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*CELL DIVISION IN ISOLATED SINGLE PLANT CELLS IN VITRO**

BY JOHN G. TORREY†

STRANGEWAYS RESEARCH LABORATORY, CAMBRIDGE, ENGLAND

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Introduction.—Ideally, a tissue culture should be started from a single isolated cell, maintained under cultural conditions which allow it to divide repeatedly to form a tissue in vitro. This ideal has been achieved with animal cells, first by Sanford *et al.*,[1] starting with single cells of L-strain mouse fibroblasts cultivated in capillary tubes, and then by Puck and Marcus,[2] using suspensions of HeLa strain human carcinoma cells in which individual cells attached to glass were seen to establish colonies under appropriate nutrient conditions. Both these methods of culture allowed precise and accurate microscopic observation during cell isolation and subsequent division.

Parallel studies of isolated plant cells have been less conclusive. Muir *et al.*[3] reported the establishment of tissue cultures from single isolated plant cells of marigold and tobacco-stem tissues by placing the isolated cell atop a piece of filter paper, which, in turn, had been placed upon a piece of callus tissue which acted as "host." Such single cells formed tissue masses 4 mm. in diameter in 6–10 weeks and could then be transferred to agar medium for further cultivation. The method unfortunately did not allow careful and continuous scrutiny of the cell either immediately after isolation or during subsequent development of the tissue. De Ropp[4] attempted to culture single isolated plant cells from several different plant callus tissues, using a modification of the capillary technique of Sanford *et al.*,[1] as well as a modified "sitting-drop" culture in which the cell was immersed in a fluid medium.

De Ropp, following isolated cells microscopically, was unable to obtain cell division in any of the mature vacuolated single cells he studied, although such cells could be maintained alive up to 4 months in a coconut-milk medium.

In the present study a method has been devised which combines the features of the hanging-drop technique and the "host" arrangement which permits observation of isolated cells and of their behavior in vitro. By this method, it has been possible to prove conclusively that mature vacuolated plant cells are capable of undergoing cell division in isolation. The method and the early stages in the development of callus-tissue cultures from single isolated cells are described.

Materials and Methods.—Callus tissue derived from the vascular cambium region of roots of the garden pea, *Pisum sativum*, was grown on a pea-root medium containing the auxin, 2,4-dichlorophenoxyacetic acid at 10^{-6} *M* supplemented with 1 gm/l powdered yeast extract by methods already described by Torrey and Shigemura.[5] Most cell isolations were from agar-grown callus tissues of an extremely friable type which had been maintained in continuous cultivation for over 2 years. Similar cells were studied from liquid-grown cultures. Cells were initially suspended in a small liquid drop of the nutrient medium on a cover slip over a moist chamber from which individual cells could be selected and then isolated by use of a deFonbrune micromanipulator equipped with a finely drawn micropipette. The isolation of cells was followed under the microscope at a magnification of 90×. Cells varying in size from 40 to 200 μ and in shape from spherical to elliptical and gourd-shaped were isolated.

The individual cells were transferred from the pipette to an agar hanging drop. Maximow's double-cover-slip method was used (cf. White[6]) and may be described briefly as follows. A warm 0.7 per cent agar drop of the nutrient medium (the M6 medium of Torrey and Shigemura[5] was used throughout) was pipetted onto a sterile 16 × 16-mm.-square cover slip, spread thin, and allowed to cool. In the center of the solidified drop was placed a small piece (about 1 mm. in diameter) of callus tissue. The cover slip was placed on the center of a second sterile $1^1/_4$-inch-square No. 1 cover slip, to which it adhered by the surface tension of a drop of paraffin oil. The double cover slip with the agar drop and callus culture in place was inverted over a standard $1^1/_2$ × 3-inch hollow-ground slide and sealed with a melted 3:1 paraffin-vaseline mixture. The culture was incubated from 2 to 4 days in the dark at 25° C. At the end of this period, the preparation was opened, the smaller cover slip slipped off with sterile forceps, and stored temporarily in a sterile moist chamber. The cover slip with its agar drop was then carried up with sterile forceps to the manipulator, and the isolated cell, held in the micropipette, was squirted out of the capillary onto the peripheral region of the agar drop surrounding the central callus mass. Usually six such cells were isolated and placed individually around the callus, then the cover slip was replaced on a fresh large cover slip, inverted over a new cavity slide, and resealed.

