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MORPHOGENESIS IN CELL COLONIES GROWN FROM CONVOLVULUS CELL SUSPENSIONS PLATED ON SYNTHETIC MEDIA[1,2]

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ABSTRACT

Filtered cell suspensions of cultured callus tissue derived from the roots of *Convolvulus arvensis* L. were plated out on synthetic agar nutrient media in petri plates. Cell colonies which formed from the single cells or small cell groups in the suspension showed a considerable range of developmental patterns depending upon the physical and chemical environment to which they were exposed. Variation of the auxin and kinin concentrations and the nature and concentration of the source of reduced N compounds had the most profound effects on colony development. High auxin favored cell enlargement, high kinin favored the development of compact colonies composed of many small cells. Both auxin and kinin were required for cell colony formation. Cell differentiation responses which were observed but not subject to experimental control included formation of starch- and crystal-storing cells, differentiation of tracheary elements, formation of cellular filaments, and development of chlorophyllous tissue. Organ initiation was studied in cell colonies developed directly from plated cell suspensions and in cell colonies subcultured on various nutrient media. Bud initiation was produced repeatedly on media containing NAA at 10^{-8} to 10^{-6} M combined with kinetin at 10^{-6} M. Root initiation was induced infrequently and unpredictably. Once roots had been formed from cell colonies derived from cell suspensions, the roots could be subcultured and induced to form buds; these in turn grew into whole plants. Subculture of young cell colonies to media containing different combinations of growth substances made possible a study of the effects of auxin and kinin on organization of primordia by the cell colonies. By following marked single cells plated on synthetic media, it was possible to produce single-cell clones which under proper nutrient conditions were induced to form buds. The value of the combined techniques of cell suspension culture and cell plating for the study of the physical and chemical factors influencing cell differentiation and organized development are pointed out.

SUCCESS IN obtaining multiplication of isolated somatic plant cells in vitro has made possible the study of the chemical and physical factors which control the initiation and development of cell colonies into unorganized callus tissues or into organized plant structures. Using the elegant cell suspension culture techniques developed by Steward and Shantz (1956), Steward et al. (1952, 1958a, 1958b), Nickell (1956), Muir et al. (1958), Torrey and Reinert (1961) and others, together with the cell-plating technique of Bergmann (1960), it is now possible to pursue the definition of chemical requirements for cell differentiation and organ initiation under rigorously defined nutrient conditions such as those demonstrated for organ initiation in tissue cultures of tobacco callus by Skoog and Miller (1957). That organized structures can develop from cell suspension cultures was amply demonstrated from studies with *Daucus* by Steward et al. (1958a, 1963) and Kato and Takeuchi (1963), with *Digitalis* by Lamba and Staba (1963), and with *Chicorium* by Vasil et al. (1964a, 1964b). Similar reports were made by Erikkson with *Haplopappus*, and by Earle with *Convolvulus* (Fourth Plant Tissue Culture Conference, Pennsylvania State University, May, 1963).

With these procedures now available it should be possible to work out in specific detail the chemical and physical environments which single somatic cells must be exposed to sequentially in order that, first, the cell may divide and form a tissue and, second, that the tissue in turn may be directed into specific types of cellular behavior leading to the differentiation of distinctive cell types, or to organized structures such as shoots, leaves, roots or whole embryos. Success in such an endeavor would give us considerable insight into the controlling processes involved in the initiation of organization in plants. Control of such extrinsic factors would allow us to explore more precisely the nature of the intrinsic genetic control mechanisms acting within the developing plant.

In an earlier paper (Earle and Torrey, 1965) the procedures involved in the cultivation of *Convolvulus* root callus tissue and the preparation of cell suspensions for cell plating on synthetic media were given. In the work described here an analysis of the chemical and physical conditions determining the course of development of cell colonies from plated *Convolvulus* cell populations is given, together with results concerned with cell differentiation and organ initiation by such cell colonies.

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MATERIALS AND METHODS—The defined medium used in these studies was developed for cell suspension cultures and is given below. Modifications described in these studies consisted of changes in the levels of reduced nitrogen compounds, growth factors or *p*H. The basal medium for cell colony formation included in mg/liter: 242 $Ca(NO_3)_2 \cdot 4H_2O$; 42 $MgSO_4 \cdot 7H_2O$; 85 KNO_3; 61 KCl; 20 KH_2PO_4; 1.5 H_3BO_3; 1.5 $ZnSO_4 \cdot 7H_2O$; 4.5 $MnSO_4 \cdot H_2O$; 2.5 $FeCl_3 \cdot 6H_2O$; 0.25 $Na_2MoO_4 \cdot 2H_2O$; 0.04 $CuSO_4 \cdot 5H_2O$; 0.1 thiamin HCl; 0.5 nicotinic acid; 0.5 pyridoxine HCl; 100.0 *myo*-inositol; 40.4 adenine sulfate; 0.22 (10^{-6} M) 2,4-dichlorophenoxyacetic acid (2,4–D); 146 L-glutamine; 0.22 (10^{-6} M) 6-furfurylamino purine (kinetin); plus 40 g/liter sucrose and 6 g/liter Difco Bactoagar. The medium, lacking L-glutamine, was adjusted to *p*H 5.5 and then autoclaved for 15 min at 15 psi. L-Glutamine was sterilized by cold Millipore filtration and added after autoclaving.

