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CELL DIVISION IN RELATION TO CYTODIFFERENTIATION IN CULTURED PEA ROOT SEGMENTS[1]

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A B S T R A C T

One mm-thick segments cut 10–11 mm proximal to the root tip of germinating seeds of garden pea *Pisum sativum* were cultured in sterile nutrient medium containing auxin in the presence and absence of kinetin. In the absence of added cytokinin, pericyclic proliferation occurred, the cortical tissues showed no proliferation and were sloughed off, and a callus tissue of diploid cells was formed. In the presence of kinetin concentrations from 0.1–1.0 ppm cortical cells of the segments were induced to divide, beginning at the third day. From experiments with ^{3}H-thymidine incorporation at different times of culture, from cytological squash preparations and from histological sections it was shown that the cortical cells stimulated to divide by cytokinin underwent DNA synthesis prior to division, were polyploid, and following cell division rapidly underwent cytodifferentiation at 5–7 days to form mature tracheary elements. At 10 days, when over 300,000 new cells had been formed per segment about 16% of these cells had formed tracheary elements. It was concluded that cytokinin, together with auxin, was essential for the initiation of DNA synthesis in the cortical cells, for their subsequent division, and finally for their specific cytodifferentiation.

ONE OF THE BASIC questions of cytodifferentiation is: What sets a given cell upon a new course of cellular activities? Can one define the environmental and physiological conditions necessary for the initiation of biochemical syntheses which ultimately lead to new cytological structures resulting in a distinctive cell type? One of the most extensively studied examples of cytodifferentiation in plants is that of tracheary element formation. As a result of these studies, one can begin to understand the hormonal and nutritional factors which control tracheid and vessel element differentiation, as well as the cellular activities which are prerequisite to the initiation of this type of cytodifferentiation.

It has been known for some time that auxin limits xylem regeneration around stem wounds in *Coleus* (Jacobs, 1952, 1954). The importance of both auxin and sugar for tracheary element formation in cultured callus tissues was shown by Wetmore and co-workers (Wetmore and Sorokin, 1955; Wetmore and Rier, 1963) and confirmed by Jeffs and Northcote (1967). More recently, it has been shown in soybean callus tissues that either auxin or cytokinin, in the presence of sugar, can limit tracheary element formation (Fosket and Torrey, 1969).

It has been pointed out (Torrey, 1966) that the differentiation of tracheary elements characteristically is accompanied by or preceded by cell divisions. Cell division has been shown to be necessary for the initiation of tracheary element formation in cultured *Coleus* stem explants (Fosket, 1968). Furthermore, in soybean callus, tracheary element differentiation is correlated with a cytokinin-dependent increase in cell number (Fosket and Torrey, 1969). This work strongly suggests that the hormones auxin and cytokinin are required not only for cell divisions but also act together upon the dividing cells to direct one or both of the daughter cells into the specific path of cytodifferentiation leading to tracheary element formation. In the present work, a new system for studying hormonal control of tracheary element formation is described, and analysis is made of the state of the responding cells with respect to their stage in the cell cycle, their chromosome number, and their sensitivity to stimuli.

MATERIALS AND METHODS—Segments 1 mm thick were cut 10–11 mm proximal to the root tip of germinating seeds of the garden pea *Pisum sativum* var. Little Marvel about 60 hr after soaking in sterile distilled water. The segments were transferred aseptically to agar nutrient medium in 10 cm petri plates, 6 segments per dish, and were cultured in the dark at 24 C. In experiments with radioactive precursors, the segments were placed upon filter paper strips moistened with the nutrient medium in 25-mm diam test tubes arranged as slants. This method allowed short-term exposures to different media. A cut surface of each segment was in contact with the

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medium. The orientation of the segment with respect to its morphological origin was found to be unimportant for the responses reported here.

The basal medium was modified from earlier studies (Matthysse and Torrey, 1967b). Inorganic salts were those of Murashige and Skoog (1962) providing a high-salt content. The organic components and hormones were those used in earlier studies on pea root cytology. The constitution of the medium designated S2M was as follows in mg/liter: $CaCl_2 \cdot 2H_2O$, 440; NH_4NO_3, 1650; KNO_3, 1900; KH_2PO_4, 170; $MgSO_4 \cdot 7H_2O$, 370; $MnSO_4 \cdot H_2O$, 16.9; H_3BO_3, 6.2; $ZnSO_4 \cdot 7H_2O$, 8.6; KI, 0.83; $CoCl_2 \cdot 6H_2O$, 0.025; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.025; $FeSO_4 \cdot 7H_2O$, 27.8; Na_2EDTA, 37.3; thiamin HCl, 0.1; nicotinic acid, 0.5; indoleacetic acid, 0.175; 2,4-dichlorophenoxy-acetic acid, 1.1; myo-inositol, 100; L-glutamic acid, 147; L-aspartic acid, 133; L-arginine, 174; L-asparagine, 132; glycine, 75; urea, 60. In addition, sucrose was added at 40 g/liter and Bacto-agar at 10 g/liter. The *p*H was adjusted to 5.5 before autoclaving. The six organic nitrogen compounds were prepared in a separate stock solution adjusted to *p*H 5.5, cold sterilized by Millipore filtration, and added to the autoclaved medium during cooling.

Comparisons were made between the segment response to this medium and a medium containing low levels of added cytokinin. Kinetin, benzyladenine, and zeatin were all tested in this system, and each produced essentially the same response. The experiments reported here were carried out with kinetin as the cytokinin.