The cultures were incubated for 24 hours, and then each slide was examined under the microscope at high magnification (400×). For illumination, an incandescent lamp heavily filtered with neutral and dark-green filters, as well as a heat filter, was used. Viable cells were located accurately with the aid of a calibrated mechanical stage, and cell dimensions were recorded, together with a sketch of the cell and notes concerning distinctive features. Incubation was continued in the dark at

25° C. Re-examination of isolated cells was made at selected, fairly infrequent, intervals to avoid damage to the cells by illumination.

All the cells were mature parenchyma cells (Fig. 1, *A*) with a large centrally or peripherally located nucleus, usually surrounded by starch grains and suspended by protoplasmic strands, in which streaming of mitochondria and other cytoplasmic particles was evident. Because the cells adhered to the surface of the solidified agar drop, the viable cells had a characteristic three-dimensional appearance which dead, collapsed cells lacked. Protoplasmic streaming or gross cytoplasmic movements were the most reliable criteria of viability.

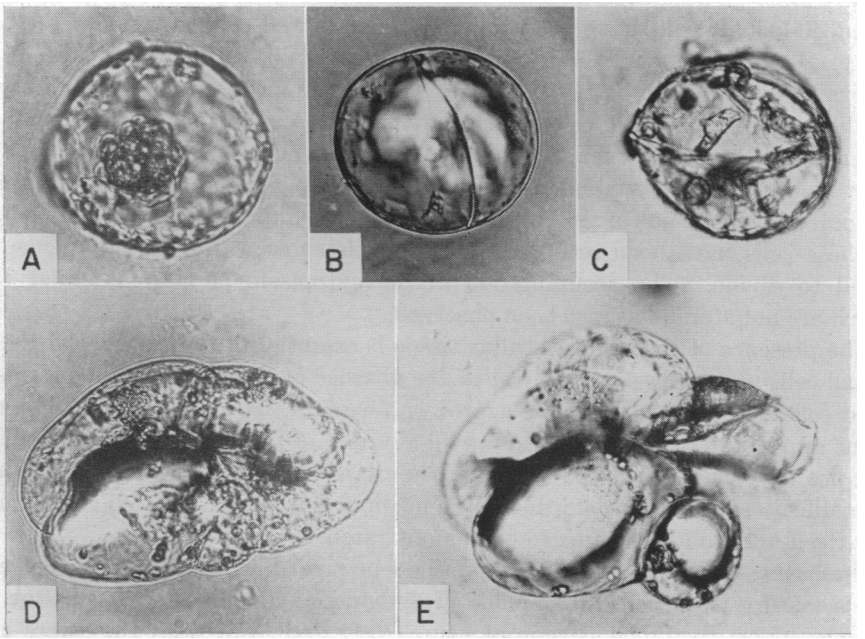

FIG. 1.—Cells and callus tissues derived from isolated single cells of pea-root callus, cultured on agar hanging drops. *A*, a single cell 24 hours after isolation, showing the large central nucleus surrounded by starch grains; *B*, a single cell after its division into two by the fifth day; *C*, a single cell after segmentation into 6 cells by the tenth day, (photographed after the culture had begun to degenerate); *D*, a clone of single-cell origin at the 5-cell stage on the nineteenth day; *E*, the same clone of single-cell origin as in *D*, at the 7-cell stage on the twenty-fourth day. Note the position of cells relative to those in *D*. The seventh cell is below the plane of focus on the right. *A–C*, 330×, *D–E*, 250×.

Experimental Results.—If a cell were going to divide, the first division usually occurred within 7 days. Figure 1, *B*, shows a cell which had divided into two by the fifth day, after which its size remained unchanged and it underwent no further development. Figure 1, *C*, illustrates a single cell which by the tenth day had divided into six, all within the original wall of the isolated cell. Although the photograph was taken after the culture had begun to degenerate, the segmentation which had occurred is evident. In these cells, division was unaccompanied by cellular enlargement, and development ceased. This segmentation behavior is reminiscent of the type of cell division which is induced experimentally with kinetin.[7]

