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Pattern of Cell Division and Wound Vessel Member Differentiation in *Coleus* Pith Explants[1]

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ABSTRACT

When pith parenchyma explants are taken from *Coleus blumei* plants and cultured on an agar medium containing sucrose and indoleacetic acid wound vessel members differentiate in 10 days. The time course of wound xylem appearance and an auxin requirement suggest that this uncomplicated system is responding in a manner comparable to wounded *Coleus* plants and cultured stem segments.

Histological examination and cell size comparisons confirm that parenchyma cells divide before differentiating. When colchicine is used to prevent mitosis no tracheary elements differentiate. Following the time course of this cytodifferentiation histologically shows that xylem differentiates from cells that are the products of several cell divisions.

The control of tracheary element differentiation in *Coleus* has been studied extensively with xylem productivity techniques in which the number of xylem cells, or xylem strands, that differentiate in response to specific, usually chemical, experimental manipulation is tabulated and compared. These investigations have yielded valuable information about the minimum requirements for xylem differentiation to take place. The pioneering work of Jacobs (10) demonstrated that auxin availability could limit regeneration of xylem around stem wounds in *Coleus*. The question as to whether auxin is an absolute requirement for xylogenesis remained unresolved since tracheary elements were found even when all sources of auxin were removed; however, the quantity of xylem produced was much diminished under these circumstances (10, *Coleus*-derived tissue culture systems also showed xylem differentiation without an exogenous source of auxin (7). A possible explanation for these results is that the endogenous levels of auxin are sufficient to support a low level of xylogenesis.

The productivity approach was used effectively by Fosket (5) to examine cell division and DNA synthesis as criteria for xylogenesis in explanted *Coleus* stem segments. Cultured stem segments were exposed to either mitotic or DNA synthesis inhibitors, and xylem productivity was determined. Either type of inhibitor diminished the differentiation of tracheary elements. Support for the view that cell division and/or DNA synthesis is required for xylem differentiation has come from work with soybean callus (8) and cultured pea root segments (16). Only a recent report by Torrey (15) provides convincing evidence that xylem differentiation can occur in the absence of any closely associated cell division.

Generally the fate change whereby a mature parenchyma cell differentiates as a tracheary element is initiated by wounding or explantation, and characteristically DNA synthesis and cell division are involved in some important way. It has been suggested that these processes alter some silent portion of the genome in such a way that new gene arrays can respond to the recently transformed environment (13). The role of the mitotic cycle in this view is as a DNA sensitizer which allows a xylogenic environment to have its effect. There is some theoretical support for this view (17). Experimental support is less readily available. From earlier work on *Coleus* there is nothing to show that dividing cells are even the same cells that subsequently differentiate (5, 7). Calculations show that cell divisions are necessary for tracheary element differentiation. Roberts (12) and Earle (2) allude to the fact that wound vessel members are often smaller than the parenchyma cells of the normal pith tissue. The fact remains that if one suggests that a cell must undergo DNA synthesis and cell division prior to differentiation it is important to demonstrate that the required events belong to the differentiating cells.

A straightforward anatomical study of a differentiating system could show the necessary relationship. To be useful it would have to be possible to recognize new cell divisions and daughter cells in the system. Pith explants, such as used by Earle (2), are sufficiently homogeneous, being wholly parenchyma, that cell divisions in this quiescent tissue would be easily discernible. Investigating the anatomical relationship between dividing cells and differentiating cells will close the gap between the physiological data showing that the two are related and the theoretical speculation about the exact nature of the relationship. It is also possible that anatomical information may provide clues to the stimulus which initiates cytodifferentiation as this stimulus is reflected in the distribution of cytokineses and tracheary elements.

MATERIALS AND METHODS

The plants used to supply tissue for this study originated as cuttings from a single *Coleus blumei* Benth. plant furnished by D. E. Fosket. Cuttings were rooted in sand for 4 weeks and then transplanted to pots of sterile soil. The potted plants were grown under greenhouse conditions with supplementary fluorescent and incandescent lighting to give 16 hr of light/day. Plants were kept uniaxial by removing axillary buds as they appeared. The plants used for each experiment were chosen for uniformity of size and coloring, and they were between 7 and 8 weeks old when tissue was excised.

All explants were obtained from the second internode of the *Coleus* plants which, according to the nomenclature of Jacobs (10), subtends the second leaf pair. Second internodes were chosen because the vascular cambium has not begun to function, and because the large amount of past research using second internodes provides information for comparison. To reduce variability plants in flower were not used.