Cell counts were based on an acid-maceration procedure modified from Brown and Rickless (1949). Depending on their age in culture, one or two tissue segments were placed directly into 1 or 2 ml volumes of a solution made of 5% chromium trioxide in 5% HCl in a small screw-cap vial and were allowed to stand at room temperature (20 C) for 24 hr. Then the solution and cells were drawn repeatedly into a 2 ml graduated glass hypodermic syringe through a #18 needle until the cells were well separated and suspended. A drop of a measured volume of suspension was placed on a slide under a 22 mm^2 cover slip and the cell number counted in representative fields or traverses of the cover slip, depending on the cell density. Polarizing filters were used, set to show secondary wall birefringence without obscuring cells with only primary walls. Cells were scored as parenchyma cells, old tracheary elements, or new tracheary elements. The latter two cell types were easily distinguished, the former being highly elongate, rectangular elements usually with scalariform secondary wall thickening. New tracheary elements were more nearly isodiametric, usually with reticulate patterning of secondary cell walls. For each sample, several drops were counted and mean values determined. From counts of the number of cells per drop, a calculation of the number of cells per segment could be made.

Histological procedures were modified from methods reported by Feder and O'Brien (1968). Tissue segments were fixed either in 10% aqueous solution of acrolein or in 3% glutaraldehyde in 0.025 M phosphate buffer, dehydrated in methoxyethanol followed by an alcohol series, and embedded in glycol methacrylate. Sections 1–2μ thick were cut with a glass knife on a Huxley ultramicrotome, mounted on glass slides, air dried with warming, and stained with 0.05% toluidine blue in benzoate buffer at *p*H 4.4 for 1–2 min, rinsed, and mounted with permount.

Cytological procedures were modified from Torrey and Barrios (1969). Segments were fixed in Carnoy's solution for 12–24 hr, rinsed in water, hydrolyzed in 1N HCl at 60 C for 10–12 min, stained with Schiff's reagent for 1–3 hr, rinsed with SO_2 water and tap water, then prepared as squashes on gelatin-coated slides. Cover slips were removed with the dry ice method, rinsed with water, air dried, and mounted with permount.

Autoradiographs of tissue sections and of tissue squashes were handled in the same way. Air-dried mounted specimens on slides were dipped in Kodak nuclear track emulsion type NTB 3 diluted 1:1 with distilled water, allowed to air dry in the dark, and stored in light-tight slide boxes for from 3 days to 2 weeks depending on the desired exposure time. In Feulgen-stained preparations, staining preceded the squash preparations. Sections were usually stained with toluidine blue after development of the emulsion, then rinsed, air dried, and mounted. Photographs were made with a Zeiss GFL microscope using planapochromatic objectives.

RESULTS—*Tissue segment proliferation on the medium lacking cytokinin*—Cell proliferation occured from root segments cultured on the S2M medium containing auxin but lacking a cytokinin. The population of cells entering division under such cultural conditions was entirely diploid (Torrey, 1961; Matthysse and Torrey, 1967a). The time course of new cell formation in root segments cultured on the medium lacking a cytokinin (S2M) is shown in Fig. 1. The initial cell number was approximately 22,000 cells/segment. After 5 days in culture, this number almost doubled to reach a value of nearly 40,000 cells. Cell number continued to increase with time, eventually producing a callus tissue which could be subcultured. However, no new tracheary elements were formed in root segments cultured on the S2M medium.

Analysis of the anatomy of the response of the root segment to culture on S2M medium showed that the dividing cells were derived largely from proliferation of the pericycle. Fig. 2 illustrates the anatomy of the 10–11 mm root segment as seen in transection at the time of tissue isolation.

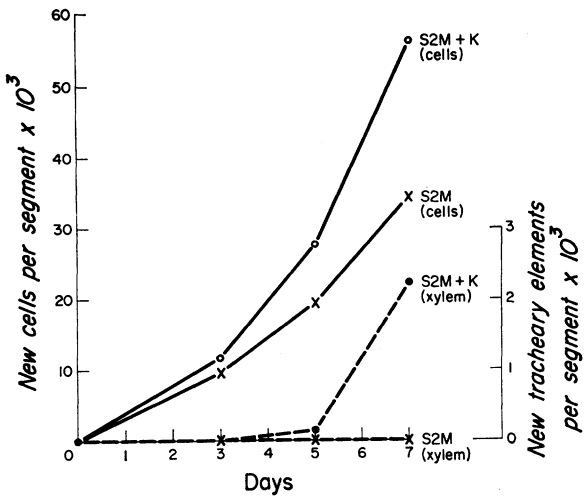


Fig. 1. Graphical representation of the increase in cells and new tracheary elements per segment in 1-mm pea root segments cultured on S2M and S2M + 0.5 ppm kinetin (K) as a function of the time in days. Unbroken lines are cell numbers; broken lines are tracheary element numbers. Initial cell number per segment was 22,500. This value has been subtracted from all cell number values before plotting.

After 3 days in culture on the medium lacking cytokinin, one may observe the beginnings of localized cell proliferation in the pericycle, without cortical cell divisions (Fig. 3). Almost no cell contents are evident in 1μ-thick sections of the highly vacuolated, mature cortical cells (Fig. 3, 5).