Sometimes cell division was followed by cell enlargement, and the early develop-

ment of a callus tissue could be observed. Figure 1, *D* and *E*, shows two stages in the formation of a callus derived from a single cell. The original cell was 100 μ in length; by the seventh day, it had formed 3 cells. At the 5-cell stage on the nineteenth day (Fig. 1, *D*), the total length was about 185 μ, and at the 7-cell stage on the twenty-fourth day (Fig. 1, *E*) the callus was more than twice the original length and several times the original volume. The culture was lost at this stage because of dehydration. Other examples of cell division and subsequent enlargement of isolated single cells have been observed, but no clone has yet been carried to the point of establishing an independent callus culture. Many cells have been studied during these experiments. Since the development of the present method, about 400 single cells have been isolated and followed critically under the microscope. Of this number, about 70 per cent were viable at the end of the first 24-hour period, and about half of these cells were still alive at the end of the first week. Of the approximately 135 cells which, after isolation in this way, were found to be viable at the end of 1 week, only 7 per cent had undergone division. Cells have been maintained in a viable condition but without division for up to 8 weeks by periodic replenishment of the culture with fresh medium. Other cytologic changes in these cells have been recorded. A few cells showed limited enlargement but failed to divide. The accumulation in the cytoplasm of large granular bodies which are not starch has also been observed.

The presence of the "nurse" callus tissue is essential to the survival of the individual cells under these conditions; in the absence of the piece of callus tissue, isolated cells do not survive on the agar drop for more than a few days. The callus tissue appears to be essential also for the division of the isolated cells, as cell division has never been observed except in the presence of the nurse tissue. Whether the callus in some way "conditions" the medium so that the isolated cell can maintain itself and divide remains to be demonstrated.

Discussion.—The success of this method in providing conclusive evidence that single mature plant cells are capable of undergoing cell division in isolation gives strong support to the conclusion of Muir *et al.*[3] and Muir[8] that they were indeed establishing callus tissues from single cells. The present method can be considered a modification of their procedure, but it has the advantage that the individual cells can be followed microscopically during the early course of callus development. Technical difficulties arise in maintaining hanging-drop cultures for the prolonged periods necessary to establish independent clones; it is expected, however, that these problems will be overcome by routine periodic replenishment and that callus-clones of undisputed single-cell origin will be established in this way.

The question arises of why only a small proportion of the viable cells isolated undergo cell division. In all previously published studies on isolated cells, both animal and plant, the same observation has been made. In the first place, the technical procedure of isolation tends to damage cells, either by physical injury or through internal chemical changes, such as loss of essential metabolites by diffusion or by light-induced destruction. Second, some cells are undoubtedly abnormal in their nuclear constitution and behavior and thus inherently incapable of cell division. Such nuclear abnormalities are not infrequent in plant callus tissues and, while preventing normal mitosis, may not manifest themselves in cells in the resting state. A third possible explanation for the low percentage of cells in division is

that cells differ in their degree of differentiation in such a way that conditions which favor cell division at one stage of differentiation may be inappropriate for the division of cells at another stage. Using a selected medium and restricted environmental conditions may thus allow only a few of the cells from the mixed-cell population of a callus tissue to undergo division. Appropriate changes in nutritional or other conditions of culture may then bring other cells into division. These and other possibilities can and should be explored by the use of this method of culturing single isolated cells.

The two types of behavior manifested by the cells which did show activity, i.e., division without enlargement and division accompanied by enlargement and followed by divisions, can best be interpreted in terms of at least two groups of limiting factors for normal callus development from isolated single cells. It is possible that these two groups of factors limit and control cell division and cell enlargement, respectively, and may involve some form of interaction such as that postulated by Skoog and his co-workers[9] between auxins and kinins. A critical study of such compounds and their effects and interactions on isolated plant cells must be made with a synthetic medium and represents a problem for future study.

Summary.—A method has been devised for culturing single isolated cells derived from a friable pea-root callus tissue, under conditions which allow cell division and the initial stages of callus tissue development to occur. An agar drop of nutrient medium on a double cover slip is inoculated with a small piece of callus tissue, sealed over a hollow-ground slide, and incubated for 2–4 days at 25° C. The culture is opened, and single cells, isolated in a micropipette with a deFonbrune micromanipulator from a cell suspension, are placed on the agar medium in the peripheral area of the drop surrounding the callus "nurse." The slide is resealed and incubated in the dark at 25° C. Microscopic observation of these cells can be readily made at a magnification of 400×. Isolated mature vacuolated single cells can divide under these conditions. Of the cells which were viable 1 week after isolation, about 7 per cent had divided; in some, division was not followed by enlargement; in others, division was followed by cell enlargement and then by further divisions. Although no independent callus-tissue clones have yet been established by this method of culture, it provides conclusive evidence that isolated single mature plant cells are capable of dividing in vitro.

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† Present address: Department of Botany, University of California, Berkeley 4, California.

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