Cell suspensions were prepared from a friable strain of agar-grown *Convolvulus* callus tissue which was shaken in a liquid basal medium for several hours, then filtered through a series of graded stainless-steel filters with the final and smallest pore size approximately 200 μ square. Aliquots of filtered suspensions were counted; then measured volumes were pipetted onto the surface of prepared agar petri plates of 6 or 10 cm diam. The dishes were usually sealed with Parafilm M (Marathon Division of American Can Co.) and cultured in the dark at 24±1 C. Intact petri plate cultures were examined periodically with a Leitz inverted microscope.

To test the effect of nutrients on cell colony growth and morphology, some colonies were transferred from the original plate to fresh medium in 25-mm-diam test tubes or to petri plates. Five to 10 colonies of 0.5 mm diam or less were transferred with watchmaker's forceps to 10 ml of medium in a 6-cm petri dish. Colonies more than 2 mm in diam were placed in separate test tubes containing 15 ml of medium.

The appearance and pigmentation of colonies formed from cells plated on different media were noted. Cell sizes were measured with an ocular micrometer or calculated from photographs. Single-cell clones were obtained by following marked cells on plates of cell suspensions until the cell colony was large enough to be subcultured. These clones developed as friable callus when maintained on a defined medium for callus (Earle and Torrey, 1965).

For examination of large colonies, a dissecting microscope with light reflected from the surface of the tissue was used. Squash preparations and intact colonies were examined also under a compound microscope, either with normal or polarized light. Squash preparations were made by teasing apart small colonies in 45% acetic acid on a microscope slide and pressing with a cover slip. Colonies were prepared for histological study by fixation in formalin-acetic-alcohol, and dehydration in a series of n-butyl alcohol solutions; they were embedded in Tissuemat, sectioned at 10 μ, and stained with Heidenhain's hematoxylin and safranin.

OBSERVATIONS AND RESULTS—*Characteristics of the cell inoculum*—The filtered cell suspension used as inoculum was heterogeneous, consisting of units made up of small or large cells or clumps of small cells. In a typical sample about 50% of the units were single cells, 91% clumps smaller than 6 cells and 98% smaller than 11 cells. The majority of cells were spherical or ellipsoidal ranging from 60–470 μ but averaging between 75–180 μ in length. The cells were highly vacuolated.

The development of cell colonies—Within 2–3 weeks after plating on agar media colonies up to 0.5 mm in diameter were visible. The size and arrangement of cells in the colonies were dependent on the constitution of the medium. Two distinct types of colonies were observed, one made up of many compact cells about 30 μ in diameter (Fig. 1), the other a group of loosely adhering cells about 70 μ in diameter (Fig. 2). The compact type of colony formed from inoculum cells which divided frequently with little enlargement. Such colonies developed on the basal medium as long as the *p*H of the medium was above 5.5. The loose, enlarged cell colonies developed in the basal medium when the *p*H was below 5.5, or if at *p*H 5.5, the auxin level was raised, e.g., the level of 2,4-D was increased above 10^{-6} M. (For a detailed discussion of the effects of *p*H on colony formation see Earle and Torrey [1965]).

The effect of various concentrations of kinetin on the morphology of the cell colonies is illustrated in Fig. 3–5. Colonies from cells plated on the defined medium containing 10^{-5} M kinetin had rounded cells of 30–60 μ arranged in tight clumps (Fig. 3). When 10^{-6} M kinetin was used, cell size was unchanged, but the cells were less tightly packed and more easily spread (Fig. 4). When no kinetin was added, plated cell units only rarely formed colonies. Some cells in such colonies were greatly enlarged, the elongated cells occasionally exceeding 500 μ in length (Fig. 5). Thus media with a high ratio of auxin to kinetin caused *Convolvulus* cells to enlarge. This promotion of enlargement by auxin and stimulation of division by kinetin was in agreement with the reported effects of these two compounds in other systems (Jablonski and Skoog, 1954; Adamson, 1962; Clutter, 1963).

Cell differentiation in cell colonies—Colonies exposed to 50 ft-c of fluorescent white light often developed uniform or localized areas of green color, and chloroplasts were visible in the cells of the green tissue. Dark-grown colonies ranged in color from bright, golden yellow to pale brown to white.