At 5 days inner cortical cells showed enlargement and the beginning of cell separation. Pericyclic proliferation added new cells which pushed the cortical cells apart and the non-dividing cortex finally sloughed away. Figure 4 shows in transection the early stage of cortical disintegration associated with continued pericyclic proliferation. Other central cylinder parenchyma cells such as xylem parenchyma or procambial cells also divided; the organization of the central cylinder was thus lost, and a disorganized, rather friable callus tissue formed. When this tissue was maintained on the S2M medium, no tracheary elements were observed the first several weeks of culture.

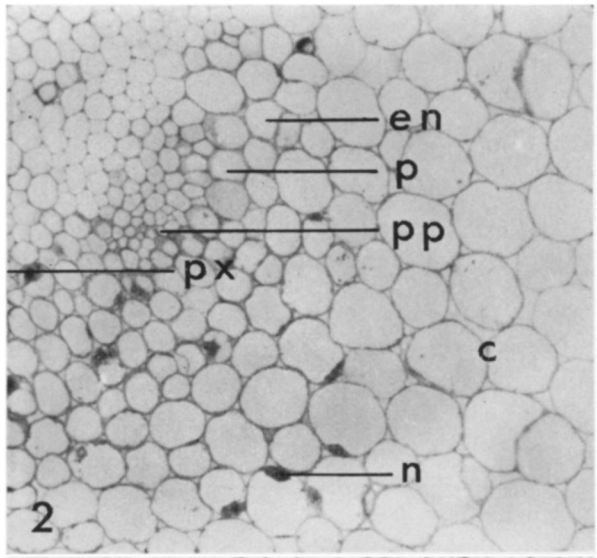
Tissue segment proliferation on the medium containing auxin and cytokinin—The behavior of the tissue segments cultured on S2M plus added kinetin was quite different. From earlier studies on the cytology of these segments, it was known (Matthysse and Torrey, 1967a, b) that over the first 2 days, cell divisions of only diploid cells occurred. However, beginning at about 72 hr, a population of polyploid cells entered division and continued to divide at least until the seventh day. This distinctive tissue response was studied in detail at the cytological level by Matthysse and Torrey (1967b) and was confirmed by Van't Hof and McMillan (1969). Our interest centered on the question of the anatomical location of the polyploid cell divisions and the subsequent fate of these cells.

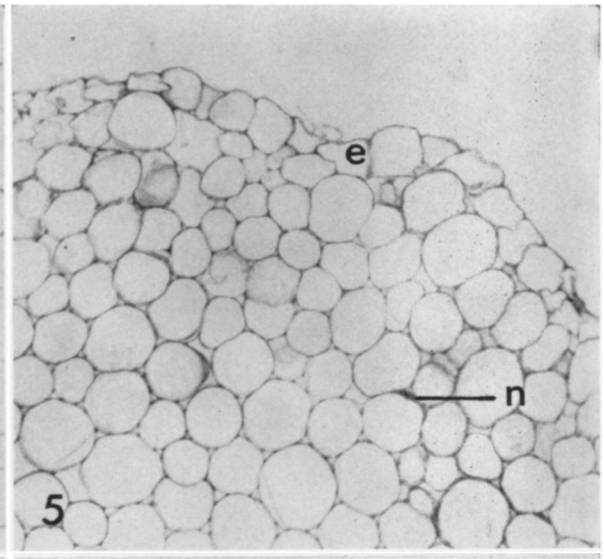
Figure 1 shows the increase in cell number with time of root segments cultured on the cytokinin-containing medium (S2M + K). It is apparent that there was no significant difference in cell number between segments cultured on S2M and S2M + K medium for the first 3 days. In both treatments, the cell population was made up of diploid cells.

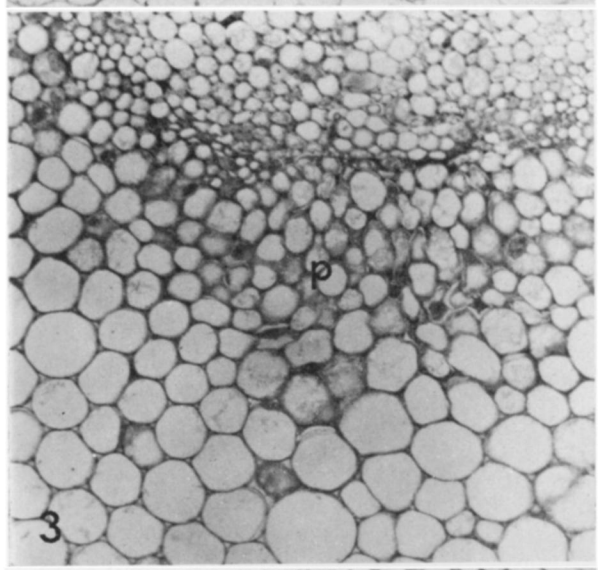
By the third day transections of pea root segments cultured on S2M + K, however, showed that marked changes had begun in the cortical cells of the root (Fig. 6). Relatively few of the cortical cells had entered division at this time. However, most cortical cells showed an increased cytoplasmic density, much less vacuolation, and large nuclei with enlarged nucleoli. Judging from the specific staining with toluidine blue, cortical cells showed marked increases in total protein and ribonucleic acid.