Second internodes were removed with a razor blade and surface-sterilized for 10 min in a 5% solution of Clorox. Sterilization was followed by three rinses with sterile distilled and deionized H_2O. The excised internodes remained in the final rinse until dissection, never more than 1 hr. All rinsing and subsequent dissection were done aseptically under a transfer hood to prevent contamination.

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A single internode was removed from the distilled H_2O to the surface of an inverted Petri plate cover which had been surface-sterilized with ethanol. The ends of the internode, which had been in contact with Clorox, were removed with a scalpel. The internode was then submerged in a sterile 2% (w/v) sucrose solution in a Petri plate on the stage of a dissecting microscope. The cortex and preexisting vascular tissue were removed from the pith with No. 11 surgical blade. The remaining pith block was then cut longitudinally into four equal pieces. These small pith blocks had approximately eight cells on each of their lateral sides and were about 2 mm in length. Not only were these blocks of tissue equal in size, but they were also physiologically equivalent; each had a corner which had been adjacent to a corner bundle and two external and two internal sides.

Regardless of the procedure used each Petri plate contained five explants. To retard drying of the agar the plates were put in clear polyethylene bags closed loosely with wire ties. The cultures were then placed in a controlled environment chamber at 24 C where they received 150 ft-c of fluorescent light on a 16:8 (light:dark) daily photocycle. Tissues were cultured for 10 days unless otherwise indicated.

Consistent with an over-all attempt at simplification an effort was made to reduce the complexity of the medium. The simplest medium used for xylem differentiation studies was that of Fosket and Roberts (7). This medium consists of 2% (w/v) sucrose in 1% (w/v) agar. In most instances I added IAA to this medium prior to autoclaving as dilutions of 100 μg/ml stock solutions.

At the completion of an experiment the pith explants were either cleared for counting tracheary elements or prepared for cryostat sectioning. Explants to be cleared were subjected to the procedure proposed by Jacobs (10). Secondary wall thickenings were stained with a dilute solution of safranin O in water.

An attempt was made to determine the number of cells in the cultured explants by means of simple geometry. Since pith cells are quite uniform in size it is possible to count the number of cells in each of the three dimensions of the explant and calculate the total number of cells by multiplying the three numbers. Triplicate counts were made of the number of cells in each dimension and a mean value was used for the subsequent computation. Since I have found that some cells in the cultured explant do not stay the same size throughout the culture period (some become subdivided) the method just outlined is not an accurate way to determine the actual number of cells in the explant after several days in culture. It does, however, provide for some significant adjustment for variation in explant size, and when used to calculate a WVM[2] percentage offers some advantage over simply using the absolute number of tracheary elements formed.

After the cell counts had been made the cleared explant was teased apart with fine-pointed forceps. A drop of water was placed on the macerated tissue on a slide and covered with a coverslip. Pressure was applied to the coverslip with a pencil eraser to squash the underlying tissue. All of the WVMs in the explant were counted and a per cent was determined from the calculated cell number.

Cell size measurements were made by one of two methods, depending upon the cell type being measured. The dimensions of differentiated tracheary elements were obtained from the cleared and squashed explants with an ocular micrometer in a compound microscope. The squashing procedure distorted the pith parenchyma cells and was therefore unsuitable for determining their size. Parenchyma cell measurements were made from cryostat sections. Unfixed explants were frozen at −20 C using Cryoform (International Equipment Company) as the supporting matrix. Sections 15 μm thick were cut at −20 C with a rotary microtome. The cut sections were picked off the microtome blade with a drop of water on an inverted microscope slide, and stained with aqueous toluidine blue (3). Parenchyma cell size was determined from these sections by measuring the maximum length or diameter for each cell measured.

Colchicine has been used in this project at 0.04% (w/v) to arrest mitosis and prevent cytokinesis in the cultured parenchyma tissue. At this concentration one would not expect DNA synthesis to be influenced in this plant material (11), although Fitzgerald and Brehaut (4) found colchicine inhibition of DNA synthesis in cultured human lymphocytes. The transfer of an explant from one type of medium to another was done by picking the piece of tissue up on the tip of a surgical blade and simply transferring it to the new medium. No attempt was made to keep the same side in contact with the agar surface. This operation was performed under a transfer hood with sterile instruments.