Occasional large crystals and numerous starch

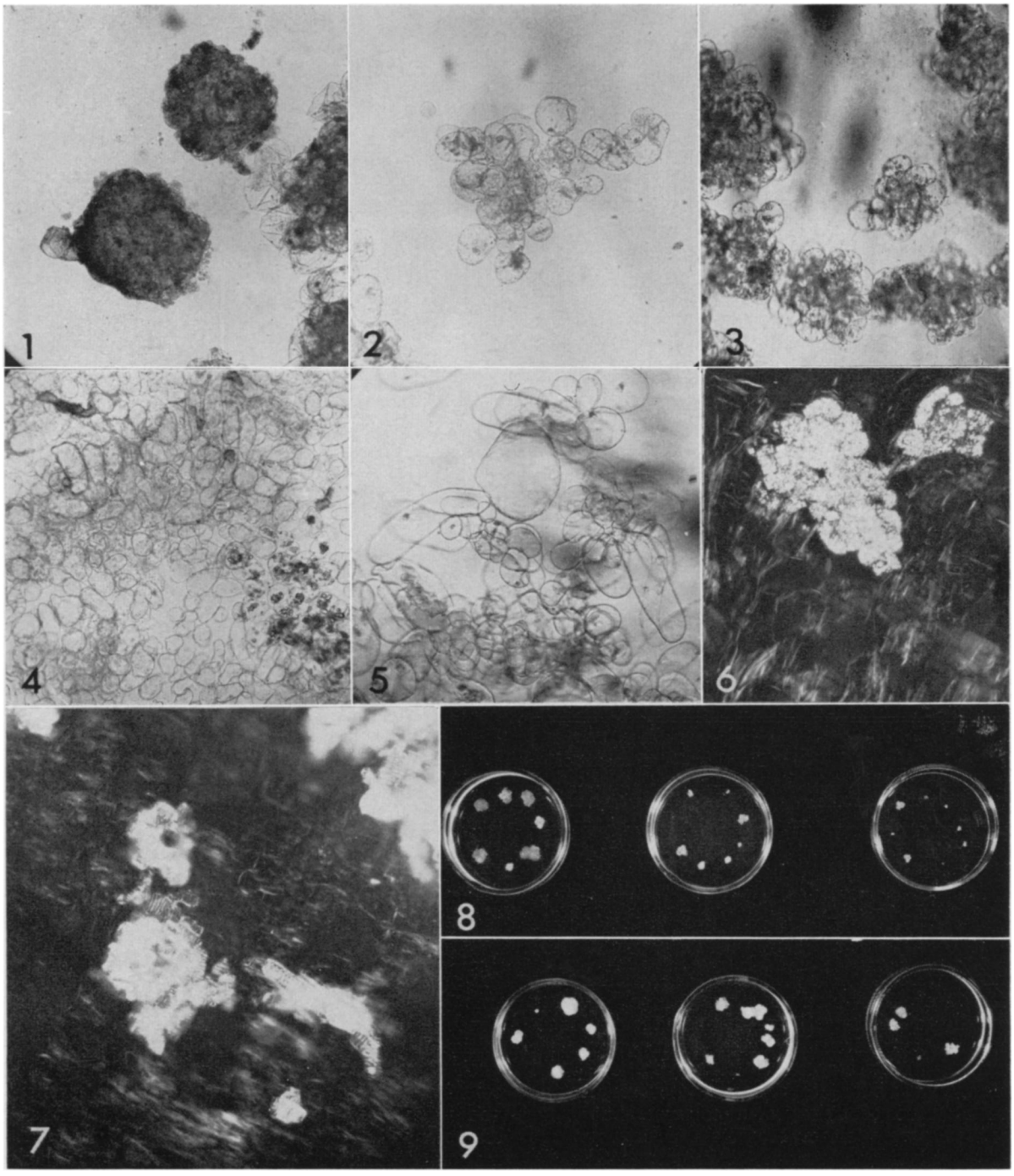


Fig. 1–9. Cell colonies derived from plated cell suspensions of *Convolvulus* callus.—Fig. 1. Nineteen-day-old colonies of small, compactly arranged cells on defined medium lacking L-glutamine at *p*H 6.0, ×130.—Fig. 2. Nineteen-day-old colony of large, loosely packed cells on defined medium lacking L-glutamine and with 5× the basal level of iron. The *p*H of the medium was 5.0, ×130.—Fig. 3. Portion of 2-month-old colony grown on defined medium containing L-glutamine and 10^{-5} M kinetin, ×130.—Fig. 4. Portion of 2-month-old colony grown on defined medium containing L-glutamine and 10^{-6} M kinetin, ×130.—Fig. 5. Portion of 2-month-old colony grown on defined medium containing L-glutamine but no kinetin, ×130.—Fig. 6. Squash preparation of cell colony photographed using polarized light, showing nest of cells containing starch surrounded by cells lacking starch, ×125.—Fig. 7. Squash preparation of cell colony photographed using polarized light, showing nest of cells differentiated into tracheid-like elements with reticulate secondary wall thickening, ×125.—Fig. 8. Inhibition of growth of 0.5-mm cell colonies by kinetin in defined medium containing 10^{-6} M 2,4-D and 10^{-3} M urea. Left dish—no kinetin; center—10^{-7} M kinetin; right—10^{-5} M kinetin. Photographed 29 days after transfer, ×0.33.—Fig. 9. Lack of kinetin inhibition of growth of 0.5-mm cell colonies growing in defined medium containing 10^{-6} M 2,4-D and the AA-mixture. Left dish—no kinetin; center—10^{-7} M kinetin; right—10^{-5} M kinetin. Photographed 29 days after transfer, ×0.33.