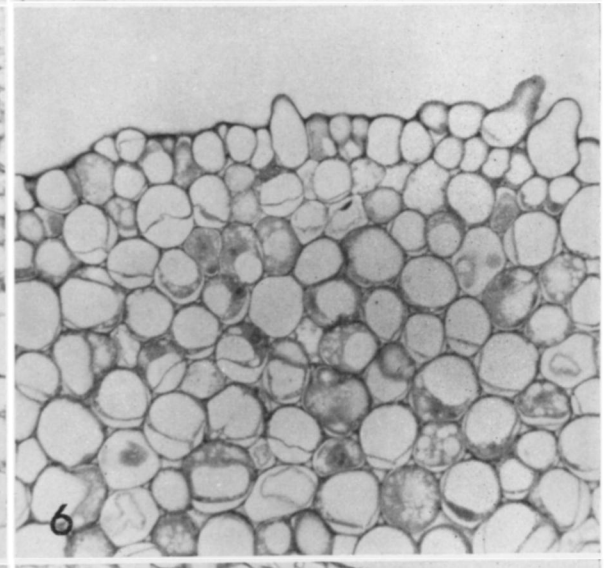
By 5 days in culture on S2M and 1 ppm kinetin, the root cortex had completely changed. Most of the cortical cells divided between the 3rd and the 5th day. The cortex was thus transformed into a complex tissue consisting of a dense, solid mass of cells integrally connected to the central cylinder (Fig. 7). The original walls of the cortical cells were easily discerned, surrounding and containing from 2–4 newly formed cells. What is most remarkable is that a high proportion of the cells derived from subdivision of cortical cells differentiated suddenly and abruptly into tracheary elements. These cells formed thickened secondary cell walls, usually in a reticulate pattern, and subsequently lost their cell contents to form mature tracheary elements (Fig. 8).

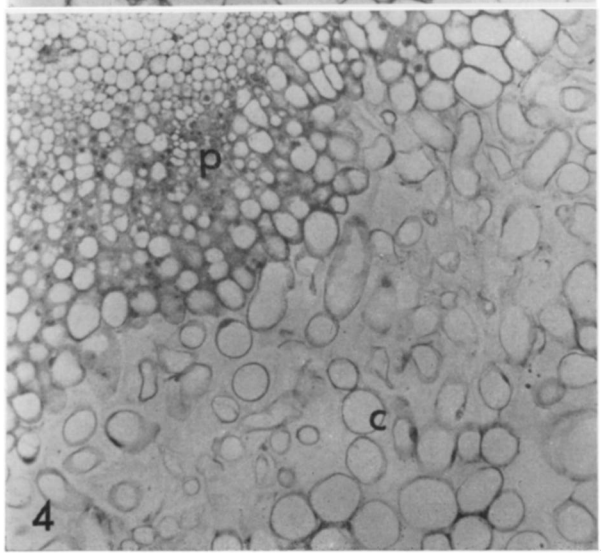
Fig. 2–7. Transections of pea root segments. All × 220.—Fig. 2. Transection of a pea root segment at the beginning of culture, showing a portion of the central cylinder and the cortex. *c*, cortex; *en*, endodermis; *n*, nucleus; *p*, pericycle; *px*, primary xylem; *pp*, primary phloem.—Fig. 3. Transection of a pea root segment cultured on S2M medium for 3 days, showing a portion of the central cylinder and pericyclic proliferation (*p*).—Fig. 4. Transection of a pea root segment after 5 days on S2M medium. The pericycle (*p*) has formed several cell layers and the elongation and separation of cortical cells (*c*) is evident.—Fig. 5. Transection of the outer cortex of a pea root segment cultured on S2M medium for 3 days. Note the scarcity of cytoplasm in these cells. An occasional nucleus (*n*) is seen closely appressed to the cell wall. *e*, epidermis.—Fig. 6. Transection of the outer cortex and epidermis of a pea root segment cultured on S2M + 1 ppm kinetin for 3 days. Note the increased cytoplasmic staining, reflecting increased cytoplasm and decreased vacuolation.—Fig. 7. Transection of the outer cortex of a pea root segment cultured on S2M + 1 ppm kinetin for 5 days. The cortical cells are subdivided into numerous smaller cells.