RESULTS

Differentiated Cell Type. The xylem cells which differentiate within cultured pith parenchyma are identical to the wound vessel members described in earlier papers (7, 8, 10). As shown in Figure 1 these cells have reticulate secondary wall thickenings, simple perforation plates, and exhibit birefringence when viewed with polarized light (Fig. 2).

The long axis of WVM differentiating in wounded whole plants or cultured stem segments is typically parallel to the long axis of the plant (14). In this way the repaired strands are, theoretically at least, functional. WVM which differentiate in cultured pith parenchyma lack much of this polarity. If strands form at all they may run with or perpendicular to the long axis of the parent plant. No matter what orientation two adjacent cells may have relative to each other there may still be a perforation plate where they meet. The individual cells comprising any one strand vary considerably in structure, size, and orientation (Fig. 3).

Auxin and Differentiation of WVM. The results of this investigation are consistent with the view that auxin is a limiting factor in the cytodifferentiation of tracheary elements in *Coleus*. Although previous studies (7, 10) have shown that there can be some differentiation of WVM even when auxin sources are removed, the experimental system utilized including existing vascular and cortical tissues, which could be a source of endogenous auxin. I have never found WVM when pith blocks were cultured on medium without auxin. Earle (2) found some minimal WVM differentiation when *Coleus* pith parenchyma was cultured on complex defined medium containing no auxin.

WVM production in response to culturing on medium containing a variety of IAA G concentrations is given in Figure 4. This curve is characteristic of many auxin response curves in that increasing auxin concentrations produce greater responses up to some maximum response level. Concentrations of auxin above this point tend to inhibit the response being measured.

Time Course of WVM Cytodifferentiation. Xylem regeneration around stem wounds (10) and xylogenesis in explanted stem segments (6) are completed in less than 10 days. When preexisting vascular tissue has been removed this developmental event takes significantly longer, as demonstrated by Earle (2) and by Clutter (1). If data are to be interpretable among the various experimental systems it is important that the time dimension be as similar as possible. Figure 5 is a graph of the appearance of WVM in cultured pith parenchyma tissue blocks. WVM differentiation in this system comes closer to approximating the *in vivo* response time than other homogeneous systems. The shape of this curve is similar to that found by Fosket (6) for *Coleus* stem segments; however, for pith blocks maximum WVM production occurs by 10 days rather than 7.

Only data through 11 days are shown; however, counts have been made through 20 days without significant increase in the

[2] Abbreviations: WVM: wound vessel members; SA: culture medium comprising sucrose, agar, and IAA; CSA: SA culture medium plus colchicine.

