Growth and Tissue Formation from Single, Isolated Tobacco Cells in Microculture

Abstract: *Single, isolated cells of tobacco divided and grew to form small colonies of over 50 cells in microcultures with a medium containing fresh liquid coconut milk, but in the absence of neighboring cells or nurse tissue. Subsequently, the mass of cells obtained from the single cell, when transferred to agar coconut milk medium, established itself as a clone of callus tissue. Some of these single-cell clones showed differentiation of many tracheid-like cells and shoots with small leaves.*

Various methods have been employed for growing single plant cells in vitro. These methods can roughly be grouped under four categories: the filter paper, nurse culture method (*1*); the hanging drop technique (*2*); the plating method (*3*); and the microculture method (*4*). Except in a few preliminary trials, in all these methods either a large number of cells in the same environment, a nurse tissue, or a "conditioned" medium (*5*) was used for growing the single cells, so that the single cell was never really free from the influence of the neighboring cells or of the medium from which it was isolated.

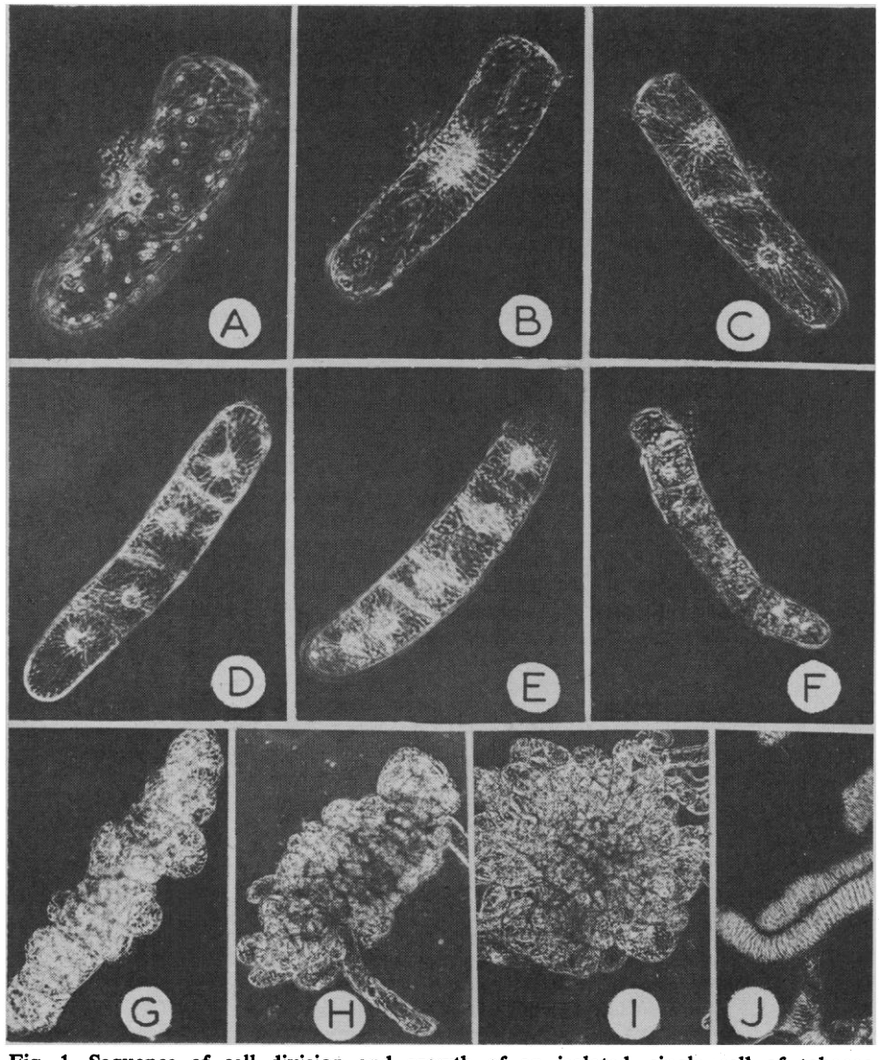

Fig. 1. Sequence of cell division and growth of an isolated, single cell of tobacco. *A*, A single cell with starch grains on the day it was isolated and placed in a microculture (× 700). *B* and *C*, The same cell in prophase and after the first cell division on the 4th day, respectively (*B*, × 560; *C*, × 448). *D–F*, Formation of a linear colony of cells on the 5th, 7th, and 9th days (*D*, × 448; *E*, × 448; *F*, × 350). *G* and *H*, Further stages in the formation of a mass of cells from the original single cell shown in *A* (*G*, × 350; *H*, × 175). *I*, Mass of cells resulting from growth by the single cell shown in *A* (× 175). All stages of development from the single cell shown in *A*. *J*, Portion of tracheid-like cells (× 525).