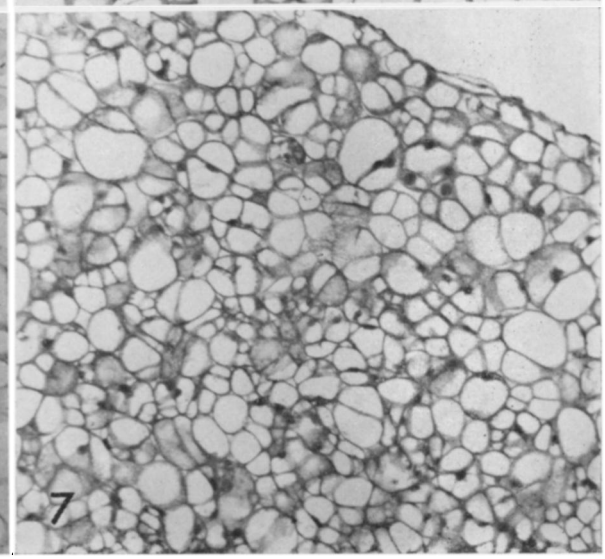


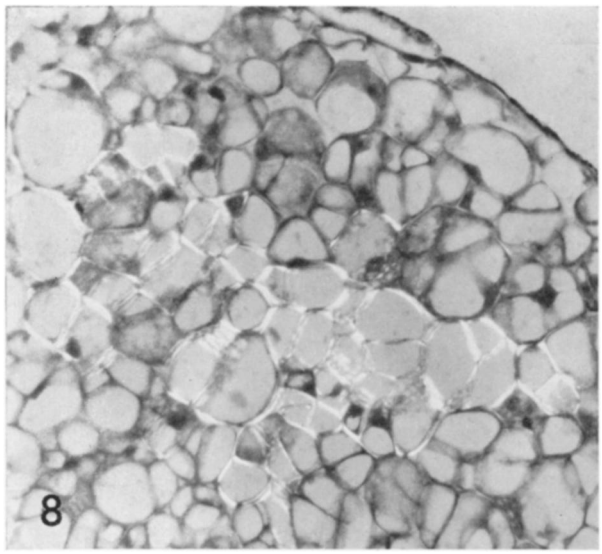


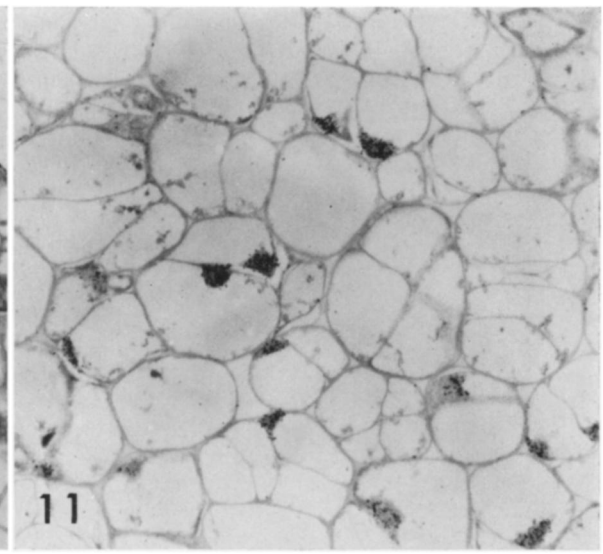


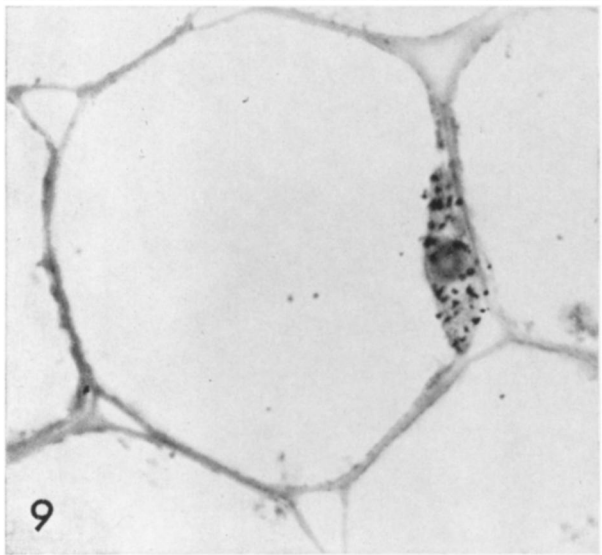


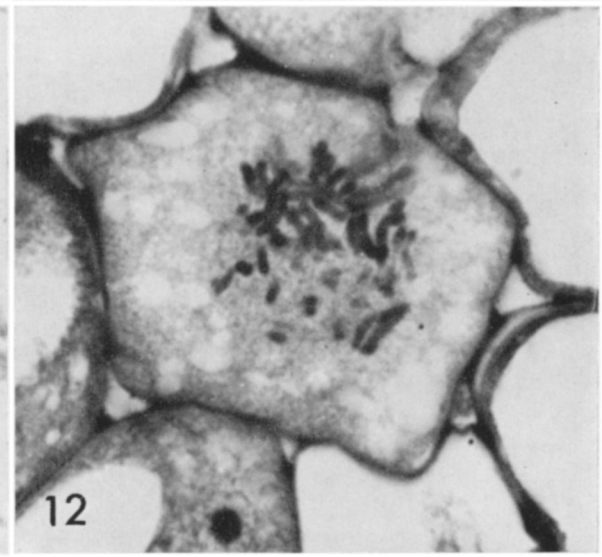


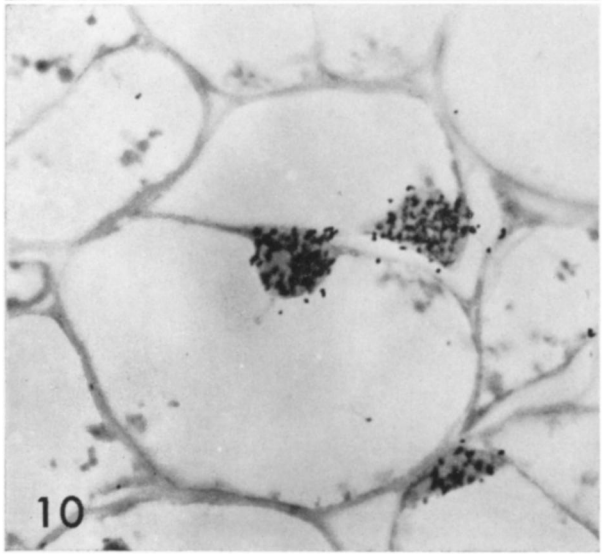


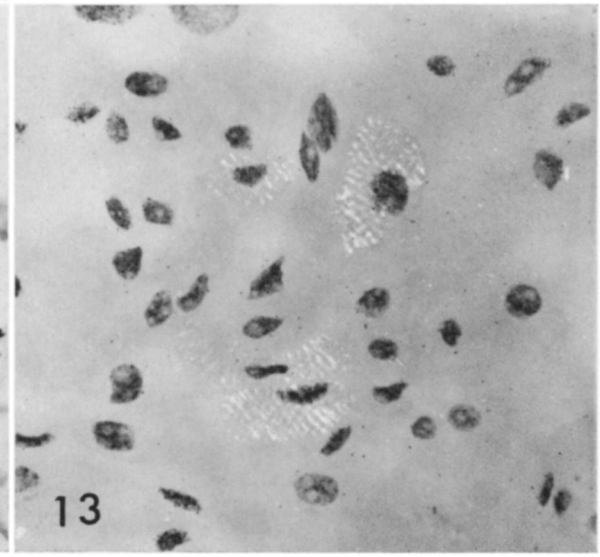












The time course of tracheary element formation as determined from cell counts of macerated segments is shown in Fig. 1. No tracheary elements were observed until the 5th day of culture at which time fewer than 100 per segment had appeared. By 7 days approximately 2500–3000 new tracheary elements had formed per segment. This value represents about 5% of the total of new cells formed. A search was made in transverse and in longitudinal sections for tracheary elements which were formed from cortical cells that did not divide during the culture period. Their frequency was extremely low (i.e., < 3%) and it was impossible to be certain in these cells that a division had not occurred in the plane perpendicular to that observed.

It was concluded that all newly differentiated tracheary elements were derived from cells produced by subdivision of pre-existing cortical cells. That is, no tracheary elements were formed which had not undergone cell division during the period of culture on the auxin-cytokinin medium. Thus, two responses were found to be dependent on the presence of cytokinin and auxin together in the medium. The first response, which occurred at 3 days and immediately thereafter, was mitosis of numerous polyploid cells. This evidence was based largely on cytological squash preparations where identification of the specific anatomical location of the responding cell was uncertain. The second response, which was restricted to cells of the cortex of the root, was the differentiation of new tracheary elements at 5–7 days in culture. It was important to establish the relationship between these two cytokinin-auxin dependent responses. Therefore we undertook studies of the incorporation of ^{3}H-thymidine to determine the time and site of DNA synthesis in these root segments.

DNA synthesis in relation to cell division and tracheary element formation—^{3}H-thymidine was provided to pea root segments grown on S2M medium or on S2M + 0.5 ppm kinetin at different periods during culture. By placing the segments on filter paper wetted by the ^{3}H-thymidine in the presence of the stimulating medium, it was possible to set relatively defined periods during which radioactive incorporation would occur. During other periods of culture the segments were grown on the equivalent medium solidified with agar lacking thymidine. Experiments were performed in which segments were exposed to ^{3}H-thymidine for the first 24 hr period in culture or for the third 24 hr period. The segments were then prepared both as autoradiographic squashes (for mitotic counts) and as histological sections to determine which cells responded and at what time in culture the respective cells showed incorporation.