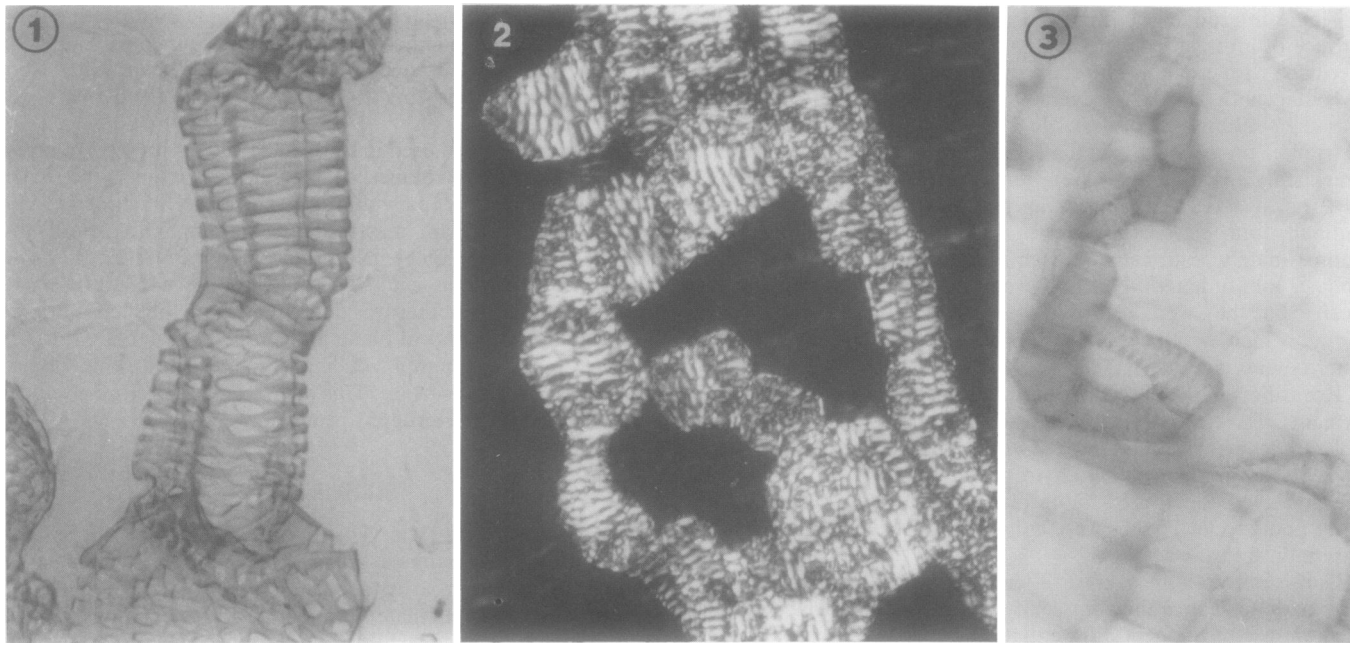

FIG. 1. Example of tracheary elements that differentiate in *Coleus* pith explants when they are cultured for 10 days on simple culture medium (× 510).

FIG. 2. Birefringence of wound vessel members when viewed with polarized light (× 260).

FIG. 3. Portion of wound vessel member strand showing a sample of variability of cell size and polarity (× 245).

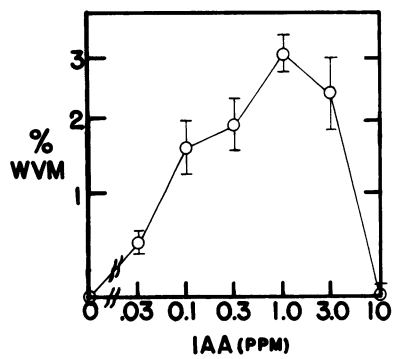

FIG. 4. Effect of IAA concentration on differentiation of wound vessel members after 10 days in culture at 25 C and a 16:8 L:D photoperiod. Data are plotted to show means and standard errors.

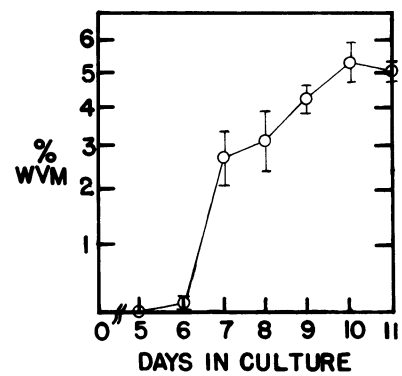

FIG. 5. Time course of wound vessel member appearance when cultured on a medium comprising agar (1%), sucrose (2%), and IAA (1 ppm). Data are plotted to show means and standard errors.

number of WVM formed. There has been no evidence of callus growth during any of these experiments. Earle (2) found that explants of *Coleus* pith formed roots within 14 to 21 days when cultured on a complex defined medium. No roots form when the culture medium is SA, even after 3 weeks.

Distribution of Cell Division. At the time of explantation pith blocks consist of fairly large parenchyma cells (Fig. 6A). Figure 6, B and C shows that the first cell divisions occur between the 3rd and 4th day in culture.

Figure 6, D through G shows the expansion of the cell division zone after 8 and then 10 days in culture. The shape of the meristematic zone has remained essentially unchanged, being definitely circular when viewed in transverse section. Undeniably many more mitoses have occurred, but not many more parenchyma cells have become involved. The majority of the new mitoses are found as further subdivision of the cells that were part of the first round of divisions.

It is possible to identify several WVM in Figure 6F within the division zone. The newly differentiated tracheary elements are found only in cells that have divided, and usually in cells that have divided more than once. Whereas the division zone has a definable and predictable pattern within the explant, the differentiating WVM do not. These cells may form strands or masses—rarely individual cells—but the location of these groups of cells within the explant is unpredictable other than that they always appear within the zone of division.

Data comparing the size of the original parenchyma cells, divided parenchyma cells, and WVM (Table I) confirm that WVM originate from those parenchyma cells which have undergone more than a single round of cell division. On the average a WVM is about one-third the volume of the original parenchyma cells.

