured cells. IV. The behaviour of the nucleus. *Amer. J. Bot.* **47**: 357–68.

STEWARD, F. C., MAPES, M. O. AND MEARS, K. 1958 *a*. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *Ibid.* **45**: 705–08.

——, —— AND SMITH, J. 1958 *b*. Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. *Ibid.* **45**: 693–703.

—— AND MOHAN RAM, H. Y. 1961. *Determining Factors in Cell Growth: Some Implications for Morphogenesis in Plants.* In "*Advances in Morphogenesis*," Vol. 1, Academic Press, New York.

—— AND SHANTZ, E. M. 1956. The chemical induction of growth in plant tissue cultures. In *Proc. Symp. Wye College* (Univ. London), (eds.) R. L. Wain and F. Wightman, Butterworth, London.
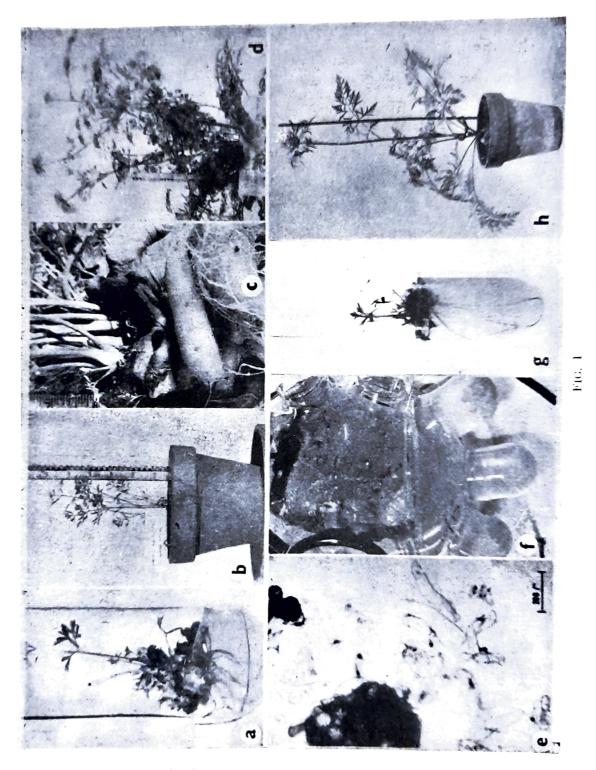
EXPLANATION OF PLATES I AND II

PLATE I

FIG. 1. Sequence of events from the Clone I-2 plant (*i.e.*, in the first vegetative cycle V_1), which was grown from freely-suspended cells and cell clusters, culminating in a plant of a second cycle (V_2) bearing an inflorescence. *a.* Plantlet grown from cell cluster derived from free cells, grown in agar medium containing coconut milk; *b.* Plantlet shown at (*a*) transferred to vermiculite and grown in the greenhouse; *c* and *d.* The same at maturity showing a massive root system at (*c*) and inflorescence at (*d*); *e.* Freely-suspended cells grown from the phloem explants taken from the tap root shown at (*c*); *f.* Cell clusters with roots grown in flask in a liquid medium containing coconut milk; *g.* Plantlet derived from an organized cell cluster grown in agar; *h.* Flowering carrot plant derived from the plantlet shown at (*g*).
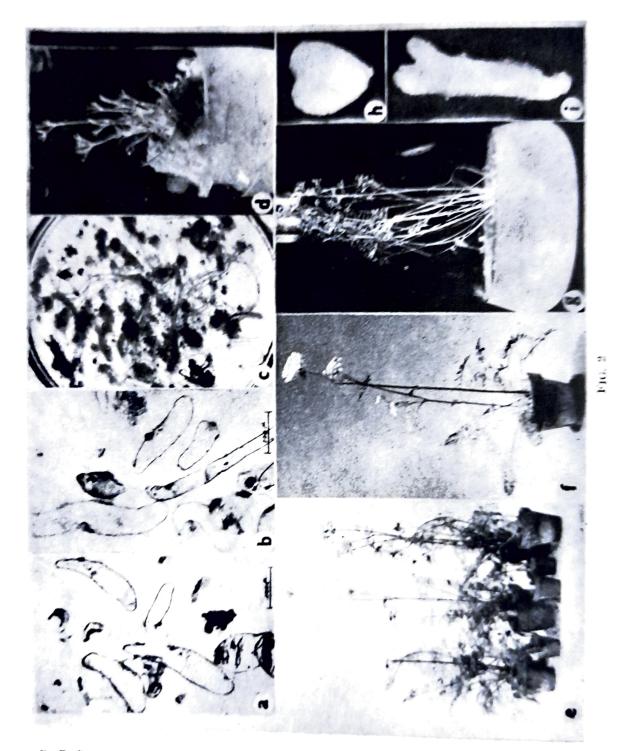
PLATE II

FIG. 2. Sequence of events from free cells, originating from the phloem of carrot with known cytology ($2n = 18$) to organized plants. Clone II series. *a.* Freely-suspended cells from the tap root (phloem) of Clone II-1 (MIT-1 plant); *b.* Freely-suspended cells from the tap root (phloem) of Clone II-1-1 (MIT-2 plant); *c.* Abundant roots formed in cell clusters

FIG. 1

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FIG. 2

from the Clone II free-cell cluster after growth in the dark and at low temperature (5° C.) for 5 months; *d.* Plantlet from an organized cell cluster derived from (*b*); *e.* Population of Clone II-1 (MIT-1 plants) of different ages and grown from free cells derived from phloem explants of Clone II plant; *f.* Clone II-1-1 (MIT-2) plant grown from free cells derived from phloem explants of Clone II-1 (MIT-1) plant; *g.* A carrot plant (MIT-3) grown from an embryo taken from a young seed on an umbel of a Clone II-1-1 plant, shown at (*f*); *h* and *i.* Heart-shaped (*h*) and torpedo-shaped (*i*) embryoids among the proliferating embryo cells derived from Clone II-1-1-1 emb. (MIT-3) liquid culture containing coconut milk.