Table 1 summarizes the results of such an experiment. On the S2M medium ^{3}H-thymidine was incorporated readily into central cylinder cells, presumably primarily pericycle cells and pericyclic derivatives. Such incorporation began at the outset and continued for the entire culture period. At 7 days a high percentage of the cells in the culture showed label since they were all derived from pericyclic proliferation. Essentially no incorporation of ^{3}H-thymidine was observed in the cells of the cortex in segments cultured on S2M medium.

On S2M + 0.5 ppm kinetin two patterns of incorporation were observed. When segments were cultured in the presence of ^{3}H-thymidine froms 0–24 hr, then transferred to medium lacking radioactivity thereafter, 96% of the cortical cells showed no label at the 5th day. Only 4% of the cortical cells were labelled. Approximately half of the cell population produced by the pericycle was labelled. If, however, segments were provided ^{3}H-thymidine from 48–72 hr and then returned to medium lacking radioactivity, at 5 days 68% of the cortical cells observed in sections were labelled and 32% were not. Thus, over two-thirds of the cortical cells had undergone DNA synthesis in the 3rd day of culture. This observation agrees almost exactly with that made by Matthysse and Torrey (1967b) concerning the time of DNA synthesis of polyploid cells in segments cultured in the presence of cytokinin and ^{3}H-thymidine. Thus it appears that the cortical cells are the source of the polyploid cells.

Figure 9 shows a cortical cell which has incorporated ^{3}H-thymidine during the third day in culture but has not yet divided. Figure 10 shows

Fig. 8–13. Fig. 8. Transection of outer cortex similar to segment in Fig. 7, photographed with polarized light. Note cortical cell subdivision. All cell walls showing the bright birefringence are maturing or fully matured tracheary elements. × 355.—Fig. 9. Autoradiograph of a transection of a cortical cell of a segment cultured on S2M + 0.5 ppm kinetin for 3 days. ^{3}H-thymidine was provided the segment for the period from 48–72 hr of culture. Silver grains are localized over the nucleus. × 1050.—Fig. 10. Cortical cell in segment treated as in Fig. 9 after cell division. × 1050.—Fig. 11. Autoradiograph of a transection of a root segment cultured 3 days on S2M + 0.5 ppm kinetin. ^{3}H-thymidine was provided from 48–72 hr during culture. Note that pairs of cells derived from cortical cell division show label as do also cells which probably have undergone two sequential divisions. × 355.—Fig. 12. Transection of a cortical cell in mitosis in a root segment cultured 5 days on S2M + 0.5 ppm kinetin. The section cuts a large percentage of the chromosomes present in the metaphase division figure. This mitosis is of a tetraploid nucleus. Note the tendency for pairing of chromosomes. × 1050.—Fig. 13. Squash preparation of pea root segment cultured for 7 days on S2M + 0.5 ppm K. ^{3}H-thymidine provided on same medium between 48 and 72 hr. Photographed with polarized light to show secondary cell wall birefringence. All tracheids showed labelling. × 355.