TABLE 1. *Growth of 0.5-mm cell colonies transferred to fresh defined media containing 10^{-6} M 2,4-D with or without kinetin and reduced nitrogen compounds and grown in the dark for 29–35 days after transfer*

Supplement to medium			Per cent of colonies of each size		
Kinetin	Reduced N[a]	Number of colonies[b]	0.5 mm[c]	0.6–5.0 mm	>5.0 mm
0	none	67 (2)	15	78	7
0	glycine	42 (1)	100	0	0
0	urea	67 (2)	12	55	33
0	AA-mixture	180 (4)	56	33	11
10^{-7} M	none	60 (2)	15	83	2
10^{-7} M	glycine	40 (1)	100	0	0
10^{-7} M	urea	79 (2)	9	87	4
10^{-7} M	AA-mixture	102 (3)	37	47	16
10^{-5} M	none	81 (2)	11	89	0
10^{-5} M	glycine	41 (1)	100	0	0
10^{-5} M	urea	80 (2)	14	86	0
10^{-5} M	AA-mixture	118 (3)	48	47	5

[a] Reduced N supplements were adjusted to *p*H 5.5, cold filter-sterilized and added at 10^{-3} M. The AA-mixture consisted of glycine, L-glutamic acid, L-aspartic acid, L-asparagine, L-arginine·HCl, and urea, each at 10^{-3} M.

[b] The number of experiments for each treatment is given in parentheses.

[c] These colonies showed no increase in size.

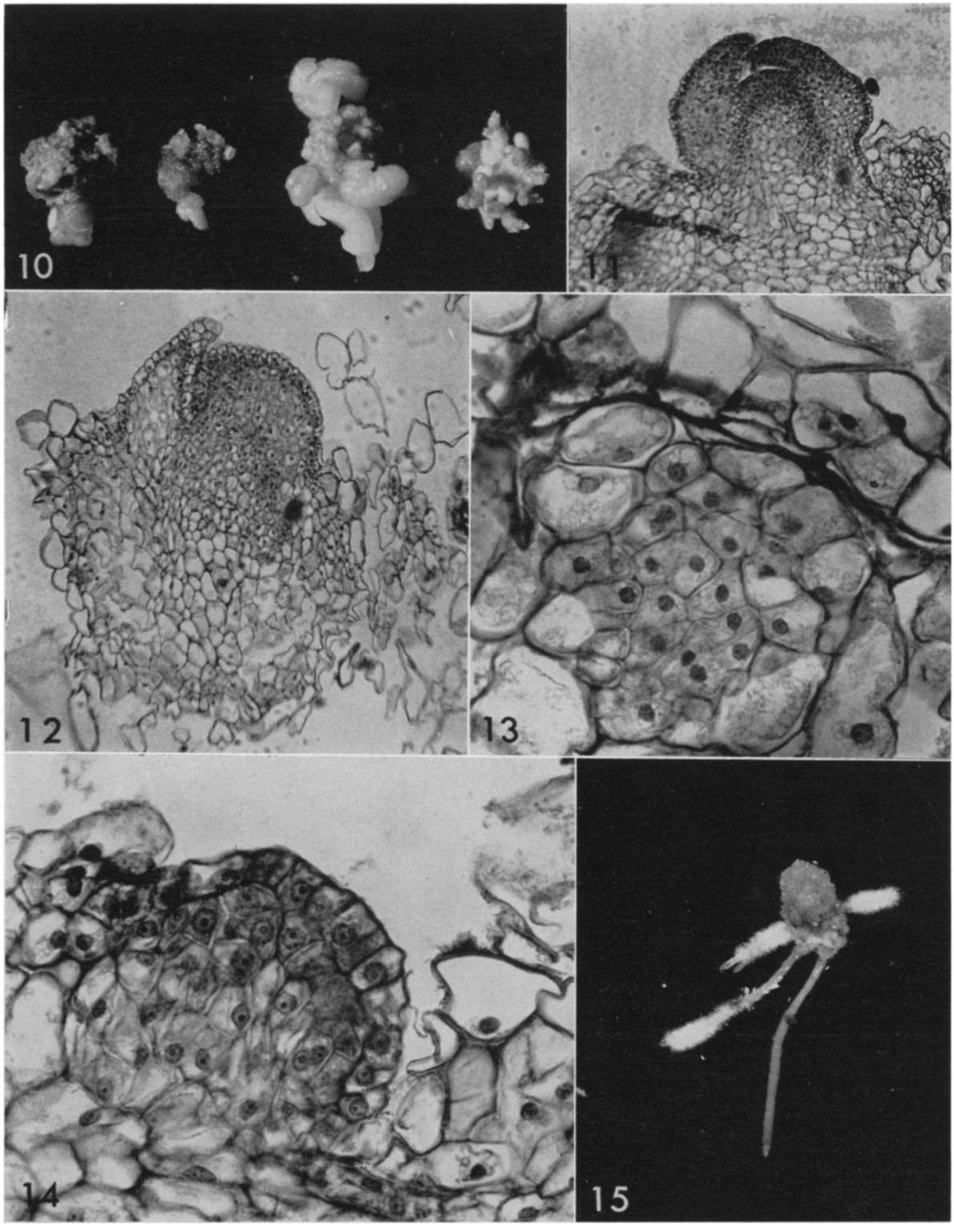
TABLE 2. *Bud formation on colonies from cells plated on defined medium containing NAA at different concentrations, kinetin at 10^{-6} M with L-glutamine omitted. Measurements from several experiments 3–4 months after plating are combined*