The plating method has been used (*6*) for studying the growth of colonies from free cells, and the behavior of individual cells of carrot and *Haplopappus*. Pieces of tissue from the secondary phloem of carrot roots in liquid medium produced freely suspended single cells which grew and divided to give rise to groups of cells (*7*). These cell aggregates then formed organized areas or nodules from which roots were produced; when a rooted nodule was transferred to agar medium, it produced shoots. The plants grown in vitro flowered and produced viable seeds when transferred to the greenhouse. In most of these instances, coconut milk was an important constituent of the nutrient medium. Plantlets with shoots and roots were obtained recently in suspension cultures of endive embryo callus tissue in a chemically defined medium (*8*). Entire plants or even continuously growing masses have not been produced from isolated, single cells grown in vitro without nurse tissue, neighboring cells, or "conditioned" medium.

We, therefore, attempted to grow single, completely isolated cells in the absence of any nurse tissue, neighboring cells, or "conditioned" medium and to obtain a continuously growing callus from each cell. These tests were additionally important since the results would throw light on the ability of a single plant cell to form a tissue and to differentiate into a plant.

The plant tissue used was hybrid tobacco (*Nicotiana tabacum* × *N. glutinosa*) secondary single-cell clone H241-18 (*9*) maintained for 8 years on agar *D* medium (inorganic salts, sucrose, coconut milk, vitamins, calcium pantothenate, α-naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid) (see *10*). Several media were tried, with or without coconut milk and 2,4-dichlorophenoxyacetic acid (2,4-D). The callus grew and dissociated best in liquid *C* medium (same as *D* medium, but without 2,4-D) on a reciprocating shaker (60 cycles per minute) in the dark. Pieces of callus weighing approximately 200 mg, when transferred to liquid *C* medium, produced the greatest number of single cells between 10 and 12 days after transfer to the shake-culture. Single cells were isolated after they had been in shake-cultures from 1 to 18 days to ascertain the most fa-

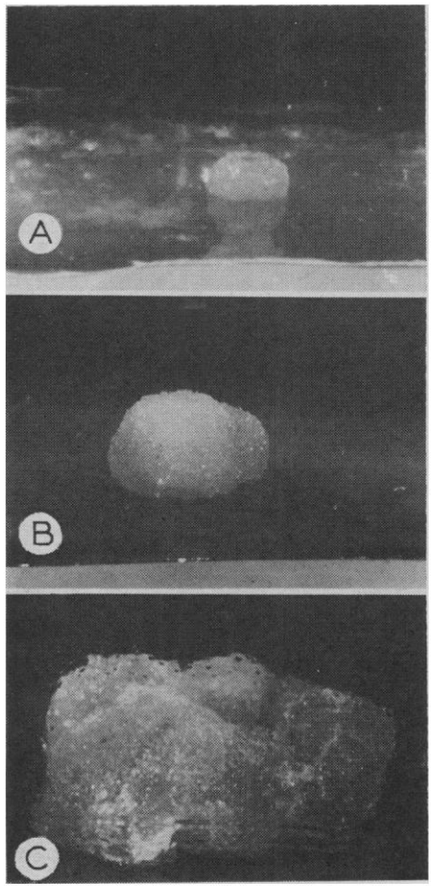

Fig. 2. *A–C*, Calluses, 2, 4, and 6 weeks old, respectively, produced by the cell masses after transfer to agar *C* medium (× 4.8).

vorable age of the single cells for isolation and subsequent growth in microculture. The best age was 10 or 11 days, which coincided with the period for the greatest dissociation of single cells.