TABLE 1. *Labelling pattern in autoradiographs of root segments cultured on S2M medium with or without added kinetin (0.5 ppm) with ^{3}H-thymidine provided during different periods of the culture time*

A. Autoradiographs of sectioned segments

Time of Sample	Medium	Period of ^{3}H-thymidine addition (hr)	% of cells: Central cylinder With label	Central cylinder Without label	Cortex With label	Cortex Without label
DAY 5	S2M	0–24	36	64	0	100
			47	53		
"	S2M	48–72	65	35	14	86
			50	50		
"	S2M + K	0–24	33	67	4	96
"	S2M + K	48–72	36	64	68	32

B. Autoradiographs of cytological squashes of segments

			DIFFERENTIATING TRACHEARY ELEMENTS: % with labelled nuclei	% without labelled nuclei	% without nuclei
DAY 7	S2M + K	Day 3–7	90	5	5

a divided cortical cell with each nucleus labelled. Figure 11 shows a region of the cortex of a pea root segment cultured on S2M medium plus 0.5 ppm kinetin for 3 days with ^{3}H-thymidine provided for the period from 48–72 hr of culture. In the field are shown a pair of cells derived from a cortical cell with both nuclei labelled. In the upper right of Fig. 11 is another cortical cell which shows four or five smaller cells within it, with two nuclei showing in the plane of the section, both clearly labelled.

Figures 9, 10, and 11 were all taken from the same root section, indicating that the cell division cycle is not synchronous. Thus one cell may still be in its S period while another in the same cortical region has already divided. However, after a relatively long lag period of about 60 hr, the majority of the cortical cells undergo one or two cell divisions and a portion of the cortical derivatives pass on into terminal stages of cytodifferentiation.

That cortical cells give rise to polyploid mitoses is suggested by their large nuclear size compared to cells in the pericycle or central cylinder and by their labelling pattern. Counts of silver grains over interphase nuclei showed that pericycle cells caused deposit of as little as a quarter the number of silver grains as those on the same slide over cortical nuclei. Clear evidence of polyploid mitoses in cortical cells was obtained in a few of the sectioned segments. Since the sections were quite thin, it was difficult to find many division figures where chromosome counts could be made. Figure 12 shows a section cut parallel to a metaphase plate in a tetraploid cortical cell. Adjacent sections allowed a determination of the chromosome number in this cell, which was 28, i.e., 4n.

A final test was made to demonstrate that the tracheary elements were derived from cortical cells which were polyploid and had undergone DNA synthesis and cell division during the course of the treatment period. Segments were cultured on S2M + 0.5 ppm kinetin. For the period from 48 hr onward they were provided ^{3}H-thymidine. Segments were then fixed and squashed at days 5, 6, and 7 in order to be sure to fix the newly differentiating tracheary elements when they had begun definitive and specific cytodifferentiation as judged from the characteristic reticulate secondary wall formation but before they had lost their cell contents or nuclei. According to earlier experiments, in autoradiographic preparations of such cells prepared as squashes, one should find differentiating tracheary elements with nuclei which are clearly labelled due to incorporated ^{3}H-thymidine. Figure 13 shows a photograph of a squash preparation autoradiograph photographed with polarized light; the nuclei still present in the differentiating elements all show superimposed silver grains. Such labelling clearly shows that the nucleus still present in the differentiating tracheary element underwent DNA synthesis followed by division during the period from 48 hr after the beginning of treatment to the time of fixation. Of over 200 such immature tracheary elements containing nuclei which were counted in squashes made at 7 days, 96% of the nuclei showed label. The total cell population showed only 74% labelling. This observation provides further evidence that a specific cytodifferentiation has been induced by the auxin-cytokinin treatment which affects only certain cells of the original segment. These cells are polyploid at division and the products of the division either immediately or after a subsequent division undergo cytodifferentiation to form tracheary elements which promptly lose their nuclei.

The effect of cytokinin concentration on cell proliferation and tracheary element formation— Increasing levels of cytokinin led to the formation of larger numbers of cells and tracheary elements.

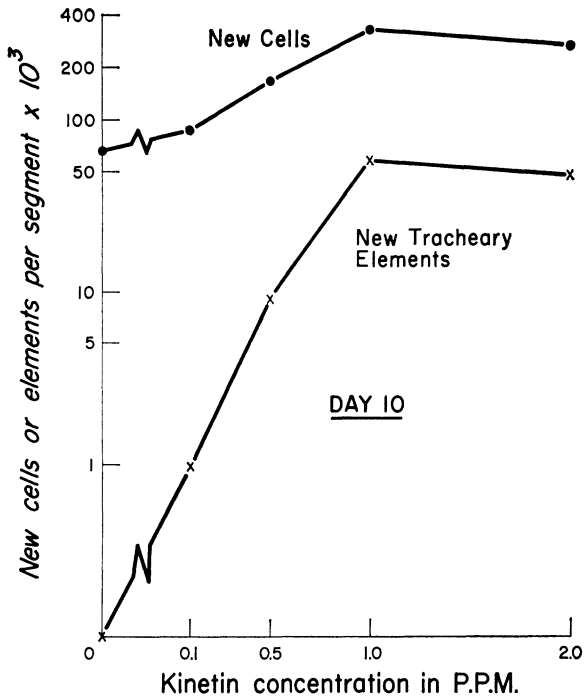


Fig. 14. A graphical comparison between the increase in cell number and new tracheary elements per segment at 10 days in relation to kinetin concentration. Note that the ordinate is on a log scale.