NAA concentration	Number of colonies	Number of colonies with buds	Per cent of colonies with buds
10^{-5} M	1	0	0
10^{-6} M	208	14	6.7
10^{-7} M	186	21	11.3
10^{-8} M	40	11	27.5

grains were observed in cells. Tracheids with reticulately thickened walls were also observed in squash preparations. Starch grains, varying widely in size, were often localized in groups of cells surrounded by starch-free cells (Fig. 6). Some strands of elongated tracheids occurred in the green tissue of light-grown colonies, but most tracheids were either scattered throughout the tissue at random or arranged in groups (Fig. 7). This clustering of tracheids and starch grains could be due to the repeated division of a precursor cell or to the existence of small regions where conditions favor starch synthesis or tracheid differentiation. Some colonies showed preferential development of tracheids or starch-containing cells. No clear correlation was found between differentiation of specific cell types and nutrient medium. Tracheids could be found either on the upper surfaces of colonies or deep within the tissue; tracheids occurred in colonies as small as 1–2 mm in diameter or were lacking in colonies as large as 10 mm in diameter. Tracheids seemed most likely to be found in green colonies and colonies with white surface tissues.

The growth of cell colonies—Within 4 weeks after original inoculation of cell suspensions on agar plates, many cell colonies approximately 0.5 mm in diameter, each weighing about 25 μg, were to be found on plates of defined medium. Such colonies served in turn as inocula to fresh media, allowing a study of nutrient requirements by small multicellular clumps. From such colonies organ initiation could be induced by manipulation of the components of the medium. In Table 1 are presented representative data on the growth of 0.5-mm cell colonies on various combinations of kinetin and reduced nitrogen compounds in the presence of a constant 2,4-D level. (From other experiments with transferred 0.5-mm-diameter cell colonies, it was clear that cell colony development was dependent on an external auxin supply.) No organ initiation occurred in this experiment.

Under favorable nutrient conditions, 90% of the colonies were capable of increasing in cell number and in cell diameter, some exceeding 5 mm in diameter in 4–5 weeks after transfer. Glycine was markedly inhibitory to colony development, when provided as the only source of reduced nitrogen. In the presence of a mixture of reduced nitrogen compounds, the glycine inhibition was much reduced. However, urea added alone favored diameter increase. In Fig. 8 and 9 are compared the effects of different kinetin concentrations in the presence of urea or the organic nitrogen mixture on colony development. It should be noted that in the absence of reduced nitrogen the presence of kinetin results in consistently decreased diameter growth of the colonies, an effect which was reflected in the morphology and texture of the colonies. On media containing less than 10^{-6} M



See page 896 for caption.

kinetin, colonies were soft, friable and pale brown, similar to stock cultures, with a dry weight about 7–8% of fresh weight. In the presence of 10^{-6} or 10^{-5} M kinetin, colonies were smooth and hard, usually yellow in color, and showed a dry weight of 17–18% of the fresh weight.

Organ formation by cell colonies from plated cell suspensions—Organ initiation by callus tissues in culture is well known and its induction by manipulation of components of the nutrient medium, especially the auxin, kinin and organic nitrogen levels, has been studied by Skoog and Miller (1957), Pilet (1961), and in complex media by Steward et al. (1958a), Torrey and Shigemura (1957), Reinert (1959) and others. Thus organ initiation from *Convolvulus* cell colonies under different cultural conditions was anticipated. Organ initiation, especially bud formation, was studied in cell colonies developed directly from plated cell suspensions as well as from 0.5-mm colonies transferred to different nutrient media. The results of these experiments are summarized briefly below.