Effects of Colchicine Treatment. If cell divisions are required for WVM differentiation, then inhibiting cell division should diminish the number of tracheary elements formed. This has been examined by Fosket (5) and found to be the case for stem segments in culture. The same holds true for pith explants cultured in SA to which colchicine had been added. If pith parenchyma blocks are maintained for 10 days on CSA no cell divisions are found, and no WVM are formed.

Since many more cell divisions are occurring than xylem differentiations it was of interest to test whether there is any propor-

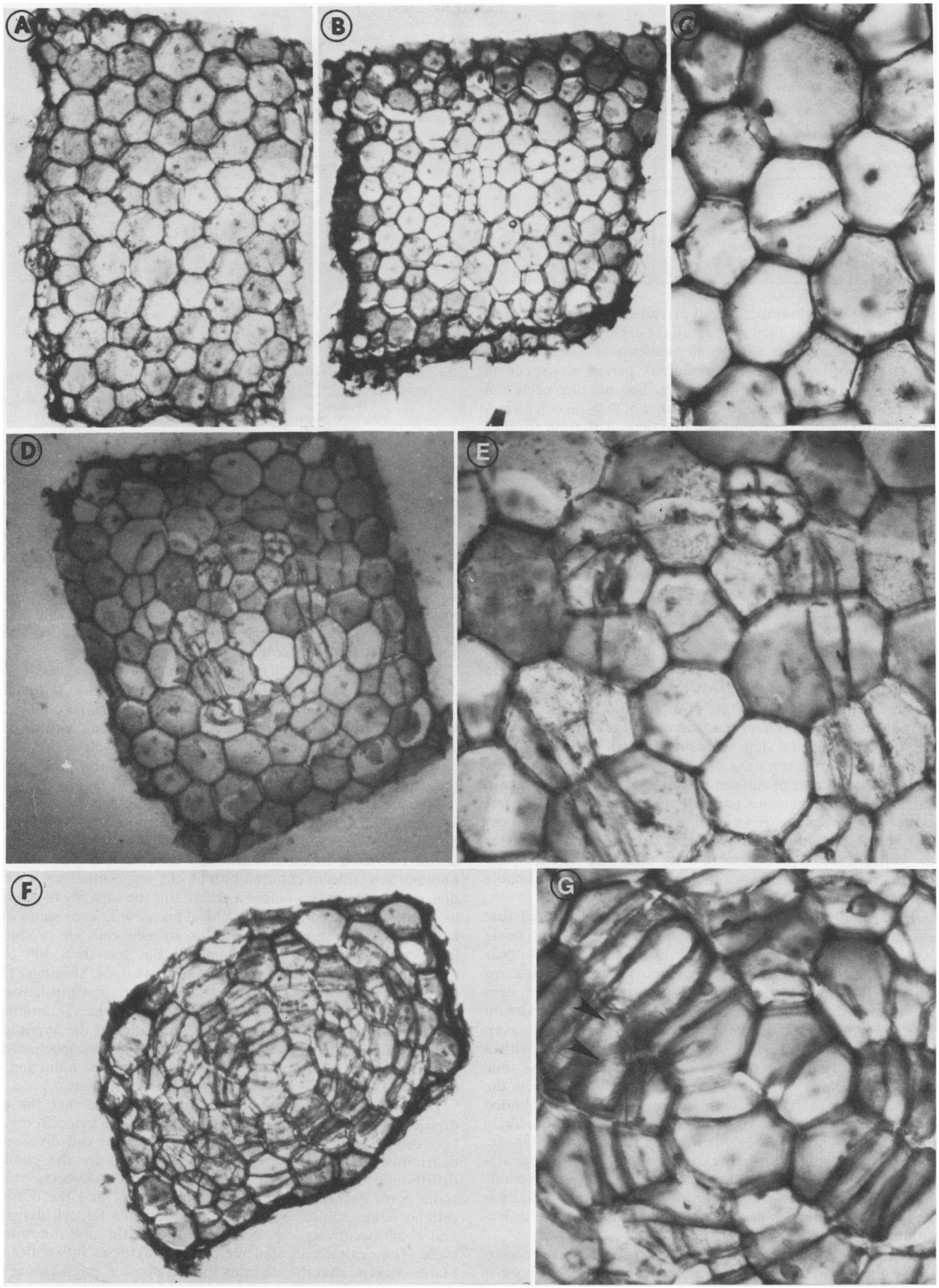

FIG. 6. Cryostat cross-sections of pith explants cultured on sucrose agar supplemented with 1 ppm IAA. A: at the time of explantation (× 110); B and C: on the 5th day of culture (B, × 75; C, × 250); D and E: on the 8th day of culture (D, × 100; E, × 280); F and G: on the 10th day of culture (F, × 75; C, × 280) with arrows pointing to differentiated WVM.