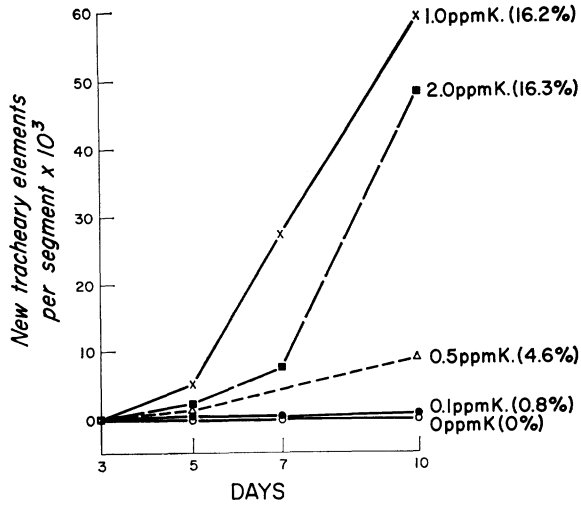


Fig. 15. Graphical representation of new tracheary element formation by pea root segments cultured for 10 days on S2M medium with different concentrations of kinetin. Within parentheses opposite each treatment is indicated the percentage of the total cell population represented by the newly formed tracheary elements.

As is shown in Fig. 14, a 10-fold increase in kinetin concentration, from 0.1 ppm to 1.0 ppm, resulted in a 5-fold increase in the number of new cells produced per segment in 10 days and approximately a 50-fold increase in tracheary element formation. Concentrations of kinetin above 1.0 ppm appeared to be supraoptimal.

Although exogenous cytokinin is not required for pericyclic proliferation in the cultured root segments, a certain threshold cytokinin concentration is required for the proliferation of the cortical cells and for tracheary element formation. The actual threshold is difficult to determine with accuracy. However, Torrey (1961) reported that polyploid divisions (cortical cells) were induced by 0.1 ppm kinetin (5×10^{-8}M). The threshold concentration of kinetin necessary for xylogenesis also appears to lie in this range (Fosket and Torrey, 1969).

Figure 15 shows the effect of kinetin concentration upon the time of appearance of tracheary elements over 10 days. Clearly, the concentration of cytokinin did not influence the onset of cytodifferentiation. The first tracheary elements were observed after 5 days of culture with all concentrations of kinetin tested. There appeared to be a fixed time-lag which was determined largely by the course of the events which led to the division of the polyploid cortical cells. Auxin and cytokinin together initiate cell division in the manner already described by Das, Patau, and Skoog (1956), and their determinative roles center on the components of cell division, viz. DNA synthesis, mitosis, and cytokinesis. In the pea segment response of cortical cells all of these components of cell division are prerequisite for the cytodifferentiation which follows.

Cytokinin appeared to have a specific role in the initiation of tracheary elements beyond its role in the initiation of polyploid cell division. It may be seen from the data of Fig. 15 that increasing concentrations of cytokinin not only increased the number of tracheary elements formed in 10 days but, more significantly, increased the percent of cells initiating differentiation. At the optimal concentration of kinetin (1.0 ppm) 16% of the total cell population consisted of newly differentiated tracheary elements. Histological evidence presents an even more striking picture; virtually all of the outer cortical derivatives become tracheary elements with this level of kinetin.

Conclusion—This study confirms and extends earlier observations concerning the specific role of auxin and cytokinin in the induction of a specific type of cytodifferentiation, the formation of tracheary elements. Auxin and cytokinin requirements for formation of tracheary elements have been determined for soybean callus (Fosket and Torrey, 1969), for pea root callus (Torrey, 1968), and for tobacco stem callus (Bergmann, 1964). Thus far, there are no reports of a cytokinin dependence for tracheary element formation other than in tissues cultured in vitro. In cases where auxin limits xylem formation as in the *Coleus* wound regeneration response or in xylem forma-

tion in cultured tissue segments provided auxin and sugar in a polar fashion, it may be presumed that cytokinin is already present in non-limiting amounts, since in these cases the evidence is strong that cell division is a prerequisite to cytodifferentiation (Fosket, 1968; Wetmore and Rier, 1963).

In the soybean callus system (Fosket and Torrey, 1969) it was demonstrated that division in a cell population was not in itself sufficient to induce tracheary element formation. Low levels of cytokinin allowed cell division to proceed but did not lead to tracheary element formation. At increased cytokinin concentrations, some of these cells responded by undergoing cytodifferentiation. The same relationship is found in pea segments: proliferation proceeds in the absence of exogenous cytokinin (perhaps dependent upon low levels of endogenous cytokinin), but cytodifferentiation is dependent upon a higher threshold level of cytokinin. Thus, in analyzing the sequential steps in specific cytodifferentiation it can be argued that cell division with all of its components is required and that subsequent events also occur which are dependent upon the hormonal milieu. In the pea root segment system a unique feature is the involvement of polyploid cells. They represent a population of cells with a different sensitivity to hormonal stimulation from the rest of the population. The basis of their special responsiveness remains to be determined.

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