Bud initiation was apparent in colonies 1–2 mm in diameter and, therefore, depended upon the development of a cell colony. Only once was a bud observed on cell colonies which developed on media containing 2,4-D. Bud initiation was best on defined media which contained kinetin and either NAA or IAA as the auxin. Since IAA is relatively ineffective in causing cell colonies to develop (Earle and Torrey, 1965), NAA produced the best response measured in terms of numbers of cell colonies formed and number of colonies with buds. In Table 2 are presented data showing the effects of NAA concentration on bud initiation.

The buds on cell colonies kept in darkness were white, but they became green when transferred to low-intensity white light, either before or after buds were visible. The rest of the colony usually remained white. Such buds seldom elongated more than a few mm (Fig. 10). No roots were seen in association with the buds, and histological sections showed that the structures initiated were organized shoot apices rather than embryos or roots. Figure 11 shows a median longitudinal section of an apex initiated in a cell colony developed from a plated cell suspension on 10^{-7} M NAA. An apical meristem with two leaf primordia is apparent but the shoot base ends blindly in the callus tissue. Quite small tissue pieces may form buds as is seen in Fig. 12, and such primordia may arise superficially on the callus surface (Fig. 13) or endogenously. Such primordia can be traced back in time to small meristematic centers at the surface of the callus or embedded in cells near the surface (Fig. 14). Because of the random orientation of cells in callus, it has not been possible to trace these primordia back to a single-cell origin, as has been suggested by Reinert (1959) for carrot embryos.

Although the *Convolvulus* cells used in these experiments were derived from root tissue, no combination of hormones and nutrients tried in the plating medium consistently produced root initiation. Two colonies which developed on defined medium containing 10^{-6} M 2,4-D, 10^{-6} M kinetin and 10 mg/liter gibberellic acid (GA_3) produced roots (Fig. 15). When 5–10 mm long, the root tips were excised and transferred to liquid medium (Torrey, 1958) and grown as excised roots. Several such root tips grew well. One vigorous root was cut into segments each of which in turn developed a lateral root and bud. From such segments whole plants developed when cultivated in soil in the greenhouse. Thus in *Convolvulus*, as in carrot, a very small group of root cells may carry the information to produce the entire plant, and this expression can be followed through by manipula-

TABLE 3. *Bud formation as per cent of 3–5 mm inocula cultured on defined media containing IAA and kinetin at concentrations indicated and the AA-mixture as organic N supplement. Data are pooled results of 5 experiments with cell colonies or callus tissue pieces after 55–99 days. Figures in parentheses represent the total number of inocula observed in each treatment*

IAA concentration (M)	Kinetin concentration (M)			
	0	5×10^{-8}	5×10^{-7} to 2.5×10^{-6}[a]	5×10^{-6} to 10^{-5}[a]
0	6(34)	4(24)	26(35)	25(65)
10^{-7}	23(13)	——	36(14)	42(12)
10^{-6}	17(18)	7(15)	33(24)	27(22)
5×10^{-6a} to 10^{-5}	10(30)	——	17(18)	26(34)

[a] Data from two or more concentrations in this range were pooled since they were essentially the same.

Fig. 10–15. Organ initiation in cell colonies derived from plated cell suspensions of *Convolvulus* callus.—Fig. 10. Bud formation on colonies from cell suspensions plated on defined medium containing 10^{-7} M NAA and 10^{-6} M kinetin. After bud initiation, colonies were transferred to medium lacking NAA and kinetin and containing 100 mg/liter GA_3. The second colony from the right was derived from a single-cell clone, ×3.2.—Fig. 11. Longitudinal section of a bud from a colony grown on defined medium, containing 10^{-7} M NAA and 10^{-6} M kinetin, ×90.—Fig. 12. Section of a cell colony grown on defined medium containing NAA and kinetin, showing shoot apex with one leaf primordium. Colony was approximately spherical, ×115.—Fig. 13. Section of cell colony grown on defined medium containing NAA and kinetin, showing nest of meristematic cells formed in the early stage of bud initiation, ×520.—Fig. 14. Median longitudinal section through apical meristem of bud initiated on cell colony grown on medium containing NAA and kinetin. Note superficial nature of primordium and lack of vascular tissue, ×520.—Fig. 15. Root formation on cell colony from cell suspension plated on defined medium containing 10^{-6} M 2,4-D, 10^{-6} M kinetin, 10 mg/liter GA, and lacking L-glutamine, ×2.7.