Table I. Size comparison of differentiated WVM with parenchyma cells before and after mitoses

Undivided pith parenchyma measurements were made from cryostat sections of fresh pith tissue. Divided pith parenchyma measurements were made from cryostat sections of pith blocks cultured for eight days. Wound vessel member measurements were made from squashed explants which had been cultured for ten days.

Cell Type	Diameter	Length
	micrometers	
Undivided Pith Parenchyma	90.5 ± 3.4	35.6 ± 2.1
Divided Pith Parenchyma	31.5 ± 3.1	86.5 ± 5.4
Wound Vessel Members	36.6 ± 2.8	67.7 ± 9.3

tionality between the number of cell divisions and the number of WVM formed. Table II shows the results of experiments involving colchicine treatment after various elapsed culture times. In each case a larger portion of the 10-day culture period was spent on CSA, following an initial period on SA. The number of WVM formed is in some way related to the number of days on colchicine-free medium. These data also suggest that the cell division requirement is not a single "all or nothing" need.

DISCUSSION

The experimental system used in this study is the simplest one available for the study of tracheary element cytodifferentiation. The tissue explants consist of a single cell type, parenchyma, removed from the second internode of *Coleus* plants so that four physiologically equivalent explants are produced. These explants are cultured on medium containing sucrose, auxin, and agar in distilled H_2O.

The value of pith explants, and other culture systems, as research tools is dependent upon how closely processes in the system approximate what happens in the whole plant. The response of the system to increasing amounts of auxin and the time course study indicate that this system reacts in much the same way as the intact plant and cultured stem segments. In each of these systems auxin limits the differentiation of tracheary elements; therefore, the maximum amount of differentiation is obtained when auxin is added. This homogeneous parenchyma explant system differs from the others in that exogenous auxin is an absolute requirement for WVM differentiation. It is likely that auxin is a limiting factor in the differentiation of xylem *in vivo*, and that where xylem differentiation has occurred without added auxin it is probable that the endogenous auxin availability is adequate.

Earle (2) and Clutter (1) have previously demonstrated that xylem differentiation will proceed without preexisting xylem being present. It takes at least 2 weeks for the first xylem cells to appear in either tobacco (1) or *Coleus* (2) parenchyma explants systems which is a full week longer than the time required when stem segments are utilized (6). If one considers the time until maximum production of WVM the difference between these systems is even more striking—7 days for the stem segments compared with 3 weeks for *Coleus* pith parenchyma on callus medium. The time course of the response for the system used in this study is the closest, so far, to that found with stem segments and wounded intact plants. The more alike the rates of response, the more likely it is that the responses are working through the same mechanism, and that data are applicable between and among the experimental systems. On sucrose agar supplemented with auxin pith explants do not initiate callus growth or root formation; instead activity is limited to those processes necessary for the induction and differentiation of wound xylem cells.

This study confirms that one of the most significant processes necesary for WVM differentiation is cell division. The work of Fosket (5) provides convincing evidence that DNA synthesis and cell division are required for xylogenesis in stem segment cultures.

Table II. Percent WVM differentiating when pith blocks are cultured for varying portions of the total culture period on CSA*

The treatment schedule in the table is read from left to right, the first number being the days of culture on the indicated medium and the second number indicates the days on the alternate medium. Data are presented as means ± standard errors.

Treatment Schedule		Wound Vessel Members
days		
SA**	CSA	percent***
10		4.6 ± 0.7
	10	0
3	7	0
5	5	1.3 ± 0.6
6	4	1.4 ± 0.5
8	2	3.7 ± 0.6
CSA	SA	
3	7	2.1 ± 0.8
5	5	0

* Culture medium containing agar (1%), sucrose (2%), IAA (1 ppm), and colchicine (0.04%).
** Culture medium containing the above ingredients except colchicine.
*** Total number of WVM divided by the geometrically determined cell number.