Isolated, single cells varied in size and shape. Small cells were generally selected for culturing since a larger number of these divided. Single cells were isolated from a drop (0.5 ml) of the 10- or 11-day-old culture from the shaker and placed in a petri plate to which 15 to 20 ml of fresh liquid *C* medium had been added. Single cells were hand-picked with "Pyrex" glass micropipettes from the petri plate under a dissecting microscope. A microculture (*11*) was prepared in which the isolated cell was placed in a drop of fresh liquid *C* medium in complete isolation from any other cells or cell groups. The microcultures, between observations, were stored in petri plates that were kept in steel cans in the dark at 26° ± 1°C and 55 percent relative humidity. All observations were made under a Zeiss phase-contrast microscope.

Originally, 160 single cells were isolated from cultures grown for 10 to 11 days on the shaker and grown in microcultures in fresh liquid *C* medium. Sixty-five of these single cells divided to form colonies of over 50 cells each. Subsequently, in three separate experiments, 10, 12, and 15 single cells were grown singly in the fresh medium, and all but one single cell grew to form small colonies of more than 50 cells each.

The single cells of tobacco showed, besides the usual cell wall, nucleus, vacuoles, and cytoplasm, a large number of starch grains which were more abundant around the nucleus (Fig. 1*A*). The starch was gradually digested after the single cell was put in the microculture. In most instances, the first division of the single cell took place by the 4th day at right angles to the long axis of the cell (Fig. 1*B*). This lag or preparatory period of 3 days seemed essential for the division of the single cell. Similarly, in freely suspended cells of carrot dispersed in nutrient agar medium in petri plates, growth started after an "induction period" of about 5 days, but some cells remained apparently unchanged for a considerably longer period after inoculation before they started to grow (*6*). Unlike carrot, where 90 percent of the single cells that ultimately grew began to grow continuously after the 9th day, in tobacco, the single cells that failed to divide by the 6th day remained undivided, but continued to live for as long as a month. After the first division, the next few divisions were also in the same plane so that a filament of 8 to 12 cells would form within 7 to 9 days (Fig. 1, *C–F*). In none of the single cell cultures did the pattern of the first four divisions deviate from this plan. Later, divisions started parallel and obliquely to the walls on the long sides of the cells; gradually, the uniseriate filament was converted into a multiseriate cordlike or globose structure (Fig. 1*G*). Further divisions produced a mass of over 50 cells within 15 to 20 days (Fig. 1, *H* and *I*). This small mass of callus, if allowed to remain in the same medium, did not show any increase in cell number. However, starch grains appeared again in abundance in the cells. The small cell colony thus formed was aseptically transferred to fresh agar *C* medium in 6-ounce (170-g) prescription bottles where it continued to grow to form a large mass of callus tissue (Fig. 2, *A–C*). Many such clones of tissue have now been established; some of them showed a large number of tracheid-like cells (Fig. 1*J*). Experiments to differentiate this callus into root and shoot were not successful, probably because the original tissue from which single cells were isolated was several years old. However, clones similarly established from single cells isolated from fresh callus of hybrid tobacco pith tissue, when grown on Murashige and Skoog's chemically defined medium (*12*) with varying concentrations of indoleacetic acid and kinetin, showed shoot formation with several leaves in some instances. The balance of factors encouraging further differentiation of the single cell clones is of interest.

VIMLA VASIL
A. C. HILDEBRANDT
Department of Plant Pathology, University of Wisconsin, Madison

References and Notes

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5. The term "conditioned" refers to a medium in which tissues or cells have been grown for some time.
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11. A microculture was prepared in the following way: A droplet of paraffin oil (U.S. Pharmacopeia heavy mineral oil) was placed near each end of a standard microscope slide. A 22-mm square No. 1 coverglass was lowered onto each droplet to form "risers" on the slide. A rectangle of mineral oil was then placed on the slide connecting the two coverglass risers and covering their inner ends. Next, the isolated cell along with the drop of fresh liquid *C* medium in the micropipette was placed on the slide between the coverglass risers and a third coverglass was inverted over the microdrop and well surrounded with the mineral oil. The resulting microculture permitted microscopic observations of the isolated cell as it grew and divided to form a colony.
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