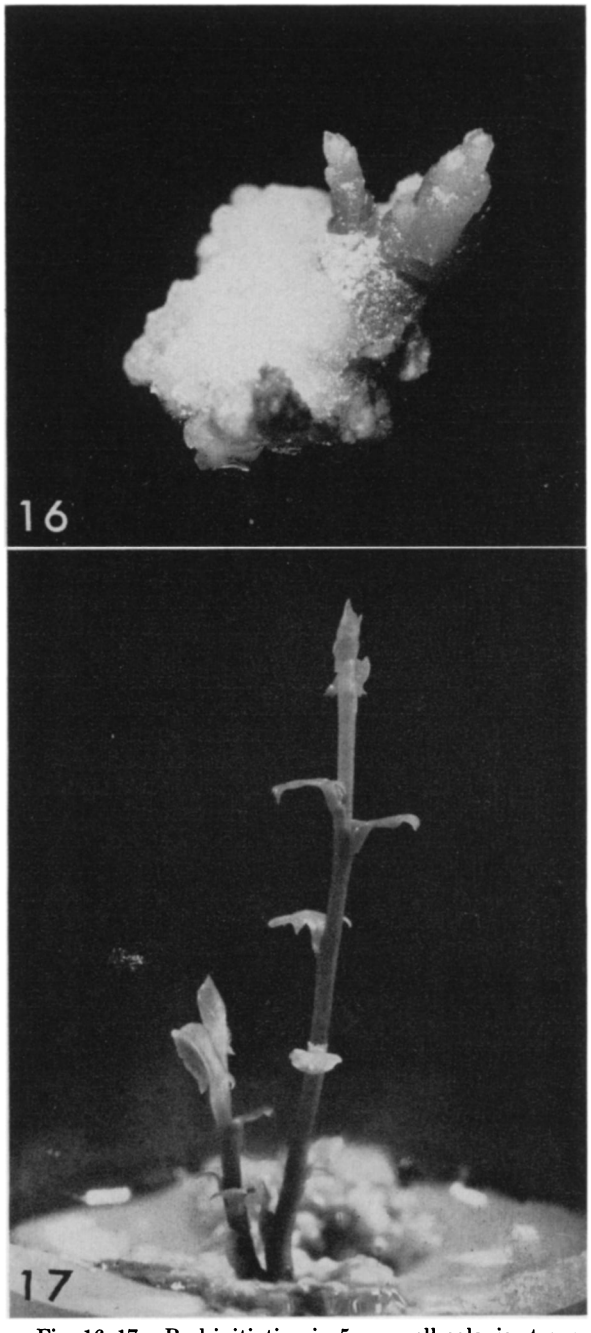


Fig. 16–17. Bud initiation in 5-mm cell colonies transferred to defined medium containing 10^{-7} M IAA and 5×10^{-6} M kinetin and grown in white fluorescent light.—Fig. 16. Cell colony with bud primordia, ×3.—Fig. 17. Cell colony with elongate shoot and expanded leaves, ×3.

tion of the cultural environment. It seems probable from the present experiment that such a sequence can be achieved starting with a single cell, since each of the steps from single cell to colony and from colony to organized structures has been demonstrated. However, the complete sequence has yet to be achieved in *Convolvulus*. The clear demonstration of single-cell origin of callus tissue which in turn gave rise to a whole plant finally came from the work of Kato and Takeuchi (1963) with carrot tissue grown on complex medium, although earlier Braun (1959) had come close to such a demonstration in his work with tobacco teratoma cell culture.

Organ formation in 3–5 mm cell colonies and callus—The establishment of cell colonies from cell suspensions required the presence in the medium of kinetin and auxin (Earle and Torrey, 1965). Thus it was difficult to test the role of kinetin in bud initiation independent of its role in stimulating cell proliferation. Attempts were made therefore to induce organ formation in callus tissue pieces and in cell colonies 3–5 mm in diameter previously developed on the defined medium. Colonies were transferred to defined media to which various levels of auxin and kinetin were added and then were maintained at 24 C in a 12-hour photoperiod with 50 ft-c white fluorescent light. The effects of IAA and kinetin at different concentrations on bud initiation by 3–5-mm cell colonies are shown in Table 3.

No combination of growth substances tested produced bud formation in all treated inocula. A few buds developed in the absence of both added IAA and kinetin, but the addition of either in the absence of the other tended to increase bud initiation. Kinetin at concentrations below 5×10^{-7} M had essentially no effect, but addition of higher amounts up to 10^{-5} M promoted bud initiation at all IAA levels. Although maximum bud initiation depended upon externally supplied auxin and kinin, there was no obvious quantitative interaction between IAA and kinetin as Skoog and Miller (1957) found in tobacco tissues.

Figure 16 shows a cultured cell colony with induced buds following treatment with kinetin and IAA. Leaf-like appendages were visible, but little internodal elongation occurred. Occasionally buds spontaneously elongated to form shoots (Fig. 17), but systematic attempts to cause elongation of buds induced on cell colonies or callus pieces proved unsuccessful.

Differentiation and variation in single-cell clones—Although manipulation of the nutrient medium affected the growth and differentiation of cell colonies in predictable ways, the variation between colonies in a given plate was often striking. Such variation was evident also in cell colonies derived from tissues of single-cell clones. Colonies derived from clonal tissues grew to different sizes on the poured plates and grew at different rates when subcultured. Similar results have been reported by Muir et al. (1958), Blakely and Steward (1964a, 1964b), Arya et al. (1962) and Sievert et al. (1963). On medium containing 10^{-5} M kinetin and 10^{-7} M IAA, 6 of the 11 clones studied formed buds. However, not all of the subcultured pieces of the bud-forming clones produced buds. When plated out on complex or defined media, clones, known to

be capable of forming tracheids and buds, produced-cell colonies many of which lacked tracheids or buds. Thus the colonies from replated single-cell clones were as variable as colonies from suspensions of stock callus tissues.