His calculations indicate that there is more DNA synthesis than cell division over the culture period of 7 days. His data demonstrate that the number of cells in the stem segments increases by about one-third, while the amount of DNA approximately doubles, on a per cell basis. It is unclear from Fosket's data (5) whether the one-third of the cell population that divided had also undergone endoreduplication, or if the replicating cell population was larger than the dividing population. The uncertainty about which cells are dividing, which are replicating, and which are differentiating makes interpretation of the role of cell division and/or DNA synthesis correspondingly equivocal.

What is lacking from that study is information tying the WVM directly to the cells that divide and replicate DNA. It remains unclear where within a stem segment the divisions take place, or even where the WVM are found. Any theory dealing with the role of cell division must account for the spatial as well as the physiological relationship between cell division and the product cell type, the tracheary element.

The present study offers evidence that as expected, the cell divisions that occur are associated with those cells that are to differentiate as WVM. It is significant, however, that cytokinesis is not restricted to those cells. The data on cell size and the photomicrographs of cultured pith blocks suggest that several cell divisions are necessary before a cell attains the capacity to redirect its fate toward becoming a WVM. This view is consistent with Fosket's data (5) showing that 139×10^3 new cells are produced in 7 culture days for stem segments, but less than 500 cells differentiate as tracheary elements—less than 0.4%. Shininger (13) has proposed a working hypothesis for the relationship between the mitotic cycle and cytodifferentiation that provides for multiple mitoses during the induction period. The thrust of the hypothesis is that only during DNA synthesis are the control mechanisms determining the array of active genes available for induction. A cell in DNA synthesis is *sensitized* to the environment.

Data from pith explants add further evidence that the cell division control mechanism is different from the cytodifferentiation control mechanism. The limiting factor for cell division is distributed radially in the explant, as shown by the circular distribution of divided cells in the pith block cross-sections. At some point between the center of the explant and the outside, cellular circumstances satisfy the requirements for cell division, and a cell occupying that position proceeds through the mitotic cycle. The centrally located region of cytokineses in smaller explants suggests that the stimulus for cell division originates at the air-tissue interface. This finds historical support in the careful observations of Sinnott and Bloch (14) who mention that when

xylem regenerates around a wound a region of cell division is established at a constant distance from the surface of the wound.

Unlike the cell division factor the distribution of the WVM differentiation controller is not reflected in a pattern of WVM appearance. Without any discernible pattern it is difficult to identify the source of the stimulus of differentiation. Presumably conditions are sufficient for xylem induction throughout the tissue block, and all that is lacking is for capable cells to become available. Part of attaining this capability is the passing of a parenchyma cell through more than a single cell division, usually three.

The multiple cell division requirement shown in this study is to be expected, according to Shininger (13) if genes must be sequentially derepressed. By this view certain parenchyma cell genes are made inducible by the first round of DNA replication after the start of the culture period. Some product of these genes, perhaps in concert with other cellular factors, serves as an inducer for a second set of genes when the cell once again replicates DNA. Data from this study suggest that three rounds of DNA synthesis are needed to activate the genes for tracheary element differentiation. Experimental support for a DNA replication requirement for xylem induction is readily available (5, 13, 16). The only data inconsistent with this view are from colchicine inhibition studies (5). If colchicine prevents only anaphase of mitosis it is hard to reconcile its inhibition of xylem differentiation with the view that it is DNA synthesis that is the necessary feature of the "quantal" mitosis (9). If, however, colchicine is shown to have an adverse effect on DNA synthesis in this system, as it has in others (4), then the available data and the available theory are compatible. Until then consideration must be given to what cell division itself may contribute to the differentiating system. Several rounds of mitosis would generate new plasma membrane and cell walls. their composition and activity reflecting the altered microenvironment of the tissue culture and perhaps leading to the xylogenic response.

Cultured *Coleus* pith parenchyma explants can be induced to differentiate WVM by hormonal and nutritional conditions similar to those required by cultured stem segments (7) and wounded whole plants (10). The time course of this response is about the same. It is likely that data from this system are meaningful for the intact plant. Evidence has been presented that directly links cell divisions with differentiating tracheary elements. I had not expected to find so much mitosis that was not directly associated with xylem differentiation. Clearly WVM come from cells which are the products of several cell divisions. It is just as clear, however, that there is much mitotic activity beyond that specifically associated with future xylem cells. The requirement for multiple cell division is consistent with the theory of eukaryotic induction proposed by Shininger (13). Final decision, however, awaits clarification of the activity of the mitotic inhibitor colchicine on DNA replication.

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