DISCUSSION—When a relatively uniform population of parenchyma cells derived from a filtered callus tissue culture grown in synthetic medium was plated out on an appropriate defined medium, cell colonies developed. Such colonies showed elaborate morphological and histological differentiation, including cells of widely different size, cells storing starch or crystals, differentiated tracheids, and cells comprising organized meristems of either roots or shoots. These forms of differentiation could be partially controlled by manipulation of the components of the medium. Thus, for example, in a relatively high auxin concentration, colonies were composed of large, loosely packed cells while at relatively high kinetin concentrations, colonies were made of small, compactly arranged cells.

Whether buds form in colonies depends on the type and concentration of auxin in the medium, acting in the presence of added kinetin. Concentrations of 2,4-D which stimulated colony development inhibited bud initiation. IAA stimulated bud development on colonies once formed, but it was relatively poor in stimulating the initial development of colonies. NAA concentrations of 10^{-8} to 10^{-6} M in the presence of 10^{-6} M kinetin stimulated both colony formation and bud initiation; this combination provided the best balance for colony and bud development.

Several types of differentiation shown by cell colonies have not yet been experimentally controlled, including tracheid formation, starch deposition, the production of cell filaments and the initiation of roots. The failure to induce root initiation reproducibly was puzzling, although this deficiency may reflect the inherent tendency of *Convolvulus* roots to form laterals sparsely. Further work on root initiation is being pursued.

These studies point out the changing requirements of cells and cell colonies during their development. Nutrient conditions favoring initial cell division of plated cell suspensions are not necessarily the best conditions for the subsequent development of multicellular colonies as unorganized callus, nor are these conditions necessarily most favorable to subsequent differentiation of cells or to organogenesis. The idea of a sequential change in exposure of cultured tissues to different nutrient environments was used by Reinert (1959) in experiments leading to induction of embryos in carrot tissue. It is not unexpected that changing nutrients and hormonal balance will influence development, as such changes must occur within the plant itself during development. It should be pointed out that during relatively long periods of subculture running several weeks, tissue cultures are not static but are also changing systems in which the tissue pieces drain the medium of certain nutrients, lower hormone levels and dump metabolic products into the medium. The prolonged periods of culture required to induce differentiation of organs in some culture systems may in fact reflect the requirement for progressive change in nutrient conditions.

Plated cells, 0.5-mm cell colonies, 5-mm cell colonies and callus tissue pieces represent a graded series with respect to nutrient requirements and sensitivity to chemical or physical agents. Plated cells, which were most sensitive, showed an absolute requirement for kinetin for cell division while callus tissues did not require added kinetin. Under the most favorable nutrient conditions, virtually all 0.5-mm colonies grew, while at most, 50% of the plated cells divided. On the other hand, cell colonies were more sensitive to adverse conditions than were callus tissue pieces, e.g., many 0.5-mm colonies failed to grow on certain reduced-N compounds which had supported excellent growth of callus tissue inocula. Thus some of the nutritional and hormonal requirements of *Convolvulus* cells depended markedly on the relationships between cells.

Variation among colonies which developed under apparently identical conditions is a matter requiring explanation. Colony variation could arise from a number of sources. Some cells, especially those which grow on generally unfavorable media, might be true permanent variants or mutants. Blakely and Steward (1962) found that aberrant characteristics in *Haplopappus* and *Daucus* cells persisted after subculture. They were able to correlate these aberrancies with various degrees of chromosomal abnormality. A second source of variation would be due to the physiological state of cells in the suspension, related to the past history of the cells in the callus and their stage of differentiation due to position relative to other cells. Thus cells may have already been set in a differentiation course prior to suspension. A third source of variability in colony formation lies in differences in size of the initial cell suspension components, which varied from single, small cells to single, large cells to small clumps of small cells. The chance of survival of the single, small cells or cell clumps is better than that of single, large cells (Blakely and Steward, 1964b). Competition among components of the inoculum might lead to selection of certain types of cells in the colony population. Interaction among cells and cell colonies of different sizes was reported by Blakely and Steward (1964a).

In devising technical procedures designed to permit one to explore the underlying basic causes of cellular differentiation and organized development, one seeks cellular responses closely tied to the experimental manipulation. In the cell-suspension, plating technique one can study rather directly the anatomical or morphological responses of large numbers of cells exposed to a range of selected physical, chemical environments. In some

types of development, for example, cell enlargement, the response can be observed in the cells treated. Other responses, such as initiation of organized primordia, by their nature require numerous consecutive cell multiplications, but these too can be observed in timed sequence. Thus the technique offers a useful approach to a study of the chemical control of plant development.

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